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Elevated Expression of Interleukin-6 Correlates with Malignant Progression of Human Gastric Cancer

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ABSTRACT

Interleukin-6 (IL-6) is a pleiotropic cytokine that play a central role in immune responses and inflammatory reactions. In addition, IL-6 has been implicated in the pathology of a variety of malignant tumors. To explore the possible implication of *IL-6* in gastric tumor progression, we performed expression analysis of *IL-6* and its receptor *(IL-6R)* in 119 gastric specimens including 15 carcinoma cell lines. Semi-quantitative reverse-transcriptase-polymerase chain reaction analysis demonstrated that low but detectable levels of *IL-6* transcript are expressed in a majority of normal and benign gastric tissues [95.5% (46/48) and 93.8% (15/16), respectively]. Compared to noncancerous tissues, significantly increased expressions of *IL-6* transcript were found in 11 of 15 carcinoma cell lines. ELISA assay of secreted IL-6 protein also showed that *IL-6* transcript levels are closely associated with protein concentrations. Expression of *IL-6* was detectable in 51 of 55 (92.7%) primary carcinomas and 58.2% (32/55) of the tumors expressed abnormally high levels of *IL-6* transcript. Tumor-specific elevation of *IL-6* was identified in 44 of 48 (91.7%) matched sets. Furthermore, abnormal overexpression of *IL-6* was more frequent in advanced tumors (26/30, 86.7%) compared to early stage tumors (6/25, 24.0%) whereas it showed no association with grade and histopathologic types of tumors. Expression of *IL-6R* transcript was observed in all cell line and tissue specimens we analyzed and showed no correlation with histopathologic characteristics of tumors. Using cell number counting and [^{3}H]thymidine uptake assays, we also found that cellular proliferation of gastric tumor cells is markedly enhanced by exogenous IL-6 treatment in a dose-dependent manner while treatment of anti-IL-6 neutralizing antibody or transfection of antisense IL-6 oligonucletides suppresses results in cell cycle arrest of the gastric tumor cells. Together, our study shows that *IL-6* is abnormally elevated in a substantial fraction of gastric cancers and enhanced expression of IL-6 stimulates cell cycle progression of gastric tumors, suggesting that elevated *IL-6* may contribute to gastric tumorigenesis in part via its mitogenic action.

Key words : IL-6, IL-6R, Cytokine. Gastric cancer, Cell proliferation

Introduction

The interaction of growth factors, cytokines and hormones with specific membrane receptors triggers a cascade of intracellular signals, resulting in the activation and/or repression of various subsets of genes. Genetic aberrations in growth factor signaling pathways are linked to a variety of chronic diseases, including cancer. It is widely accepted that one of the mechanisms by which cancer cells are thought to acquire a growth advantage is through the autocrine or paracrine loop formation of mitogenic growth factors.

Interleukin-6 (IL-6) is a 26 kDa protein released by a wide range of immune, inflammatory, and tissue cells (1). This cytokine is an important element of the immune reaction through its ability to stimulate both humoral and cellular defence mechanisms (2). It is frequently associated with the early stages of host defense in response to infection or injury (3). IL-6, as other cytokines, has pleiotropic effects. The diversity of biological activities of IL-6 include B-cell terminal differentiation, stimulation of plasmacytoma and hybridoma,

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induction of acute-phase protein synthesis, stimulation of hematopoietic progenitors, and activation of T cells and thymocytes. IL-6 exerts its action through a receptor complex consisting of two membrane-bound glycoproteins: the IL-6-binding chain (IL-6R) and the signal transducting chain, gp130 (4). In the IL-6 signaling pathway, the stimulation of target cells with complex of soluble IL-6R and IL-6 induces the homodimerization of gp130, and the tyrosine-specific phosphorylation of gp130 (5). Upon binding of IL-6, IL-6R became associated extracellularly with gp130 to form high-affinity IL-6 binding sites, and gp130 transduces the signal.

In addition to its physiological properties, IL-6 is involved in the pathogenesis of various neoplasias such as multiple myeloma, non-Hodgkin's lymphoma, and kaposi's sarcoma (6). HIV-Tat protein has a growth –promoting activity in AIDS-Kaposi's sarcoma (KS) cells (7). The Tat-induced growth of KS cells can be specifically inhibited by antisense oligonucleotides, suggesting that Tat induces KS cell proliferation, at least in part, through an IL-6-dependent autocrine mechanism. IL-6 is produced by various tumors and cell lines and has been implicated in the growth regulation of several malignancies, in the either an autocrine or a paracrine fashion (6, 8, 9). Administration of a monoclonal antibody against human IL-6 was effective in inhibiting the growth in vivo of myeloma cells, thus confirming the role of IL-6 in the growth of myeloma cells (10). Interestingly, other nonmyeloma tumors have been shown to be directly stimulated by IL-6 and it has been presumed that the common factor among these diverse tumors is the presence of IL-6R. IL-6 has been reported as being an autocrine growth factor for renal cell carcinomas (8). However, the great majority of murine and human tumor lines do not have IL-6R and with these, IL-6 has been shown either to be inhibitory or have no effect (11). Experiments in vitro showed that the growth of human breast carcinoma cell line MCF-7, SK-BR3, T47D, and ZR-75.1 was inhibited by human rIL-6 (12). Experiments with murine sarcoma cell lines (MCA105, 106, and 203) and a colon carcinoma line (MC-38) showed that systemic sdministration of human rIL-6 reduced substabntially the number of metastatic lesions (13). Lu et al. (14) reported that fibroblast-derived IL-6 behaves as an inhibitor for early-stage melanoma cells but as a mitogenic agent for more malignant cancers.

Gastric adenocarcinoma is one of the most commonly diagnosed malignancies world-wide and a leading cause of cancer mortality in certain areas such as Korea, Japan, South America, and Eastern Europe (15). Although evidence has accumulated indicating the involvement of the alterations of multiple genes such as *p53*, *K-ras*, *c-erbB2*, *K-sam*, and *E-Cadherin*, the underlying molecular events that drive the neoplastic process in gastric cancer are largely undefined (16). In the present study, we have investigated the expression status of IL-6 and IL-6R mRNA in human gastric cell lines and primary tumors to evaluate the role of this cytokine in the process of human gastric cancer development. We report here that IL-6 expression is abnormally elevated in a substantial fraction of human gastric carcinoma. Abnormal overexpression of IL-6 is more frequently detected in advanced stages of cancer suggesting the involvement of IL-6 production in the progression of human gastric cancer.

Materials and Methods

Tissue specimens and cell lines Total 119 gastric tissues including 55 adenocarcinomas, 3 adenomas, 6 hamartomas, 7 hyperplastic polyps, and 48 normal gastric tissues were obtained from 55 gastric cancer patients and 16 noncancer patients by surgical resection in the Kyung Hee University Medical Center (Seoul, Korea). Tissue specimens were snap-frozen immediately in liquid N_2 and stored at –70°C until used. Adjacent portions of each tumor were fixed for histopathological examination. Fifteen gastric carcinoma cell lines (SNU1, SNU5, SNU16, SNU216, SNU484, SNU601, SNU620, SNU638, SNU719, MKN1, MKN28, MKN45, MKN74, AGS, and KATO-III) were obtained from Korea Cell Line Bank (Seoul National University, Seoul, Korea) or American Type Culture Collection (Rockville, MD). Three human prostate carcinoma cell lines (LNCaP, DU145, and PC3) were included to validate the quantitative PCR approach for the genomic and mRNA level of *IL-6*. Total cellular RNA was extracted from tissues and cell lines as described previously (17). Genomic DNA was extracted from the same cells by di-

alysis of the DNA phase after RNA was extracted.

Quantitative PCR analysis Our PCR-based strategies were previously described (18, 19). Briefly, 1 μg of DNase1-treated RNA was converted to cDNA by reverse transcription using random hexamer primers and MoMuLV reverse transcriptase. PCR was initially performed over a range of cycles (24, 27, 30, 33, 36, and 39 cycles) and 2 μl of 1:4 diluted cDNA (12.5 ng/50 μl PCR reaction) undergoing 27-33 cycles was observed to be within the logarithmic phase of amplification with primer set, AW148B/AW150B for *IL-6*, AW192B/AW194B for *IL-6R*, and G2/G3 for *GAPDH*, an endogenous expression control. The sequences of oligonucleotide primers used for quantitation of *IL-6(R)* were shown in Table 1. PCR was performed for 34 cycles at 95°C (1 min), 60°C (0.5 min), and 95°C (1 min) in 1.5 mM $MgCl_2$-containing reaction buffer (PCR buffer II, Perkin Elmer). Ten μl of RT-PCR products were resolved on 2% agarose gels. For quantitative genomic PCR, exon 1 region of *IL-6* was amplified with intron-specific primers IL6E1F and IL6E1R (Table 1). The *c-N-Ras* gene used for an endogenous control for quantitative genomic PCR was amplified with primers RS60 and RS61 Quantitation was achieved by densitometric scanning of the ethidium bromide-stained gels and absolute area integrations of the curves representing each specimen were compared after adjustment for *GAPDH*. Integration and analysis were performed using Molecular Analyst software program (Bio-Rad, Hercules, CA). Quantitative PCR was repeated at least three times for each specimen and the mean was obtained.

Table 1. Oligonucleotide primers used for quantitative RT- and genomic PC

Gene	Primer	Sequence (5′ to 3′)	Orientation
IL-6	AW148B	CCTTCTCCACAAGCGCCTTC	Forward
	AW150B	GGCAAGTCTCCTCATTGAATC	Reverse
	IL6E1F	TCACATTGCACAATCTTAAT	Forward
	IL6E1R	GTGACTGACAGCACAGCTGGG	Reverse
IL-6R	AW192B	CATTGCCATTGTTCTGAGGTTC	Forward
	AW194B	AGTAGTCTGTATTGCTGATGTC	Reverse
GAPDH	G2	CATGTGGGCCATGAGGTCCACC	Forward
	G3	AACCATGAGAAGTATGACAACA	Reverse
c-N-Ras	RS60	GTTATAGATGGTGAAACCT	Forward
	RS61	TATGTGTCTCCTTCGGAAGC	Reverse

IL-6 ELISA The levels of IL-6 protein in the supernatant of cultured cells were quantitated using human ELISA system (Amersham Pharmacia Biotech, Piscataway, NJ), which is based on sandwich enzyme immunoassay technique. Briefly, 1 x 10^4 cells were incubated in RPMI for 24 h and the supernatants were collected. Fifty μl of antibody to human IL-6 conjugated to biotin and 50 μl of the supernatant or IL-6 standard provided by the manufacturer was added to the well coated with an antibody against human IL-6. After incubation for 2 h at room temperature, streptavidin conjugated to horseradish peroxidase was added to each well with TMB substrate. The absorbance at 450 nm was determined using a microplate reader.

Cell Proliferation Assay Cell growth assays were performed in triplicate as followed: AGS and MKN1 cell lines were seeded at the density of 0.75 x 10^4 cells/well in 6 well plates and maintained in medium with 10% FBS for 24 h. Cells were washed twice with phosphate buffered saline (PBS) and medium containing 1% FBS with various concentrations of recombinant human IL-6 or monoclonal anti-human IL-6 antibody (R & D Systems, Inc., Minneapolis, MN) was added. Cell numbers were counted using a hemocytometer for 3 days at 24 h intervals.

[^{3}H]Thymidine Incorporation Assay DNA synthesis was measured by determining the incorporation of [^{3}H]thymidine. Cells seeded at the density of 2 x 10^4 cells/well were cultured on 24-well multiplates and maintained in the presence of 10% serum for 24 h. The cells were washed twice with phosphate buffered saline, and medium various concentrations of anti-IL-6 neutralizing antibody was added in the absence of serum and incubated for 20 h. Cells were then pulse-labeled for 4 h with 1 μCi/ml of [^{3}H]thymidine (Amersham Pharmacia Biotech) and the radioactivity incorporated into trichloroacetic acid-precipitable materials was counted by a liquid scintillation counter.

Results

***IL-6* Expression in Gastric Carcinoma Cell Lines** To ex-

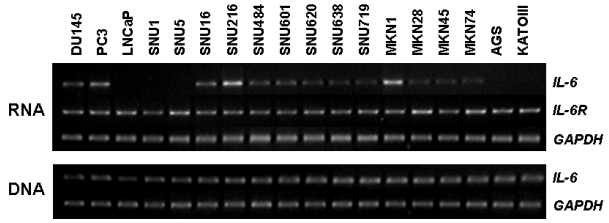

Fig 1. Expression and genomic status of *IL-6* and *IL-6R* in gastric carcinoma cell lines. For expression analysis, *IL-6* and *IL-6R* transcripts were amplified by RT-PCR using exon-specific primers. Ten μl of the PCR products were resolved on a 2% agarose gel. For analysis of IL-6 gene levels, the exon 1 region of the *IL-6* gene was amplified using intron-specific primers using the genomic DNA isolated from the same cells used for mRNA expression analysis as templates. *GAPDH* was used as an endogenous control.

plore the implication of *IL-6* overexpression in gastric carcinogenesis, we initially evaluated expression levels of *IL-6* mRNA in 15 gastric carcinoma cell lines. To avoid the variations in PCR amplification and gel electrophoresis, three human prostate carcinoma cell lines (LNCaP, DU145, and PC3) whose *IL-6* expression status has been previously characterized were used as standard negative and positive controls. The same concentration of cDNA from these control cell lines was subjected to every RT-PCR along with gastric samples under the same conditions. As shown in Fig. 1, predicted levels of *IL-6* (*IL-6/GAPDH*) were observed in two expression controls, DU145 (1.05) and PC3 (1.98) whereas no *IL-6* transcript was detected in a nonexpressor control LNCaP (0.00), indicating that the expression level determined by our RT-PCR assay is well consistent with previously characterized expression status of the gene. In addition, ELISA assay showed that secreted IL-6 protein concentrations (254 ± 21 and 104 ± 17 pg/ml/1 x 10^4 cells in PC3 and DU145, respectively) correlate with mRNA expression levels of *IL-6*. Expression of *IL-6* mRNA was detectable in all of the 15 gastric carcinoma cell lines we examined but its expression levels were variable in a range of 0.12-2.45. *IL-6R* was expressed in all of the 15 cell lines and no significant difference in its level (0.77-1.34) was recognized among specimens. ELISA assay also revealed that variable levels of IL-6 protein are produced in these cell lines (Table 2). Secreted IL-6 protein concentrations showed a strong correlation with expression levels of *IL-6* mRNA.

Table 2. Expression status of IL-6(R) in human gastric carcinoma cell line

Cell lines	mRNA Expression levels		Protein levels
	IL-6	IL-6R	IL-6 (pg/ml)
SNU1	0.12	0.64	72 ± 11
SNU5	0.15	0.67	78 ± 13
SNU16	0.55	0.94	103 ± 16
SNU216	2.33	0.90	219 ± 15
SNU484	1.09	0.99	111 ± 13
SNU601	1.27	1.18	125 ± 16
SNU620	1.01	1.05	118 ± 15
SNU638	1.19	1.03	128 ± 19
SNU-719	0.89	1.34	118 ± 14
MKN1	2.45	1.22	263 ± 21
MKN28	1.17	0.98	159 ± 16
MKN45	0.99	1.25	112 ± 16
MKN74	0.98	0.96	120 ± 12
AGS	0.19	0.79	81 ± 8
KATO-III	0.20	1.09	79 ± 11

Elevated Expression of IL-6 in Primary Gastric Carcinomas To elucidate the *IL-6* status in gastric cancer pathogenesis, we analyzed expression levels of *IL-6* and *IL-6R* in 48 normal and 16 benign tumor tissues including 3 adenomas, 6 hamartomas, and 7 hyperplastic polyps by quantitative RT-PCR. As shown in Fig. 2, expression of *IL-6* mRNA was detectable in a majority of normal and benign gastric tissues [95.5% (46/48) and 93.8% (15/16), respectively], but its levels were low (0.00-0.34). Expression of *IL-6R* mRNA was easily detectable and were not significantly variable in its levels among specimens. (0.73-1.09). Next we analyzed *IL-6* expression in 55 primary gastric carcinomas including 48 matched sets. *IL-6* mRNA expression was detected in 51 of 55 (92.7%) primary carcinoma and its expression levels were observed in the ranges of 0.00-2.23. As summarized in Fig 3, *IL-6* expression levels in primary

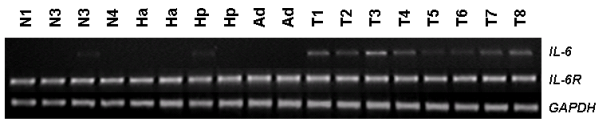

Fig 2. Quantitative RT-PCR analysis of *IL-6* and *IL-6R* expression in human gastric tissues. Expression levels of *IL-6* and *IL-6R* in primary cancer (T1-T8) and adjacent noncancerous tissues (N1-N4) were compared using matched tissue sets obtained from the same gastric cancer patients. *GAPDH* was used as an endogenous control. N, normal tissues; T, tumor tissues. Ha, hamartoma; Hp, hyperplastic polyps; Ad, adenomas

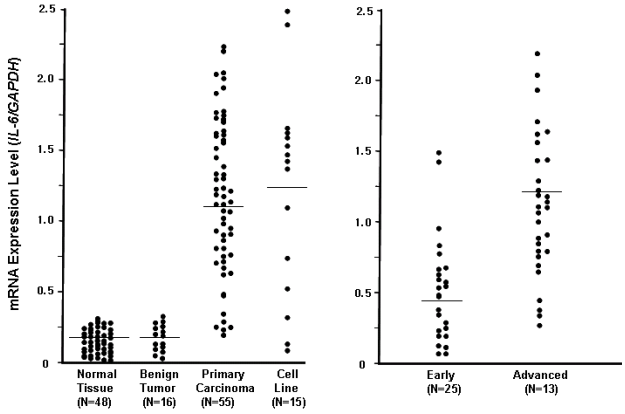

Fig 3. Expression levels of *IL-6* transcript in primary gastric carcinomas and cell lines. Quantitation was achieved by densitometric scanning of *IL-6* RT-PCR products in ethidium bromide-stained gels and absolute area integrations of the curves representing each specimen were compared after adjustment for *GAPDH*. Quantitative PCR was repeated at least three times for each specimen and the means were obtained. Bar indicates the mean expression level of each specimen group. Early, early stage tumors; Advanced, advanced stage tumors

carcinomas were significantly high compared to normal gastric tissues ($P < 0.001$). Based on the *IL-6* expression in normal gastric tissues, we arbitrarily set expression levels more than three-fold (0.63) of normal means (0.21) as abnormally high. Abnormal elevation of *IL-6* expression was detected in 58.2% (32/55) of primary carcinomas. Moreover, among 48 matched sets from the same patients, tumor-specific elevation of *IL-6* was found in 44 (91.7%) cases (Fig. 2). Furthermore, abnormal overexpression of *IL-6* was significantly high in advanced tumors (26/30, 86.7%) compared to early stage tumors (6/25, 24.0%) ($P < 0.001$). However, no significant difference was identified between poorly differentiated tumors (17/28, 60.7%) and well or moderately differentiated tumors (15/27, 55.6%). Abnormal elevation of *IL-6* expression showed no association with histologic types of tumor [diffused, 52.6% (10/19); intestinal, 61.1% (22/36)]. In contrast to *IL-6*, *IL-6R* expression was found in all tissue specimens we analyzed and showed no correlation with histopathologic characteristics of tumors (data not shown). Collectively, our results indicate that abnormal elevation of *IL-6* is a frequent event in gastric tumorigenesis and may contribute to the malignant progression of human gastric cancers

Absence of Genomic Amplification of *IL-6* To address that abnormal elevation of IL-6 expression results from gene amplification, genomic level of *IL-6* was determined by quantitative genomic PCR. However, no significant difference was recognized in *IL-6* gene levels among the 15 cell lines and 48 matched tumor sets (Fig. 1). Thus, these data suggest that IL-6 is frequently overexpressed in gastric carcinoma tissues, possibly due to the transcriptional activation of the gene.

Stimulation of Tumor Cell Growth by IL-6 To investigate

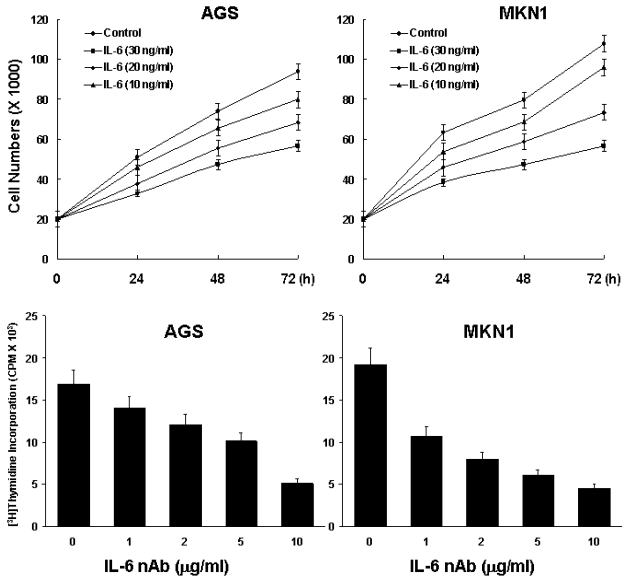

Fig 4. Growth responses of gastric carcinoma cell lines (AGS and MKN1) to IL-6. A. Growth stimulation of gastric tumor cells by exogenous IL-6 treatment. Cells were plated at an initial density of 0.75×10^4 cells/well and treated with IL-6 (0, 10, 20, and 30 ng/ml) in the presence of 1% FBS. Cell numbers were counted every 24 h using a hemocytometer. Data represent means and standard deviations of triplicate assays. B, [^{3}H]thymidine uptake analysis of anti-IL-6 neutralizing antibody (IL-6 nAb) effect on DNA synthesis. 2×10^4 cells/well were treated with IL-6 nAb (0, 1, 2, 5, and 10 μg/ml) for 20 h in the absence of FBS and then pulse-labeled for 4 h with 1 μCi/ml of [^{3}H]thymidine. The radioactivity incorporated into trichloroacetic acid-precipitable materials was counted by a liquid scintillation counter. Data represent means and standard deviations of triplicate assays.

the IL-6 effect on gastric tumor cell growth, the AGS and MKN1 cell lines were treated with various concentrations of IL-6 (1, 10, 30, and 50 ng/ml) in the presence of 1% FBS, and cell numbers were analyzed at 24 h intervals. As shown in Fig 4A, the cell proliferation of AGS and MKN1 was stimulated by IL-6 in a dose-dependent manner. To further verify whether increased IL-6 expression in these cancer cells acts as an autocrine growth factor, we treated the cells with anti-IL-6 neutralizing antibody (IL-6 nAb) and its effect on cell cycle progression was analyzed using [^{3}H]thymidine uptake assay. Compared to untreated controls, DNA synthesis of the IL-6 nAb-treated cells was severely inhibited (Fig. 4B). Although its effect was less significant than IL-6 nAb, antisense IL-6 transfection also led to growth suppression (data not shown). These findings suggest that elevated IL-6 acts as a growth-stimulating factor for gastric tumor cells and might contribute to the malignant progression of human gastric carcinomas.

Discussion

IL-6 is a pleiotropic cytokine that play a central role in antigen-specific immune responses and inflammatory reactions (1). In addition, IL-6 has been implicated in the pathology of a variety of malignant tumors including myeloma, renal cell carcinoma, and cervical carcinoma (6, 8, 9). IL-6 expression is up-regulated in several solid and hematopoietic neoplasms and elevated levels of circulating IL-6 have been reported to be associated with poor outcome of the diseases (20, 21) Autocrine growth stimulation and/or apoptosis inhibition via PI3K activation have been suggested as the possible mechanisms for the oncogenic action of IL-6 (8, 9). Despite a significant role of IL-6 in tumorigenesis, the signaling mechanisms by which IL-6 expression is activated in human tumors have been poorly understood. Recently, TGF-β1 was suggested as a potent regulator of IL-6 in human lung fibroblast and osteoblast (22, 23). It was also reported that both IL-6 and TGF-β1 levels are elevated in patients with metastatic prostate carcinoma and correlate with tumor burden or clinically evident metastases (24). Recently, we demonstrated that the *IL-6* gene is a direct transcription target of TGF-β1 in malignant prostate carcinoma cells. It was observed that TGF-β1-mediated IL-6 gene expression is Smad2-dependent and occurs through the functional collaboration between multiple signaling pathways including p38, NF-&;B, SAPK/JNK, and Ras.

In the gastric carcinoma, high levels of IL-6 secretion were found in the sera of patients and increased serum IL-6 levels correlate with disease status of gastric (25, 26). It was also reported that helicobacter pylori infection is associated with increased gastric mucosal levels of proinflammatory cytokines including IL-6 (27). In the present study, we demonstrate that high levels of *IL-6* are expressed in a majority of gastric carcinoma whereas no or barely detectable levels of *IL-6* are expressed in normal or benign gastric tissues. *IL-6R* mRNA expression was detected in all tissue and cell line specimens we tested. This observation suggests that an autocrine mechanism may be involved in the oncogenesis of gastric cancer. However, it should be noted that tumor cell growth could also be affected by adjacent stromal fibroblasts through the paracrine effect. The response of gastric tumor cells to fibroblast-derived IL-6, which can be sensitive or resistant in the process of gastric carcinogenesis, should be further studied. It has been reported that growth of human breast tumor cell MCF-7 is markedly stimulated by a paracrine effect of breast fibroblast-derived growth factors. It is now identified that fibroblast-derived stimulating factor is IL-6.

Elevated and autocrine production of IL-6 correlates with progression and metastasis of many human tumors including gastric cancer. Previous studies demonstrated that IL-6 is a protective resistance factor to prostate tumor cell killing by chemotherapeutic drugs such as cis-diamminedichloroplatinum and etoposide, and promotes anchorage-independent growth of immortalized, nontumorigenic human prostate epithelial cells (28, 29). Recently, IL-6 was also suggested to stimulate tumor invasion and angiogenesis by induction of vascular endothelial growth factor and matrix metalloproteinases (30-32). However, the molecular mechanism by which IL-6 contributes to gastric tumor progression remains largely undefined. Recently, it was demonstrated that IL-6 stimulates gastric tumor growth and effectively protects gastric cancer cells from the apoptosis induced by hydrogen peroxide and the an-

ti-apoptotic effect of IL-6 is, at least in part, due to the up-regulation of mcl-l (33, 34). In the present study, we found that inactivation of the IL-6 signaling by treatment of anti-IL-6 neutralizing antibody or by transfection of anti-sense IL-6 suppresses proliferation of gastric carcinoma cells while treatment of IL-6 stimulates tumor cell growth. Although we did not address the molecular mechanism of IL-6 action in the present work, our data strongly indicate that IL-6 acts as an autocrine growth factor in gastric carcinomas.

In conclusion, this study demonstrates that *IL-6* mRNA is expressed in a substantial fraction of human gastric carcinoma tissues and cell lines and elevated expression of *IL-6* correlates with more advanced stages of tumors. Moreover, gastric tumor cell growth was stimulated by IL-6 while anti-IL-6 antibody inhibited growth. These results thus suggest that IL-6 may play an important role in the progression of human gastric cancer.

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