Pergamon

European Journal of Cancer Vol. 30A, No. 14, pp. 2130–2136, 1994
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0959-8049/94 $7.00+0.00

0959-8049(94)00347-5

Colony-stimulating Factor-producing Tumours: Production of Granulocyte Colony-stimulating Factor and Interleukin-6 is Secondary to Interleukin-1 Production

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We have reported that tumours producing colony-stimulating factor (CSF) also secrete interleukin-1 (IL-1) and IL-6. In the present study, we characterised the role played by IL-1 in CSF production. IL-1 receptor antagonist inhibited the production of granulocyte (G)-CSF and IL-6 by approximately 90% in CSF-producing human lung carcinoma cell lines. Similar results were obtained with hydrocortisone, which suppresses IL-1 gene expression. In contrast, 15 non-CSF-producing human lung carcinoma cell lines did not show detectable IL-1 production, although seven of them were induced to produce G-CSF and IL-6 by exogenous IL-1α. Cell lines that responded to IL-1α, including the CSF-producing lines, expressed receptors for IL-1α. These results indicate that CSF-producing tumours can be characterised by their constitutive IL-1 production, IL-1 receptor expression and IL-1-dependent excess production of G-CSF and IL-6. Furthermore, transcription factor(s) may be involved in the abnormal IL-1α production.

Key words: CSF-producing tumour, lung carcinoma cell line, G-CSF, IL-6, IL-1 receptor, IL-1 receptor antagonist, transcription factor

Eur J Cancer, Vol. 30A, No. 14, pp. 2130–2136, 1994

INTRODUCTION

COLONY-STIMULATING FACTOR (CSF)-producing tumours are characterised by the production of CSF by tumour cells, and marked leucocytosis in the tumour-bearing patients. We have previously demonstrated that CSF-producing tumours are multi-cytokine-producing tumours which secrete interleukin-1 (IL-1) and IL-6, in addition to CSFs [1–3]. Furthermore, we have also observed the inhibitory effect of anti-IL-1α antiserum on the production of granulocyte (G)-CSF and IL-6 [3]. Consequently, the question arose whether the IL-1 production is the primary event in the abnormal production of these cytokines. In the present study, to clarify whether or not the production of G-CSF and IL-6 is secondary to that of IL-1, we attempted to block the action of IL-1 and to inhibit IL-1 gene expression in cell lines derived from tumours that produce these three cytokines. For this purpose, we added recombinant IL-1 receptor antagonist (IL-1ra), which inhibits the binding of both IL-1α and IL-1β to IL-1 receptors [4–6], or hydrocortisone (HC), which has been reported to inhibit IL-1 gene expression in normal monocytes [7], to cultures of CSF-producing cell lines and examined the changes in G-CSF and IL-6 production.

As approximately 40% of CSF-producing tumours are lung carcinomas [8]; we examined 15 non-CSF-producing human lung carcinoma cell lines in terms of their potential for IL-1 production and IL-1α receptor expression in comparison with three CSF-producing lines. These studies revealed the characteristic features of CSF-producing tumours based on their IL-1 production, IL-1 receptor expression and dependence on IL-1 for the production of G-CSF and IL-6. We also investigated the cause of the abnormal IL-1 production in CSF-producing tumours.

MATERIALS AND METHODS

Cell lines

Eighteen cell lines from human non-small cell lung carcinomas were examined. KHC287 [1–3, 9] and Lu-Y1 [9] were established in our laboratory. PC-3 [10], A549 [11], Lu99 [12], ABC-1 [13], Lc-1sq [14], LK-2, RERF-LC-MS, RERF-LC-OK and VMRC-LCD were provided by the Japanese Cancer Research

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Revised 8 July 1994; accepted 16 July 1994.

Resources Bank. HLC-1 [15], SQ-5 and HL111783 were provided by Riken Cell Bank (Tsukuba, Japan). Sq-19, 86-2 [16], LK-79 and 11-18 were provided by the Cancer Cell Repository (Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai, Japan). Of the 18 cell lines, KHC287, Lu-Y1 and Lu-99 produce CSF. KHC287, Lu99 and 86-2 were from large cell carcinomas; Lu-Y1, A549, RERF-LC-MS, RERF-LC-OK, PC-3, 11-18, ABC-1, HLC-1 and VMRC-LCD were from adenocarcinomas and Sq-19, LK-2, SQ-5 and Lc-1sq were from squamous cell carcinomas. The histological subtypes of original tumours of LK-79 and HL-111783 were not available.

Cell culture

A549 and RERF-LC-MS cells were cultured in modified Eagle's medium (MEM) (Nissui, Tokyo, Japan) supplemented with 1% non-essential amino acid (Gibco, Grand Island, New York, U.S.A.) and 10% fetal calf serum (FCS) (Gibco). ABC-1, RERF-LC-OK and VMRC-LCD cells were cultured in MEM supplemented with 1% non-essential amino acids, 4 mM HEPES (Sigma, St Louis, Missouri, U.S.A.), 10% FCS, 2 mM L-Gln and 0.18% $NaHCO_3$. Cells from the remaining 13 cell lines were cultured in RPMI 1640 (Nissui) with 10% FCS. Cells were cultured at an initial cell density of 5×10^5/ml, and after 48–72 h, when the cells were subconfluent, culture supernatants were collected and kept frozen (−20°C) until examination.

Human recombinant cytokines

IL-1α and IL-1ra were kindly provided by Dainippon Pharmaceutical Co. (Osaka, Japan) and Otsuka Pharmaceutical Co. (Tokushima, Japan), respectively.

Enzyme-linked immunosorbent assay (ELISA)

G-CSF concentration was measured by ELISA, as described previously [17], the lower limit of detection for G-CSF being 30 pg/ml. IL-1α and IL-1β concentrations were measured with ELISA kits purchased from Otsuka Pharmaceutical Co. and IL-6 was measured with an ELISA kit purchased from Research and Diagnostics System (Minneapolis, Minnesota, U.S.A.). The lower limits of detection for IL-1α, IL-1β and IL-6 were 8, 20 and 5 pg/ml, respectively. IL-1ra concentration was measured by ELISA as described previously [18], the lower limit of detection for IL-1ra being 200 pg/ml. All the cytokine assays were performed in triplicate.

Northern blot analysis

One hundred nanograms per millilitre of IL-1ra were added to the culture of KHC287 cells on day 0, and RNA was extracted at 48 h of culture. One hundred units per millilitre of IL-1α (4 ng/ml IL-1α) were added to the culture of KHC287 or A549 cells on day 0 and RNA was extracted at 48 h. Northern blot analysis was performed as described previously [1]. The cDNA probes for IL-1α, IL-6 and G-CSF were kindly provided by Dainippon Pharmaceutical Co., Dr T. Hirano (Osaka University, Japan) and Chugai Pharmaceutical Co., respectively.

IL-1α binding assay

Cells from each line were allowed to attach to a 24-well culture plate (Corning, New York, U.S.A.) at an initial cell density of 4×10^5 per well in 2 ml of respective complete medium. After 48 h, the culture medium was removed and the cells were washed three times with ice-cold medium. The cells were then treated with 50 mM glycine hydrochloride (Nakalai Tesque, Kyoto, Japan) (pH 3.0, distilled water with 150 mM NaCl) for 60 s at 4°C to detach the ligands from the IL-1α receptor. After washing twice with ice-cold medium, the cells were incubated for 3 h at 4°C in a binding buffer (RPMI 1640 with 25 mM HEPES and 10 mg/ml bovine serum albumin) with varying concentrations of 3-[^{125}I]iodotyrosyl IL-1α (human, recombinant) (Amersham), in the presence or absence of a 400-fold excess of unlabelled IL-1α, in a final volume of 500 μl. Thereafter, the binding buffer was removed and the cells were rinsed twice with ice-cold binding buffer and detached from the

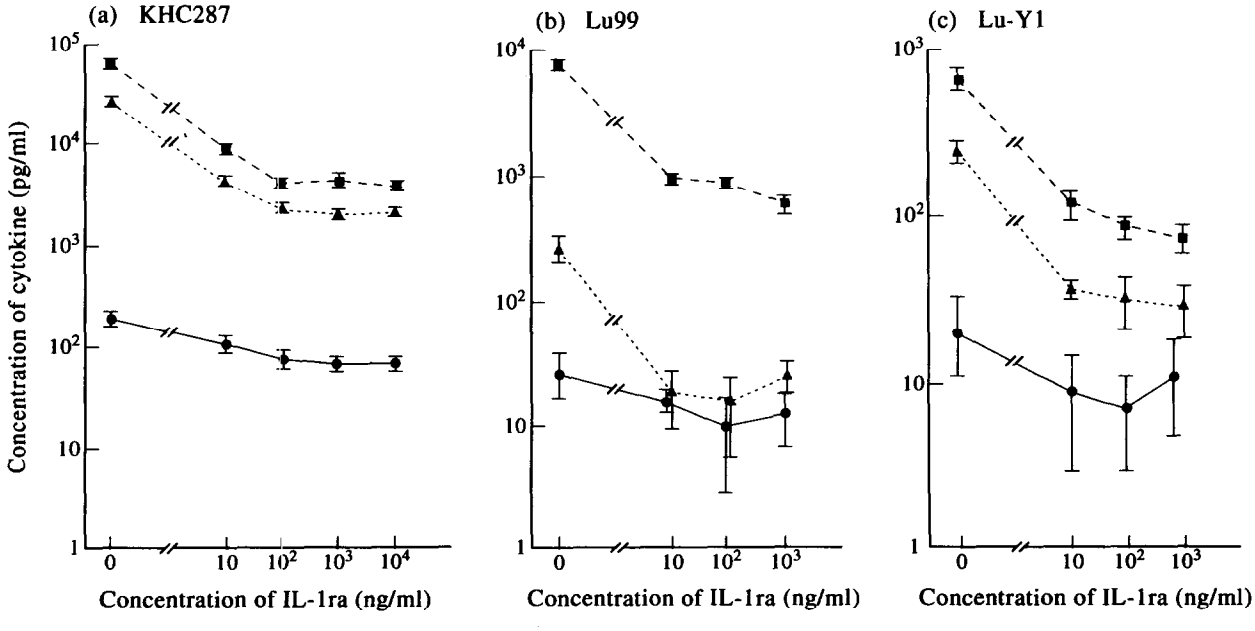

Figure 1. Effects of IL-1 receptor antagonist (IL-1ra) on cytokine production by CSF-producing tumour cell lines. Values for cytokine concentration are expressed as mean ± S.E. (n= 3). ■ — — ■, G-CSF; ▲ - - - - ▲, IL-6; ● —— ●, IL-1α.

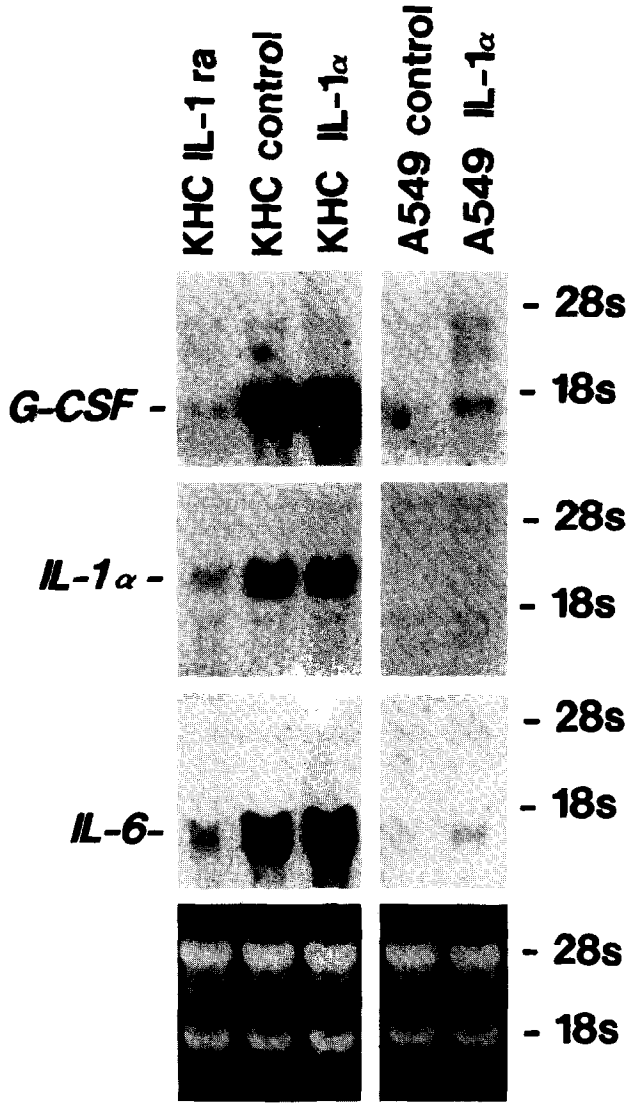

Figure 2. Northern blot analysis of RNA from KHC287 and A549 cells. The amount of RNA electrophoresed was normalised by determining the ethidium bromide profile after electrophoresis.

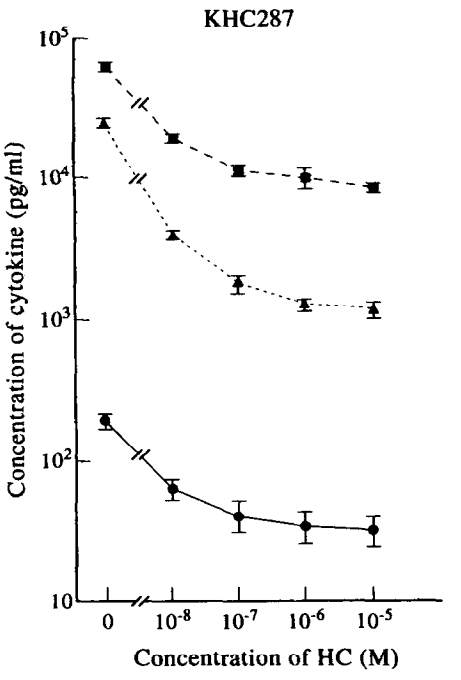

Figure 3. Effects of hydrocortisone (HC) on cytokine production by KHC287 cells. Values for cytokine concentration are expressed as mean ± S.E. (n=3). ■ — — ■, G-CSF; ▲ - - - - ▲, IL-6; ● —— ●, IL-1α.

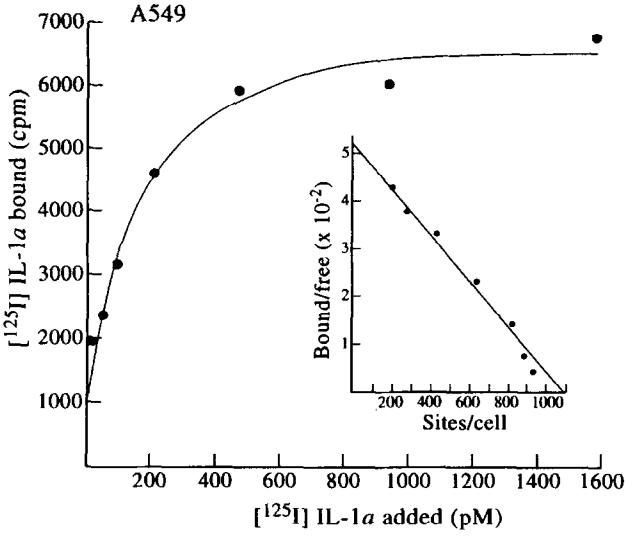

Figure 4. Specific binding of [^{125}I] IL-1α as a function of its concentration. [^{125}I] IL-1α binding to A549 cells was determined at the indicated concentrations at 4°C. Data represent specific IL-1α binding. The inset shows Scatchard analysis of the binding data.

culture plates, using 0.1% trypsin EDTA (Gibco). Both the entire cell suspension and binding buffer were individually transferred to counting vials and the radioactivity was determined in a γ-counter. To evaluate cell numbers, additional plates for each line examined were cultured. Cells were detached using trypsin EDTA and the cell number counted with a haemocytometer. Cell-bound radioactivity in the presence of unlabelled IL-1α gave the non-specific binding. Specific binding was calculated by subtracting this value from the total bound radioactivity in the absence of unlabelled IL-1α. Receptor number and binding affinities were derived by Scatchard analysis. All the binding assays were performed in triplicate.

Chloramphenicol acetyltransferase (CAT) assay

The 400-bp upstream region of the IL-1α gene was amplified by polymerase chain reaction (PCR) from peripheral white blood cell DNA obtained from a healthy person. The pair of primers used was, sense primer: CAGCCAGAACACAACTACAG and antisense primer: AGCCAGAGAGGGAGTCATTT. The amplified gene was ligated into the Hind III site of the pSV00CAT plasmid [19] with Hind III linkers. The plasmid containing the IL-1α upstream region in the direction of the sense strand was designated ILV00CATS, and that in the direction of the anti-sense strand, ILV00CATAS.

DNA of the CAT and the RSV-galactosidase (β-gal) genes was transfected by electroporation and CAT and β-gal activity was measured and corrected for the efficiency of the transfection by β-gal assay described previously [1].

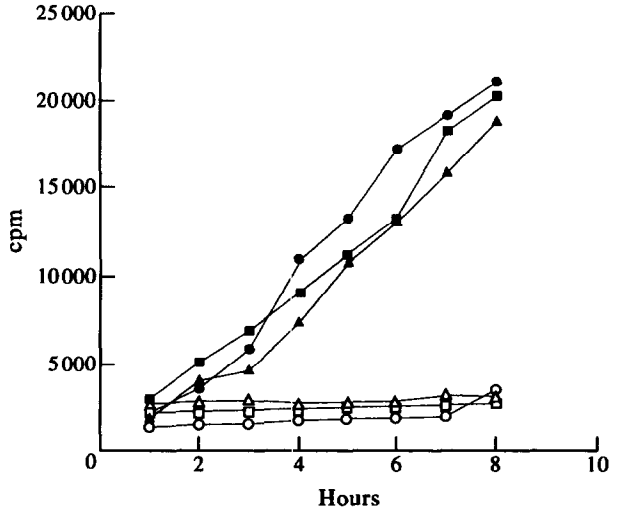

Figure 5. CAT activity in KHC287, LK-2, HLC-1 and VMRC-LCD cells transfected with ILV00CAT. ● —— ●, KHC287 cells; ■ —— ■, KHC287; cells with IL-1α (100 U/ml); ▲ —— ▲, KHC287 cells with IL-1ra (100 ng/ml); ○ —— ○ LK-2 cells; □ —— □ HLC-1 cells; △ —— △ VMRC-LCD cells.

RESULTS

Effects of IL-1ra and HC on cytokine production by CSF-producing cell lines

As shown in Figure 1a, IL-1ra inhibited the production of IL-1α, G-CSF and IL-6 by KHC287 cells in a dose-dependent fashion. IL-1α production was moderately inhibited (62% reduction), while the inhibition of G-CSF and IL-6 production was marked; the reduction was 94 and 91%, respectively, at 100 ng/ml of added IL-1ra. The plateau of inhibition of each cytokine production occurred at 100 ng/ml of added IL-1ra. These results were confirmed at the mRNA level. Figure 2 shows the northern blot analysis of RNA from KHC287 cells. The expression of the IL-1α, G-CSF and IL-6 genes was markedly diminished by the addition of 100 ng/ml of IL-1ra. A similar effect of IL-1ra was observed in the other CSF-producing human lung carcinoma cell lines (Lu99, Lu-Y1) examined (Figure 1b, c). In this experiment, the plateau of inhibition of these three cytokines was observed at 10 ng/ml of added IL-1ra. These experiments were repeated three times and representative results are shown in Figures 1 and 2. IL-1β was detectable only in the culture supernatant of KHC287 cells (71 pg/ml). IL-1ra was not detected in the culture supernatant of control cultures of KHC287, Lu99 and Lu-Y1 cells. IL-1ra did not affect the proliferation of these three cell lines, as evaluated by cell counting (data not shown).

We then examined the effects of HC on cytokine production by KHC287 cells (Figure 3). The reduction of IL-1α (79%) and G-CSF (83%) production by HC (10^{-7}) was similar, and the reduction of IL-6 production (93%) was more prominent ($P<0.02$, G-CSF versus IL-6 by χ^2 test). The plateau of inhibition of each cytokine production occurred at 10^{-7} M of added HC. HC did not affect the proliferation of KHC287 cells. This experiment was repeated three times, and similar results were obtained on each occasion.

Cytokine production by human lung carcinoma cell lines with or without exogenous IL-1α

Table 1 shows the production of IL-1α, G-CSF and IL-6 by the three CSF-producing and 15 non-CSF-producing lung carcinoma cell lines with or without exogenous IL-1α stimulation. This experiment was repeated twice and similar results were obtained on each occasion. These 18 lines could be classified into three categories based on the production of the three cytokines and on their responsiveness to exogenous IL-1α. Group 1, CSF-producing cell lines, showed augmentation of G-CSF and IL-6 production by exogenous IL-1α, group 2 showed no production of these three cytokines (except for low level IL-6 production in two lines), but G-CSF and IL-6 production was induced by exogenous IL-1α and group 3 showed neither production of these cytokines nor responsiveness to exogenous IL-1α. IL-1β was not detected in any cell lines except for KHC287. The manner of cytokine production or responsiveness to exogenous IL-1α was not characteristic of any particular histological subtype. Exogenous IL-1α did not affect the cell growth of any cell lines.

We confirmed these results at the mRNA level. As shown in Figure 2, the levels of G-CSF and IL-6 mRNA expression in KHC cells from group 1 and A549 cells from group 2 were clearly enhanced by exogenous IL-1α. By northern blot analysis, LK-2 (group 3) did not express any mRNA of these cytokines, even when the cells were stimulated by exogenous IL-1α (data not shown).

Laboratory and clinical findings of patients from whom lung carcinoma cell lines were derived

As shown in Table 2, marked leucocytosis was observed in the patients from whom the group 1 cell lines were established. Leucocytosis was not observed in any group 2 patients, irrespective of G-CSF production in response to exogenous IL-1α. Levels of C-reactive protein (CRP) were generally proportional to the amount of IL-6 produced by the respective cell lines without exogenous IL-1α. Fever of unknown origin was observed only in the patient from whom KHC287 was derived, the cell line which produced the greatest amount of IL-1α.

Expression of IL-1α receptor in lung carcinoma cells

As shown in Table 3, cells classified in groups 1 and 2 expressed IL-1α receptors. Representative binding capacity to IL-1α in a cell line (A549) is shown in Figure 4. Alternatively, four of five cell lines examined in group 3 did not express IL-1α receptors. There were no significant differences in binding sites or affinities between groups 1 and 2, as evaluated by Anova analysis. This experiment was repeated three times and similar results were obtained on each occasion.

CAT assay

The plasmid containing 400 bp of the upstream region of the IL-1α gene in the direction of the sense strand (ILV00CATS) produced CAT activity in KHC287, in group 1, but did not have this effect in LK-2, HLC-1 and VMRC-LCD cells, in group 3 (Figure 5). The addition of IL-1α or IL-1ra to the KHC287 culture did not change the level of CAT activity. ILV00CATAS, the pSV00CAT ligated with the IL-1α upstream region (400 bp) in the anti-sense direction, did not show any detectable CAT activity in any of the four cell lines examined (data not shown).

DISCUSSION

In the present study, we first examined whether the production of G-CSF or IL-6 is secondary to the IL-1α production (endogenous IL-1) in CSF-producing human lung carcinoma cell lines because, in our previous study, we observed the suppression of G-CSF and IL-6 production by anti-IL-1α anti-

Table 1. Classification of human lung carcinoma cell lines based on their ability to produce G-CSF and IL-6 in relation to their IL-1α production and responsiveness to exogenous IL-1α

Classification	Cell line	Exogenous IL-1α added (U/ml)	Cytokine concentration in the culture supernatant: IL-1α (pg/ml)	G-CSF (pg/ml)	IL-6 (pg/ml)
Group 1	KHC287	0	181 ± 33	61256 ± 2050	22100 ± 3826
		100	—	183617 ± 15257	73253 ± 3557
	Lu99	0	21 ± 10	8397 ± 353	233 ± 17
		100	—	17781 ± 1250	672 ± 156
	Lu-Y1	0	24 ± 3	641 ± 39	231 ± 66
		100	—	12802 ± 1950	1017 ± 115
Group 2	A549	0	<8	<30	<5
		100	—	3199 ± 243	226 ± 23
	RERF-LC-MS	0	<8	<30	106 ± 17
		100	—	2462 ± 155	1508 ± 201
	RERF-LC-OK	0	<8	<30	432 ± 88
		100	—	3378 ± 287	5406 ± 627
	PC-3	0	<8	<30	<5
		100	—	228 ± 31	191 ± 26
	Sq-19	0	<8	<30	<5
		100	—	203 ± 16	180 ± 69
	86-2	0	<8	<30	<5
		100	—	264 ± 31	11 ± 4
	11-18	0	<8	<30	<5
		100	—	<30	149 ± 17
Group 3	ABC-1, LK-2, HLC-1, VMRC-LCD, LK-79, HL-111783, SQ-5, Lc-1sq	0	<8	<30	<5
		100	—	<30	<5

Values for cytokine concentration are expressed as mean ± S.E. (n=3). One hundred units of IL-1α corresponds to 4 ng IL-1α protein. No data (—) indicates that IL-1α measurement was not done.

serum in KHC287, CHU2 and T24 cells [3]. In the present study, we used IL-1ra and HC. The specificity of IL-1ra for IL-1 activity appears to be established; to our knowledge, interaction between IL-1ra and other cytokines has never been reported. Furthermore, it is unlikely that cytokines other than IL-1 and IL-1ra bind to IL-1 receptors because both IL-1 receptor type I and type II belong to an immunoglobulin superfamily [20, 21], unlike receptors for other cytokines [22]. Regarding HC, a glucocorticoid responsive element (GRE) has been reported to exist in intron 6 of the IL-1α gene [23] and intron 5 of the IL-1β gene [24].

IL-1ra suppressed G-CSF and IL-6 production by approximately 90% in the three CSF-producing cell lines examined. Therefore, it could be concluded that G-CSF and IL-6 production is largely dependent on endogenous IL-1 (secondary to IL-1) in these three cell lines. The plateau of suppression occurred at 10 ng/ml (Lu99, Lu-Y1) and 100 ng/ml (KHC287) of added IL-1ra. The amount of IL-1ra that produced the plateau value of suppression corresponded approximately to 500-fold the amount of IL-1α produced by the KHC287 cell line. It has been reported that 50% of IL-1 binding to its receptors is blocked by equimolar IL-1ra; however, to block the biological activities of IL-1, a 10- to 100-fold molar excess of IL-1ra is required [25]. Because KHC287 cells produce almost equal amounts of IL-1α and IL-β, the requirement of 500-fold IL-1ra may be reasonable. In addition, IL-1ra, IL-1α and IL-1β are almost equal in molecular weight; IL-1ra 17.3, IL-1α 18.1 and IL-1β 17.5 kDa. In Lu99 and Lu-Y1, 500-fold IL-1ra was also required to obtain maximal inhibition regardless of no IL-1β production, although the precise reason for this is unclear. Interestingly, IL-1α production was moderately reduced by the addition of IL-1ra in these three cell lines. It has been reported that IL-1 itself induces IL-1 gene expression [26]. Therefore, it is possible that IL-1ra suppressed, to some extent, the loop of autostimulation by IL-1 in these three cell lines.

The addition of HC to the culture of KHC287 cells suppressed the production of IL-1α, G-CSF and IL-6. This may also support the dependence of G-CSF and IL-6 production on endogenous IL-1. However, the suppression of IL-6 production was more remarkable than that of IL-1α or G-CSF. It is probable that IL-6 production was reduced not only by diminished IL-1α stimulation, but also by the direct inhibition of IL-6 gene expression by HC, since the GRE has been identified in the promoter region of the IL-6 gene [27, 28].

We examined the 15 other cell lines to determine whether every tumour can produce G-CSF or IL-6 in response to IL-1

Table 2. Clinical and laboratory findings of patients from whom lung carcinoma cell lines were derived

	Cell line	Peak WBC count (/ml)	Neutrophils (%)	Peak CRP level (mg/dl)
Group 1	KHC287	123 200	94	27.0
	Lu99	86 400	96	9.2
	Lu-Y1	96 400	88	4.2
Group 2	A549	—	—	—
	RERF-LC-MS	8600	72	4.6
	RERF-LC-OK	3800	56	8.2
	PC-3	—	—	—
	Sq-19	8400	61	—
	86-2	6100	61	—
	11-18	8700	65	0.3
Group 3	ABC-1	8100	50	2.4
	LK-2	4000	40	0.3
	HLC-1	6750	—	4.6
	VMRC-LCD	—	—	—
	LK-79	9300	68	—
	HL-111783	—	—	—
	SQ-5	—	—	—
	Lc-1sq	5900	—	4.6

No data (—) indicates that clinical or laboratory data were not available.

compared with CSF-producing lines. Of the 15 cell lines examined, seven lines produced G-CSF or IL-6 in response to exogenous IL-1α. However, the levels of G-CSF or IL-6 production were generally low, and we could not detect the production of IL-1α or IL-1β in any of the 15 cell lines, as evaluated by ELISA or northern blotting. CSF-producing tumours, therefore, in terms of exhibiting constitutive IL-1 production and continuous stimulation of G-CSF and IL-6 production by endogenous IL-1, appear to be distinctly different from these tumours. Some group 2 cell lines produced G-CSF at levels of more than 1000 pg/ml in response to IL-1α; however, patients from whom these lines were established did not show any clinical features of CSF-producing tumours, such as leucocytosis, fever or high CRP levels [9].

Table 3. Binding capacity of lung carcinoma cell lines to IL-1α

Group	Cell line	Binding sites / cell	Affinity (10^{-12}M)
Group 1	KHC287	290	33
	Lu-Y1	160	52
Group 2	A549	1075	98
	RERF-LC-MS	3200	1400
	PC-3	90	6
		925	150
	Sq-19	350	260
	86-2	95	100
	11-18	400	300
Group 3	ABC-1	—	—
	LK-2	—	—
	VMRC-LCD	225	45
	LK-79	—	—
	HL-111783	—	—

No data (—) indicates no specific binding to IL-α.
Only PC-3 cells had both high and low affinity binding sites for IL-1α.

To clarify differences in the amounts of G-CSF and IL-6 produced in response to IL-1, we examined the binding capacity of lung carcinoma cells to IL-1α. All group 1 and 2 carcinoma cells examined had receptors for IL-1α, although there were no significant differences in binding sites or affinities between groups 1 and 2. It has been reported that signals for the biological activities of IL-1 can be transduced when part of IL-1 receptors are bound to their ligands [29]. This feature could account for the lack of differences between groups 1 and 2. As another possibility, chronic exposure to IL-1 in group 1 carcinoma cells may have downregulated the IL-1 receptor expression by destabilising its mRNA [30] or internalisation of IL-1 receptors [31]. VMRC-LCD of group 3 failed to respond to exogenous IL-1α, regardless of its receptor expression. Like THP-1 cells [32], VMRC-LCD cells might have expressed only IL-1 receptor type II, which is not supposed to be a signalling receptor. Nevertheless, IL-1 receptors appear to be very important when a tumour initiates IL-1 production and subsequently becomes CSF-producing. For instance, some group 2 cell lines could be CSF-producing tumours if they acquired the ability to produce large amounts of IL-1.

Finally, we examined the mechanism responsible for the abnormal expression of the IL-1 gene in KHC287 cells. Southern blot analysis showed no major rearrangement or amplification of the IL-1α gene (data not shown). The 400-bp upstream region of the IL-1α gene contains TATA-like sequences [20], a tetradecanoyl phorbol acetate responsive element and a GRE-like sequence (our sequence analysis). CAT activity was detected in KHC287 cells but not in group 3 cell lines examined when the CAT gene containing this 400-bp upstream region was transfected to these cells. These results suggest that transcription factor(s) binding to the region is one of the causes which enhance

the transcription of the IL-1α gene, however, we did not show direct evidence for the binding of any factors. Exogenous IL-1α or IL-1ra did not change the level of CAT activity. There may be more complicated factor(s), including a *cis*-acting mechanism, involved in the regulation of IL-1 gene expression [23], and determination of the regulatory elements in the upstream region of the IL-1 gene is required.

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Acknowledgements—This work was supported in part by a Grant-in-Aid for Scientific Research 04671521 from the Ministry of Education, Science and Culture of Japan, Dr Simizu Foundation for the Promotion of Immunology Research Grant for 1992 and Dr Sasaki Foundation for the Promotion of Leukemia Research.