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Production of Multiple Growth Factors by a Newly Established Human Thyroid Carcinoma Cell Line

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A multiple growth factor-producing tumor cell line (NIM-1) was newly established from a patient with thyroid cancer and remarkable neutrophilia. NIM-1 cells also caused severe neutrophilia in nude mice bearing tumors. NIM-1-conditioned medium (NIM-1CM) contained activities that supported not only granulocyte, macrophage and eosinophil colony formation of human bone marrow cells but also the growth of colony-stimulating factor (CSF)-dependent cell lines, NFS60-KX and TF-1. Northern blot hybridization analysis revealed the constitutive expression of granulocyte-CSF (G-CSF), granulocyte/macrophage-CSF (GM-CSF) and interleukin(IL)-6 mRNAs in NIM-1 cells. Enzyme-linked immunosorbent assays (ELISA) using NIM-1CM also confirmed the production of IL-1α and a small amount of IL-1β besides G-CSF, GM-CSF and IL-6 in NIM-1 cells. In addition, unexpected production of IL-11 in NIM-1 cells was detected by northern blot hybridization analysis and by bioassay using an IL-11-dependent cell line. Therefore, NIM-1 cell line is shown to produce multiple cytokines including potentially megakaryopoietic growth factors such as GM-CSF, IL-6 and IL-11.

Key words: Thyroid carcinoma cell line — Hemopoietic growth factor — Leukocytosis

Various humoral factors are known to be concerned in hemopoiesis.[1)] Some of these hemopoietic growth factors have recently been characterized by the use of culture techniques and methods of molecular cloning.

Patients with malignant tumors sometimes reveal an abnormal leukocytosis[2)] and it has been verified that an ectopic production of colony-stimulating factors (CSFs) by the tumor cells is often responsible for the pathogenesis of leukocytosis.

In this paper, we report on a newly established thyroid cancer cell line which produces granulocyte-CSF (G-CSF), granulocyte/macrophage-CSF (GM-CSF), interleukin (IL)-1α, IL-1β, IL-6 and IL-11.

MATERIALS AND METHODS

Isolation of the tumor cells A 59-year-old Japanese female with an unresectable thyroid papillary adenocarcinoma was admitted to our hospital. Although she had received radiotherapy about one year before this admission, she suffered from lumbago due to metastatic tumor of the sacral bone. Her peripheral white blood cell count on admission was 41,300/μl (97.5% mature neutrophils). The hemoglobin concentration and the platelet count were almost normal. Her general condition became worse with progression of leukocytosis to 141,300/μl just before death (Fig. 1). Her course was complicated by hypercalcemia (up to 16.0 mg/dl). The tumor tissue was isolated from the metastasis to the sacral bone and inoculated subcutaneously into female nude mice of Balb/c strain (Shizuoka Laboratory Animal Center, Hamamatsu). The *in vitro* culture of the tumor cells was performed using RPMI 1640 medium (Flow Laboratories, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Irvine Scientific, Santa Ana, CA), 100 U/ml of penicillin G and 100 μg/ml of streptomycin.

Clonogenic assays of human CFU-GM Bone marrow cells were obtained from hematologically normal patients who had given informed consent. Non-adherent mononuclear cell fraction was isolated by density gradient separation (Ficoll-Paque; Pharmacia Inc., Piscataway, NJ) and by plating the cells on plastic dishes (Corning #25020, NY) for 60 min. These cells were plated at 4×10^4 cells per dish in 0.3% Bactoagar (Difco Laboratories, Detroit, MI) containing Iscove's modified Dulbecco's medium (IMDM) and 20% FBS. As a CSF source, 20% human placental conditioned medium (HPCM), 20 ng/ml of recombinant human G-CSF (rhG-CSF; Chugai Pharmaceutical Co., Tokyo), 20 ng/ml of recombinant human GM-CSF (rhGM-CSF; Sumitomo Pharmaceutical Co., Osaka) or 10% NIM-1-conditioned medium (NIM-1CM) was added. The cells were incubated for 8 days at 37°C, under 5% CO_2 in humidified air. Colonies with more than fifty cells

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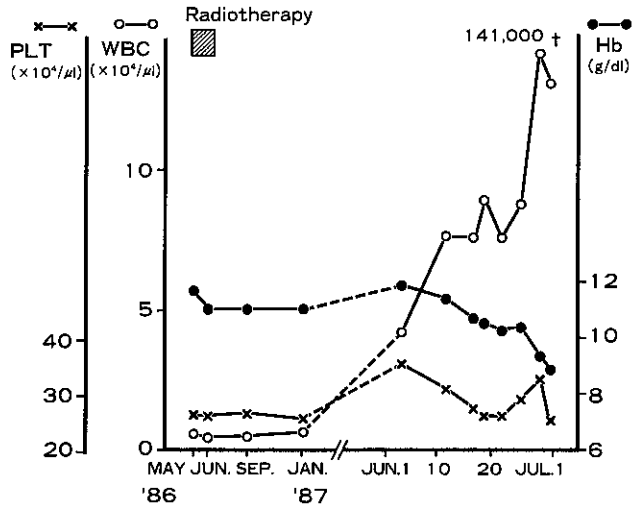

Fig. 1. Clinical course of the patient. Radiotherapy means ^{131}I injection therapy. (– – –) means a blank period as regards hematological examinations. Hb, hemoglobin; WBC, white blood cell count; PLT, platelet count of her peripheral blood.

(twenty cells in the case of eosinophil colonies) were enumerated and colony morphology was determined by May-Grünwald-Giemsa and dual esterase staining (Mutoh Pure Chemicals, Tokyo).

Cell lines and proliferation assays NFS60 cell line, a murine IL-3 and murine GM-CSF dependent multilineage hemopoietic cell line[3,4] was generously provided by Dr. Ihle (St. Jude Children's Research Hospital, TN). This cell line can become responsive to hG-CSF but not to hGM-CSF, hM-CSF or hIL-3.[5] We previously established a subline of NFS60 which grew rapidly in the presence of hG-CSF and was designated as NFS60-KX.[5] TF-1, an erythroleukemia cell line that depends on hIL-3 and hGM-CSF for its growth,[6] was kindly provided by Dr. T. Kitamura (University of Tokyo). This cell line was cultured using bacterial graded noncoated plates to avoid cell adherence to the plates according to Ref. 6.

Cell growth was measured by counting viable cells using a trypan blue dye exclusion assay. A highly IL-11-responsive subline of T1165 murine plasmacytoma cell line,[7] developed at our laboratory, was used for a bioassay detecting IL-11 activity and its growth was measured by MTT assay.[5] The original T1165 cell line had been provided by Dr. Revel (Weissmann Institute, Israel).

Northern blot hybridization Total RNA was isolated by using guanidine thiocyanate-cesium chloride, and poly(A)$^+$RNA was purified by using an oligo(dT)-cellulose column. Poly(A)$^+$RNA (3 μg on each lane) was electrophoresed on agarose gels and transferred to nylon filters (Hybond N, Amersham, Buckinghamshire, UK). Northern blot hybridization[8] was carried out using nucleotide fragments labeled with [α-^{32}P]dCTP as probes, and hybridized filters were autoradiographed. Probes used were: 1.5-kb DNA fragment including a full-length hG-CSF cDNA for G-CSF[9,10]; the synthesized C-terminal oligonucleotide (21mer) of hGM-CSF cDNA for GM-CSF[11]; probe cocktails including several exons of hIL-3,[12] hIL-4,[13] hIL-5[14] and hIL-6[15] for each interleukin, respectively (Probe Cocktail BPR 18, BPR 22, BPR 27, BPR 32; British Bio-technology, Oxford, UK). As to IL-11, the transferred filter was hybridized with IL-11 oligomer probe (GACCAGGCGGCAAACACAGTTCAT) whose 5′ end had been labeled using [γ-^{32}P]ATP. After washing, the filter was exposed to the imaging plate for 2 h at room temperature and analyzed using Bioimage Analyzer, BAS 2000 (Fuji Photo Film Co., Tokyo).

ELISA for G-CSF, GM-CSF, IL-1, IL-1 and IL-6 hG-CSF was kindly determined by Chugai Pharmaceutical Co. (Tokyo) using the enzyme-linked immunosorbent assay (ELISA) developed by them. hGM-CSF, hIL-1α and hIL-1β were determined by using GM-CSF ELISA Kit (Genzyme, Boston, MA), IL-1 Detecting Kit (Otsuka Pharmaceuticals, Tokyo) and IL-1 EASIA Kit (Medgeni x Diagnostics, Brussel, Belgium) according to the manufacturers' instructions, respectively. For hIL-6, an ELISA system established by N. Ida (Toray Basic Research Laboratories, Kamakura; paper in preparation) using anti-IL-6 monoclonal antibody[16] was employed.

RESULTS

Establishment of NIM-1 cell line Tumor tissue isolated from the patient was inoculated subcutaneously into nude mice. After about 4 weeks, the tumor grew and was subsequently transplanted into another mouse. The peripheral blood of the tumor-bearing mice revealed severe granulocytosis (200,000–300,000/μl, more than 90% mature neutrophils) compared with intact nude mice (about 4,000/μl).

Such expanded tumor cells were transferred to *in vitro* culture in RPMI 1640 medium supplemented with 10% FBS. These cells continued to grow, adhering completely to the flask wall, for more than one year, and were designated as NIM-1 cell line (Fig. 2). NIM-1 cells are routinely maintained in *in vitro* culture.

Colony-stimulating activity detected by clonogenic assays of human bone marrow cells To test whether or not NIM-1 cells release any colony-stimulating activity into the culture medium, clonogenic assays were performed using human bone marrow cells as described in "Materials and Methods." The results are shown in Table I. NIM-1-conditioned medium (NIM-1CM) supported

CFU-GM colony formation. Unlike rhG-CSF, NIM-1CM stimulated not only granulocyte but also macrophage and eosinophil colony formation, resembling GM-CSF in its activity. Therefore, these data suggested that NIM-1 cells produce GM-CSF.

NIM-1CM supports growth of CSF-dependent cell lines

Some CSF-dependent cell lines have been reported, derived from both mice and humans. Among them, we chose NFS60-KX (derived from NFS-60) and TF-1 to test whether NIM-1CM supports their growth. As shown in Fig. 3, NIM-1CM clearly supported the growth of NFS60-KX cells, as did rhG-CSF and the conditioned medium of KX-87, which is a G-CSF-producing hepatoma cell line established in our laboratory (KX-87CM).[5] To our knowledge, NFS60-KX can only respond to G-CSF and IL-6 (and erythropoietin (Ep) to some extent) among human cytokines. Therefore, it was expected that NIM-1CM exhibits G-CSF activity. NIM-1CM also supported the growth of TF-1 cells as shown in Fig. 4. As TF-1 cells respond to hIL-3, hGM-CSF and hEp but not to G-CSF,[6] NIM-1CM was considered to contain other growth factor(s) in addition to G-CSF.

Gene expression of several growth factors in NIM-1 cells

To survey gene expression of the colony-stimulating activities expected from the clonogenic assays of bone marrow cells and the responses of the two cell lines, northern blot hybridization assays were performed using probes for several growth factors. Among them, the mRNAs of G-CSF, GM-CSF and IL-6 were detected and their sizes corresponded to those reported, respectively (G-CSF, 1.5 kb; GM-CSF, 0.8 kb; IL-6, 1.3 kb) (Fig. 5).

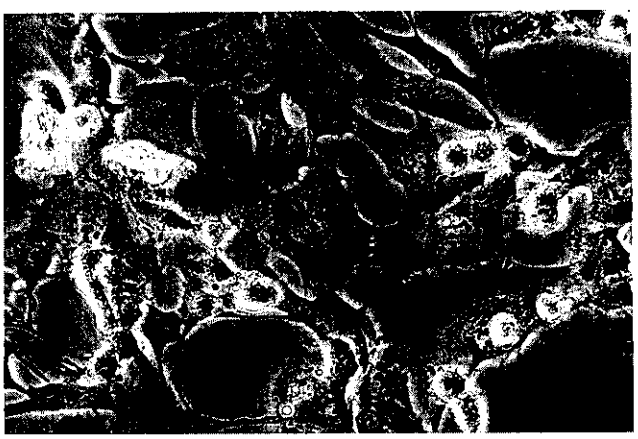

Fig. 2. The morphology of NIM-1 cells as seen by phase-contrast microscopy (×200, bar is 50 μm).

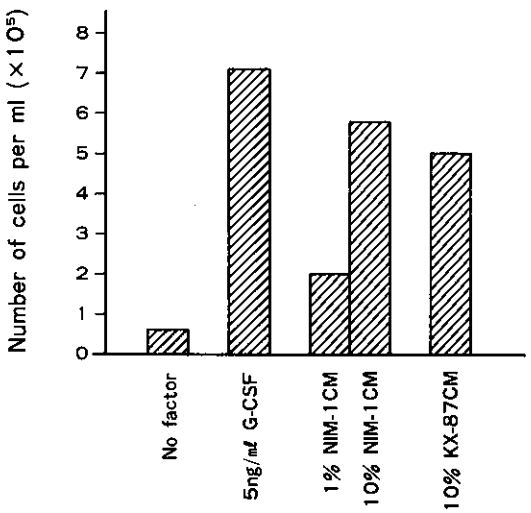

Fig. 3. Effects of NIM-1CM on growth of NFS60-KX cells. NFS60-KX cells (5×10^4 cells/ml) were incubated with 5 ng/ml rG-CSF, 1% or 10% NIM-1CM, 10% KX-87CM or no factor. Viable cells were counted after 48 h culture.

Table I. Comparison of CSA for Human Bone Marrow Cultures

Case No.	Colony stimulator		CFU-GM per 4×10^4 cells	Colony morphology (%)[a]			
				G	GM	M	Eo
1	None		0±0[b]	–	–	–	–
	HPCM	20%	123±20	65±8	30±7	4±2	1±1
	NIM-1CM	10%	129±11	37±7	38±10	17±5	8±2
	rG-CSF	20 ng/ml	133±18	62±10	31±8	7±3	0±0
	rGM-CSF	20 ng/ml	155±25	20±5	40±11	16±5	24±4
2	None		0±0	–	–	–	–
	HPCM	20%	46±5	55±10	40±10	5±3	0±0
	NIM-1CM	10%	119±16	66±11	28±6	4±2	2±1

a) G, >90% granulocytes; M, >90% macrophages; GM, mixed granulocytes and macrophages; Eo, eosinophils.
b) Values are the mean±SE of triplicate experiments.

In addition, expression of IL-11 mRNA was unexpectedly detected, as shown in Fig. 6. IL-11 mRNA consisted of 2.6-kb and 1.3-kb bands, showing almost the same pattern as reported.[7] These hybridizations were, of course, specific because no band was detected in the negative control. No significant mRNA expression was observed for IL-3, IL-4 and IL-5 (data not shown). Therefore, the genes of G-CSF, GM-CSF, IL-6 and IL-11 were constitutively expressed in NIM-1 cells.

Detection of several growth factors by ELISA and IL-11 by bioassay To confirm that NIM-1 cells actually secrete several growth factors into the culture medium, ELISA

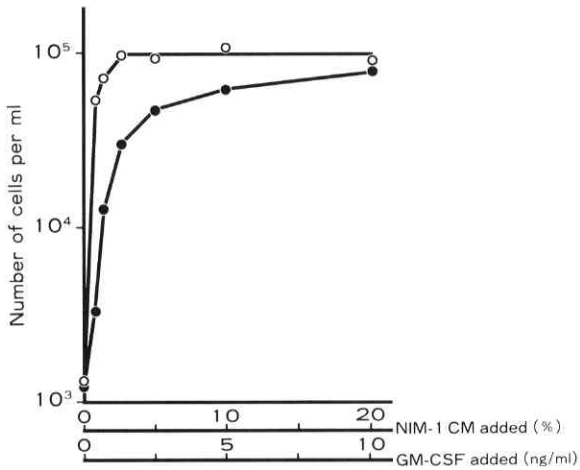

Fig. 4. Effects of NIM-1CM on growth of TF-1 cells. TF-1 cells (4×10^4 cells/ml) were incubated with 0.63–20% NIM-1CM (●), 0.32–10 ng/ml rGM-CSF (○) or no factor. Viable cells were counted after 48 h culture.

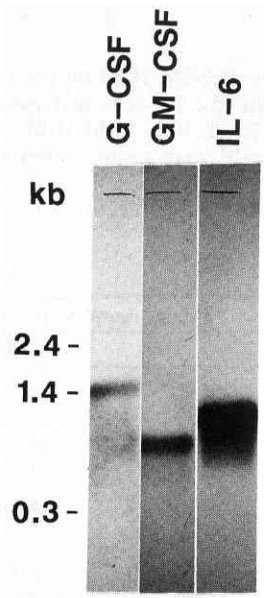

Fig. 5. Expression of G-CSF, GM-CSF and IL-6 mRNA in NIM-1 cells. Poly(A)$^+$RNA (3 μg) was applied to each lane. Probes used are described in "Materials and Methods."

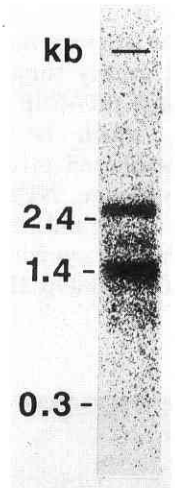

Fig. 6. Expression of IL-11 mRNA in NIM-1 cells. Poly(A)$^+$RNA (1.5 μg) was applied to the lane. The probe used is described in "Materials and Methods."

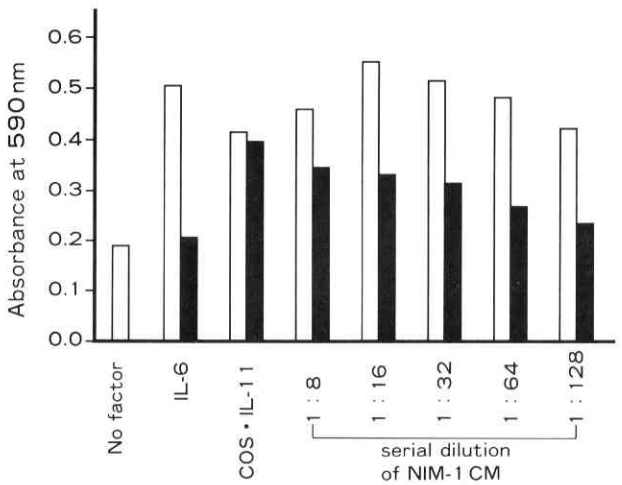

Fig. 7. Effects of NIM-1CM on growth of T1165 cells. T1165 cells (5000 cells per well) were incubated with 16 ng/ml purified human IL-6, 12.5% culture supernatant of COS 1 cells transfected with human IL-11 cDNA, serial dilutions of NIM-1CM 1:8 to 1:128) or no factor. Anti-IL-6 monoclonal antibody, IG 61 (2.5 μg/ml), was added for comparison and the results are shown by the black columns. MTT assay was performed after 42 h culture and the absorbance (590 nm) was measured.

was carried out on each cytokine anticipated to exist in NIM-1CM. NIM-1CM contained 36 ng/ml G-CSF, 1 ng/ml GM-CSF, 750 pg/ml IL-1α, 33 pg/ml IL-1β and 59 ng/ml IL-6. There is no immunoassay system available for IL-11. Therefore, we utilized T1165 cell line, which we have found to proliferate in the presence of IL-6 or IL-11. As shown in Fig. 7, NIM-1CM supplemented with a sufficient amount of anti-IL-6 neutralizing antibody still supported the growth of T1165 cells in a dose-dependent fashion. These results confirmed the presence of IL-11 activity in NIM-1CM.

DISCUSSION

We established a new cell line, NIM-1, which had originated from thyroid carcinoma tissue. Several lines of evidence suggested that this cell line produces multiple growth factors. First, the original patient exhibited remarkable neutrophilia, and nude mice bearing the tumor also showed severe neutrophilia. Second, the clonogenic assays of human bone marrow cells showed the existence of CSA in NIM-1CM that supported colony formation of not only granulocytic lineage but also macrophage and eosinophilic lineages. Third, NIM-1CM supported the growth of CSF-dependent cell lines such as NFS60-KX and TF-1. As NFS60-KX can proliferate only with G-CSF among human CSFs, NIM-1CM undoubtedly contains G-CSF activity. On the other hand, TF-1 cells do not respond well to G-CSF but do respond to IL-3, GM-CSF, IL-4, IL-5, IL-6, and Ep. Therefore, NIM-1CM may contain some of the growth factors described above in addition to G-CSF.

We confirmed that NIM-1 cells produce G-CSF, GM-CSF and IL-6 at the molecular level, by northern blot hybridization analysis and ELISA. Furthermore, ELISA revealed the production of IL-1α and a small amount of IL-1β by NIM-1 cells. IL-1 may also influence hemopoiesis positively.[17, 18] In addition, Dr. S. Etoh *et al.* (University of Occupational and Environmental Health, Fukuoka) also examined NIM-1CM and detected IL-1 and parathyroid hormone-related peptide (PTH-RP), which had probably caused the hypercalcemia in the patient (personal communication).

It is notable that NIM-1 cells produce IL-11 in addition to several other growth factors. IL-11 is a newly proposed interleukin which is, like IL-6, considered to support megakaryocyte colony formation as well as B cell development and plasmacytoma proliferation.[7] NIM-1 is the first cancer cell line of human origin that has been shown to produce IL-11 constitutively. Therefore it is concluded that NIM-1 cells produce at least three cytokines that might be related to megakaryopoiesis — GM-CSF, IL-6 and IL-11. It is of course unclear whether the original tumor actually exerted such growth factor activities *in vivo*. Nevertheless, as shown in Fig. 1, peripheral blood of the patient revealed severe leukocytosis with the tumor progression, while the platelet count showed no remarkable change. As to M-CSF, we have no data at present. Little or no Ep may be contained in NIM-1CM because no erythroid colony was detected by routine clonogenic assay of human bone marrow cells (data not shown).

Several CSF-producing tumor cell lines have been reported.[5, 19–23] Some lines such as CHU-2 and 5637 produce both G-CSF and GM-CSF constitutively. NIM-1 also produces both CSFs. It is suspected that the activation of G-CSF gene in such cell lines is not due to gene amplification or rearrangement, but to the transactivator on the promoter region of the gene.[24] A similar mechanism might also be involved in the activation of GM-CSF gene. Since NIM-1 cell produces many kinds of growth factors including two CSFs constitutively, this cell line should be of potential use for research on several cytokines (including megakaryopoietic growth factors or unknown cytokines), as well as on the mechanisms of multiple cytokine gene expression and carcinogenesis.

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