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In vivo Tumor Growth Enhancement by Granulocyte Colony-stimulating Factor

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The intraperitoneal administration of human recombinant granulocyte colony-stimulating factor (G-CSF) enhanced the growth of intradermally inoculated tumor in mice; in a Meth A fibrosarcoma model, G-CSF administration significantly shortened the latency before tumor appearance, accelerated the increase of tumor size, shortened the survival time of tumor-bearing mice and increased the incidence of lethal tumor growth. A similar growth-enhancing effect of G-CSF was observed in models employing Meth 1 fibrosarcoma, colon carcinoma 26, and L1210 leukemia, although not all the effects were statistically significant. *In vitro* study showed that G-CSF did not enhance Meth A growth in suspension culture or in soft agar. These data suggest that G-CSF enhances the Meth A growth not directly but through the mediation of host factors. The accumulation of neutrophils was histologically observed in the tumor nodule, the blood, and the spleen in mice given G-CSF repeatedly. The spleen cells and the peripheral blood leukocytes of G-CSF-injected mice enhanced Meth A growth *in vitro* as compared with those of mice injected with physiological saline. These results suggest the possibility that the *in vivo* growth of tumor cells was enhanced by G-CSF-induced overproduction of cells including neutrophils.

Key words: Granulocyte colony-stimulating factor — Neutrophil — Spleen cell — Peripheral blood leukocyte — Tumor cell line

Colony-stimulating factors are a class of glycoproteins characterized by their capacity to support the survival, proliferation, and differentiation of hematopoietic progenitor cells *in vitro*.[1,2] Recently recombinant human colony-stimulating factors have been produced and have been used as anti-leukopenia agents.

Recombinant human granulocyte colony-stimulating factor (G-CSF) has been produced in *E. coli*[3–5] and has been expected to be a useful agent for protection against myelosuppression during chemotherapy[6–9] and radiotherapy.[10] Although the effects of G-CSF on the growth of hematopoietic cells and nonhematopoietic cells have been investigated *in vitro*,[11–13] the *in vivo* effects of G-CSF on the tumor growth have not yet been evaluated extensively.

Our previous work demonstrated that G-CSF administration partially nullified the eradication of Meth A fibrosarcoma by interferon α A/D (IFN) therapy and inhibited the antiproliferative activity of monocytes induced by IFN administration.[14] In this study, we showed that under the present experimental conditions G-CSF enhanced the *in vivo* growth of several murine tumor cell lines; Meth A fibrosarcoma, Meth 1 fibrosarcoma, colon carcinoma 26, and L1210 leukemia.

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MATERIALS AND METHODS

Mice and tumors Female BALB/c mice and male BALB/c×DBA/2 Cr ($CD2F_1$) mice were obtained from Japan Charles River Co., Ltd. (Atsugi, Kanagawa). Meth A fibrosarcoma was supplied by Dr. Y. Hashimoto. Meth 1 fibrosarcoma was furnished in ascitic form by Dr. M. Morimoto. L1210 leukemia was from National Cancer Institute (Bethesda, MD). Colon carcinoma 26 (C26) was from Simonsen Laboratories, Inc. (Gilroy, CA) and was passed *in vitro* in RPMI medium supplemented with 10% fetal bovine serum (FBS).

G-CSF Recombinant human granulocyte colony-stimulating factor (G-CSF) (KRN8601, Kirin Amgen) purified from *E. coli*[3] was kindly provided by Kirin Brewery Co., Ltd. (Tokyo). It had a specific activity of 10^8 units/mg when assayed in a granulocyte/macrophage colony-forming unit assay.[7]

Tumor inoculation Tumor cells as indicated were inoculated intradermally (i.d.) in the flank of BALB/c mice (Meth A, Meth 1 and C26) or $CD2F_1$ mice (L1210) (Day 0). The mice were given 500 μg of G-CSF/kg per day or physiological saline (PS) intraperitoneally (i.p.) at indicated intervals. Tumor sizes were measured at the longest (a) and shortest (b) arms and expressed as $\sqrt{ab}$ (mm). Lethal tumor growth was judged on Day 60 or more after tumor inoculation.

Preparation of peripheral blood leukocytes At the indicated intervals, mice were anesthetized with chloroform, then killed and bled by heart puncture. Whole peripheral blood leukocytes including polymorphonuclear leukocytes (PBL) were purified from heparinized blood by using dextran as described below. Blood was diluted with an equal volume of Dulbecco's phosphate-buffered saline (Nissui Pharmaceutical Co. Ltd., Tokyo), pH 7.2 (PBS) and a half volume of 6% dextran (M_r 200,000–300,000; Wako Pure Chemical Industries, Ltd., Tokyo) dissolved in PBS. After a 45-min incubation at room temperature, the leukocytes recovered in the upper layer were used.

Culture medium and other reagents RPMI-1640 medium containing 100 μg kanamycin/ml (Banyu Pharmaceutical Co., Ltd., Tokyo) and either the indicated concentration of normal mouse serum prepared from female BALB/c mouse (NMS) or 10% FBS (GIBCO) was used.

***In vitro* effect of spleen cells of G-CSF-injected mice** Spleen cells or PBL were prepared from the Meth A-bearing mice injected i.p. with 500 μg of G-CSF/kg per day or PS for the indicated intervals. Meth A cells (5×10^2 cells/0.1 ml/well) and spleen cells or PBL (5×10^4–1×10^5 cells/0.1 ml/well) were cocultured in RPMI medium supplemented with 0.5–4.0% NMS. After three days, the growing Meth A cells were measured by regrowth assay; an aliquot of the incubation mixture was transferred into 1 ml of RPMI medium supplemented with 10% FBS and the incubation was continued for another two days. The cell concentrations were determined by using a Model Zb Coulter Counter (Coulter Electronics Inc., Hialeah, FL). Under these experimental conditions, cell growth in the culture of the highest cell concentration was in the logarithmic phase. The cell counts of the tumor cell-free incubation groups was negligible in this regrowth assay.

Tissue staining The tissue sections were fixed with 3.7% formaldehyde and were stained with hematoxylin and eosin.

Statistics The results on *in vivo* incidence of palpable tumor growth and incidence of lethal tumor growth were analyzed by the use of Fisher's exact test. The data on *in vivo* and *in vitro* growth of different tumors, organ weight and the cell numbers of PBL and spleen cells were analyzed by using Student's t test. Differences in the latency time between tumor inoculation and palpable tumor appearance and in the survival time of tumor-bearing mice were analyzed by using the Mann-Whitney U-test.

Table I. Effect of i.p. Administration of G-CSF on the Growth of i.d.-Inoculated Tumors[a)]

Tumor	No. of cells	Schedule	Drug	Days of tumor appearance, median (range)	$P^{c)}$	Survival days, median (range)	$P^{d)}$	Lethal growth/total[b)]	$P^{e)}$
Meth A	5×10^3	d −2–10	G-CSF	7 (7–10)	<u>≤0.01</u>	46 (37–58)	>0.1	10/10	<u>0.043</u>
Meth A	5×10^3	d −2–10	PS	18 (7–20, >90)		53 (39–56, >90)		6/10	
Meth A	1×10^3	d −2–10	G-CSF	7 (7–56, >90)	<u>≤0.05</u>	42 (35–89, >90)	<u>≤0.02</u>	9/10	<u>0.010</u>
Meth A	1×10^3	d −2–10	PS	>90 (7–23, >90)		>90 (37–48, >90)		3/10	
Meth 1	1×10^4	d −3–10	G-CSF	6 (6–8, >90)	>0.1	64 (59–66, >90)	>0.1	7/10	0.156
Meth 1	1×10^4	d −3–10	PS	>90 (6–11, >90)		>90 (59–80, >90)		4/10	
C26[f)]	1×10^3	d −1–10	G-CSF	10 (10)	<u>≤0.01</u>	64 (46–107)	>0.1	10/10	<u>0.043</u>
C26	1×10^3	d −1–10	PS	>120 (13–47, >120)		82 (42–82, >120)		6/10	
C26	1×10^2	d −1–13	G-CSF	>88 (13–55, >120)	>0.1	>105 (80–89, >120)	>0.1	5/10	0.32
C26	1×10^2	d −1–13	PS	>120 (31–33, >120)		>120 (101–116, >120)		3/10	
L1210	1×10^2	d −1–8	G-CSF	8 (8–9)	>0.05	14 (14–15)	<u>≤0.02</u>	5/5	1.0
L1210	1×10^2	d −1–8	PS	9 (8–9)		16 (15–16)		5/5	
L1210	1×10^1	d −1–8	G-CSF	>60 (9, >60)	>0.1	>60 (18, >60)	>0.1	2/5	0.53
L1210	1×10^1	d−1–8	PS	>60 (9, >60)		>60 (18, >60)		2/6	

a) The mice were inoculated i.d. with the indicated tumors on Day 0 and were given G-CSF (500 μg/kg per day) on the indicated days starting 1–3 days before tumor inoculation.
b) No. of mice with lethal tumor growth/total no. of mice.
c), *d*), *e*) Statistical analysis of G-CSF group vs. PS group by using the Mann-Whitney U-test[c, d)] and Fisher's exact test.[e)] Significant differences are indicated by underlining.
f) Colon carcinoma 26.

RESULTS

Tumor growth enhancement by G-CSF *in vivo* The incidence of lethal growth of i.d.-inoculated Meth A fibrosarcoma was 60% in mice inoculated with a small number (5×10^3) of tumor cells (Table I and Fig. 1D). The i.p. administration of G-CSF significantly shortened the latency time between tumor inoculation and tumor appearance and increased the incidence of palpable tumors from Day 7 after tumor inoculation and final lethal tumor growth (Fig. 1D, Table I). Fig. 1A showed that the increase of tumor size was accelerated by G-CSF administration. In the case of 1×10^3 Meth A cells, G-CSF administration significantly enhanced all the indicators examined for tumor growth, including shortening of median survival time (Table I). Table I and Fig. 1 showed that the i.p. administration of G-CSF significantly enhanced the growth of the i.d.-inoculated (1×10^3 cells) C26 as judged from all the indicators except median survival time. In the case of Meth 1 fibrosarcoma and L1210 leukemia, G-CSF administration significantly enhanced tumor growth in terms of one indicator for each tumor; increase of tumor size (Meth 1, Fig. 1C) or shortening of the median survival time of tumor-bearing mice (L1210, Table I). Other indicators consistently showed the tendency of tumor growth enhancement by G-CSF although the changes were statistically not significant.

In the dose-response study, 32 μg/kg or a higher dose of G-CSF administration significantly enhanced i.d.-inoculated (5×10^3 cells) Meth A fibrosarcoma, but 13 μg/kg of G-CSF slightly enhanced tumor growth (Fig. 2). In a comparison between the "all G-CSF group except 13 μg/kg group" and the "PS group," each indicator was significantly different: mean tumor size on Days 14–28; median survival time, Day 46 vs. >Day 120 (P=0.03); incidence of lethal tumor growth, 30/39 (73%) vs. 3/10 (30%) (P=0.008).

The *in vitro* effect of G-CSF on tumor cell growth The direct effect of G-CSF on the growth of Meth A cells as a tumor growth factor was tested in the culture medium

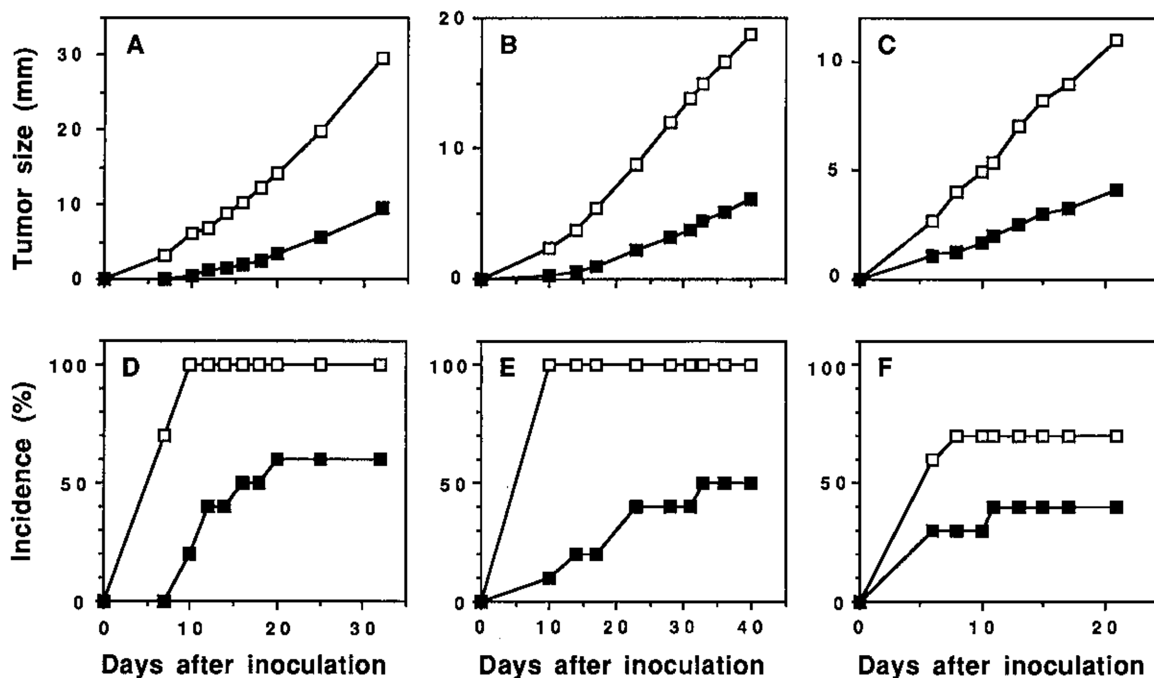

Fig. 1. Growth of Meth A fibrosarcoma (A, D), colon carcinoma 26 (C26) (B, E), and Meth 1 fibrosarcoma (C, F) in the G-CSF-injected mice. Female BALB/c mice (10 mice per group) were i.d.-inoculated with 5×10^3 cells/mouse of Meth A cells, 1×10^3 cells/mouse of C26 cells and 1×10^4 cells/mouse of Meth 1 cells on Day 0 and were given 500 μg of G-CSF/kg per day (□) or PS (■) on Day −2 to Day 10 (Meth A), Day −1 to Day 10 (C26), and Day −3 to Day 10 (Meth 1) after tumor inoculation. Tumor growth is shown in terms of mean tumor size (A, B, C) and incidence of palpable tumors (D, E, F). Statistically significant differences were found at $P<0.05$ for tumor size in the G-CSF group vs. PS group by Student's t test (for intervals of: A, Day 7 to Day 32; B, Day 10 to Day 40; C, Day 6 to Day 21) and for incidence of palpable tumors by Fisher's exact test (for intervals of: D, Day 7 to Day 18; E, Day 10 to Day 40; F, no significant).

supplemented with various doses of NMS. In our preliminary experiment, the maximum concentration of G-CSF in blood was supposed to be about 2 μg/ml as determined by radioimmunoassay when 500 μg/kg G-CSF was injected i.p. into mice, and it has been reported that G-CSF stimulated G-CSF-responding tumor cell growth below 0.1 μg/ml.[11-13] Fig. 3 showed that the growth of Meth A cells was unchanged in the presence of G-CSF of 10 μg/ml and less in 4% NMS and lower concentrations of NMS (0.05–1%, data not shown). In soft agar, G-CSF did not enhance the colony formation of Meth A cells either (data not shown). These data indicate that G-CSF did not directly enhance the growth of these tumors under these conditions.

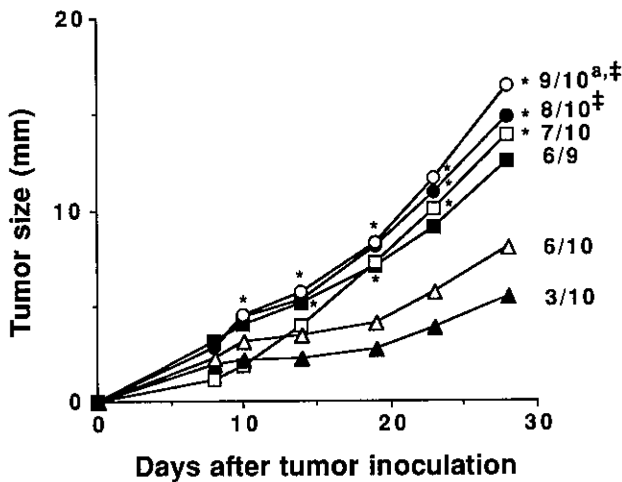

Fig. 2. Dose-dependence of the effect of G-CSF on the growth of i.d.-inoculated Meth A fibrosarcoma. Female BALB/c mice (10 or 9 mice per group) were i.d.-inoculated with 5×10^3 cells/mouse of Meth A cells on Day 0 and were given various doses of G-CSF or PS on Day 2 to Day 10. Doses of G-CSF were: □, 500 μg/kg; ■, 200 μg/kg; ○, 80 μg/kg; ● 32 μg/kg; △ 13 μg/kg; ▲, PS. * Statistically significant difference in tumor size at $P<0.05$ vs. the PS group (one-tailed Student's t test): "500 μg/kg group," Days 19–28; "200 μg/kg group," Days 14–21; "80 μg/kg group," Days 10–28; "32 μg/kg group," Days 10–28. a. Number of mice with lethal tumor growth/total no. of mice. ‡ Statistically significant difference at $P<0.05$ vs. the PS group (Fisher's exact test).

Effect of G-CSF administration on the weight and the cell number of the organs We supposed that G-CSF does not enhance the tumor growth directly but through the mediation of host factors. We investigated the changes of organ weight and cell number upon G-CSF administration. Multiple G-CSF administration increased the weight of liver and spleen, and the number of PBL and spleen cells (Table II), but decreased bone marrow cells.[15] Table III shows that the percentage of mature

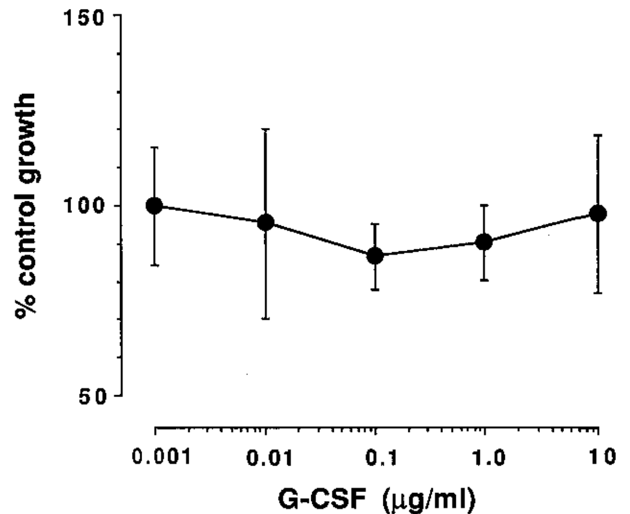

Fig. 3. Effect of G-CSF on *in vitro* tumor growth. Tumor cells were cultured in 4% BALB/c normal mouse serum with or without G-CSF. The growing cells were counted with a Coulter counter. The results are presented as percent of control growth of G-CSF-free culture.

Table II. Effect of i.p.-Administered G-CSF on the Organs of Mice

Treatment	Organ weight and cell no.							
	Experiment 1 (5-day treatment)					Experiment 2 (29-day treatment)		
	Spleen (mg)	Thymus (mg)	Lymph node (mg)	Bone marrow cells ($\times10^7$/femur)	Total PBL[b] ($\times10^7$/ml)	Spleen (mg)	Liver (mg)	Kidney (mg)
G-CSF[a]	227±9[c, d]	50±10	12±2	1.1±0.1[d]	3.2±0.6[d]	764±141[d]	1453±65[d]	196±12
PS	105±4	50±2	12±0	2.1±0.2	0.6±0.1	112±4	1260±64	178±17

a) G-CSF was injected i.p. at 500 μg/kg per day.
b) Peripheral blood leukocytes. The cell count in the hearts of mice was performed in Turk solution.
c) Each value is the mean±SD of four mice (Experiment 1) or three mice (Experiment 2).
d) Statistically significant difference at $P<0.05$ vs. the PS group (Student's t test).

Table III. Effect of G-CSF Administration on the Cell Populations of Spleen and PBL

Spleen or PBL[a]	Treatment of mice[b]	Weight (mg)	Cell no.[c]	Cell composition (%)[d]		
				Neutrophils	Lymphocytes	Others
Experiment 1			(/mouse)			
Spleen	G-CSF	212±30[e,f]	4.7×10^8	30±3[f]	57±6[f]	14±3[f]
Spleen	PS	114±1	2.4×10^8	2±1	92±3	6±3
Experiment 2						
Spleen	G-CSF	208±6[f]	4.0×10^8	37±4[f]	53±5[f]	9±1
Spleen	PS	127±11	1.8×10^8	1±1	93±5	5±4
Experiment 3			(/mm³)			
PBL[a]	G-CSF	–	22000±3900[f]	54±9[f]	40±9[f]	6±1
PBL	PS	–	6200±520	17±6	77±7	6±2

a) Peripheral blood leukocytes.
b) Mice were injected i.p. with 500 μg of G-CSF/kg per day or PS for four days (Experiments 2 and 3) and five days (Experiment 1). The numbers of mice were two (Experiments 1 and 2) and four (Experiment 3).
c) The spleens and the heart blood of the mice were collected on the day after the last injection. The total spleen cell counts and total PBL counts were made using Turk solution. The cell counting was done with pooled spleen cells and with PBL of individual mice.
d) Stamp samples of the spleen and blood smears on slide glass were prepared and stained with Wright's-Giemsa stain. The differential counts of spleen cells and PBL were done with the stained samples of individual mice.
e) Mean±SD.
f) Statistically significant difference at $P<0.05$ vs. the PS group (Student's *t* test).

neutrophils increased but that of lymphocytes was decreased by G-CSF administration for four or five days.

Effect of the spleen cells and PBL of G-CSF-injected mice on *in vitro* tumor growth We investigated the effect of spleen cells and PBL of G-CSF-injected mice on Meth A growth, because neutrophil accumulation was observed in spleen and PBL of the G-CSF-injected mice. The growth rate of Meth A cells co-cultured with the spleen cells or PBLs of G-CSF-injected mice was higher than that in the case of PS-injected mice (Tables IV and V). These results suggested that the cells induced by G-CSF, including neutrophils, enhanced the *in vivo* growth of Meth A cells.

Pathological study of G-CSF-injected mice The tumor was not palpable at Day 7 after inoculation of 5×10^3 Meth A cells, but the repeated injection of G-CSF after such inoculation resulted in tumor nodule formation during the same period of time. At this time, many neutrophils had infiltrated into the tumor nodules of G-CSF-injected mice (Fig. 4). At a later period, neutrophils infiltrated into the tumor nodules of PS-injected mice (data not shown). These data indicated that the administration of G-CSF accelerated the accumulation of neutrophils into tumor nodules. These results, together with *in vitro* Meth A growth enhancement in the presence of neutrophil-enriched cell mixtures (Tables IV and V), suggested that the cells that accumulated in tumor tissue, neutrophils in particular, enhanced the tumor growth.

DISCUSSION

In the present study, we showed that G-CSF administration enhanced the growth of the i.d.-inoculated tumor cell lines without tumor selectivity (Meth A fibrosarcoma, Meth 1 fibrosarcoma, colon carcinoma 26 and L1210 leukemia were used). It was reported that the growth of several tumor cell lines was directly enhanced by G-CSF; these were leukemic cell lines and small lung cancer cell lines.[11-13] In this study, however, Meth A fibrosarcoma did not respond to G-CSF supplemented in culture medium. These data indicated the possible mediation of host factors in the *in vivo* growth enhancement of tumors by G-CSF. There are two possible mechanisms for the enhancement of *in vivo* tumor growth by G-CSF administration, other than direct growth acceleration of tumor cells by G-CSF: 1) neutrophils induced by G-CSF or G-CSF itself might suppress the host antitumor immunity; 2) neutrophils induced by G-CSF might stimulate the tumor growth. Regarding the former possibility, we have reported that G-CSF administration partially nullified the eradication of Meth A fibrosarcoma by interferon α A/D (IFN) therapy and inhibited the antiproliferative activity of monocytes of peripheral blood induced by IFN.[14] It was also reported that neutrophils inhibited natural killer- and lymphokine-activated killer-mediated cytotoxicity.[16-18]

Table IV. Effect of the Spleen Cells of G-CSF-administered Mice on Meth A Growth *in vitro*

NMS%	Spleen cells[a] (×10⁶/ml)	Treatment of mice	Meth A growth in the presence of spleen cells: Cell counts[b]
Experiment 1			
4.0	1.0	G-CSF	1623±271[c]
		PS	701±40
4.0	0.5	G-CSF	1217±140[c]
		PS	205±49
4.0	None		1040±13
0.25	1.0	G-CSF	2967±568
		PS	3093±86
0.25	0.5	G-CSF	3147±248
		PS	2902±187
0.25	None		1752±227
Experiment 2			
4.0	1.0	G-CSF	2686±565[c]
		PS	701±40
4.0	0.5	G-CSF	1217±140[c]
		PS	205±49
4.0	None		2681±645

a) Spleen cells of mice that had been injected i.p. with G-CSF (500 μg/kg per day) or PS for five days.
b) Meth A cells were co-cultured with the spleen cells or cultured alone in the NMS. The growing Meth A cells were measured by means of regrowth assay. The results are presented as the final counts with the Coulter counter. Each value is the mean±SD of more than quadruplicate determinations.
c) Statistically significant difference at $P<0.05$ vs. the PS group (Student's *t* test).

Table V. Effect of the Peripheral Blood Leukocytes of G-CSF-administered Mice on Meth A Growth *in vitro*

NMS%	PBL[a] (×10⁶/ml)	Treatment of mice	Meth A growth in the presence of PBL: Cell counts[b]
Experiment 1			
4.0	1.0	G-CSF	1608±213[c]
		PS	1163±192
4.0	0.5	G-CSF	1126±191[c]
		PS	249±73
4.0	None		70±30
0.5	1.0	G-CSF	2507±76
		PS	2553±283
0.5	0.5	G-CSF	1769±333
		PS	1824±167
0.5	None		1117±93
Experiment 2			
4.0	1.0	G-CSF	3624±799[c]
		PS	1463±428
4.0	0.5	G-CSF	1631±311[c]
		PS	605±219
4.0	None		1521±272

a) Peripheral blood leukocytes containing polymorphonuclear leukocytes were prepared by the use of dextran from heparinized blood of mice that had been injected i.p. with G-CSF (500 μg/kg per day) or PS for four days.
b) Meth A cells were co-cultured with the spleen cells or cultured alone in the NMS. The growing Meth A cells were measured by means of regrowth assay. The results are presented as the final counts with the Coulter counter. Each value is the mean±SD of more than quadruplicate determinations.
c) Statistically significant difference at $P<0.05$ vs. the PS group (Student's *t* test).

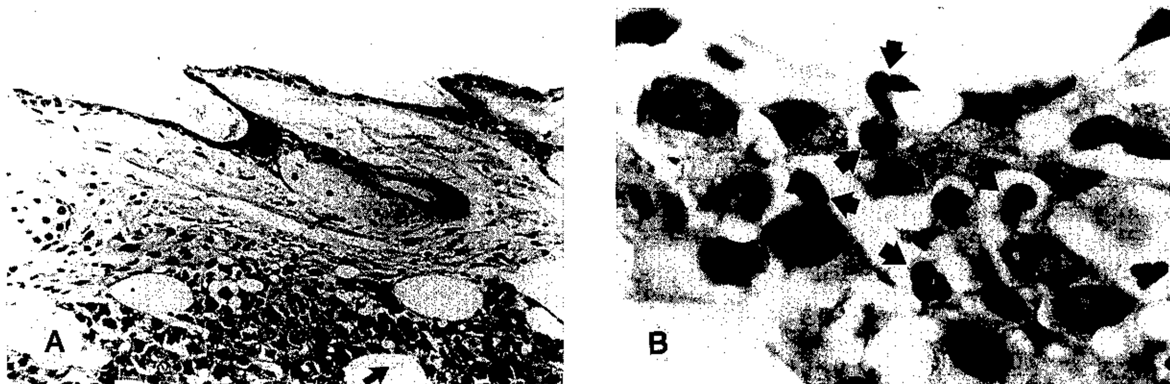

Fig. 4. Histology of a tumor nodule of a G-CSF-injected mouse. Mice were inoculated i.d. with 5×10^3 Meth A cells on Day 0 and were repeatedly injected i.p. with 500 μg G-CSF/kg per day or PS starting from Day −2. Tumor nodules appeared only in G-CSF-injected mice on Day 7. The sections of tumor nodule were stained with hematoxylin and eosin. B (×1000) is an enlarged view of the indicated area in A (A, ×100). Several neutrophils have infiltrated among the tumor cells (arrows).

In this paper we focussed our attention on the latter possibility. We found that the spleen cells and peripheral blood leukocytes of G-CSF-injected mice enhanced tumor growth *in vitro*. As regards nonimmunological effects of netrophils, several reports have shown that neutrophils enhance tumor malignancy; they include malignant transformation[19] and stimulation of tumor invasion and metastasis.[20-22] In accordance with this, the pathological study showed the infiltration of neutrophils into the tumor tissue, suggesting that the neutrophils associated with tumor nodules enhanced tumor growth. The mechanism of tumor growth acceleration by neutrophils at the molecular level remains uncertain. It is possible that inflammation-related factors produced by neutrophils,[23] such as proteinase or prostaglandins, mediate the tumor growth acceleration, because it has been suggested that these factors could stimulate cell growth.[24-26] Furthermore, the previous study[27] showed infiltration of neutrophils in the liver of G-CSF-injected mice. These observations favor the possibility that by supplying neutrophils G-CSF enhances the growth of tumors of a small size, either latent tumors or tumors at an early stage after metastatic colonization, which otherwise may be eradicated through the host immunological and non-immunological surveillance systems.

In conclusion it is possible that the overproduced neutrophils enhance tumor growth and, under some circumstances, the reduction of neutrophils may contribute to tumor regression during the course of cancer therapy. The results of the present study warrant further investigation aimed at identifying the cell population responsible for enhancement of tumor growth and analyzing the mechanism of the tumor growth enhancement by those cells.

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