Jpn. J. Cancer Res. 82, 1299–1308, November 1991

Murine Tumor Cells Metastasizing Selectively in the Liver: Ability to Produce Hepatocyte-activating Cytokines Interleukin-1 and/or -6

Kazuyoshi Takeda, Noriyuki Fujii, Yasutaka Nitta, Hiroshi Sakihara, Katsutoshi Nakayama, Hidemi Rikiishi and Katsuo Kumagai[1]

Department of Microbiology, Tohoku University School of Dentistry, 4-1 Seiryo-machi, Aoba-ku, Sendai 980

Increasing evidence suggests that an intimate correlation may exist between the production of a cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF) and the ability to metastasize spontaneously in the lungs in murine transplantable tumors. In the present study, we further examined the cytokine production by tumor cells with the ability to metastasize in the liver. Four out of 8 test tumors, which produced metastasis in the lungs but not in the liver, exhibited the ability to produce GM-CSF activity in culture. Three other tumors produced metastasis in the liver but not in the lungs. These tumor cells exhibited no ability to produce GM-CSF, but two of them expressed an interleukin-6 (IL-6) mRNA and also produced IL-6 activity in the culture fluids. One of the two IL-6-producing tumors and the remaining liver metastatic tumor produced interleukin-1 (IL-1) as revealed by bioassay and neutralization test. In the tumor cells producing pulmonary metastasis, neither IL-6 gene expression nor IL-1 production could be detected. The last test tumor, which produced no metastasis either in the lungs or liver, produced neither GM-CSF, IL-1 nor IL-6. Furthermore, injection of antisera reactive to recombinant murine IL-6 caused a marked decrease of the number of liver metastases of an IL-6-producing tumor, but not lung metastases of a GM-CSF-producing tumor, which could be markedly inhibited by injection of anti-recombinant murine GM-CSF sera. These results suggest the possibility that there may be a correlation between the cytokines produced by tumor cells and their organ specificity in spontaneous metastasis, and also indicate that these tumor models may provide a useful tool for studies on the role of cytokines in tumor metastasis.

Key words: Metastasis — Liver — Interleukin-6 — Interleukin-1 — Granulocyte-macrophage colony-stimulating factor

Cytokines, a group of protein cell regulators which are produced by a wide variety of cells in the body, play an important role in many physiological responses. Current studies on structure-function relationships, ligand-receptor interactions, and intracellular mechanisms of action should enable the effective use of these important biological mediators. On the other hand, however, recent investigations on the production of cytokines in diseases have revealed that certain cytokines may be involved in the pathophysiology of a range of diseases (reviews in Refs. 1, 2 and 3).

It has been reported that cytokine activities can be detected in conditioned media from cell lines of murine transplantable tumors and established human tumor cell lines.[4, 5] Using a model of highly and weakly metastatic tumor variants, it has been shown that a correlation may exist between the production of a cytokine, colony-stimulating factor(s) (CSF)[2] and the metastatic ability of tumor cells.[6, 7] We also have shown that some, but not other, transplantable murine tumor strains in culture produced CSF activity, which was identified as granulocyte-macrophage (GM)-CSF by its reactivity to a specific antibody to murine recombinant GM-CSF.[8] Subsequently, we have shown that a series of murine transplantable tumor cells can be divided into two groups according to their ability to develop metastasis in mice: (1) tumors developing macroscopical or microscopical metastasis in the lungs and lymph nodes and expressing GM-CSF mRNA in the cell, and (2) tumors developing no metastasis in those organs and expressing no GM-CSF mRNA. These results suggest that an intimate relation may exist between the ability of tumor cells to produce GM-CSF and development of tumors in certain organs such as the lungs.[9] During such investigations, we found three tumors, which developed no pulmonary metastasis and expressed no GM-CSF mRNA, but developed severe nodular metastasis in the liver. We were, therefore, interested to know whether these tumor cells had the capacity to produce certain cytokine(s) other than GM-CSF, which might be correlated to the development of metastasis in the liver.

[1] To whom requests for reprints should be addressed.

[2] Abbreviations: CSF, colony-stimulating factor; GM-CSF, granulocyte/macrophage CSF; IL-1, interleukin-1; IL-6, interleukin-6; rmGM-CSF or rmIL-6, recombinant murine GM-CSF or IL-6; rmIL-1 or rhIL-1, recombinant murine or human IL-1; CHX, cycloheximide.

In the present paper, we show that tumors with metastatic capability in the liver produce the hepatocyte-activating cytokines interleukin-6 (IL-6) and/or interleukin-1 (IL-1), in place of GM-CSF found in the tumor cells metastatic to the lungs. In addition, we show that injection of anti-IL-6 sera in mice caused a marked decrease in the number of liver metastases of an IL-6-producing tumor, while injection of anti-GM-CSF sera resulted in a marked inhibition of lung metastasis of a GM-CSF-producing tumor.

MATERIALS AND METHODS

Mice and tumors Table I shows the tumor cells used for the experiments (5 ascitic and 3 solid forms), X5563,[10] MM48,[11] NR-S1,[12] 3LL-SA,[13] B16,[6] RL♀1,[13] P815[14] and L5178Y,[15] with their histological classifications, described for the original tumors developed in the mouse and mouse strains bearing the respective tumors. All the mice and tumors transplanted were syngeneic with each other. Mice, specific pathogen-free and 5–7 weeks old, were purchased from our university farm or Funabashi Farm Laboratories, Tokyo. Ascitic tumors were maintained by weekly transplantation of ascitic into the peritoneal cavity of recipient mice. Solid tumors were passaged by subcutaneous inoculation of finely chopped fragments into the recipient mice using a syringe with an 18 gauge needle.

Preparation of tumor cells For preparation of ascitic tumor cells, the ascitic fluids were harvested from the peritoneal cavity of tumor-bearing mice into a syringe containing heparin. The tumor cells were washed three times by centrifugation (800 rpm) for 1 or 2 min in phosphate-buffered saline (PBS). With this repeated washing, tumor cells with markedly decreased contamination by leukocytes were obtained. The washed cells were then suspended in PBS and left to stand at 4°C for 20 min. After careful removal of the supernatants containing cell debris, the sedimented tumor cells were collected, suspended in PBS, and adjusted to the required concentration for use in experiments.

To prepare single cell suspensions from solid tumors, the finely chopped tissue fragments were enzymatically treated at 37°C for 15 min with collagenase (Sigma type IV, 600 units/ml) and DNase (Sigma type I, 0.2 mg/ml), followed by centrifugation three times at 1,500 rpm for 5 min in PBS, as previously described.[8, 9, 16] The final single cell preparations were suspended in RPMI1640 medium at the required concentration.

These preparation methods generally yielded homogeneous tumor cell suspensions with a purity of nearly 95% and viability exceeding 95%. The purified tumor cells contained less than 2 or 3% macrophages detected by non-specific esterase staining. A few T lymphocytes enumerated by immunofluorescence staining with anti-Thy1 serum were also present. In some experiments, tumor cells, from which contaminating macrophages or granulocytes or T lymphocytes were further removed, were prepared as previously described.[9, 16]

Evaluation of tumor metastasis In the present study, s.c. inoculation of tumor cells was principally used. The method can result in local growth and subsequent dissemination to other organs (lungs and liver in most test systems). It can be considered a model for the whole, multistep metastatic process and is usually referred to as spontaneous metastasis. The root of the left thigh was inoculated s.c. with a suspension of 2.5×10^6 tumor cells prepared as described above. Four or 5 weeks later, when the diameters of local tumors had reached 15 to 20 mm, lungs, liver and other organs were removed and fixed in Bouin's solution to facilitate visualization of tumor colonies.[8] The number of tumor colonies in the lungs and liver was determined with a dissecting microscope. Grades of metastasis were designated as ++, + and −, representing >50, 1–50 and 0 colonies, respectively.

Lung and liver tissues were also routinely processed, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin-eosin and Gomori's reticulum staining solution for microscopical examination. Fifty microscopical fields were selected at random and viewed at a magnification of ×40 to score tumor cell metastases. Microscopical metastases were graded according to numbers of tumor cells detected in the 50 microscopical fields: "poor" for undetectable in all the fields, "low" for detectable only as microfoci in all or part of the fields, "high" for detectable as large foci in all or part of the fields.

In all the recipients, tumor metastases in other organs such as regional lymph nodes or spleen were examined macroscopically or sometimes microscopically.

Detection of cytokine production in tumor cell culture To test the production of cytokines by tumor cells, sus-

Table I. Test Tumors, Forms of Tumor Mass, Histological Classification and Tumor-bearing Mouse Strains

Tumor	Form of tumor: ascites or solid[a]	Histological classification	Mouse strain bearing tumor
X5563	A	Plasmacytoma	C3H/He
MM48	A	Mammary carcinoma	C3H/He
NR-S1	S	Squamous carcinoma	C3H/He
3LL-SA	A	Lung carcinoma	C57BL/6
B16	S	Melanoma	C57BL/6
RL♀1	A	Lymphoma	BALB/c
P815	A	Mastocytoma	DBA/2
L5178Y	S	Lymphoma	DBA/2

a) A=ascites, S=solid.

pensions of tumor cells at a concentration of 5×10^4/ml in RPMI1640 containing 1% fresh syngeneic normal mouse serum (NMS) were cultured at 37°C for desired periods. Supernatants were then collected, passed through a membrane filter, and assayed for cytokine activities. IL-6 activity was measured by using the IL-6-dependent cell line, PIL-6.[17] PIL-6 cells were cultured in 96-well flat-bottomed microtiter plates at 2×10^3 cells per well in triplicate cultures with serial twofold dilutions of samples for 5 days. Cells were pulsed with ^{3}H-TdR for 4 h, and the incorporated radioactivity was measured. IL-1 activity was measured by ConA-comitogenic assay using murine thymocytes.[18]

Other cytokines in the culture supernatants of tumor cells, IL-2, IL-3 and IL-5 were assayed by using cell lines dependent upon the cytokines, CTLL-2 (IL-2),[13] FDC-P2 (IL-3)[19] and T88-M (IL-5),[20] respectively.

Cytokines and antisera Recombinant murine GM-CSF (rmGM-CSF) was supplied by Dr. K. Irie (Sumitomo, Takarazuka). Rabbit antiserum for rmGM-CSF was prepared by injecting rabbits in multiple intradermal sites with purified rmGM-CSF in complete Freund's adjuvant.[9] Recombinant murine IL-6 (rmIL-6) and human IL-6 (rhIL-6) were supplied by the Genetics Institute (G.I., Cambridge, MA). Goat antiserum specifically reactive to rmIL-6 was supplied by Drs. T. Matsuda and T. Hirano, Osaka University. Recombinant murine IL-1 (rmIL-1) and rabbit anti-mouse IL-1α serum were purchased from Genzyme Co. Ltd. (Boston, MA).

Northern blot analysis For the isolation of RNA from the tumor cells, purified tumor cells were cultured in RPMI1640 medium containing 10% fetal calf serum (FCS) at 37°C for 3 h, in the presence of cycloheximide (CHX) at 10 μg/ml.[9] Total RNA was prepared as previously described,[9] and the mRNA was hybridized with IL-6 or GM-CSF cDNA probe. cDNA probes for murine IL-6 and GM-CSF were generous gifts from the Genetics Institute.

Assay for inhibition of tumor metastasis by anti-rmGM-CSF and rmIL-6 For the experiments, a suspension of 1.5×10^5 tumor cells in 0.2 ml of 3LL-SA and P815 tumor cells were inoculated i.v. into C57BL/6 and DBA/2 mice, respectively. Mice were given 0.2 ml i.p. injections of antisera, which were started 24 h before tumor inoculation[21] and continued once a day for 13 days (Day -1 to Day $+13$). Mice were killed one day after termination of treatment with antisera. The number of tumor colonies in the lungs and liver was determined with a dissecting microscope.

RESULTS

Metastasis of tumor cells in the liver Mice were s.c. inoculated with 2.5×10^6 cells per mouse of test tumors.

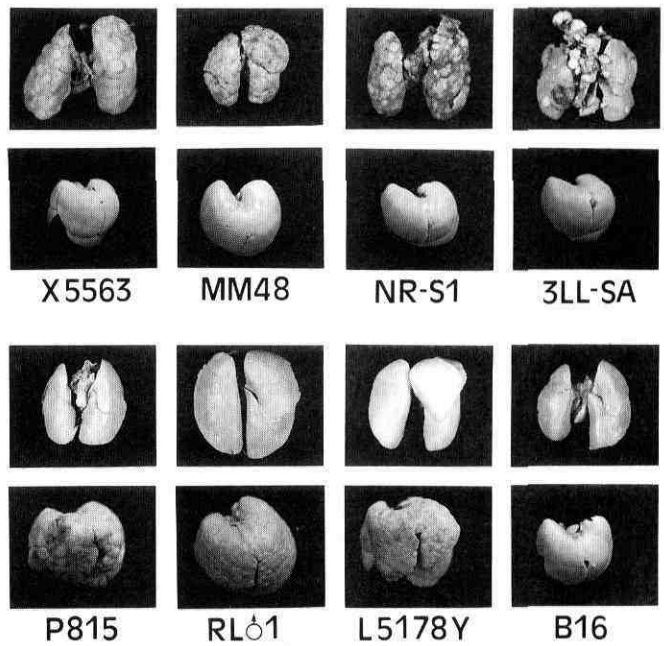

Fig. 1. Metastatic growth in the lungs (upper figures) and liver (lower figures) of mice inoculated s.c. with test tumor cells. Lungs and liver were removed from the mice 35–40 days after inoculation with the tumor cells indicated and fixed with Bouin's solution.

Table II. Incidence of Spontaneous Metastasis of Test Tumors in the Lungs and Liver of Mice[a]

Tumor	No. positive/No. tested			
	Metastatic nodules		Microscopical metastatic foci	
	Lung	Liver	Lung	Liver
X5563	12/12[b]	0/12	High	Poor
MM48	12/12[c]	0/12	High	Poor
NR-S1	12/12[b]	0/12	High	Poor
3LL-SA	12/12	0/12	High	Poor
P815	0/12	12/12[d]	Poor	High
RL♂1	0/12	12/12[d]	Poor	High
L5178Y	0/12	12/12[d]	Poor	High
B16	0/15	0/15	Poor	Poor

a) 28–35 days after inoculation with 2.5×10^6 tumor cells.
b) Grades of metastasis were ++ in all the mice.
c) Grades of metastasis were ++ in 10 and + in 2 out of 12 mice.
d) Grades of metastasis were ++ in all the mice.

All the tumors developed locally in all the inoculated animals. Autopsies were performed on the mice that died of tumors or were killed in the terminal stage of tumors,

and the lungs, liver and other tissues were assayed for visible metastasis. A representative result of the experiments is shown in Fig. 1, which shows the lungs and liver of mice inoculated with the 8 test tumor strains. As shown in the figure, four tumor cells, namely, X5563, MM48, NR-S1 and 3LL-SA produced spontaneous metastatic nodules in the lungs, as previously described,[8] whereas none of these tumor cells produced obvious metastatic nodules in the liver. On the other hand, three tumor strains, namely, P815, RL♂1 and L5178Y, produced severe metastatic nodules in the liver, whereas none of these strains produced obvious pulmonary metastasis during the entire observation period, even in the terminal stages when the mice died of tumors. As shown in Table II, repeated experiments revealed that such an organ specificity of tumor metastasis for each tumor was obtained with all the individual mice tested. Table II also shows that P815, RL♂1 and L5178Y produced numerous microscopical metastatic foci in the liver but not in the lungs, while X5563, MM48, NR-S1 and 3LL-SA produced such foci in the lungs but not in the liver. No visible metastases appeared either macroscopically or microscopically either in the lungs or in the liver of the mice inoculated s.c. with the strain of B16 tumor cells used, as previously described.[8]

Detection and characterization of mitogenic activities in the culture supernatants of tumor cells Our previous reports[8,9] indicated that there may be an intimate correlation between GM-CSF gene expression in tumor cells and spontaneous metastasis in the lungs. Therefore, we examined whether there may also be a correlation between metastasis in the liver and the production of certain cytokines by P815, RL♂1 and L5178Y. Figure 2 shows the results of experiments, in which the culture supernatants of each tumor were examined for stimulat-

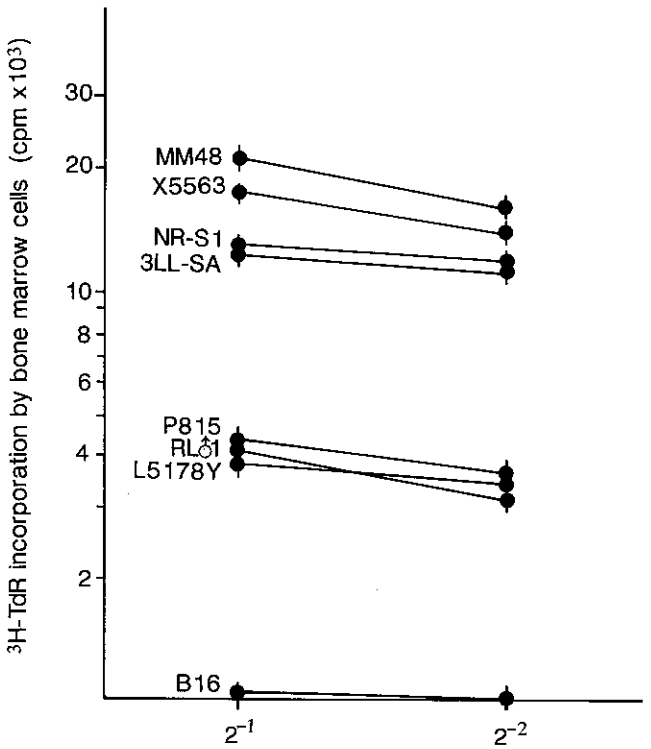

Fig. 2. Proliferative response of bone marrow cells incubated with the supernatants of tumor cell cultures. The culture fluids were collected 48 h after culture of cells at a concentration of 5×10^4 cells/ml in RPMI1640 containing 1% syngeneic normal mouse serum. The supernatants were passed through a membrane filter, and added to bone marrow cells (1×10^5) removed from the syngeneic mice, then triplicate cultures were made at the indicated concentrations. The mean±SD of background ^{3}H-TdR incorporation by bone marrow cells (1×10^5) in the absence of tumor-culture supernatants was 1,020±180.

Table III. Neutralization Tests by Anti-rmGM-CSF of Proliferative Response of Bone-marrow Cells to Culture Supernatants of Tumor Cells

Test culture fluid	Concentration	^{3}H-TdR incorporation, cpm (% inhibition) with anti-rmGM-CSF		
		0	1/125	1/1000
X5563	25%	10,648±52	1,079±321 (90)	3,145±432 (70)
NR-S1	10%	12,916±382	1,177±54 (91)	2,759±94 (79)
MM48	25%	33,616±4,780	1,744±352 (95)	2,289±54 (93)
P815	25%	2,785±915	2,864±85 (−3)	ND
RL♂1	25%	2,419±639	2,206±191 (9)	ND
L5178Y	25%	3,850±284	3,574±170 (7)	ND
rmGM-CSF	1 ng/ml (500 U/ml)	13,844±530	1,380±57 (90)	2,411±50 (83)

Bone-marrow cells (5×10^5/ml) were incubated with culture fluids of tumor cells, or rmGM-CSF at the concentrations indicated in the presence of rabbit anti-rmGM-CSF serum at 1/125 or 1/1,000 in a volume of 200 μl for 3 days. ^{3}H-TdR (0.5 μCi/well) was added to each well 8 h before the termination of culture, and radioactivity was counted. The values represent the mean of triplicate cultures.

Table IV. Neutralization Tests by Anti-rmIL-6 Antibody of Proliferative Response of Bone-marrow Cells to Culture Supernatants of P815, RL♂1 and L5178Y Tumor Cells

Test culture fluid	Concentration	^{3}H-TdR incorporation, cpm (% inhibition) with anti-rmIL-6		
		0	1/500	1/1000
P815	25%	4,267±228	1,235±115 (90)	1,580±258 (80)
RL♂1	25%	4,001±186	1,583±134 (88)	1,680±350 (75)
L5178Y	25%	3,541±285	2,872±295 (27)	3,141±175 (15)
rmIL-6	50 U	3,572±417	885±100 (100)	1,041±67 (96)

Bone-marrow cells (5×10^5/ml) were incubated with culture fluids of tumor cells, or rmIL-6 at the concentrations indicated in the presence of goat anti-rmIL-6 serum at 1/500 or 1/1000 in a volume of 200 μl for 3 days. ^{3}H-TdR (0.5 μCi/well) was added to each well 8 h before the termination of culture, and radioactivity was counted. The values represent the mean of triplicate cultures.

ing activity on the bone marrow cells. The results clearly indicate that X5563, MM48, NR-S1 and 3LL-SA (producing pulmonary metastasis in the lungs) produced high mitogenic activity against bone marrow cells in culture. It was also found that the culture supernatants of P815, RL♂1 and L5178Y tumor cells contained mitogenic activities, though less than those of tumors producing pulmonary metastasis. No mitogenic activity could be detected in the remaining tumor cell line B16, which lacked definite spontaneous metastatic ability.

As can be seen in Table III, when the mitogenic activities of culture supernatants were examined in the presence of a specific antiserum against rmGM-CSF, the activities produced by X5563, MM48, NR-S1 and 3LL-SA were neutralized by the serum, as shown in our previous studies.[8,9] On the other hand, the mitogenic activities produced by P815, RL♂1 and L5178Y tumor cells were found not to be neutralized by the GM-CSF antiserum. As previously shown,[9] GM-CSF mRNA can be detected in all of these pulmonary metastatic tumors, but not in P815, RL♂1 and L5178Y tumor cells (data not shown).

Detection of IL-6 gene expression in P815 and RL♂1 cells As shown in Table IV, we further found that the mitogenic activities against bone marrow cells detectable in the culture fluids of P815 and RL♂1 but not L5178Y cells, all producing liver metastasis, were almost completely neutralized by a specific rabbit antiserum against murine recombinant IL-6, as was the rmIL-6. Figure 3 also shows the results of experiments in which the culture supernatants of test tumors were examined for their mitogenic activities on IL-6-dependent cell line, PIL-6. The results clearly indicate that a high mitogenic response of PIL-6 cells was induced by the culture supernatants of P815 and RL♂1 but not by those of L5178Y and other tumor cells. On the other hand, none of the culture supernatants of P815 and RL♂1 showed any mitogenic activity against CTLL-2, FDC-P2 and T88-M cell lines (data not shown). These results indicate that

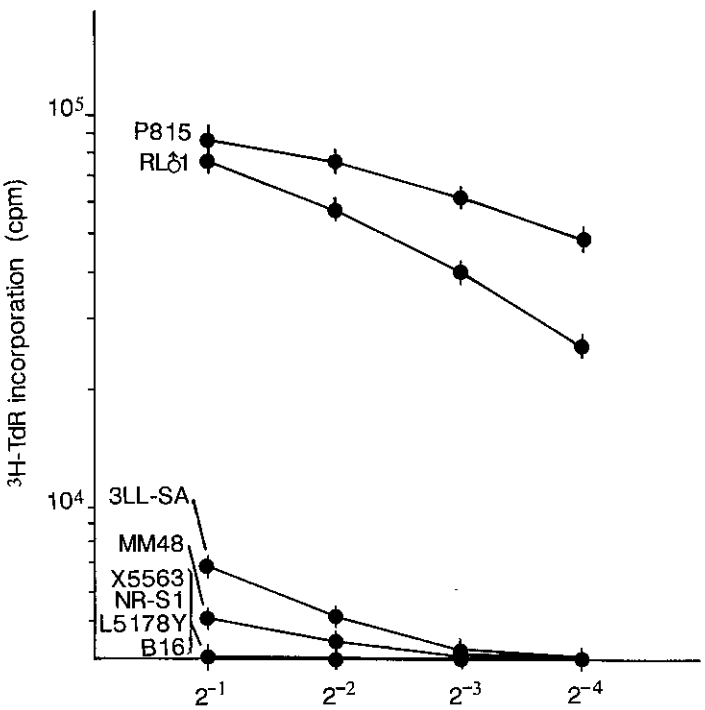

Fig. 3. Proliferative response of PIL-6 cells incubated with the supernatants of tumor cell cultures. The culture fluids were collected, passed through a membrane filter, added to the PIL-6 cells, and assayed for growth-stimulating activity as described in "Materials and Methods." The data represent the mean±SD of triplicate cultures at the indicated dilutions of the supernatants.

the tumor cells, P815 and RL♂1 had the ability to produce IL-6 in culture, but produced no detectable levels of IL-2, IL-3 or IL-5.

Furthermore, detection of IL-6 mRNA from the test tumor cells was carried out by Northern hybridization

(Fig. 4). Transcripts corresponding to the IL-6 mRNA were detected only in P815 and RL♂1 tumors among the 8 test tumors. Further experiments, in which mRNA was extracted from the tumor cells (P815) depleted of adherent cells and T lymphocytes and then hybridized with IL-6 cDNA probe, showed that IL-6 transcripts could be obtained at similar levels to those detected in the original preparations (data not shown). These results may exclude the possibility that the cells transcribing IL-6 are host cells such as macrophages or lymphocytes contaminating tumor tissues, but not tumor cells themselves. These results are quite similar to those for secretion of GM-CSF from the tumor cells with metastatic capability in the lungs.[8]

Detection of IL-1 in the culture fluids of RL♂1 and L5178Y tumor cells Next, we characterized the mitogenic activity of L5178Y tumor cell culture fluids using other bioassay systems. As shown in Table V, a high ConA-comitogenic activity against murine thymocytes was found in the culture fluids of L5178Y. Although low or variable levels of IL-1 activity were found in one other strain (RL♂1) that metastasized to the liver, no activity was found in any of the tumors producing pulmonary metastasis or in the liver metastatic tumor, P815. Table VI shows the results of experiments in which the comitogenic activity against murine thymocytes found in L5178Y culture fluids was examined in the presence of a specific antiserum against murine IL-1 (α), as compared with the mitogenic activities of murine and human rIL-1. The mitogenic activities of L5178Y and rmIL-1α were specifically neutralized by the antiserum. The mitogenic activity against murine bone marrow cells found in L5178Y culture fluids was also neutralized by a specific anti-IL-1 serum (data not shown).

These results indicate that the three tumor cells, P815, RL♂1 and L5178Y with metastatic capability in the liver, produce IL-6 (P815) or IL-1 (L5178Y) or both (RL♂1), in contrast with production of GM-CSF by X5563, MM48, NR-S1 and 3LL-SA, all of which exhibit pulmonary metastasis. B16 tumor cells without spontaneous metastatic capability showed no production of any of IL-1, IL-6 and GM-CSF.

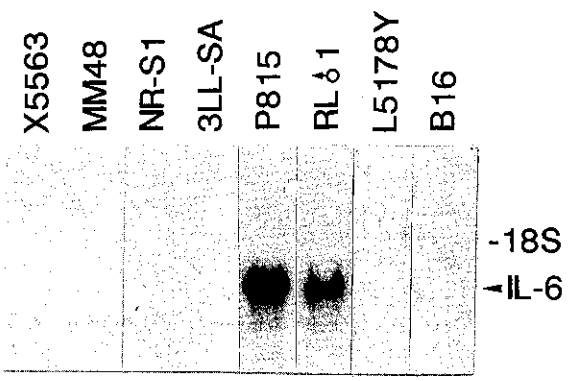

Fig. 4. Northern blots showing expression of IL-6 mRNA in tumor cells. Each lane contained 10 μg of total RNA extracted by CHX treatment from the source cited. The size marker was 18 S rRNA.

Table V. ConA-comitogenic Assay for Murine Thymocytes in the Culture Supernatants of Tumor Cells

Culture supernatant of tumor cells	^{3}H-TdR incorporation (cpm)	
	Concentration of culture supernatants	
	1/4	1/8
X5563	693±68	780±126
MM48	849±246	921±157
NR-S1	953±143	850±202
3LL-SA	876±202	861±165
P815	44±14	305±86
RL♂1	2,209±478	1,239±155
L5178Y	10,006±1,713	8,795±1,078
B16	825±101	652±69
rmIL-1, 50 U	10,012±673	

The culture supernatants were collected 48 h after culture of cells, passed through a filter membrane, added to the thymocytes in the presence of ConA, and subjected to ConA-comitogenic assays. The data represent the mean±SD of triplicate cultures at the indicated dilutions of the culture fluids.

Table VI. Neutralization Tests by Anti-rmIL-1α of ConA-comitogenic Activity by L5178Y Culture Fluids

Test culture fluid	Concentration	^{3}H-TdR incorporation, cpm (% inhibition) with anti-rmIL-1α		
		0	1/20	1/40
L5178Y	50%	37,531±8,243	387±50 (99)	4,337±695 (88)
rmIL-1α	100 U	20,633±1,434	701±30 (97)	873±14 (96)
rhIL-1α	100 U	88,785±5,704	31,271±1,863 (65)	71,286±6,817 (20)

ConA-comitogenic activity of L5178Y culture fluids or rmIL-1α or rhIL-1α at the concentrations indicated was assayed using thymocytes of C3H/He mice in the presence of anti-rmIL-1α at the indicated dilutions.

Table VII. Effect of Anti-rmGM-CSF on Experimental Lung Metastasis of 3LL-SA Tumor Cells and That of Anti-rmIL-6 on Experimental Liver Metastasis of P815 Tumor Cells

Tumor	Treatment	No. of lung metastases on day 15 mean±SD (range)	Inhibition (%)	No. of liver metastases on day 15 mean±SD (range)	Inhibition (%)
3LL-SA	α-rmGM-CSF	10.2±4.1 (15–4)	83	/	
	normal rabbit serum	59.0±21.4 (84–55)	—	/	
P815	α-rmIL-6	/		42.0±17.0 (54–30)	75
	normal goat serum	/		170.4±17.0 (199–155)	—
	α-rmGM-CSF	/		207.2±46.6 (280–163)	−22

Five mice per group inoculated with 3LL-SA or P815 tumor cells were given 0.2 ml of anti-rmGM-CSF (at 1:60 dilution of serum) or anti-rmIL-6 (at 1:60 dilution of serum) as described in "Materials and Methods." As a control serum, normal rabbit or goat serum at 1:60 dilution was used.

Inhibition by anti-IL-6 or -GM-CSF sera of tumor metastasis To test the possibility that GM-CSF and IL-6 production may be involved in the selective metastasis of the test tumors in the lungs and liver, we examined whether antibodies capable of neutralizing specifically the growth-stimulating activities of GM-CSF and IL-6 would inhibit formation of metastatic foci in the respective organs. C57BL/6 mice inoculated i.v. with 3LL-SA were injected with anti-rmGM-CSF for two weeks and the lung tumor colonies were counted 15 days after tumor inoculation. Data from a representative experiment are shown in Table VII. The number of lung tumor colonies was significantly reduced by injection of anti-GM-CSF. On the other hand, DBA/2 mice inoculated i.v. with P815, as well as C57BL/6 mice with 3LL-SA, were treated with anti-IL-6. The results (Table VII lower column) showed that anti-rmIL-6 was effective for the inhibition of liver metastasis of P815 tumor cells but not lung metastasis of 3LL-SA tumor cells. These results suggest that the anti-rmGM-CSF and -rmIL-6 sera specifically inhibited the metastasis of 3LL-SA in the lungs and that of P815 in the liver, respectively.

DISCUSSION

The development of tumor metastasis is a complex biological phenomenon, the outcome of which depends on the interplay between host elements and intrinsic factors of the tumor cells.[22] Bioactive substances produced by tumor cells may be one of the factors involved in the regulation of tumor growth, by influencing cellular metabolism of a factor produced by the tumor cells itself. Such substances may also play an important role in the regulation of tumor metastasis by altering cellular interactions between tumor cells and their surrounding tissues.

In the previous report,[8] we investigated the relationship between the spontaneous metastatic potential of murine tumor cells and their ability to produce cytokines. The results indicated that all of the tumor cells producing nodular metastasis in the lungs of mice expressed GM-CSF mRNA in the cells and produced GM-CSF in the culture fluids, suggesting the possibility that GM-CSF gene expression in tumor cells may be correlated with ability to metastasize to the lungs. In the present report, we further investigated the cytokine production of a group of tumors, which had the ability to produce metastasis in the liver, but not in the lungs. It was found that none of the tumors expressed GM-CSF mRNA or produced GM-CSF activity in the cultures. They did, however, produce mitogenic activity against bone marrow cells in the culture fluids, which appeared to be distinct from GM-CSF. One of the factors involved was identified as IL-6, and another was IL-1. In contrast, tumor cells producing pulmonary metastasis produced neither IL-6 nor IL-1. These results suggest the possibility that the ability of murine transplantable tumors to metastasize in the liver correlates with production of IL-6 and/or IL-1, as the ability to metastasize in the lungs correlates with production of GM-CSF.[8]

Recently, it has been recognized that a variety of murine and human tumor cells produce at least one hematopoietic growth factor that can stimulate their own proliferation *in vitro*.[23] For example, most human acute myelomonocytic leukemia (AMML) cells produce GM-CSF and respond to it.[24] It has also been reported that more than 80% of primary human acute myeloid leukemia (AML) cells release significant quantities of IL-1α.[25] Leukemic cells isolated from patients with adult T cell leukemia (ATL) also produced IL-1-like factors.[26] Specific antibodies for IL-1 inhibited spontaneous cell proliferation in certain cases of AML. These results

suggest that GM-CSF and IL-1 may act as autocrine growth factors in most cases of AML. It has also been shown that IL-6 may be important in the pathology of human myeloma,[27] chronic lymphocytic leukemia of B-cell origin (B-CLL)[28] and prostate cancer,[29] where IL-6 may play a role as an autocrine growth factor. Thus, it appears that both IL-1 and IL-6, as well as GM-CSF, may be important elements in the pathogenesis of some lymphoma or lymphocytic leukemia or other tumors, although other lymphoid growth factors may also be involved.

In the present work, we have shown that production of IL-1 and IL-6 can be detected only in tumor cells with the ability to metastasize to the liver. Preliminary experiments (Table VII) also showed that injection of anti-rmIL-6 sera in mice markedly inhibited the liver metastasis of an IL-6-producing tumor, P815, but not lung metastasis of a GM-CSF-producing tumor, 3LL-SA. These results suggest the possibility that production of IL-6 may be involved in the liver metastasis of IL-6-producing tumor cells, as may be GM-CSF in the lung metastasis of GM-CSF-producing tumors.

The role played by tumor IL-6 and IL-1, if any, in the production of tumor metastasis in the liver remains undetermined. It has, however, been shown that IL-6 is functionally and immunologically related to hepatocyte-stimulating factor (HSF) derived from human monocytes [30, 31] and that injection of IL-6 into adult male rats elicited cytokine-specific changes in the liver expression of acute-phase proteins.[32–34] IL-6 was also found to be the factor that stimulates hepatocyte production of fibronectin.[35]

IL-1, which is produced by a variety of cells, is a cytokine with a multifunctional activity.[36] *In vivo* or *in vitro* stimulation with IL-1 induces hepatocytes or hepatoma cells to produce a range of acute-phase proteins.[37, 38] It was suggested that IL-1 may be a cytokine capable of inducing IL-6 production.[3] In fact, in the present study, the culture supernatant of L5178Y tumor cells containing IL-1 activity was found to induce IL-6 production in the lymphoid tissues of mice (data not shown). Thus, IL-1, as well as IL-6, is an HSF. IL-1 is a potent cytokine, which is one of the crucial mediators of the systemic interaction between vascular cells and leukocytes.[39] IL-1 also promotes tumor cell adhesion to cultured human endothelial cells.[40]

Thus, it is possible that the stimulation of liver tissues by IL-6 or IL-1 secreted by tumor cells produces certain molecules which facilitate the formation of linking or adhesion between tumor cells and liver tissues such as capillary endothelium, resulting in formation of metastatic foci. If this is true, our tumor models developing metastasis selectively in the liver and producing IL-6 and/or IL-1 appear suitable for the study of the mechanisms involved in organ-specific tumor metastasis, as do those developing pulmonary metastasis and producing GM-CSF. We plan to characterize adhesive proteins and their receptor molecules[41, 42] in these liver and pulmonary metastasis models and to investigate possible regulation of the factors by IL-6/IL-1 and GM-CSF, respectively.

ACKNOWLEDGMENTS

We are grateful to Dr. K. Ohya and Dr. A. Abe of the Dental School, Tohoku University, for taking photographs. This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, and the Ministry of Health and Welfare, Japan.

(Received June 6, 1991/Accepted August 9, 1991)

REFERENCES

1) Fong, Y. and Lowry, S. F. Tumor necrosis factor in the pathophysiology of infection and sepsis. *Clin. Immunol. Immunopathol.*, **55**, 157–170 (1990).
2) DiGiovine, F. S. and Duff, G. W. Interleukin 1: the first interleukin. *Immunol. Today*, **11**, 13–20 (1990).
3) Balkwill, F. R. and Burke, F. The cytokine network. *Immunol. Today*, **10**, 299–304 (1989).
4) Milas, L., Faykus, M. H., McBride, W. H., Hunter, N. and Peters, L. J. Concomitant development of granulocytosis and enhancement of metastases-formation in tumor-bearing mice. *Clin. Exp. Metastasis*, **2**, 181–190 (1984).
5) Okabe, T., Nomura, H. and Osawa, N. Establishment and characterization of a human colony-stimulating-factor-producing cell line from a squamous-cell carcinoma of the thyroid gland. *J. Natl. Cancer Inst.*, **69**, 1235–1244 (1982).
6) Ishikawa, M., Koga, Y., Hosokawa, M. and Kobayashi, H. Augmentation of B16 melanoma lung colony formation in C57BL/6 mice having marked granulocytosis. *Int. J. Cancer*, **37**, 919–924 (1986).
7) Nicoletti, G., Lollini, P-I., Bagnara, G. P., De Giovanni, C., Re, Del B., Bons, L., Prodi, G. and Nanni, P. Are colony-stimulating factor-producing cells facilitated in the metastatic process? *Anticancer Res.*, **7**, 695–700 (1987).
8) Tsuchiya, Y., Igarashi, M., Suzuki, R. and Kumagai, K. Production of colony-stimulating factor by tumor cells and the factor-mediated induction of suppressor cells. *J. Immunol.*, **141**, 699–709 (1988).
9) Takeda, K., Hatakeyama, K., Tsuchiya, Y., Rikiishi, H. and Kumagai, K. A correlation between GM-CSF gene expression and metastases in murine tumors. *Int. J.*

Cancer, **47,** 413–420 (1991).
10) Nakajima, H., Fujiwara, H., Takai, Y., Izumi, Y., Sano, S., Tsuchida, T. and Hamaoka, T. Studies on macrophage-activating factor (MAF) in antitumor immune responses. I. Tumor-specific Lyt-1^+2^- T cells are required for producing MAF able to generate cytolytic as well as cytostatic macrophages. *J. Immunol.,* **135,** 2199–2205 (1985).
11) Yoshida, K. and Tachibana, T. Studies on lymphatic metastasis. I. Primary immunoregulatory role of regional lymph nodes in the establishment of lymphatic metastases. *J. Natl. Cancer Inst.,* **75,** 1049–1058 (1985).
12) Urano, M., Nesumi, N., Ando, K., Koike, S. and Ohnuma, N. Repair of potentially lethal radiation damage in acute and chronically hypoxic tumor cells *in vivo. Radiology,* **118,** 447–451 (1976).
13) Sato, H., Boyse, E. A., Aoki, T., Iritani, C. and Old, L. J. Leukemia-associated transplantation antigens related to murine leukemia virus. The X.1 system: immune response controlled by a locus linked to H-2. *J. Exp. Med.,* **138,** 593–606 (1973).
14) Gabius, H-J., Randlow, G., Schirrmacher, V., Nagel, G. A. and Vehmeyer, K. Differential expression of endogenous sugar-binding proteins (lectins) in murine tumor model systems with metastatic capacity. *Int. J. Cancer,* **39,** 643–648 (1987).
15) Watanabe, Y., Okura, A., Naito, K. and Kobayashi, M. Murine liver metastasis model using L5178Y-ML lymphoma and the effect of antitumor agents on the metastasis. *Jpn. J. Cancer Res.,* **79,** 1208–1216 (1988).
16) Shiiba, K., Suzuki, R., Kawakami, K., Ohuchi, A. and Kumagai, K. Interleukin 2-activated killer cells: generation in collaboration with interferon γ and its suppression in cancer patients. *Cancer Immunol. Immunother.,* **21,** 119–128 (1986).
17) Takai, Y., Seki, N., Senoh, H., Yokota, T., Lee, F., Hamaoka, T. and Fujiwara, H. Enhanced production of interleukin 6 in mice with type II collagen-induced arthritis. *Arthritis Rheum.,* **32,** 594–600 (1989).
18) Matsushima, K., Durum, S. K., Kimball, E. S. and Oppenheim, J. J. Purification of human interleukin 1 from human monocyte culture supernatants and identity of thymocyte comitogenic factor, fibroblast-proliferation factor, acute-phase protein-inducing factor, and endogenous pyrogen. *Cell. Immunol.,* **92,** 290–301 (1985).
19) Suzuki, R., Suzuki, S., Igarashi, M. and Kumagai, K. Induction of interleukin 3 but not interleukin 2 or interferon production in the syngeneic mixed lymphocyte reaction. *J. Immunol.,* **137,** 1564–1572 (1986).
20) Hitoshi, Y., Yamaguchi, N., Mita, S., Sonoda, E., Takaki, S., Tominaga, A. and Takatsu, K. Distribution of IL-5 receptor-positive B cells. Expression of IL-5 receptor on Ly-1 $(CD5)^+$ B cells. *J. Immunol.,* **144,** 4218–4225 (1990).
21) Vink, A., Coulie, P., Warnier, G., Renauld, J-C., Stevens, M., Donckers, D. and Van Snick, J. Mouse plasmacytoma growth *in vivo*: enhancement by interleukin 6 (IL-6) and inhibition by antibodies directed against IL-6 or its receptor. *J. Exp. Med.,* **172,** 977–1000 (1990).
22) Fidler, I. J. Critical factors in the biology of human cancer metastasis: twenty-eighth. *Cancer Res.,* **50,** 6130–6138 (1990).
23) Lang, R. A. and Burgess, A. W. Autocrine growth factors and tumorigenic transformation. *Immunol. Today,* **11,** 244–249 (1990).
24) Young, D. C., Wagner, K. and Griffin, J. D. Constitutive expression of the granulocyte-macrophage colony stimulating factor gene in acute myeloblastic leukemia. *J. Clin. Invest.,* **79,** 100–106 (1987).
25) Cozzolino, F., Rubartelli, A., Aldinucci, D., Sitia, R., Torcia, M., Shaw, A. and Guglielmo, R. D. Interleukin 1 as an autocrine growth factor for acute myeloid leukemia cells. *Proc. Natl. Acad. Sci. USA,* **86,** 2369–2373 (1989).
26) Shirakawa, F., Uki, Y. and Yoshiya, T. Production of bone-resolving activity corresponding to interleukin-1α by adult T-cell leukemia cells in humans. *Cancer Res.,* **48,** 4284–4287 (1988).
27) Kawano, M., Hirano, T., Matsuda, T., Taga, T., Horii, Y., Iwato, K., Asaoku, H., Tang, B., Tanabe, O., Tanaka, H., Kuramoto, A. and Kishimoto, T. Autocrine generation and essential requirement of BSF-2/IL-6 for human multiple myelomas. *Nature,* **332,** 83–85 (1988).
28) Hirano, T. and Kishimoto, T. Interleukin 6 and plasma cell neoplasia. *Prog. Growth Factor Res.,* **1,** 133–141 (1989).
29) Siegall, C. B., Schwab, G., Nordan, R. P., FitzGerald, D. J. and Pastan, I. Expression of the interleukin 6 receptor and interleukin 6 in prostate carcinoma cells. *Cancer Res.,* **50,** 7786–7788 (1990).
30) Marinkovic, S., Jahreis, G. P., Wong, G. G. and Baumann, H. IL-6 modulates the synthesis of a specific set of acute phase plasma proteins *in vivo. J. Immunol.,* **142,** 808–812 (1989).
31) Castell, J. V., Andus, T., Geiger, T., Lechón, M. G., David, M., Klapproth, J., Hirano, T., Kishimoto, T. and Heinrich, P. C. Interleukin-6 is the major regulator of acute phase protein synthesis in rat and man. *Adv. Immunopharmacol.,* **4,** 191–201 (1988).
32) Gaulide, J., Richards, C., Harnish, D., Lansdorp, P. and Baumann, H. Interferon beta 2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. *Proc. Natl. Acad. Sci. USA,* **84,** 7251–7255 (1987).
33) Gauldie, J., Northemann, W. and Fey, G. H. IL-6 functions as an exocrine hormone in inflammation hepatocytes undergoing acute phase responses require exogenous IL-6. *J. Immunol.,* **144,** 3804–3808 (1990).
34) Bereta, J., Kurdowsda, A., Koj, A., Hirano, T., Kishimoto, T., Content, J., Fiers, W., van Damme, J. and Gauldie, J. Different preparations of natural and recombinant human interleukin-6 (IFN-β2, BSF-2) similarly stimulate acute phase protein synthesis and uptake of

α-aminoisobutyric acid by cultured rat hepatocytes. *Int. J. Biochem.*, **21**, 361–366 (1989).
35) Lanser, M. E. and Brown, G. E. Stimulation of rat hepatocyte fibronectin production by monocyte-conditioned medium is due to interleukin 6. *J. Exp. Med.*, **170**, 1781–1786 (1989).
36) Oppenheim, J. J., Kovacs, E. J., Matsushima, K. and Durum, S. K. There is more than one interleukin 1. *Immunol. Today*, **7**, 45–56 (1986).
37) Geiger, T., Andus, T., Klapproth, J., Northoff, H. and Heinrich, P. C. Induction of alpha 1-acid glycoprotein by recombinant human interleukin 1 rat hepatoma cells. *J. Biol. Chem.*, **263**, 7141–7146 (1988).
38) Darlington, G. J., Wilson, D. R. and Lachman, L. B. Monocyte-conditioned medium, interleukin 1, and tumour necrosis factor stimulate the acute phase response in human hepatoma cells *in vitro*. *J. Cell Biol.*, **103**, 787–793 (1986).
39) Mantovani, A. and Dojana, E. Cytokines as communication signals between leukocytes and endothelial cells. *Immunol. Today*, **10**, 370–375 (1989).
40) Dejana, E., Bertocchi, F. and Bortolami, M. C. Interleukin 1 promotes tumor cell adhesion to cultured human endothelial cells. *J. Clin. Invest.*, **82**, 1466–1470 (1988).
41) Horst, E., Meijer, C. J. L. M., Radaszkiewicz, T., Ossekoppele, G. J., Van Krieken, J. H. J. M. and Pals, S. T. Adhesion molecules in the prognosis of diffuse large-cell lymphoma: expression of a lymphocyte homing receptor (CD44), LFA-1 (CD11a/18), and ICAM-1 (CD54). *Leukemia*, **4**, 595–599 (1990).
42) Springer, T. A. Adhesion receptors of the immune system. *Nature*, **346**, 425–434 (1990).