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Expression of Interleukin-6 and Its Effect on the Cell Growth of Gastric Carcinoma Cell Lines

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The expression and the effect of IL-6 were examined in human gastric carcinoma cell lines to determine whether IL-6 serves as a growth stimulator. The expression of IL-6 mRNA was detected in three (TMK-1, MKN-1, MKN-7) of 8 gastric carcinoma cell lines. All three cell lines secreted IL-6 into the culture fluid, in large amounts in the cases of MKN-1 and MKN-7 cells. Scatchard plot analysis of IL-6 binding revealed that MKN-1 and MKN-7 cells had both high- and low-affinity receptors. Cell growth of MKN-1 and MKN-7 cells was stimulated by IL-6, while anti-IL-6 antibody inhibited growth. The expression of IL-1α mRNA by these three cell lines was induced by IL-6. IL-1α increased the expression of mRNA for IL-6 by TMK-1 cells. These findings indicate that IL-6 induced by IL-1α is an autocrine growth factor for some gastric carcinomas.

Key words: IL-6 — Gastric carcinoma cell line — Autocrine — Cytokine

Some carcinomas express a variety of growth factors/cytokines and their receptors to form multi-autocrine loops by which their growth is regulated.[1] The growth factors/cytokines also mediate the interaction between carcinoma cells and stromal cells in the microenvironment around the carcinoma tissue. Gastrointestinal carcinomas express multiple growth factors, such as EGF, TGF-α, TGF-β and FGF, that stimulate cell growth in an autocrine manner, while they function as paracrine factors for stromal fibroblasts and endothelial cells to promote fibrosis and angiogenesis.[2–4] We have reported that IL-1α is secreted by gastric carcinoma cells and stimulates their growth *in vitro*.[5]

IL-6 is a cytokine with a multiplicity of functions that play a central role in both the cellular and humoral immune response.[6–8] There is increasing evidence that IL-6 deregulation is involved in a variety of diseases, including malignancies.[9] IL-6 can enhance or inhibit proliferation of carcinoma cells.[10–12] For instance, IL-6 stimulates the growth of hematological malignancies[9, 13, 14] and renal cell carcinoma.[11] In certain of these tumors, IL-6 acts in an autocrine fashion. However, no study has been conducted to clarify the role of IL-6 in gastric carcinomas.

In the present study, therefore, we examined the expression of IL-6 in several gastric carcinoma cell lines. Furthermore, the effect of IL-6 on cell proliferation and the expression of IL-1α gene were studied.

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The abbreviations used are: EGF, epidermal growth factor; TGF, transforming growth factor; FGF, fibroblast growth factor; IL, interleukin; FBS, fetal bovine serum; Ab, antibody; BSA, bovine serum albumin.

MATERIALS AND METHODS

Cell culture Eight cell lines derived from human gastric carcinomas were used. The TMK-1 cell line was established from poorly differentiated adenocarcinoma in our laboratory.[15] Five gastric carcinoma cell lines of the MKN series (MKN-1, adenosquamous cell carcinoma; MKN-7, MKN-28 and MKN-74, well differentiated adenocarcinoma; MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr. T. Suzuki (Fukushima Medical University, Fukushima). The KATO-III and HSC-39 cell lines which were established from signet-ring-cell carcinoma were kindly provided by Dr. M. Sekiguchi (The University of Tokyo, Tokyo) and Dr. K. Yanagihara (Hiroshima University, Hiroshima),[16] respectively. All the cell lines were routinely maintained in RPMI-1640 (Nissui Seiyaku, Tokyo) containing 10% FBS (Whittaker, Walkersville, MA) under conditions of humidified 5% CO_2 in air at 37°C. They were grown to subconfluence in the medium described above.

Cell growth The cells were seeded in 12 well dishes (Costar, Cambridge, MA) at 1×10^4 cells per well and cultured in RPMI 1640 containing 0.5% FBS in the presence of IL-6 (10 U/ml) and/or anti-IL-6 antibody (IL-6 Ab) (1 μg/ml or 10 μg/ml). Recombinant human IL-6 and IL-6 Ab were purchased from Genzyme Corporation (Boston, MA). Normal rabbit IgG at the same concentration was used as a control for IL-6 Ab. To examine the growth stimulation by IL-6, the medium was changed every 2 days and the number of cells was counted using a Neubauer-type counting chamber every 2 days. To examine growth inhibition by IL-6 Ab, the cells were counted after treatment for 3 days.

Northern blot analysis RNA was prepared by the cesium chloride guanidine-thiocyanate method with slight modifications as described elsewhere.[17] Total RNAs or poly $(A)^+$-selected RNAs (5 μg) were electrophoresed on 1% agarose gel containing 6% formaldehyde and transferred to a nylon membrane filter. Hybridization using a ^{32}P-labeled probe and washing were performed as described previously,[18] and filters were exposed to X-ray film. The 1.3 kb human IL-6 oligonucleotide probe was purchased from Oncogene Science, Inc. (Cambridge, MA) and a β-actin probe from Oncor (Gaithersburg, MD).

Measurement of IL-6 The cells were seeded in 12-well dishes at 1×10^5 cells per well and grown to 70% confluency in RPMI 1640 containing 10% FBS. The cells were then cultured with serum-free medium for 72 h and the content of IL-6 in the conditioned medium was measured by using a human IL-6 immunoassay kit (R&D Systems Ltd., Minneapolis, MN) according to manufacturer's instructions. The experiments were performed in duplicate and repeated twice.

IL-6 binding assay The cells were seeded in 6-well dishes (Falcon, Lincoln Park, NJ) at 1×10^6 cells per well. After 24 h, the cells were washed with the binding medium (RPMI 1640 containing 1% bovine serum albumin) and various dilutions of [^{125}I]-IL-6 or unlabeled IL-6 in 0.4 ml of the binding medium were added. Nonspecific binding was estimated by using 100 nM unlabeled IL-6. Binding reaction was carried out for 4 h at 4°C. Data were obtained from triplicate experiments.

Cytokine treatment of gastric carcinoma cells After 24 h of serum starvation, 10 U/ml IL-6 or 10 U/ml IL-1α was added. The cells were treated for 0 h (control), 1 h, 3 h, 12 h and 24 h, and RNAs were extracted. Recombinant human IL-1α was kindly donated by Dr. M. Yamada (Dainippon Pharm. Co., Ltd., Osaka).

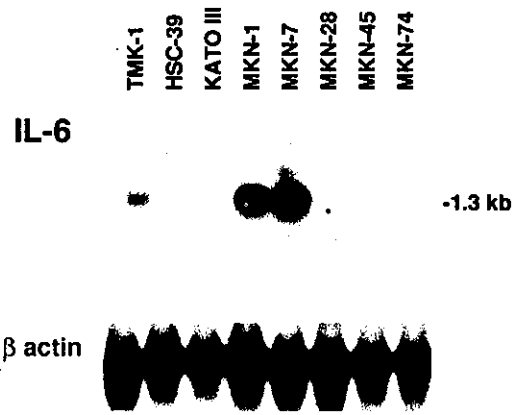

Fig. 1. Expression of IL-6 mRNA by gastric carcinoma cell lines. Five micrograms of poly(A)$^+$-selected RNA was subjected to northern blot analysis using ^{32}P-labeled IL-6 cDNA as described in "Materials and Methods." A β-actin probe was employed as an internal control.

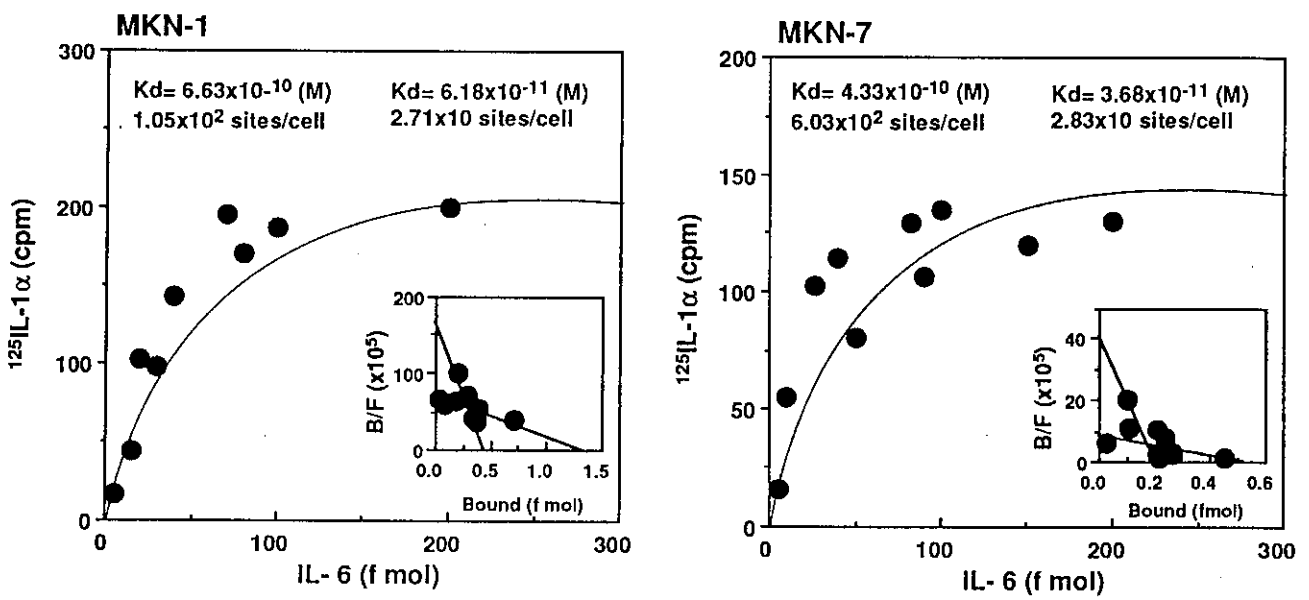

Fig. 2. Specific binding of ^{125}I-labeled IL-6 to gastric carcinoma cell lines. The nonspecific binding was defined as the portion of the binding which was displaced by 100 nM unlabeled IL-6. Points are means of triplicate measurements. Inset, Scatchard plot analysis.

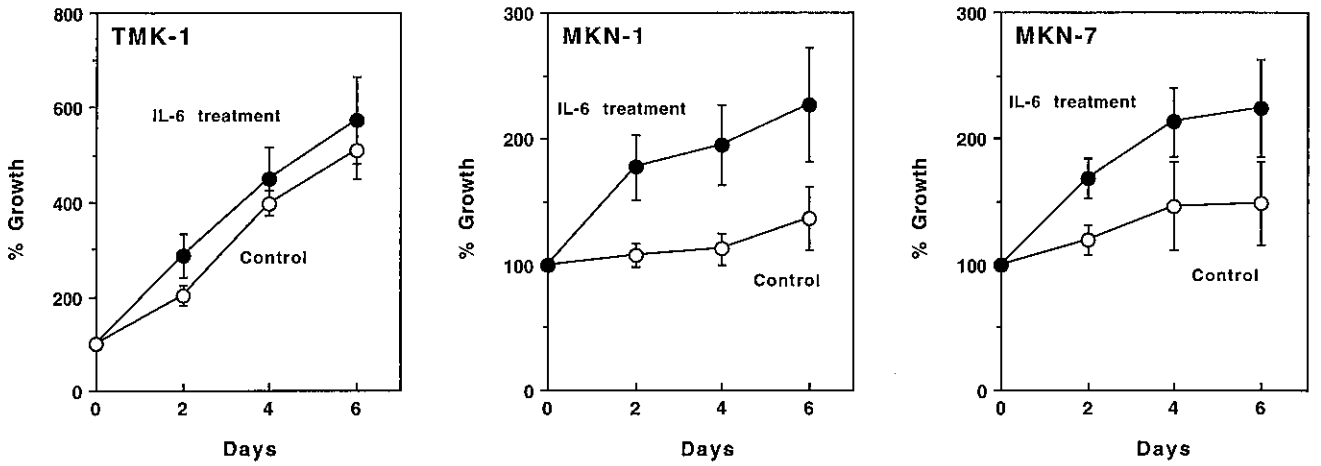

Fig. 3. Stimulatory effect of IL-6 on cell growth of gastric carcinoma cell lines. TMK-1, MKN-1 and MKN-7 cells were cultured with 0.5% FBS in the presence or absence of 10 U/ml IL-6. Points are each the average±SE of six independent experiments.

Table I. Effect of Anti-IL-6 Ab on the Cell Growth of Gastric Carcinoma Cell Lines

	% Growth[a]		
	TMK-1	MKN-1	MKN-7
Control	100	100	100
Normal IgG (10 μg/ml)	97.3±4.6	103.2±3.2	95.0±2.4
IL-6 Ab (1 μg/ml)	84.0±6.9[b]	79.6±7.2[b]	89.1±8.7
(10 μg/ml)	68.5±6.3[b]	62.7±9.4[b]	78.5±9.5[b]

a) Gastric carcinoma cell lines (TMK-1, MKN-1 and MKN-7) were cultured for 3 days in the absence (control) or presence of normal IgG (10 μg/ml) or IL-6 Ab (1 μg/ml or 10 μg/ml). The data represent the average±SE of six independent experiments.
b) The values were significantly different from that of the control as well as that of normal IgG ($P<0.01$ by Student's t test).

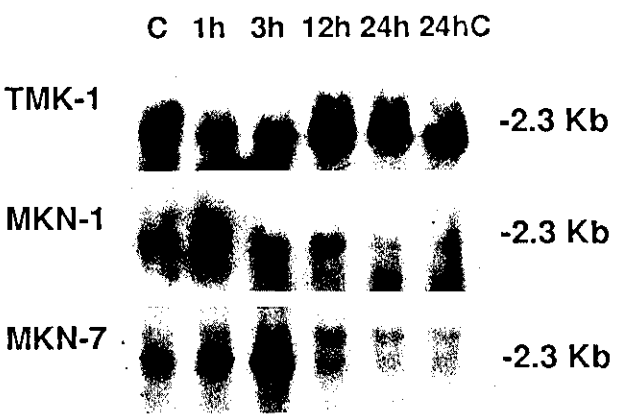

Fig. 4. Time course of the effect of IL-6 on the expression of IL-1α by gastric carcinoma cell lines. TMK-1, MKN-1 and MKN-7 cells were treated with 10 U/ml IL-6 for the periods indicated. Five micrograms of poly(A)$^+$ RNA was analyzed as in Fig. 1.

RESULTS

Expression and secretion of IL-6 by gastric carcinoma cell lines The expression of IL-6 mRNA by gastric carcinoma cell lines was examined by northern blot analysis. The IL-6 transcript of 1.3 kb was clearly detected in 3 of 8 human gastric carcinoma cell lines at various levels (Fig. 1). MKN-1 and MKN-7 cells expressed it at extremely high levels, while TMK-1 cells expressed it at a low level.

To find out whether gastric carcinoma cells secrete IL-6 protein, IL-6 contents in the culture fluid were measured by immunoassay. The amounts of IL-6 in the culture fluid of MKN-1, MKN-7, MKN-28 and TMK-1 were 3340 pg/ml, 5900 pg/ml, 37 pg/ml, and 220 pg/ml, respectively, and these values are consistent with the results of northern blotting. IL-6 was undetectable in the culture fluid of the other 4 cell lines.

Southern blot analysis revealed that neither amplification nor rearrangement was observed in any of the gastric carcinoma cell lines (data not shown).

Binding of IL-6 to gastric carcinoma cell lines The specific binding of [^{125}I]-IL-6 to the cell surface receptors

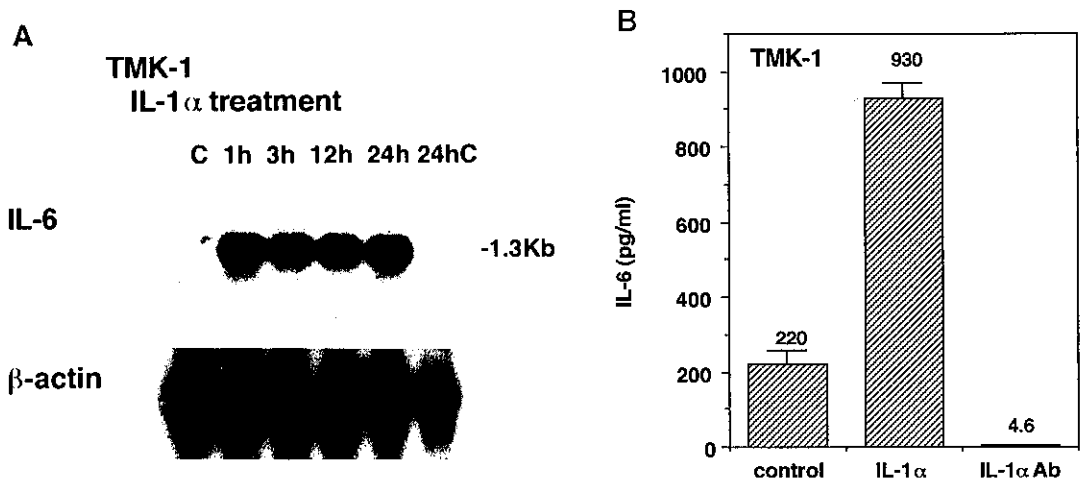

Fig. 5. A, Time course of the effect of IL-1α on the expression of IL-6 mRNA by TMK-1. The cells were treated with 10 U/ml of IL-1α for the periods indicated. B, IL-6 protein secretion by TMK-1 cells. The cells were cultured for 3 days in serum-free medium with or without IL-1α or IL-1α Ab and the content of IL-6 in the culture fluid was measured as described in "Materials and Methods." Values are each the average$\pm$SE of six independent experiments.

of MKN-1 and MKN-7 is shown in Fig. 2. The specific binding was saturable, and Scatchard plot analysis revealed that MKN-1 and MKN-7 had both high- and low-binding-affinity receptors. The apparent dissociation constants (Kd) were 6.63×10^{-10} M and 6.18×10^{-11} M for MKN-1, and 4.33×10^{-10} M and 3.68×10^{-11} M for MKN-7. The numbers of IL-6 binding sites per cell were 1.05×10^{2} and 2.71×10 in MKN-1 and 6.03×10^{2} and $2.83\times$ 10 in MKN-7, respectively. The specific binding of IL-6 to TMK-1 cells was too low to allow Scatchard plot analysis (data not shown).

Effect of IL-6 on cell growth of gastric carcinoma cell lines We next examined the effect of exogenous IL-6 on the cell growth of TMK-1, MKN-1 and MKN-7 cells. Ten U/ml of IL-6 significantly stimulated the cell growth of MKN-1 and MKN-7 cell lines ($P<0.05$), whereas no clear effect was found in TMK-1 (Fig. 3).

To examine whether IL-6 acts as an autocrine growth stimulator, we blocked the effect of endogenous IL-6 by using anti-IL-6 antibody (IL-6 Ab) and examined the cell growth. As shown in Table I, IL-6 Ab (10 μg/ml) inhibited the cell growth of MKN-1 by 37.3%, that of MKN-7 by 21.5% and that of TMK-1 by 31.5%, the inhibitory effect being significant ($P<0.01$).

Mutual induction of IL-6 and IL-1α expression We have previously reported that IL-1α induces the expression of various growth factor/receptor genes in gastric carcinoma cell lines. To determine whether IL-6 modulates the expression of IL-1α, TMK-1, MKN-1 and MKN-7 cells were treated with IL-6 and the expression of IL-1α mRNA was examined. The expression of IL-1α mRNA was induced by IL-6, and the peak of induction was observed at 12 h, 1 h and 3 h in TMK-1, MKN-1 and MKN-7 cells, respectively (Fig. 4).

We next examined the effect of IL-1α on the mRNA expression and protein secretion of IL-6 in TMK-1 cells. As shown in Fig. 5, IL-1α strongly induced IL-6 mRNA in TMK-1. Moreover, the secretion of IL-6 was significantly induced by IL-1α, while it was blocked by anti-IL-1α Abs.

DISCUSSION

In the present study, we found that IL-6 mRNA was expressed by 3 of 8 gastric carcinoma cell lines, and all of these 3 cell lines secreted IL-6 into the culture media. Both MKN-1 and MKN-7 cell lines had significant numbers of high- and low-affinity receptors for IL-6. Cell growth was stimulated by exogenous IL-6. Furthermore, anti-IL-6 Ab suppressed the cell growth of these two cell lines. These results strongly suggest that IL-6 functions as an autocrine growth stimulator for MKN-1 and MKN-7.

On the other hand, the IL-6 binding activity of TMK-1 cells was undetectable, and exogenous IL-6 did not induce the cell growth of TMK-1. However, in this cell line, anti-IL-6 Ab suppressed growth. It is possible that a small number of IL-6 receptors on the cell surface of TMK-1 is saturated by pre-existing IL-6, and exogenous IL-6 cannot stimulate the cell growth further.

IL-1 is known to induce IL-6 expression in certain cells.[19] We have already demonstrated that IL-1α acts as growth stimulator for TMK-1 and induces EGF and TGF-α. Moreover, it has been reported that cell growth of gastric carcinomas is regulated by multi-autocrine and -paracrine loops involving various growth factors such as EGF and TGF-α. The present study revealed that IL-6 induces IL-1α mRNA and *vice versa*. Therefore, IL-6 may be involved in the multiple autocrine and paracrine loops of the growth factor/cytokine system in gastric carcinomas. The stimulatory effect of IL-6 on the cell growth may be brought about not only through a direct pathway, but also indirectly via a paracrine pathway with IL-1α and other cytokines.

IL-6 is a pleiotropic cytokine involved in various physiological processes, such as host defense, bone metabolism, and acute-phase response.[20,21] Overproduction of IL-6 is associated with several pathological conditions, including autoimmune disorders and postmenopausal osteoporosis.[22-24] IL-6 may also be important in the pathogenesis of human myeloma,[14] chronic lymphocytic leukemia of B-cell origin,[25] cervical carcinoma[26] and prostate cancer,[27,28] where it may function as an autocrine or paracrine growth factor. Furthermore, IL-6 has been suggested to function as an cell adhesion molecule for some carcinoma cells and to affect their metastasis.[29] Clinical trials with monoclonal antibodies to human IL-6 in terminal multiple myeloma patients have provided evidence that *in vivo* neutralization of the cytokine is therapeutically effective.[30,31] In this study, we demonstrated that IL-6 is an autocrine growth stimulator for some gastric carcinoma cells. IL-6 may be a suitable target for a new therapeutic approach to gastric cancer.

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