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Clinicopathological study of involvement of granulocyte colony stimulating factor and granulocyte-macrophage colony stimulating factor in non-lymphohematopoietic malignant tumors accompanied by leukocytosis

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Summary. Involvement of granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) in non-lymphohematopoietic malignant tumors accompanied by leukocytosis was clinicopathologically investigated. Among 1,778 autopsy cases in the last 20 years, 485 lesions of 439 cases with non-lymphohematopoietic malignant tumors accompanied by leukocytosis with a white blood cell count of 10,000/mm^3 or greater during the course were immunohistologically examined for G-CSF and GM-CSF. Three (0.7%) and two cases (0.5%) were G-CSF- and GM-CSF-positive, respectively. GM-CSF mRNA was confirmed by using non-fixed cryopreserved tumor tissues in one case positive for GM-CSF. G-CSF-positive cases were large cell carcinoma of the lung, adenocarcinoma of the colon, and adenocarcinoma of the stomach, and GM-CSF-positive cases were spindle cell carcinoma of the lung and malignant thymoma. In the case with stomach carcinoma, the primary lesion showing moderately differentiated adenocarcinoma was negative, but the lung metastatic lesion showing less differentiated adenocarcinoma was G-CSF-positive. The survival period was six months or less in four out of five positive cases. The highest white blood cell count in five CSF-positive cases was markedly elevated: 29,400-103,500/mm^3 (mean: 59,700/mm^3). In four cases, excluding one case which may have been markedly affected by chemotherapy, the bone marrow showed hyperplasia, and the number of the granulocyte series cells significantly increased. There were three cases (0.7%) negative for both G-CSF and GM-CSF, although they showed marked leukocytosis (60,000/mm^3 or higher) which were higher than the mean count of CSF-positive cases and was not observed in autopsy cases with non-tumorous diseases. Other stimulating factors may be involved in the development of leukocytosis in such cases.

Key words: Malignant tumor, Non-lymphohematopoietic, Leukocytosis, Granulocyte colony stimulating factor, Granulocyte-macrophage colony stimulating factor

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Introduction

Leukocytosis can be seen as one of the symptoms accompanied by malignant tumors. It is known that the increase in leukocytes is so marked that it should be differentiated from that in leukemia (Hughes and Higley, 1952). Recently, the involvement of various factors produced by tumors that stimulate leukocyte proliferation have been shown as a cause of leukocytosis. Granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor, tumor necrosis factor α, interleukin 1, interleukin 3, and interleukin 6 have been reported as factors that are considered to induce marked leukocytosis by stimulating bone marrow hematopoietic cells to proliferate (Suzuki et al., 1993). However, the actual frequency of malignant tumors that produce these growth factors is not clear. Moreover, most studies reported G-CSF as a tumor-produced leukocyte proliferation stimulating factor, and GM-CSF-producing malignant tumors have rarely been reported. There have been a few reports in which multiple numbers of cases with G-CSF- and GM-CSF-producing malignant tumors were clinicopathologically analyzed. Therefore, in this study, patients with non-lymphohematopoietic malignant tumors accompanied by leukocytosis who underwent pathological autopsy in our Department in the last 20 years were immuno-histochemically examined for G-CSF and GM-CSF, and the colony stimulating factor (CSF)-producing malignant

tumors were clinicopathologically investigated.

Materials and methods

Subjects

The study was performed in 1,778 patients who were autopsied at the Department of Molecular and Cellular Pathology, Graduate School of Medical Science, Kanazawa University between 1980 and 1999. Among these patients, those with non-lymphohematopoietic malignant tumors who developed leukocytosis with a white blood cell count of 10,000/mm^3 or higher (including patients with multiple carcinomas and excluding those in whom tumors disappeared due to therapy) were investigated. As the control, the degree of leukocytosis in autopsy cases with non-tumorous diseases (cases without tumorous lesions in the main pathological findings) were examined.

Immunohistochemistry

Two or three representative primary lesions or metastatic tissues were selected from each case, and immunohistochemistry for G-CSF and GM-CSF was performed by the streptoavidin-biotin method. Preparations for immunohistological staining of G-CSF were treated with Target Unmasking Fluid (Kreatech Diagnostics, Amsterdam, Netherlands) to activate antigens. For the primary antibodies, mouse anti-human G-CSF monoclonal antibody (50-fold diluted, Biochemical Industry Co., Ltd., Tokyo, Japan) and rabbit anti-human GM-CSF polyclonal antibody (50-fold diluted, R&D Systems Inc., Minneapolis, MN, USA) were used. After color development, the tissues were stained with methylgreen or hematoxylin. As negative controls, non-immunized mouse IgG (DAKO Japan, Kyoto, Japan) and rabbit immunoglobulin fraction (DAKO Japan) were used instead of the primary antibodies against G-CSF and GM-CSF, respectively. The non-immunized immunoglobulins were diluted to adjust the protein concentration to that of the corresponding primary antibody.

Detection of G-CSF and GM-CSF mRNAs by reverse transcription- polymerase chain reaction (RT-PCR)

From formalin-fixed paraffin-embedded tissues of cases which were judged positive by immunohistochemistry, total RNA was extracted using Paraffin Block RNA Isolation Kit (AMBION Inc., Austin, TX, USA). The condition of the extracted RNA was estimated by 2% agarose gel electrophoresis. In a case whose tissues were cryopreserved without fixation, total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (Sambrook et al., 1989).

Using total RNA extracted, RT-PCR was performed using Reverse Transcription Series (Promega Corporation, Madison, WI, USA). The base sequences of PCR primers used in detecting G-CSF and GM-CSF transcription products and the sizes of the PCR products are shown in Table 1. Four pairs of primers for G-CSF (Nagata et al., 1986) and GM-CSF (Miyatake et al., 1985) were prepared based on the genomic DNA sequences. The primers were designed to contain introns in the region to distinguish PCR products of the RNAs from those of a minute contaminant of the genomic DNAs. To confirm whether cDNA was successfully synthesized from formalin-fixed paraffin block-embedded tissue blocks and non-fixed frozen tissues, $ß_2$-microglobulin was also amplified (Noonan et al., 1990). In PCR for G-CSF and GM-CSF, a cycle consisting of one minute at 94 °C, one minute at 60 °C, and one minute at 72 °C was repeated for 30 cycles. For ß2-microglobulin, a cycle consisting of one minute at 94 °C, one minute at 57 °C, and one minute at 72 °C was repeated for 25 cycles. The PCR product (9 μl) was electrophoresed on a 4.5% agarose gel (3% agarose and 1.5% Nusieve GTG agarose) (FMC Bioproducts, Rockland, ME, USA) containing ethidium bromide, and the PCR products were visualized by illumination using UV light.

Analysis of bone marrow

In cases with tumors positive for G-CSF or GM-CSF on immunohistochemistry, the bone marrow was examined as follows. As controls, the bone marrow of the autopsy patients in whom the white blood cell count was 10,000/mm^3 or below, the red blood cell count was 350 x 10^4/mm^3 or greater, and the platelet count was 15x10^4/mm^3 or greater without clinical hematological disorders was examined by the same procedure (six autopsy cases with non-lymphohematopoietic malignant tumors and six autopsy cases with non-tumorous diseases).

Cellularity of hematopoietic cells in the bone marrow: tissue sections prepared from formalin-fixed

Table 1. Sequences of oligonucleotide primers for PCR.

PRIMER NAME	FORWARD PRIMER	REVERSE PRIMER	LENGHT OF PCR PRODUCT (bp)
$ß_2$-microglobulin	5'-ACCCCCACTGAAAAAGATGA-3'	5'-ATCTTCAAACCTCCATGATG-3'	114
G-CSF 1	5'-CAGAGCCCCATGAAGCTGATG-3'	5'-CTTCCTGCACTGTCCAGAGTG-3'	70
G-CSF 2	5'-TTGGGTCCCACCTTGGACACA-3	'5'-GGCCATTCCCAGTTCTTCCAT-3'	87
GM-CSF 1	5'-CTGAACCTGAGTAGAGACACTG-3'	5'-CTGGAGGTCAAACATTTCTGAG-3'	75
GM-CSF 2	5'-CATGATGGCCAGCCACTACAAG-3'	5'-GGTGATAATCTGGGTTGCACAG-3'	73

paraffin-embedded bone marrow (the lumbar vertebra or costa) were stained with HE. Five fields were randomly selected from tissue sections and photographed (direct magnification: x100). The bone trabeculas were excluded from the field. The photographed 35-mm films were entered into a Power Mac G4/500 computer (Apple Computer Co., Tokyo, Japan) using a Quickscan 35 (Minolta Co., Osaka, Japan), and the bone marrow images were digitized. The captured bone marrow images were analyzed using an image processing software Adobe Photoshop 5.5 (Adobe systems Co., Tokyo, Japan) and an image analysis software NIH Image 1.62 (NIH, Bethesda, MD). The area occupied by hematopoietic cells was divided by the whole captured image area excluding bone trabeculas, and the value was regarded as the cellularity of hematopoietic cells in the bone marrow.

Histochemistry and immunohistochemistry of bone marrow: four types of cells (granulocyte, erythrocyte, histiocyte/monocyte and megakaryocyte series) were distinguished by histochemistry or immunohistochemistry. Cells of the granulocyte series were identified by chloroacetate-esterase staining. Cells of the erythrocyte, histiocyte/monocyte and megakaryocyte series were labeled by immunohistochemistry using anti-glycophorin A antibody (Greaves et al., 1983) (200-fold diluted, DAKO Japan), anti-CD68 antibody (Pulford et al., 1989) (100-fold diluted, DAKO Japan), and anti-von Willebrand factor antibody (Chuang et al., 2000) (1,600-fold diluted, DAKO Japan), respectively. In immunohistochemistry, the streptoavidin-biotin method was used as for G-CSF and GM-CSF. For activation of antigens, sections were treated with Target Unmasking Fluid or microwave. Five fields (direct magnification: x50) were randomly selected from tissue sections and photographed. The photographs were printed in color (final magnification: x200), and the labeled cells were counted. All nucleated cells in the color print were also counted, and the labeled cell count was divided by the nucleated cell count to calculate the relative constitutive ratio of each cell series.

The cellularity of hematopoietic cells was multiplied by the relative constitutive ratio of each cell series to calculate the absolute constitutive ratio of each cell series in the bone marrow.

Statistical analysis

The cellularity of hematopoietic cells and relative and absolute constitutive ratios of each cell series in the bone marrow were compared between the controls and CSF-positive cases by Student t-test.

Results

Clinicopathological findings

Of 1,778 autopsied patients, 869 patients had non-lymphohematopoietic malignant tumors. Among these cases, 439 cases with 485 lesions developed leukocytosis with a white blood cell count of 10,000/mm^3 or greater (single carcinoma, 397 cases; double carcinoma, 39 cases; triple carcinoma, 2 cases; quadruple carcinoma, 1 case: excluding cases in which tumors disappeared due to therapy) (Tables 2, 3). The major primary organs of carcinomas were the lung, liver, stomach, pancreas, gallbladder, and large intestine. The highest white blood cell count during the course was less than 20,000/mm^3 in most cases (307, 70.0%). The highest white blood cell count was 103,500/mm^3 in the case with giant-cell type large cell carcinoma of the lung. On the other hand, there were 365 autopsy cases with non-tumorous diseases which developed leukocytosis with a white blood cell count of 10,000/mm^3 or higher during the course (Table 2). The highest white blood cell count was less than 20,000/mm^3 in most cases (275, 75.3%). The case with the highest white blood cell count (54,700/mm^3) was lung abscess complicated by sepsis. No autopsy cases with non-tumorous diseases had a white blood cell count of 60,000/mm^3 or higher.

Immunohistochemistry

All cases with non-lymphohematopoietic malignant tumors which developed leukocytosis with a white blood cell count of 10,000/mm^3 or higher during the course

Table 2. Items of 804 cases with leukocytosis among1778 autopsy cases examined.

CASES	WHITE BLOOD CELL (/mm^3)								TOTAL
	10000≤ <20000	20000≤ <30000	30000≤ <40000	40000≤ <50000	50000≤ <60000	60000≤ <70000	90000≤ <100000	100000≤ <110000	
Cases with non-lymphohematopoietic	307	84	30	9	4	3	1	1	439
Malignant tumor									
Single cancer	275	76	30(1)	8(1)	4	2	1(1)	1(1)	397
Double cancer	29	8(1)		1		1			39
Triple cancer	2								2
Quadruple cancer	1								1
Cases without malignant tumor	275	59	21	9	1				365

Numbers in parentheses represent CSF-positive cases.

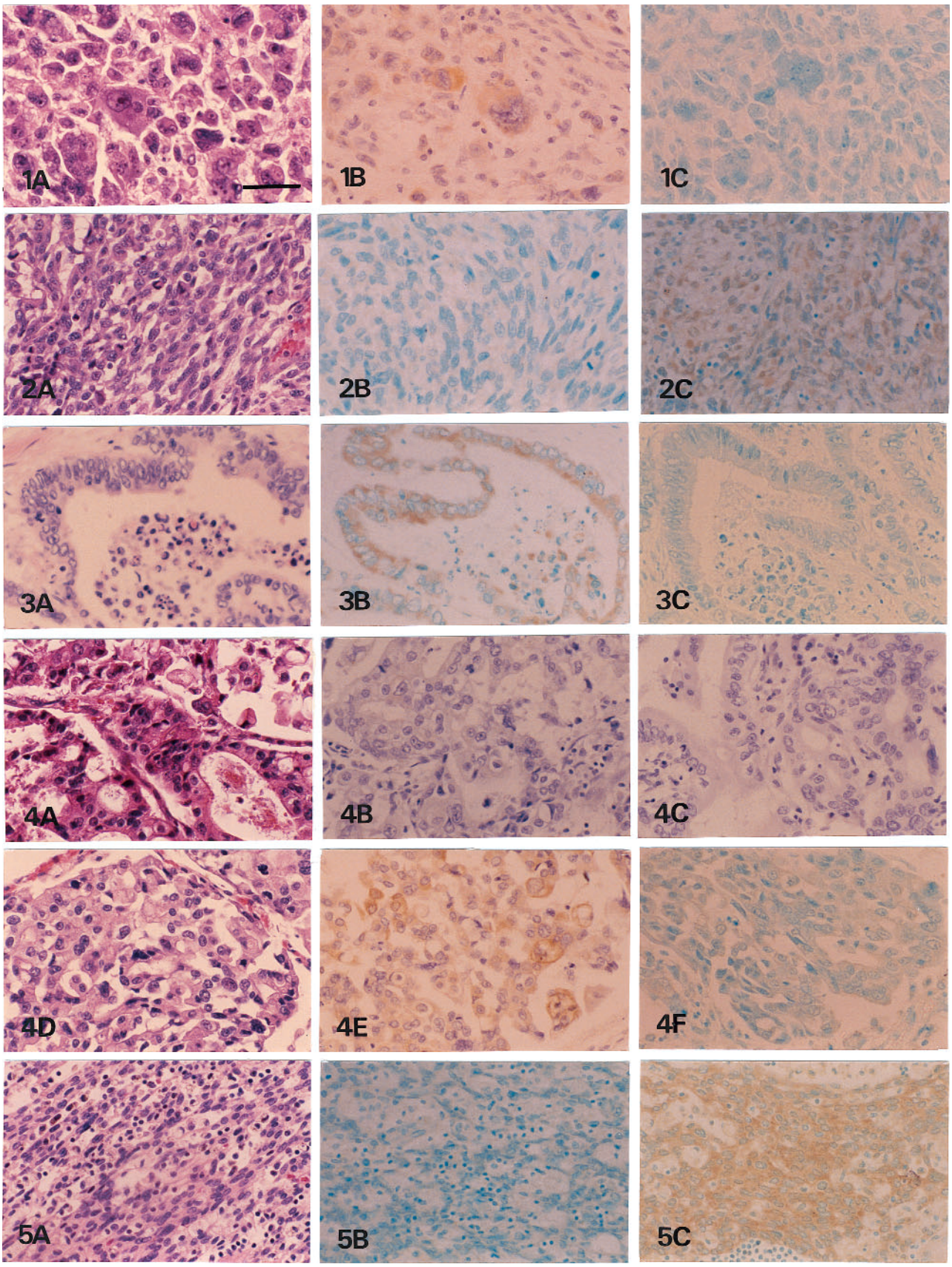

Fig. 1. Histopathology (**A and D**) and immunohistochemistry for G-CSF (**B and E**) and GM-CSF (**C and F)** of 5 positive cases. **1A-1C** represent Case 1 (giant cell- type large cell carcinoma of the lung); **2A-2C**, Case 4 (spindle cell carcinoma of the lung); **3A-3C**, Case2 (adenocarcinoma of the ascending colon); **4A-4F**, Case 3 (**4A-4C**, adenocarcinoma of the stomach; **4D-4F**, metastasis to the lung); **5A-5C**, Case 5 (malignant thymoma). Bar: 100 μm.

were examined, and three and two cases had G-CSF-positive and GM-CSF-positive tumors, respectively (Table 4). The primary organs were the lung, ascending colon, stomach, and thymus. The histological types of lung carcinomas were giant cell-type large cell carcinoma (G-CSF-positive) and spindle cell carcinoma (GM-CSF-positive). In the former, the cytoplasm of some large tumor cells was stained with anti-G-CSF antibody (Fig. 1-1). In the latter, the cytoplasm of many spindle cells was stained with anti-GM-CSF antibody (Figs. 1-2). The G-CSF-positive ascending colon carcinoma was moderately differentiated adenocarcinoma, in which some tubular adenocarcinoma cells were positively stained (Figs. 1-3). This case had also moderately differentiated tubular adenocarcinoma of the stomach (double carcinoma case) which was negative for both G-CSF and GM-CSF. In a case with G-CSF-positive stomach carcinoma (Case 3), the primary lesion showing moderately differentiated tubular adenocarcinoma was negative for both G-CSF and GM-CSF, but the lung metastatic lesion showing less differentiation was G-CSF-positive (Figs. 1-4). The GM-

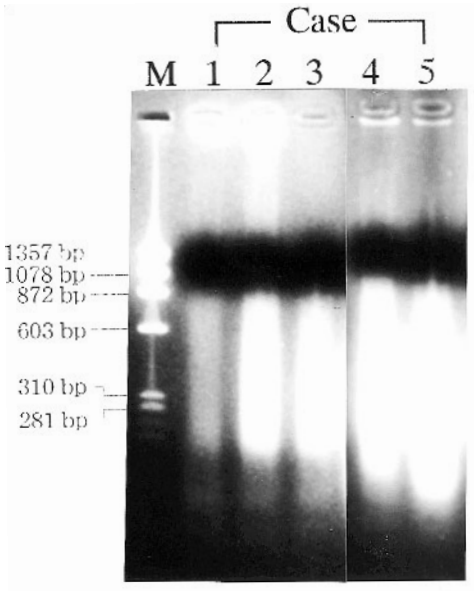

Fig. 2. Agarose-gel electrophoresis of total RNAs isolated from formalin-fixed, paraffin-embedded tissues. Various amounts of degraded RNAs were isolated in all CSF-positive cases. M, DNA size marker (ϕx174 digested with Hae III).

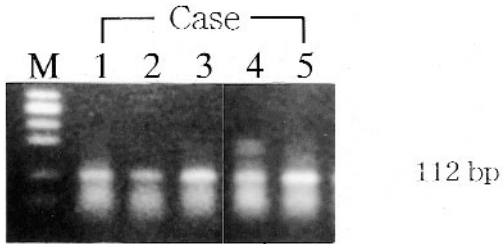

Fig. 3. Agarose-gel electrophoresis of reverse transcription-PCR products for $ß_2$-microglobulin by using total RNAs isolated from formalin-fixed, paraffin-embedded tissues. The $ß_2$-microglobulin was successfully amplified in all CFS-positive cases. M, DNA size marker øX174 digested with Hae III).

Table 3. Correlation between primary organs of malignant tumors and degrees of leukocytosis in 485 lesions of 439 cases examined.

PRIMARY ORGANS	WHITE BLOOD CELL (/mm^3)								NUMBER
	10000≤ <20000	20000≤ <30000	30000≤ <40000	40000≤ <50000	50000≤ <60000	60000≤ <70000	90000≤ <100000	100000≤ <110000	OF LESIONS
Lung	62	20	5	2			1(1)	1(1)	91
Liver	70	12	2	2					86
Stomach	59	11	7(1)	1	2				80
Pancreas	25	14	6		1	3			49
Gallbladder	30	11	4	3					48
Colon	19	7(1)	1						27
Thyroid gland	19	5	1						25
Esophagus	10	2	1	1	1				15
Prostate	10	2	2	10		1			10
Kidney	11	1							12
Urinary bladder	8	2							10
Renal pelvis	4	1							5
Ureter	3		1						4
Breast	3								3
Rectum	3								3
Uterus		3							3
Ovary	1	1							2
Soft tissue	2								2
Thymus	1			1(1)					2
Duodenum	1								1
Anus	1								1
Unknown	1								1
Total	343	92(1)	30(1)	10(1)	4	4	1(1)	1(1)	485

Numbers in parentheses represent CSF-positive cases.

CSF-positive malignant thymoma was spindle cell type (Fig. 1-5). No cases were positive for either GM-CSF and GM-CSF. The highest white blood cell count in these CSF-positive cases was 29,400-103,500/mm3 (mean: 59,700/mm^3). Of five cases which developed marked leukocytosis with a white blood cell count of 60,000/mm^3 or higher, which was not observed in autopsy cases with non-tumorous diseases, two cases were CSF-positive (Table 2). Cases 1, 3, and 5 underwent chemotherapy, and the white blood cell counts decreased to 33,600 and 11,200/mm^3 before death in cases 1 and 3, respectively.

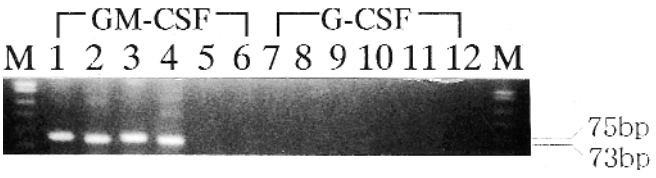

Fig. 4. Agarose-gel electrophoresis of reverse transcription-PCR products for GM-CSF (lanes 1-6) and G-CSF (lanes 7-12) by using total RNAs isolated from unfixed, frozen tissues of the lung carcinoma (1, 2, 7 and 8), liver metastasis (3, 4, 9 and 10) and liver (5, 6, 11 and 12) of Case 4. Lanes 1, 3 and 5 show PCR reactions with GM-CSF 1 forward and reverse primers; lanes 2, 4 and 6, with GM-CSF 2 forward and reverse primers; lanes 7, 9 and 11, with G-CSF 1 forward and reverse primers; lanes 8, 10 and 12, with G-CSF 2 forward and reverse primers. PCR products of 75 or 73bp in length for GM-CSF are observed in the lung carcinoma (lanes 1and 2) and liver metastasis (lanes 3 and 4). M, DNA size marker (ΦX174 digested with Hae III).

Detection of G-CSF and GM-CSF mRNAs by RT-PCR

Formalin-fixed paraffin-embedded tissues: as shown in Fig. 2, in all five G-CSF- or GM-CSF-positive cases, total RNA could be extracted from formalin-fixed paraffin-embedded tumor tissues. The amount varied among the cases. In all cases, the extracted RNA was degraded to a length less than 1 kb, but ß2-microglobulin mRNA was detected by RT-PCR in all cases (Fig. 3). However, neither G-CSF nor GM-CSF mRNA was amplified in any case.

Non-fixed frozen tissues: among five cases positive for CSF, the lung primary lesion, liver metastatic lesion, and liver tissues from a case with GM-CSF-positive lung spindle cell carcinoma (Case 4) were cryopreserved without fixation. In this case, ß2-microglobulin mRNA was amplified in all tissues. GM-CSF mRNA was detected by RT-PCR in the lung primary and liver metastatic lesions, but G-CSF mRNA was not detected (Fig. 4). In the liver tissue without metastasis, neither G-CSF mRNA nor GM-CSF mRNA was detected.

Analysis of bone marrow

Fig. 5 shows the HE staining, histochemistry, and immunohistochemistry of the bone marrow tissues in representative control and CSF-positive cases. Table 5

Table 4. Clinicopathological findings of 5 cases with non-lymphohematopoietic malignant tumors positively stained for G-CSF or GM-CSF.

CASE	AGE (year)	SEX	PRIMARY TUMOR (histopathology)	WHITE BLOOD CELL MAXIMUM/mm^3 (% of eosinophils)	CHEMOTHERAPY	INFLAMMATORY LESION AT TERMINAL STAGE	CAUSE OF DEATH	SURVIVAL TIME	IMMUNOHISTO-CHEMICAL STAINING	
									G-CSF	GM-CSF
1	67	M	Lung cancer (large cell carcinoma, giant cell type)	103500 (5)	+	Focal pulmonary abscess	Tumor	2 months	+	-
2	71	M	Colon cancer (moderately differentiated adenocarcinoma)	29400 (1)	-	Sepsis and broncho-pneumonia	Multiple organ failure	5 months	+	-
			Gastric cancer (moderately differentiated adenocarcinoma)						-	-
3	74	M	Gastric cancer (moderately differentiated adenocarcinoma)	32300 (0)	+	-	Tumor	6 months	- (primary)	-
									+ (metastatic)	-
4	74	M	Lung cancer (spindle cell carcinoma)	93000 (12)	-	Broncho-pneumonia	Broncho-pneumonia	1 month	-	+
5	64	F	Malignant thymoma (spindle cell type)	40200 (0.2)	+	-	Tumor	6 years	-	+

M, male: F, female.

summarizes the cellularity and relative constitutive ratios of bone marrow hematopoietic cells in 12 control and five CSF-positive cases, and Table 6 summarizes the absolute constitutive ratios.

Cellularity of hematopoietic cells: in 12 cases examined as controls, the cellularity of hematopoietic

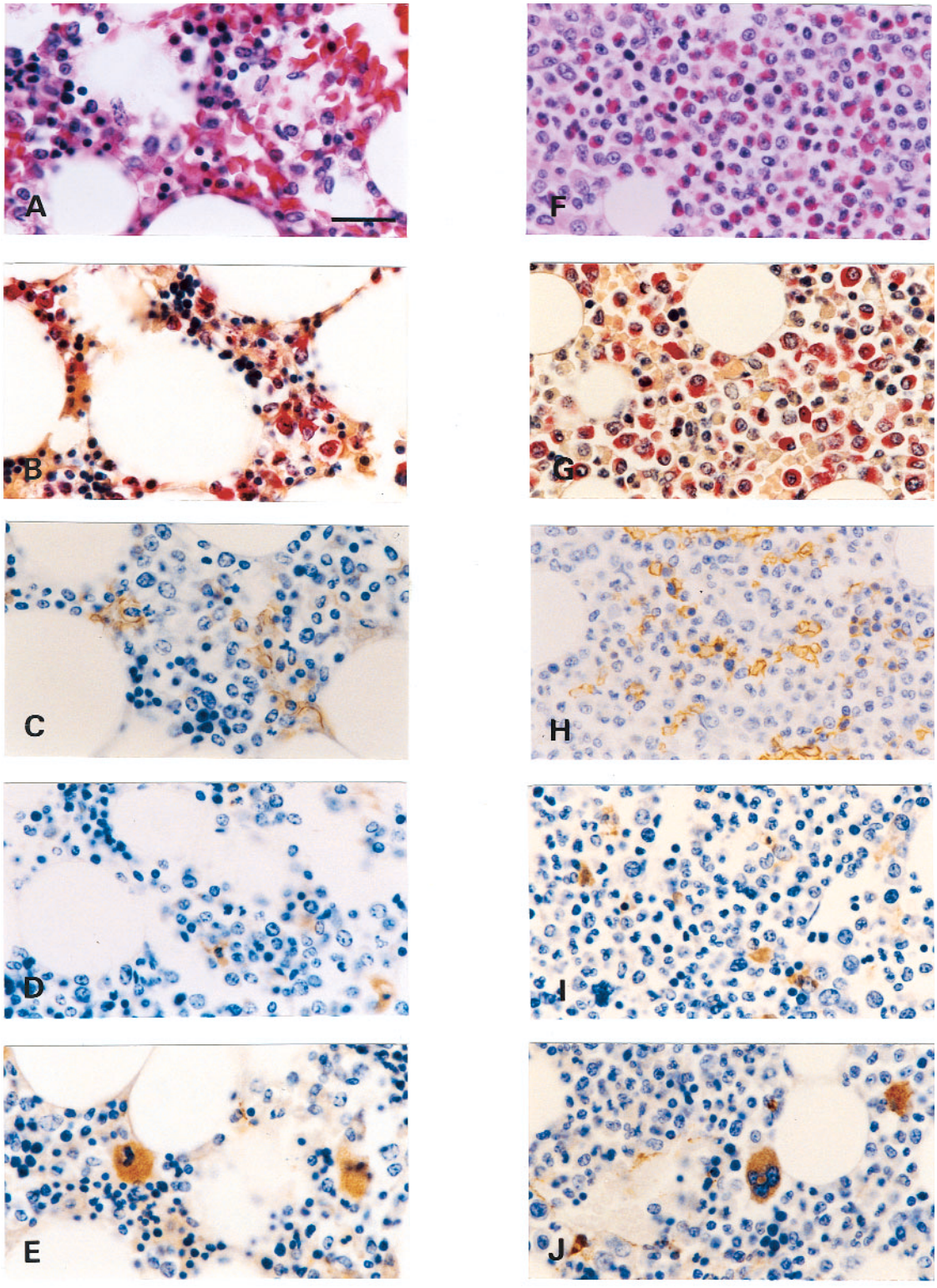

Fig. 5. The bone marrow of Control 14 **(A-E)** and Case 4 (**F-J**). **A and F**, HE staining; **B and G**, chloroacetate-esterase staining; **C and H**, immunohistochemistry for glycophorin A; **D and I**, immunohistochemistry for CD68; **E and J**, immunohistochemistry for von Willbrand factor. Bar: 100 μm.

cells was 31.5-56.2% (43.5±8.1%). In contrast, the cellularity of hematopoietic cells was 29.0-76.7% in five cases which were positive for CSF. Excluding one case (Case 1) with the bone marrow in which hematopoiesis was deeply suppressed by chemotherapy, the cellularity of bone marrow hematopoietic cells was 67.4-76.7%

Table 5. Cellularity and relative ratios of granulocyte, erythrocyte, monocyte/macrophage and megakaryocyte in bone marrow of G-CSF- or GM-CSF-positive cases and controls.

CASE	CELLULARITY	GRANULOCYTE	ERYTHROCYTE	MONOCYTE/ MACROPHAGE	MEGAKARYOCYTE
G-CSF-positive case					
1	29.0%	7.2%	8.9%	28.2%	4.2%
2	67.4%	68.9%	16.7%	3.2%	0.7%
3	76.7%	57.3%	18.9%	8.6%	0.6%
GM-CSF-positive case	72.6±4.0%	60.9±8.0%	17.4±2.4%	5.2±2.4%	0.7±0.3%
4	72.0%	51.5%	19.5%	4.4%	0.4%
5	74.3%	65.9%	14.3%	4.6%	1.0%
Control without leukocytosis	*				
Case with malignant tumor					
1	53.0%	58.0%	26.9%	0.8%	0.4%
2	32.9%	42.0%	27.3%	1.7%	0.6%
3	42.5%	33.3%	18.5%	4.0%	0.5%
4	43.5%	52.7%	12.9%	1.7%	0.5%
5	55.2%	54.6%	22.5%	1.8%	0.6%
6	42.6%	62.8%	28.3%	2.2%	1.4%
Case without malignant tumor	43.5±8.1%	49.4±9.6%	22.1±5.8%	2.2±1.1%	0.6±0.3%
7	31.5%	55.8%	24.8%	ND	0.7%
8	41.0%	32.1%	18.9%	0.8%	0.6%
9	56.2%	57.7%	ND	3.1%	0.2%
10	39.6%	47.9%	ND	2.3%	0.6%
11	37.2%	48.7%	27.3%	3.8%	ND
12	47.0%	47.0%	13.6%	1.7%	0.4%

ND: not done. *: significant difference (P=0.016<0.05).

Table 6. Absolute ratios of granulocyte, erythrocyte, monocyte/macrophage and megakaryocyte in bone marrow of G-CSF - or GM-CSF-positive cases and controls .

CASE	GRANULOCYTE	ERYTHROCYTE	MONOCYTE/ MACROPHAGE	MEGAKARYOCYTE
G-CSF-positive case				
1	2.1%	2.6%	8.2%	1.2%
2	46.4%	11.2%	2.2%	0.5%
3	44.0%	14.5%	6.6%	0.4%
GM-CSF-positive case	44.1±5.1%	12.6±2.0%	3.8±1.9%	0.5±0.2%
4	37.0%	14.0%	3.2%	0.3%
5	49.0%	10.6%	3.4%	0.8%
Control without leukocytosis				
Case with malignant tumor	*			
1	30.8%	14.3%	0.4%	0.2%
2	13.8%	9.0%	0.6%	0.2%
3	14.1%	7.8%	1.7%	0.2%
4	22.9%	5.6%	0.7%	0.2%
5	30.1%	12.4%	1.0%	0.3%
6	26.8%	12.1%	0.9%	0.6%
Case without malignant tumor	21.7±6.9%	9.3±2.8%	0.9±0.5%	0.3±0.1%
7	17.5%	7.8%	ND	0.2%
8	13.1%	7.7%	0.3%	0.3%
9	32.4%	ND	1.7%	0.1%
10	19.0%	ND	0.9%	0.2%
11	18.1%	10.1%	1.4%	ND
12	22.1%	6.4%	0.8%	0.2%

ND: not done. *: significant difference (P=0.019<0.05).

(72.6±4.0%), which was significantly higher than that in the controls ($p=0.016<0.05$).

Relative constitutive ratios of hematopoietic cells: in 12 cases examined as the controls, the relative ratios of the granulocyte, erythrocyte, monocyte/macrophage, and megakaryocyte series were 32.1-62.8% (49.4±9.6%), 12.9-28.3% (22.1±5.8%), 0.8-4.0% (2.2±1.1%), and 0.2-1.4% (0.6±0.3%), respectively. In Case 1 with G-CSF-positivity, the relative ratios of the monocyte/macrophage and megakaryocyte series were high, while the ratios of the other two series were low, compared with the controls. In CSF-positive cases excluding Case 1, the ratio of the granulocyte series was 51.5-68.9% (60.9±8.0%), which tended to be higher than that in the controls, but the difference was not significant. The ratio of the erythrocyte series was 14.3-19.5% (17.4±2.4%), which tended to be lower than that in the controls, but not significant. The ratio of the monocyte/macrophage series was 3.2-8.6% (5.2±2.4%), which tended to be higher than that in the controls, but not significant. The ratio of the megakaryocyte series was 0.4-1.0% (0.7±0.3%), which was similar to that in the controls. Other nucleated cells such as undifferentiated stem cells, adipose cells, fibroblasts, vascular endothelial cells, and vascular pericytes and smooth muscle cells are present in the bone marrow, but these cells were not measured in this study. Therefore, the total of the ratios of the four cell series investigated did not reach 100%.

Absolute constitutive ratios of hematopoietic cells: in 12 cases examined as controls, the absolute ratios of the granulocyte, erythrocyte, monocyte/macrophage, and megakaryocyte series to were 13.1-32.4% (21.7±6.9%), 5.6-14.3% (9.3±2.8%), 0.4-1.7% (0.9±0.5%), and 0.1-0.6% (0.3±0.1%), respectively. In CSF-positive cases excluding Case 1, the absolute ratio of the granulocyte series was 44.0-49.0% (44.1±5.1%), which was significantly higher than that in the controls ($p=0.019<0.05$). In contrast, the absolute ratio of the erythrocyte series was 10.6-14.5% (12.6±2.0%), which tended to be higher than that in the controls, but the difference was not significant. The ratio of the monocyte/macrophage series was 2.2-6.6% (3.8±1.9%), which tended to be higher than that in the controls, but not significant. The ratio of the megakaryocyte series was 0.4-0.8% (0.5±0.1%), which tended to be higher than that in the controls, but not significant.

Discussion

As a result of immunohistochemical investigation in this study, G-CSF-positive (3 cases, 0.7%) and GM-CSF-positive cases (2 cases, 0.5%) were observed in five (approximately 1.1%) out of 439 cases with non-lymphohematopoietic malignant tumors accompanied by leukocytosis. RT-PCR was performed using formalin-fixed paraffin-embedded tissues prepared from these positive cases. Although $ß_2$-microglobulin mRNA was confirmed, neither G-CSF mRNA nor GM-CSF mRNA was detected. However, in Case 4, which was GM-CSF-positive and whose non-fixed tissues were cryopreserved, GM-CSF mRNA was detected. Although the reason why G-CSF or GM-CSF mRNA was not detected in formalin-fixed paraffin-embedded tissues was not clear, the followings were considered: 1) while $ß_2$-microglobulin was constantly expressed in almost all cells (Nagata et al., 1986), CSF was produced only by a portion of tumor cells, and the amount may have been very minute, 2) as shown in Fig. 2, mRNA extracted from formalin-fixed paraffin-embedded tissues was markedly degraded, and 3) the efficiencies of conversion to cDNA and PCR amplification of mRNA extracted from formalin-fixed paraffin block-embedded tissues were low (de Franchis et al., 1988; Lo et al., 1989).

In this study, G-CSF-positive cases were lung giant cell-type large cell carcinoma, colon adenocarcinoma, and stomach adenocarcinoma, and GM-CSF-positive cases were lung spindle cell carcinoma and malignant thymoma. Among G-CSF-producing malignant tumors, which were relatively frequently reported, such as carcinomas of the oral cavity (Yoneda et al., 1991), thyroid gland (Yazawa et al., 1995), lung (Kodama et al., 1984; Shijubo et al., 1992; Furihata et al., 1996; Asano et al., 1997; Omura et al., 1999), esophagus (Ota et al., 1998), stomach (Obara et al., 1985), gallbladder (Furihata et al., 1999), pancreas (Umematsu et al., 1996), intrahepatic bile duct (Aizawa et al., 1997), urinary bladder (Sato et al., 1994), and so on, the frequency of lung carcinoma is high. In contrast, there have only been a few reports of GM-CSF-producing malignant tumors. To our knowledge, only 10 cases consisting of four thyroid carcinoma (Nakada et al., 1996), four lung carcinoma (Sawyers et al., 1992), one renal pelvic carcinoma (Wetzler et al., 1993), and one renal carcinoma (Kan et al., 1996) have been reported in the English literature. However, the results of this study suggested that GM-CSF-producing malignant tumors are not extremely rare. When the investigation of GM-CSF by blood chemistry becomes more common in the future, the frequency of GM-CSF-producing malignant tumors reported may increase. As shown in Case 4, patients with GM-CSF producing tumors often show eosinophilia (Sawyers et al., 1992; Watanabe et al., 1998). Thus, marked leukocytosis with eosinophilia is one of the useful clinical indicators for suspecting GM-CSF producing neoplasms to measure the serum GM-CSF level of patients. Among the previous reports of non-lymphohematopoietic malignant tumors, there has been only one case of renal carcinoma in which the expression of GM-CSF was confirmed at the mRNA level (Kan et al., 1996) as in Case 4 of this study.

Regarding the histological types of G-CSF-producing carcinomas, undifferentiated carcinoma (Robinson, 1974; Kodama et al., 1984; Shijubo et al., 1992; Nakada et al., 1996; Oshika et al., 1998; Omura et al., 1999), squamous cell carcinoma (Asono et al., 1977; Satoh et al., 1993; Furihata et al., 1996; Uematsu et al., 1996; Ota et al., 1998) and adenocarcinoma (Furihata et al., 1999) were reported, and the majority were

undifferentiated carcinomas. Approximately half of the cases of GM-CSF-producing carcinomas reported were undifferentiated carcinomas (Wetzler et al., 1993). In this study, G-CSF-positive lung carcinoma was undifferentiated large cell carcinoma, and GM-CSF-positive lung carcinoma was spindle cell carcinoma which was considered to be a poorly differentiated non-small cell carcinoma (Travis et al., 1999). In a case with stomach carcinoma (Case 3) in which the primary lesion was negative but the lung metastatic lesion was G-CSF-positive, the metastatic lesion was less differentiated compared with the primary lesion, suggesting that as the carcinoma becomes less differentiated, CSF becomes more likely to be produced. Although biological mechanisms are unclear in the production of these CSFs by tumor cells, gene mutation, gene amplification, and the activation of gene regulatory factors were noted for G-CSF (Ota et al., 1998).

In this study, the survival period of four CSF-positive cases excluding a case with malignant thymoma was six months or less, and the prognosis was poor. A similar tendency was noted in papers that summarized and analyzed cases reported in the past. According to Furihata et al. (1999), five cases with G-CSF-producing gallbladder carcinoma, including one case reported by them, died within one year, and the survival period was six months or less in four cases. Watanabe et al. (1998) retrospectively investigated six cases with rare GM-CSF-producing tumors (three cases of lung carcinoma, two cases of thyroid carcinoma, and one case of renal carcinoma) and noted that three out of four patients for whom the prognosis was described died within four months. Although it is not simple because there are several factors that determine the prognosis, CSF may be one of the factors. Actually, it has been reported that in a culture experiment using a G-CSF-producing cell line established from human undifferentiated thyroid carcinoma, G-CSF production by the tumor was increased by stimulation with tumor necrosis factor, interleukin 1α, and interleukin 1ß, and the proliferation of the tumor cells was promoted (Oka et al., 1993). Autocrine promotion of proliferation via G-CSF receptors was noted as a mechanism (Tachibana et al., 1995). Furthermore, it has been shown that metastasis to the lung was more likely to occur when the CSF productivity of the tumor was high in a mouse experimental subcutaneous tumor transplantation model (Nicoletti et al., 1987). Therefore, CSFs produced by tumors stimulate the proliferation of tumor cells, as well as bone marrow hematopoietic cells, which may facilitate growth and metastasis, resulting in a poor prognosis. As described above, however, CSF generally tends to be expressed in poor prognostic undifferentiated carcinomas. The prognosis of patients depends on the original strength of the invasive growth of the carcinoma, and it cannot be excluded that CSF production may be a phenomenon accompanied by the tumor (a simple phenotype). Whether CSF produced by the carcinoma strongly affects the prognosis is an issue to be investigated.

There have been no reports of studies that investigated how CSF produced by tumors actually has an influence on hematopoiesis in the bone marrow. Generally, G-CSF facilitates the proliferation and differentiation of neutrophils and the mobilization of mature neutrophils to peripheral blood (Morstyn and Burgess, 1988). GM-CSF also stimulates the proliferation of the monocyte/macrophage series, erythrocyte and megakaryocyte series, as well as the granulocyte series (Clark and Kamen, 1987). In this study, changes in the bone marrow in five cases with CSF-positive malignant tumors were compared with the controls. In general histopathology, the ratio of bone marrow hematopoietic cells was calculated by visual observation in the microscopic fields (Peel and Krause, 1981). Approximate constitutive ratios of hematopoietic cells are obtained based on morphological differences in HE-stained preparations. Since the values estimated vary depending on the microscopic examiners and the conditions in these measurement methods, the bone marrow cannot be accurately evaluated. Therefore, in this study, imaging analysis was used for the ratio of bone marrow hematopoietic cells, and the constitutive ratios of hematopoietic cells were objectively calculated by histochemistry and immunohistochemistry. Case 1 finally exhibited hypoplastic bone marrow; granulocyte and erythrocyte series cells decreased while cells of the monocyte/macrophage series increased. These changes were caused by chemotherapy, and the active involvement of G-CSF was unlikely. In the other four cases with CSF-positive malignant tumors, the cellularity of hematopoietic cells was higher and the absolute constitutive ratio of the granulocyte series was significantly higher than those in the controls, indicating that the absolute number of cells of the granulocyte series increased. In the other cell series, although the absolute constitutive ratios tended to be high, the differences were not significant. G-CSF and GM-CSF produced by tumors may have had physiological activity and promoted mainly the proliferation of cells of the granulocyte series. Cases 2 and 4 were complicated by sepsis and bronchial pneumonia, and the degree of increase in cells of the granulocyte series may have been affected by these infectious diseases. For Cases 3 and 5, the effects of chemotherapy should be considered.

In this study, the white blood cell count increased to above 50,000/mm^3 in some cases with non-tumorous lesions, although it was rare, indicating that leukocytosis to this degree may occur in infectious diseases. It was very interesting that five cases with tumors developed leukocytosis with a white blood cell count higher than the above count, and two of them were CSF-positive in this study. Therefore, in cases with non-lymphohematopoietic malignant tumors accompanied by leukocytosis with a white blood cell count of 60,000/mm^3 or higher, some white blood cell increasing factors may exist with a high frequency. Inversely, it should be emphasized that three cases (0.7%) were

negative for both G-CSF and GM-CSF, in spite of marked leukocytosis (60,000/mm^3 or higher), which was higher than the mean white blood cell count in the CSF-positive cases and was not observed in cases with non-tumorous diseases. In these cases, it cannot be completely excluded that: 1) G-CSF and GM-CSF produced by tumor cells were not retained in the tumor cells, and were secreted during the early phase, which resulted in negativity on immunohistochemical staining (Akasyuka et al., 1991), 2) the amount of CSF produced by each tumor cell was less than the sensitivity of the immunohistochemistry, and 3) the ratio of CSF-producing cells in the tumor was so small that it could not be identified in the tissue excised. Actually, some cases were CSF-negative on immunohistochemistry, despite the high clinical blood levels of G-CSF and GM-CSF (Furihata et al., 1999). However, it is quite possible that other stimulating factors produced by tumors such as macrophage colony stimulating factor and interleukins 1, 3, and 6 are involved. Further clinicopathological studies are necessary for cases with malignant tumors accompanied by leukocytosis, considering the involvement of not only G-CSF and GM-CSF, but also other white blood cell proliferation stimulating factors.

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