Jpn. J. Cancer Res. 85, 179–186, February 1994

Autonomous Expressions of Cytokine Genes by Human Lung Cancer Cells and Their Paracrine Regulation

Kazuto Mizuno, Saburo Sone,[1] Etsuko Orino, Akihiko Nii and Takeshi Ogura

Third Department of Internal Medicine, The University of Tokushima, School of Medicine, Kuramoto-cho 3-18-15, Tokushima 770

Cell-to-cell interaction between tumors and host inflammatory cells is important for the subsequent cancer progression or regression. We examined the expressions of mRNAs for various proinflammatory cytokines by nine human lung cancer cell lines and the influences of cytokines on their gene expressions. The cytokines used were interleukin 1β (IL-1β), interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), granulocyte-macrophage colony stimulating factor (GM-CSF) and monocyte chemotactic and activating factor. Gene expressions of cytokines were measured by Northern blot analysis. Substantial expressions of cytokine genes were detected in several lung cancer cell lines such as RERF-LC-MS, RERF-LC-OK and VMRC-LCD, although the levels of expression of each cytokine varied in different cell lines. Four lung cancer cell lines (RERF-LC-MS, RERF-LC-OK, A549 and YO-88) were used to examine the effects of exogenous cytokines (IL-1β, TNF-α and GM-CSF) on cytokine gene expressions by the cells. TNF-α and IL-1β caused significant changes in the levels of mRNA expressions of certain cytokines. Moreover, on stimulation with TNF-α, RERF-LC-OK cells produced IL-6 extracellularly. These extensive differences in the levels of gene expressions and productions of cytokines could have profound effects on the interactions between human lung cancer cells and the corresponding host cells.

Key words: Lung cancer — Cytokine — TNF-α — IL-1 — Paracrine

It has long been appreciated that complex interactions occurring between tumors and host inflammatory cells, particularly macrophages may influence progression or regression of the tumors.[1–3] Recently, much progress has been made in identification of various cytokines responsible for internal and external signalings that affect the growth of tumor cells. Several cytokines (TNF-α, IL-6, IL-10, GM-CSF and IFN-γ)[2] detectable in tumor-infiltrating inflammatory cells have been found to be involved in tumor cell-host cell interactions.[4–8] On the other hand, autonomous productions of cytokines such as M-CSF,[9, 10] IL-6[11–13] and IL-10 and GM-CSF[14, 15] have been reported in several human tumors of nonhematopoietic origin. Several human lung cancer cell lines have also been found to produce cytokines such as IL-8,[16] CSF and IL-6[17–19] constitutively. The products of these cytokines could influence the functions of infiltrating host inflammatory cells such as macrophages and lymphocytes. In particular, tumor-infiltrating macrophages, which produce various cytokines (TNF, IL-1, IL-6, GM-CSF), are known to be involved in tumor progression or regression.[20]

Recently, much attention has been paid to certain cytokines which stimulate growth of human tumor cells and induce secondary cytokine productions by the cells.[21–24] These findings raise the possibility that one cytokine produced by tumor cells and/or tumor-infiltrating host cells may induce secondary production of multiple other cytokines. Little is known, however, about autocrine and/or paracrine mechanisms which may regulate human lung tumor growth. Tumor-derived cytokines such as IL-1, TNF-α, G-CSF and IL-6 may also be important in the development of paraneoplastic syndromes associated with symptoms such as leukocytosis, thrombocytosis, hypercalcemia and/or loss of body weight.[25, 26] To elucidate complex interactions between lung cancer and macrophages, we examined the gene expressions of macrophage-related cytokines and their regulations by proinflammatory cytokines in nine human lung cancer cell lines. We found that all the lung cancer cell lines expressed at least one cytokine spontaneously. Moreover, addition of exogenous TNF-α or IL-1 affected the gene expressions of some cytokines.

MATERIALS AND METHODS

Cell lines The human lung cancer cell lines RERF-LC-MS (adenocarcinoma), RERF-LC-OK (adenocarcinoma), VMRC-LCD (adenocarcinoma), A 549 (adenocarcinoma), Lu-134-A-H (small cell carcinoma), N 291

[1] To whom all correspondence should addressed.

[2] Abbreviations used are: GM-CSF, granulocyte-macrophage colony stimulating factor; IFN-γ, interferon γ; IL-1β, interleukin-1β; MCAF, monocyte chemotactic and activating factor; TNF-α, tumor necrosis factor α.

(small cell carcinoma) and H 69 (small cell carcinoma) were obtained from ATCC.

Establishment of lung cancer cell lines The cell lines YO-88 (adenocarcinoma) and T-SC89 (small cell carcinoma) were established from malignant pleural effusions of a 65-year-old Japanese man (case 1) and of a 61-year-old man (case 2), respectively, in our laboratory between 1989 and 1990. On sampling of tumor specimens these patients had recurrence of malignant pleural effusions in their clinical courses after chemotherapy alone (case 1) or with irradiation therapy (case 2), but did not have particular symptoms or signs characteristic of paraneoplastic syndrome. At that time, white blood cells in pleural effusions of cases 1 and 2 consisted of 80% and 31% macrophages, and 9% and 69% lymphocytes, respectively. Briefly, all specimens were collected during standard diagnostic procedures under aseptic conditions, and centrifuged on a Ficoll-Hypaque gradient to separate tumor cells from erythrocytes, prior to plating in culture. The cells were set up in parallel in RPMI 1640 with 20% fetal bovine serum (FBS) in 25 cm^2 culture flasks until cell lines were established. Absence of mycoplasma contamination was confirmed by using fluorescent Hoechst 33258.

Reagents FBS and RPMI 1640 medium were purchased from Gibco (Grand Island, NY) and Nissui Chemical Co. (Tokyo), respectively. RPMI medium supplemented with FBS (10%), glutamine (1 m*M*) and gentamycin (50 mg/ml) was used in all experiments. Natural human TNF-α (specific activity, 3.25×10^5 Japanese Reference Units/mg protein) was a gift from Hayashibara Institute (Okayama). Human recombinant IL-1β was supplied by Otsuka Pharmaceutical Co. (Tokushima). Human recombinant GM-CSF (specific activity, 9.3×10^6 U/mg protein) was kindly supplied by the Genetics Institute (Cambridge, MA). cDNAs for IL-1β, TNF-α and GM-CSF were obtained from Otsuka Pharmaceutical Co. Lipopolysaccharide (LPS) derived from *Escherichia coli* 055:B5 strain was purchased from Difco Laboratories, Detroit, MI. The cDNA for IL-6 was a gift from Dr. T. Hirano (Osaka University, Osaka). The cDNA for MCAF was a gift from Dr. K. Matsushima (Cancer Institute, Kanazawa University, Kanazawa).

Isolation and culture of human blood monocytes Mononuclear cells were separated on lymphocyte separation medium from the leukocyte concentrates collected from peripheral blood (200 ml) of healthy donors. Monocyte-enriched fractions were separated from the mononuclear cells by centrifugal elutriation in a Beckman JE-5.0 elutriation system.[27] More than 97% of the cells were viable, as judged by the Trypan-blue dye exclusion test. These cells were washed twice, then incubated for 7 days in medium with GM-CSF (100 U/ml) to induce monocyte-derived macrophages. Then these macrophages were plated at various concentrations for 1 h, and incubated for 6 h with LPS (1 μg/ml). Cells were fixed with 1% paraformaldehyde (PFA) for 20 min at room temperature according to the method described previously[28] for measuring membrane-bound TNF activity. Then these cells were washed three times, and incubated for 24 h in medium to eliminate the effect of remaining PFA and substances leaking from fixed cells. After three more washes, they were used for measurement of membrane-form TNF activity.

Bioassay for membrane-form TNF activity TNF activity was measured by assay of cytotoxicity to L929 cells using the MTT dye reduction method described previously.[28] Briefly, 2×10^4 L929 cells were plated into each well of 96-well plates containing fixed monocyte-derived macrophages that had been induced by incubation of monocytes for 7 days in medium with GM-CSF (100 U/ml) in the presence of actinomycin D (2 μg/ml). Natural TNF-α was also applied as a standard control. Cells were cultured at 37°C under 5% CO_2 in humidified air. After 24 h, 50 μl of MTT stock solution (5 mg/ml) was added to all wells, and the cells were incubated for 2 h at 37°C. Then the culture media were removed and 100 μl of DMSO was added to dissolve the dark blue crystals. Absorbances were measured with an MTP-32 Microplate Reader (Corona Elactric, Ibaragi) at test and reference wavelengths of 550 and 630 nm, respectively. TNF activity is expressed as percent cytotoxicity calculated by means of the following equations:

$$\%\ \text{Cytotoxicity} = (A-B)/A\times100,$$

where A is the absorbance of wells of L929 cells cultured with medium only and B is that of the well of L929 cells cultured with a test sample.

Measurement of IL-6 by ELISA Enzyme immunoassay (EIA) for human IL-6 was performed essentially as described previously.[29] The sensitivity limit of the EIA was 3.1 pg/ml.

Northern blot hybridization analysis Total RNA was obtained from cells by the acid guanidium thiocyanate-phenol-chloroform extraction method.[30] Briefly, samples of 5×10^7 cells were washed three times with ice-cold phosphate-buffered saline. Denaturing solution (4 *M* guanidium thiocyanate, 25 m*M* sodium citrate, 0.5% N-lauryl-sarcosine, 0.1 *M* 2-mercaptoethanol, pH 7.0) was then added, and the cells were scraped off the plates, transferred to sterile 2-ml Eppendorf tubes and sonicated. Then, (a) 2 *M* sodium acetate, pH 4.0, (b) water-saturated phenol, and (c) chloroform-isoamyl alcohol (49:1) were added sequentially, with mixing, to the denaturing solution at ratios by volume of 0.1:1:0.2:1. The final suspension was shaken vigorously for 10 s, then cooled on ice for 15 min. Samples were centrifuged at 10,000*g* for 20 min at 4°C. After centrifugation, the

aqueous phase was transferred to fresh tubes and mixed with 1 vol of isopropanol, and the tubes were then incubated at −20°C for at least 1 h to precipitate the RNA. The RNA pellet obtained by centrifugation at 10,000*g* for 20 min was resuspended in 0.5 ml of the denaturing solution, and precipitated by treatment with 1 vol of isopropanol at −20°C for 1 h. The resulting RNA pellet was resuspended in 70% ethanol, precipitated by recentrifugation, dried in a vacuum, and dissolved in 10–50 μl of diethyl pyrocarbonate-treated water. The quantity and purity of the RNA were estimated with a spectrophotometer (Beckman Instruments, Palo Alto, CA). Each total RNA sample (5–10 μg of RNA) was denatured in a solution of 20 m*M* 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer, pH 7.0, containing 6% formaldehyde, 50% formamide, 5 m*M* sodium acetate, and 1 m*M* EDTA at 65°C for 5 min and then separated on 1.2% agarose gel containing 6% formaldehyde. It was then transferred to a Hybond-N^+ nylon membrane (Amersham International plc, England) and hybridized with ^{32}P-labeled probes in similar conditions to those described previously.[28] The probes used were the *Pst*I-*Pvu*II fragment (0.67 kb) of cDNA for IL-1β, the *Pst*I-*Pst*I fragment (1.1 kb) of cDNA for TNF-α, the *Pst*I-*Nco*I fragment (0.5 kb) of cDNA for GM-CSF, the *Taq*I-*Ban*II fragment (0.44 kb) of cDNA for IL-6, and the *Pst*I-*Pst*I fragment (0.4 kb) of cDNA for MCAF. These probes were labeled by the multiprime DNA labeling method.[31] Before hybridization, the nylon membrane was prehybridized in 50% formamide, 5×SSPE (SSPE; 0.15 *M* NaCl, 10 m*M* NaH_2PO_4, pH 7.4, 1 m*M* EDTA), 200 μg/ml sonicated salmon sperm DNA, 0.1% SDS, and 5×Denhardt's solution at 42°C for 6 h. Hybridization was performed at 42°C for 14 h in the same solution containing 10% dextran sulfate and labeled cDNA (10^6 cpm/ml) which had a specific activity of about 10^9 cpm/μg. After hybridization, the membrane was washed four times with 2×SSPE containing 0.1% SDS for 15 min each time at 42°C and then with 1×SSPE containing 0.1% SDS for 30 min at 42°C. The membrane was autoradiographed with Kodak XAR-5 film at −70°C with an intensifying screen. Unless otherwise described, these experiments were performed 4 h after the start of tumor cell culture with or without stimulating agents. All experiments were repeated at least 3 times with similar results.

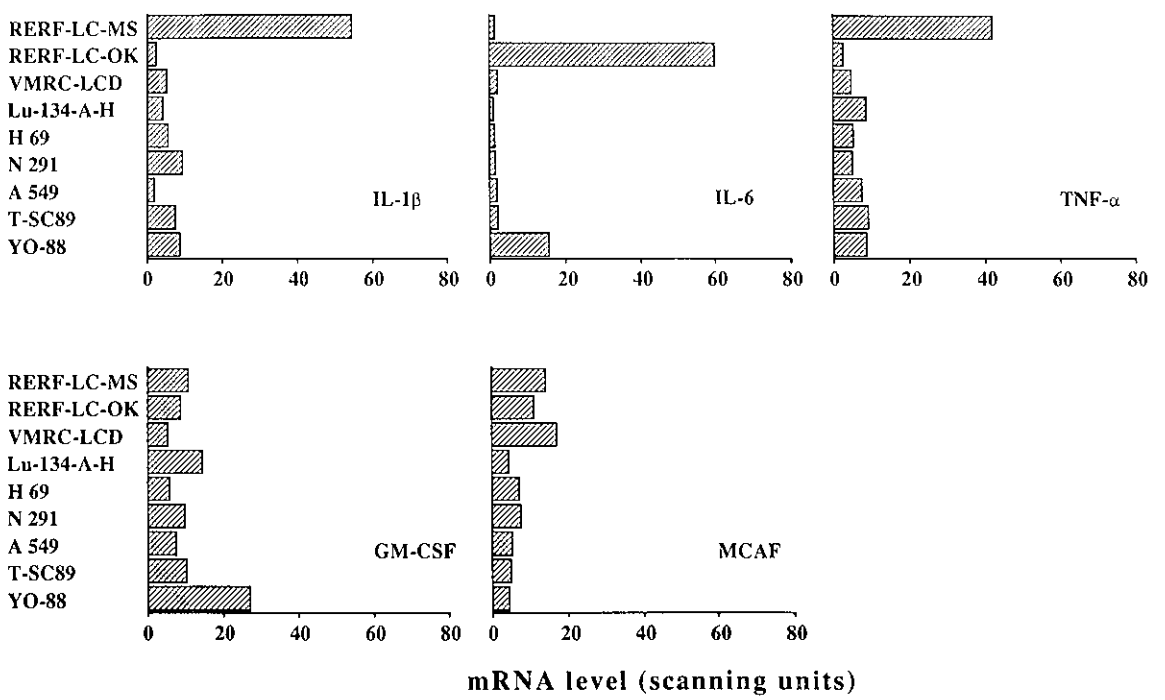

Fig. 1. Spontaneous gene expressions of various cytokines by human lung cancer cell lines. The indicated lung cancer cell lines (1×10^6 cells/ml) were incubated for 72 h. The cells were then lysed and their total RNAs were prepared. Equal amounts of total RNA (5 μg/lane) were separated by electrophoresis, blotted onto a nylon membrane, and hybridized with ^{32}P-labeled IL-1β, IL-6, TNF-α, GM-CSF, MCAF or β-actin cDNA probe. Relative mRNA levels were assessed by densitometry on the basis of β-actin.

Quantitative analysis of autoradiograms Autoradiograms obtained by Northern blot analysis were quantitated by densitometric scanning with a FUJIX Bio-Image Analyzer-BAS2000 (Fuji Photo Film Co., Tokyo).

RESULTS

Spontaneous cytokine gene expression by human lung cancer cells First, we examined whether human lung cancer cell lines constitutively express mRNAs for various cytokines (IL-1β, IL-6, GM-CSF, TNF-α and MCAF). For this, human lung cancer cells at a density of 1×10^6 cells/ml were incubated for 72 h before harvesting. Then the levels of mRNAs were quantitated by densitometric scanning, and expressed as relative scanning units as shown in Fig. 1. Spontaneous expressions of mRNAs for IL-1β and TNF-α were seen in RERF-LC-MS cells, mRNA for IL-6 in RERF-LC-OK cells and mRNA for GM-CSF in YO-88 cells. In addition, mRNA for the MCAF gene was expressed spontaneously in three lung cancer cell lines (RERF-LC-MS, RERF-LC-OK and VMRC-LCD), although at low levels.

Effects of cytokines on cytokine gene expressions in various lung cancer cell lines We examined whether the cytokines TNF-α, IL-1β and GM-CSF affected the proliferations of four lung cancer cell lines (RERF-LC-MS, RERF-LC-OK, A549 and YO-88). As judged by ^{3}H-thymidine uptake and microscopical examination, none of these three cytokines at concentrations of 100 U/ml affected the proliferation or morphology of any of the four cell lines tested (data not shown).

We next examined the effects of various cytokines on the expressions of the genes for IL-1β, TNF-α, IL-6, GM-CSF and MCAF in the same four human lung

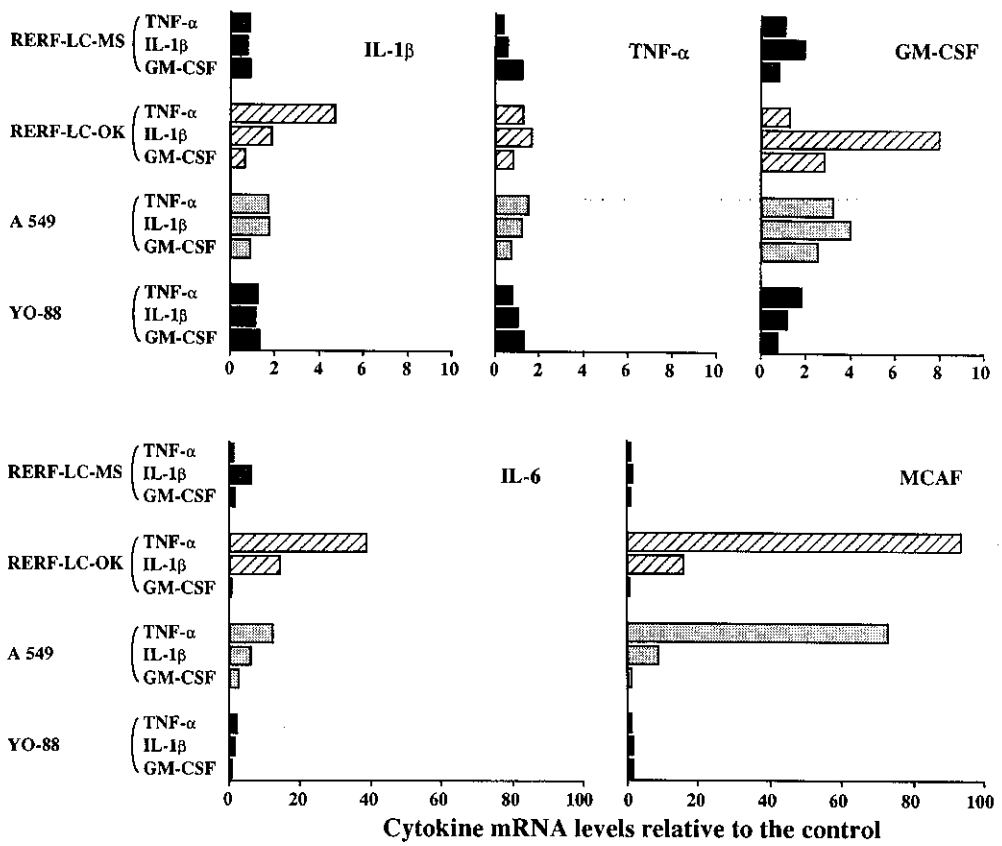

Fig. 2. Regulations by IL-1β, TNF-α and GM-CSF of cytokine gene expressions in four lung cancer cell lines. Four lung cancer cell lines (1×10^6 cell/ml) were incubated for 4 h in medium with or without TNF-α (100 U/ml), IL-1β (100 U/ml) or GM-CSF (100 U/ml). The cells were then lysed and their total RNAs were prepared. Equal amounts of total RNA (5 μg/lane) were separated by electrophoresis, blotted onto a nylon membrane, and hybridized with ^{32}P-labeled human IL-1β, IL-6, TNF-α, GM-CSF, MCAF or β-actin cDNA probe. Relative mRNA levels were assessed by densitometry on the basis of β-actin. Changes of mRNA levels on addition of exogenous cytokines were calculated by comparison with the values in cells incubated in medium alone.

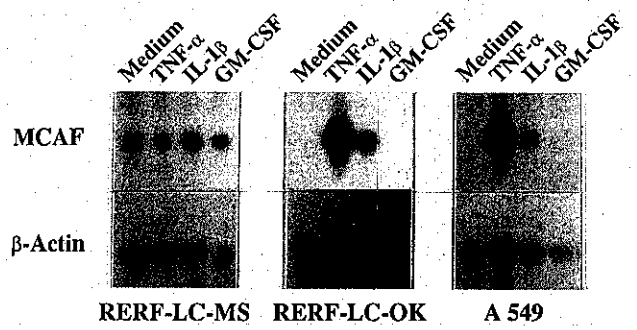

Fig. 3. Up-regulations by TNF-α, IL-1β and GM-CSF of MCAF gene expressions in three lung cancer cell lines. Lung cancer cell lines (1×10^6 cells/ml) were incubated for 4 h in medium with or without TNF-α (100 U/ml), IL-1β (100 U/ml) or GM-CSF (100 U/ml). Then the cells were lysed and their total RNAs were prepared. Equal amounts of total RNA (5 μg/lane) were separated by electrophoresis, blotted onto a nylon membrane, and hybridized with ^{32}P-labeled human MCAF or β-actin cDNA probe.

Table I. IL-6 Production by RERF-LC-OK Cells Stimulated with Natural TNF-α or Fixed Monocyte-derived Macrophages

	Sample	TNF activity (% cytotoxicity)	IL-6 production (pg/ml)
Set 1	TNF-α (U/ml)		
	0	0.0	262
	1	55.1	619
	10	81.2	843
	100	ND[a]	1006
Set 2	Monocyte-derived macrophages ($\times10^{-4}$/well)		
	0	0	558
	1	27.8	763
	3	60.9	938
	10	75.3	1032

Monocyte-derived macrophages were stimulated for 6 h with LPS (1 μg/ml) and then fixed with PFA before measurement of membrane-form TNF activity as described in "Materials and Methods." SDs were consistently $<10\%$ of means.
a) ND, not done

cancer cell lines (RERF-LC-MS, RERF-LC-OK, A549 and YO-88), by incubating these cells for 4 h in medium with various cytokines before Northern blot analysis. Changes of mRNA levels are shown in Fig. 2. GM-CSF did not affect the gene expressions of any of the cytokines examined. IL-1β mRNA expression was enhanced only in RERF-LC-OK cells on TNF-α stimulation. The TNF-α mRNA level in RERF-LC-MS cells was reduced by treatment with TNF-α or IL-1β. On the other hand, the IL-6 mRNA levels were increased by TNF-α in RERF-LC-OK and A 549 cells, but were increased by IL-1β in all the lung cancer cell lines examined, and especially RERF-LC-OK cells. The GM-CSF mRNA level was increased in RERF-LC-OK cells and also to some extent in A 549 cells by treatment with IL-1β.

Fig. 3 shows representative results of Northern blot analyses of the effects of various cytokines on the expressions of the genes for MCAF. Marked MCAF mRNA expression was seen in RERF-LC-OK and A 549 cells after TNF-α treatment. IL-1β also augmented the MCAF mRNA levels in RERF-LC-OK and A 549 cells, although less than TNF-α.

Induction of IL-6 production by lung cancer cells stimulated by cytokines We measured concentrations of IL-6 extracellularly secreted by lung cancer (RERF-LC-OK) cells stimulated with TNF-α. Natural TNF-α, which was cytotoxic to L929 cells, stimulated RERF-LC-OK cells to produce IL-6 extracellularly in a dose-dependent manner (Table I). Under these experimental conditions we examined the effect of membrane-form TNF on induction of IL-6 production by RERF-LC-OK cells. Monocyte-derived macrophages that had been treated for 6 h with LPS, also expressed TNF activity and induced production of IL-6 by the lung cancer cells depending on the density of cells added (Table I).

DISCUSSION

Complex interactions occur between tumors and host inflammatory cells, leading to either progression or regression of the tumors.[1-3] In the present study, we found that nine different human lung cancer cell lines all spontaneously expressed the genes for at least one of five cytokines examined. In particular, aberrantly high levels of cytokine gene expression were observed in human lung adenocarcinoma cell lines (RERF-LC-MS and RERF-LC-OK). Constitutive expressions of cytokine genes in human lung cancer cell lines might contribute to positive or negative feedback control of the anti-tumor defense mechanism, because infiltrating host cells such as lymphocytes and macrophages have been found in primary cancers.[32, 33]

TNF and IL-1 are produced mainly by cells of the monocyte-macrophage series.[20] TNF was also found to induce GM-CSF production by lung adenocarcinoma (SUT) and squamous cell carcinoma (SKMES) cell lines.[34] These findings were confirmed and extended in the present study on four human lung cancer cell lines (RERF-LC-MS, RERF-LC-OK, A549 and YO-88) which showed that human TNF-α induced the gene expressions of not only GM-CSF, but also IL-1β, MCAF and IL-6 (Fig. 2). IL-1β also induced expressions of the genes for IL-6, GM-CSF, MCAF. Thus, it is likely that

an aberrantly high level of expression of one cytokine gene induces the expression of multiple other cytokines in lung cancer cells.

In the present study extracellular production of IL-6 by human lung cancer (RERF-LC-OK) cells was also seen at the protein level, when the cells were stimulated with soluble form of TNF-α. This supports the findings obtained at the mRNA level (Fig. 2). Recently human macrophages were found to produce large amounts of 26 kDa membrane-form TNF-α when activated with various stimuli,[28] suggesting a possible cell-to-cell interaction between macrophages and lung cancer cells through a paracrine mechanism by which cancer cells produce secondary cytokines. This was the case in our study showing that membrane-form TNF-α expressed on human monocyte-derived macrophages induced IL-6 production by RERF-LC-OK cells in a dose-dependent manner (Table I).

GM-CSF was found to stimulate growth of several tumor cells of nonhematopoietic origin,[21] but it did not affect gene expression by lung cancer cell lines of any cytokine examined (Figs. 2 and 3). Under our experimental conditions, TNF-α mRNA expression was down-regulated only in RERF-LC-MS cells by addition of TNF-α or IL-1β.

Continuous recruitment of blood monocytes into growing tumors seems to be important for cell-to-cell interaction between inflammatory cells and tumor cells. MCAF, which is chemotactic for monocytes, is known to play an important role in *in vivo* recruitment of monocytes to inflammatory sites.[35, 36] Several human lung cancer cell lines showed variable levels of MCAF mRNA (Fig. 2). Interestingly, MCAF gene expression could be augmented by treating the cells (RERF-LC-OK and A549) with TNF-α and IL-1β; TNF-α was much more effective than IL-1β. In contrast, GM-CSF did not induce MCAF production at all.

Several cytokines such as TNF and IL-1 detectable in some human cancer cells are known to induce production of other cytokines by host inflammatory cells.[4, 5, 8] Recently fibroblasts,[37, 38] endothelial cells[39] and mesothelial cells[9] were also found to produce various cytokines. These findings indicate the possibility of complex interactions between these cytokines in terms of effects on lung cancer cells and tumor-infiltrating host cells through autocrine and/or paracrine mechanisms. Further studies on the regulations of production of cytokines at the mRNA and protein levels in this model of human lung cancer should contribute to our understanding of the roles played by such cytokines in progression or regression of lung cancer.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare of Japan.

(Received September 10, 1993/Accepted November 10, 1993)

REFERENCES

1) Fidler, I. J. Macrophages and metastasis — a biological approach to cancer therapy: Presidential address. *Cancer Res.*, **45**, 4714–4726 (1985).
2) Evans, R. The immunological network at the site of tumor rejection. *Biochim. Biophys. Acta*, **865**, 1–11 (1986).
3) Sone, S. Role of alveolar macrophages in pulmonary neoplasias. *Biochim. Biophys. Acta*, **823**, 227–245 (1986).
4) Beissert, S., Bergholz, M., Waase, I., Lepsien, G., Schauer, A., Pfizenmaier, K. and Krönke, M. Regulation of tumor necrosis factor gene expression in colorectal adenocarcinoma: *in vivo* analysis by *in situ* hybridization. *Proc. Natl. Acad. Sci. USA*, **86**, 5064–5068 (1989).
5) Naylor, M. S., Stamp, G. W. and Balkwill, F. R. Investigation of cytokine gene expression in human colorectal cancer. *Cancer Res.*, **50**, 4436–4440 (1990).
6) Hasday, J. D., Shah, E. M. and Lieberman, A. P. Macrophage tumor necrosis factor-α release is induced by contact with some tumors. *J. Immunol.*, **145**, 371–379 (1990).
7) Pisa, P., Halapi, E., Pisa, E. K., Gerdin, E. Hising, C., Bucht, A., Gerdin, B. and Kiessling, R. Selective expression of interleukin 10, interferon γ, and granulocyte-macrophage colony-stimulating factor in ovarian cancer biopsies. *Proc. Natl. Acad. Sci. USA*, **89**, 7708–7712 (1992).
8) Vitolo, D., Kanbour, A., Johnson, J. T., Herberman, R. R. and Whiteside, T. L. *In situ* hybridization for cytokine gene transcripts in the solid tumor microenvironment. *Eur. J. Cancer*, **29A**, 371–377 (1993).
9) Demetri, G. D., Zenzie, B. W., Rheinwald, J. G. and Griffin, J. D. Expression of colony-stimulating factor genes by normal human mesothelial cells and human malignant mesothelioma cells lines *in vitro*. *Blood*, **74**, 940–946 (1989).
10) Ramakrishnan, S., Xu, F. J., Brandt, S. J., Niedel, J. E., Bast, R. C., Jr. and Brown, E. L. Constitutive production of macrophage colony stimulating factor by human ovarian and breast cancer cell lines. *J. Clin. Invest.*, **83**, 921–926 (1989).
11) Matsuguchi, T., Okamura, S., Kawasaki, C. and Niho, Y. Production of interleukin 6 from human liver cell lines: production of interleukin 6 is not concurrent with the production of α-fetoprotein. *Cancer Res.*, **50**, 7457–7459 (1990).

12) 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).
13) Meyers, F. J., Gumerlock, P. H., Kawasaki, E. S., Wang, A. M., deVere White, R. W. and Erlich, H. A. Bladder cancer, human leukocyte antigen II, interleukin-6, and interleukin-6 receptor expression determined by the polymerase chain reaction. *Cancer,* **67,** 2087–2095 (1991).
14) Sabatini, M., Chavez, J., Mundy, G. R. and Bonewald, L. F. Stimulation of tumor necrosis factor release from monocytic cells by the A375 human melanoma via granulocyte-mactophage colony-stimulating factor. *Cancer Res.,* **50,** 2673–2678 (1990).
15) Frei, K., Piani, D., Malipiero, U. A., Meir, E. V., de Tribolet, N. and Fontana, A. Granulocyte-macrophage colony-stimulating factor (GM-CSF) production by glioblastoma cells: despite the presence of inducing signals GM-CSF is not expressed *in vivo. J. Immunol.,* **148,** 3140–3146 (1992).
16) Hotta, K., Hayashi, K., Ishikawa, J., Tagawa, M., Hashimoto, K., Mizuno, S. and Suzuki, K. Coding region structure of interleukin-8 gene of human lung giant cell carcinoma LU65C cells that produce LUCT/interleukin-8: homogeneity in interleukin-8 genes. *Immunol. Lett.,* **24,** 165–170 (1990).
17) Itoh, K., Fujioka, S., Igarashi, T., Nakamura, H., Endoh, N., Sugiura, Y., Wong, P., Asai, T. and Yoshida, S. Colony-stimulating factor (CSF) and CSF-stimulating activity produced by a human giant cell carcinoma of the lung. *Acta Haematol. Jpn.,* **49,** 960–968 (1986).
18) Matsuguchi, T., Okamura, S., Kawasaki, C., Shimoda, K., Omori, F., Hayashi, S., Kimura, N. and Niho, Y. Constitutive production of granulocyte colony-stimulating factor and interleukin-6 by a human lung cancer cell line, KSNY: gene amplification and increased mRNA stability. *Eur. J. Haematol.,* **47,** 128–133 (1991).
19) Yamada, T., Hirohashi, S., Shimosato, Y., Kodama, T., Hayashi, S., Ogura, T., Gamou, S. and Shimizu, N. Giant cell carcinomas of the lung producing colony-stimulating factor *in vitro* and *in vivo. Jpn. J. Cancer Res.,* **76,** 967–976 (1985).
20) Nathan, C. F. Secretory products of macrophages. *J. Clin. Invest.,* **79,** 319–326 (1987).
21) Dedhar, S., Gaboury, L., Galloway, P. and Eaves, C. Human granulocyte-macrophage colony-stimulating factor is a growth factor active on a variety of cell types of nonhematopoietic origin. *Proc. Natl. Acad. Sci. USA,* **85,** 9253–9257 (1988).
22) Berdel, W. E., Danhauser-Riedl, S., Steinhauser, G. and Winton, E. F. Various human hematopoietic growth factors (interleukin-3, GM-CSF, G-CSF) stimulate clonal growth of nonhematopoietic tumor cells. *Blood,* **73,** 80–83 (1989).
23) Van Meir, E., Sawamura, Y., Diserens, A., Hamou, M. and de Tribolet, N. Human glioblastoma cells release interleukin 6 *in vivo* and *in vitro. Cancer Res.,* **50,** 6683–6688 (1990).
24) Watson, J. M., Sensintaffar, J. L., Berek, J. S. and Martinez-Maza, O. Constitutive production of interleukin 6 by ovarian cancer cell lines and by primary ovarian tumor cultures. *Cancer Res.,* **50,** 6959–6965 (1990).
25) Bunn, P. A., Jr. and Ridgway, E. C. Neoplastic syndromes. *In* "Cancer Principles and Practice of Oncology," ed. V. T. Devita, S. Hellman and S. A. Rosenberg, pp. 1896–1940 (1989). J. B. Lippincott Co., Philadelphia.
26) Sato, K., Fujii, Y., Kakiuchi, T., Kasono, K., Imamura, H., Kondo, Y., Mano, H., Okabe, T., Asano, S., Takaku, F., Tsushima, T. and Shizume, K. Paraneoplastic syndrome of hypercalcemia and leukocytosis caused by squamous carcinoma cells (T3M-1) producing parathyroid-related protein, interleukin 1α, and granulocyte colony-stimulating factor. *Cancer Res.,* **49,** 4740–4746 (1989).
27) Orino, E., Sone, S., Nii, A. and Ogura, T. IL-4 upregulates IL-1 receptor antagonist gene expression and its production in human blood monocytes. *J. Immunol.,* **149,** 925–931 (1992).
28) Nii, A., Sone, S., Orino, E. and Ogura, T. Induction of a 26-kDa membrane-form tumor necrosis factor (TNF)-α in human alveolar macrophages. *J. Leukocyte Biol.,* **53,** 29–36 (1993).
29) Yanagawa, H., Sone, S., Munekata, M., Atagi, S., Nii, A. and Ogura, T. IL-6 in malignant pleural effusions and its augmentation by intrapleural instillation of IL-2. *Clin. Exp. Immunol.,* **88,** 207–212 (1992).
30) Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.,* **162,** 156–159 (1987).
31) Feinberg, A. P. and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.,* **132,** 6–13 (1983).
32) Takagi, S., Chen, K., Schwarz, R., Iwatsuki, S., Herberman, R. B. and Whiteside, T. L. Functional and phenotypic analysis of tumor-infiltrating lymphocytes isolated from human primary and metastatic liver tumors and cultured in recombinant interleukin-2. *Cancer,* **63,** 102–111 (1989).
33) Kabawat, S. E., Bast, R. C., Jr., Welch, W. R., Knapp, R. C. and Bhan, A. K. Expression of major histocompatibility antigens and nature of inflammatory cellular infiltrate in ovarian neoplasms. *Int. J. Cancer,* **32,** 547–554 (1983).
34) Munker, R., Gasson, J., Ogawa, M. and Koeffler, H. P. Recombinant human TNF induces production of granulocyte-monocyte colony stimulating factor. *Nature,* **323,** 79–82 (1986).
35) Matsushima, K., Karsen, C. G., DuBois, G. C. and Oppenheim, J. J. Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line. *J. Exp. Med.,* **169,** 1485–1490 (1989).

36) Zachariae, C. O. C., Anderson, A. O., Thompson, H. L., Appella, E., Mantovani, A., Oppenheim, J. J. and Matsushima, K. Properties of monocyte chemotactic and activating factor (MCAF) purified from a human fibrosarcoma cell line. *J. Exp. Med.*, **171**, 2177–2182 (1990).
37) Kaushansky, K., Lin, N. and Adamson, J. W. Interleukin 1 stimulates fibroblasts to synthesize granulocyte-macrophage and granulocyte colony-stimulating factors: mechanism for the hematopoietic response to inflammation. *J. Clin. Invest.*, **81**, 92–97 (1988).
38) Zucali, J. R., Broxmeyer, H. E., Gross, M. A. and Dinarello, C. A. Recombinant human tumor necrosis factors α and β stimulate fibroblasts to produce hemopoietic growth factors *in vitro*. *J. Immunol.*, **140**, 840–844 (1988).
39) Broudy, V. C., Kaushansky, K., Segal, G. M., Harlan, J. M. and Adamson, J. W. Tumor necrosis factor type α stimulates human endothelial cells to produce granulocyte/macrophage colony-stimulating factor. *Proc. Natl. Acad. Sci. USA*, **83**, 7467–7471 (1986).