Regulation of Disease-Progression Genes in Human Gastric Carcinoma Cells by Interleukin 8

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ABSTRACT

The expression of interleukin 8 (IL-8) by human gastric carcinomas directly correlates with tumor vascularity and disease progression. To determine whether IL-8 can act in an autocrine manner to regulate the expression of other disease-progression genes, we examined the expression of IL-8 receptors IL-8RA (CXCR1) and IL-8RB (CXCR2) in six different human gastric carcinoma cell lines and 38 surgical specimens of human gastric carcinomas. All of the gastric carcinoma cell lines expressed mRNA and protein for IL-8RA and IL-8RB protein. In all surgical specimens, the majority of the tumor cells and small vessel endothelial cells stained positive for IL-8RA and IL-8RB protein. In vitro treatment of human gastric cancer MKN-1 cells with exogenous IL-8 enhanced the expression of epidermal growth factor receptor, type IV collagenase (metalloprotease-9), vascular endothelial growth factor, and IL-8 mRNA. In contrast, treatment with exogenous IL-8 decreased expression of E-cadherin mRNA. IL-8 treatment increased invasive capacity of MKN-1 cells, which was associated with activity of metalloprotease-9. Collectively, these results demonstrate that human gastric carcinoma cells express receptors for IL-8 and that IL-8 may play a role in the progressive growth of human gastric carcinoma by autocrine/paracrine mechanisms.

INTRODUCTION

IL-8, a member of the CXC chemokine family, was initially shown to be a chemoattractant for neutrophils and lymphocytes (1, 2). Subsequent studies revealed that IL-8 can also induce haptotactic migration of tumor cells (3), proliferation of keratinocytes and melanoma cells (4, 5), and angiogenesis (6, 7). Indeed, human recombinant IL-8 has been shown to induce proliferation and migration of human umbilical vein endothelial cells, and its stimulation of vascularization in the rat cornea assay (7) has led to its implication in the induction of angiogenesis in such diverse diseases as psoriasis (8), rheumatoid arthritis (9), idiopathic pulmonary fibrosis (10), and some neoplasms (11, 12). We have recently reported that most gastric cancer cell lines express IL-8 mRNA and protein (11). Gastric cancer cells in surgical specimens of human gastric carcinomas overexpress IL-8 compared with corresponding normal mucosa, and the IL-8 mRNA level directly correlated with the vascularity of the tumors (11). Furthermore, transfection of gastric carcinoma cells with the IL-8 gene enhanced their tumorigenic and angiogenic potential in the gastric wall of nude mouse (12).

To determine whether IL-8 can interact with gastric cancer cells in an autocrine manner, we examined the presence and level of two distinct human IL-8 receptors: IL-8RA (CXCR1) and IL-8RB (CXCR2) (13, 14). The deduced amino acid sequences of both receptors predict that they belong to a family of seven transmembrane G protein-coupled receptors (15). IL-8RA and IL-8RB bind to IL-8 with high affinity, although IL-8RB also binds to melanocyte growth-stimulatory activity and neutrophil-activating peptide 2 (16). IHC analyses revealed that both IL-8 receptors are expressed on the surface of human carcinoma cells (17, 18), but the role of these receptors in cancer progression remains unknown.

In this study, we examined the expression of IL-8RA and RB in human gastric carcinoma cell lines and surgical specimens. We also examined the effects of IL-8 on the expression of disease-progression genes by treating gastric carcinoma cell lines with recombinant IL-8. Our studies clearly demonstrate that gastric carcinoma cells express both IL-8RA and IL-8RB and that the IL-8 receptor system may play a role in the processes of invasion and metastasis.

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3 The abbreviations used are: IL, interleukin; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; MMP, metalloproteinase; RT-PCR, reverse transcription-PCR; IHC, immunohistochemical; SVEC, small vessel endothelial cell; LVEC, large VEC.

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MATERIALS AND METHODS

Cell Cultures and Tumor Tissues. Six cell lines established from human gastric carcinomas were maintained in RPMI 1640 (Nissui Co., Ltd., Tokyo, Japan) with 10% fetal bovine serum (M.A. Bioproducts, Inc., Walkersville, MD). The TMK-1 cell line (poorly differentiated adenocarcinoma) was established in our laboratory (19). The other five gastric carcinoma cell lines (MKN-1, adenosquamous carcinoma; MKN-7, MKN-28, and MKN-74, well-differentiated adenocarcinomas; and MKN-45, poorly differentiated adenocarcinoma) were provided by Dr. T. Suzuki (Fukushima Medical College, Fukushima, Japan).

A total of 38 cases of gastric carcinoma, all assessed by Hiroshima University School of Medicine, were examined. The definition of stage grouping and the histological classification were made according to the criteria of the Japanese Research Society for Gastric Cancer (20).

RT-PCR. Total RNA was extracted from human gastric carcinoma cell lines using RNAzol B (Cinna/Biotex, Houston, TX) according to the manufacturer’s instructions. Two pairs of oligomers were synthesized based on the reported sequences of human IL-8 receptors (5'-CATGTCAAATATTACAGATCC-3' and 5'-TACTTGTTGAGTGTCTCAGTTT-3' for IL-8RA and 5'-CATGGAGAGTGACAGCTTTGA-3' and 5'-ACTTGTTGATTTCCAGGGATT-3' for IL-8RB) (13, 14). RT-PCR was performed using the obtained RNA and the oligomers as templates and primers, respectively (21). The cDNA was amplified by 30 PCR cycles, and the thermal cycle profile was: denaturation for 2 min at 94°C, annealing for 2 min at 55°C, and extension for 3 min at 72°C. After the reaction, the mixtures were loaded onto a 5% nondenaturing polyacrylamide gel in Tris-borate-EDTA buffer. RT-PCR reaction without the reverse transcriptase showed no specific band.

IL-8 Treatment of Gastric Carcinoma Cells. After 24 h of serum starvation, 10 ng/ml IL-8 (Otsuka, Tokushima, Japan) was added to the cultures. The six gastric carcinoma cells were treated for 0 (control), 3, and 24 h. Five micrograms of polyadenylated RNA were subjected to Northern blot analysis.

Northern Blot Analysis. Polyadenylated mRNA was extracted from gastric carcinoma cell lines and surgical specimens using the FastTrack mRNA isolation kit (Invitrogen Co., San Diego, CA). mRNA was electrophoresed on a 1% denaturing formaldehyde/agarose gel, electrotransferred at 0.6 A to a GeneScreen nylon membrane (DuPont Co., Boston, MA), and UV cross-linked with 120,000 mJ/cm² using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Hybridizations were performed as described previously (22). Nylon filters were washed at 65°C with 30 mM NaCl, 3 mM sodium citrate (pH 7.2), and 0.1% SDS (w/v).

The cDNA probes used in these analyses were a 0.5-kb human IL-8 cDNA probe, a 1.1-kb human type IV collagenase cDNA probe (kindly provided by Dr. W. G. Stetler-Stevenson, NIH, Bethesda, MD), a 0.6-kb mouse E-cadherin cDNA probe (kindly provided by Dr. M. Takeichi, Kyoto University, Kyoto, Japan), a 2.4-kb human EGFR cDNA probe (purchased from Health Science Research Resources Bank, Osaka, Japan), and a 0.7-kb human VEGF cDNA probe (kindly provided by Dr. M. Shibuya, University of Tokyo, Tokyo, Japan). The β-actin cDNA probe and glyceraldehyde-3-phosphate dehydrogenase cDNA probes were purchased from Oncor, Inc. (Gaithersburg, MD) and Clontech Inc. (Palo Alto, CA), respectively. Each cDNA fragment was purified by agarose gel electrophoresis, recovered using GeneClean (BIO 101, Inc., La Jolla, CA), and radiolabeled using the random primer technique with 32P-labeled deoxyribonucleotide triphosphates (23).

IHC Staining. Archival paraffin blocks of 38 cases were available. Consecutive 4-μm sections were cut from each study block. Sections were immunostained for IL-8RA and RB. IHC staining was performed by the immunoperoxidase technique with minor modifications (17, 18). IL-8RA- and RB-specific
antibodies (24), which were rabbit polyclonal antibodies, were used at a 1:200 dilution. These antibodies react specifically with cytoplasmic domains of IL-8RA and IL-8RB but do not cross-react with each other. The specificity of the reaction was determined as: (a) anti-IL-8RA and anti-IL-8RB antibodies were absorbed at 4°C overnight with excess GST proteins fused with extracellular domains of human IL-8RA and IL-8RB, respectively; and (b) nonimmune rabbit IgG was used in the primary reaction.

Gelatin Zymogram. TMK-1 and MKN-1 cells were incubated with 10 ng/ml IL-8 (Otsuka) for 24 h in serum-free conditions. A total of 15 μl of the culture medium were applied to a 10% polyacrylamide gel containing 2 mg/ml gelatin. After electrophoresis, the gel was washed with washing buffer [50 mM Tris-HCl (pH 7.4) and 2.5% Triton X-100] at room temperature for 30 min and then placed in incubation buffer [30 mM Tris-HCl (pH 7.4), 0.2 mM NaCl, 10 mM CaCl₂, and 0.02% NaN₃] at 37°C overnight. The gel was stained with 0.02% Coomassie brilliant blue in 20% methanol and 10% acetic acid and destained with 20% methanol and 10% acetic acid. Molecular weights of the gelatinolytic bands were estimated using molecular weight markers (Daiichi Pure Chemicals, Tokyo, Japan).

Invasion Assay. Cultured cells were harvested by a brief exposure to 0.25% trypsin at 37°C. After centrifugation, cells were resuspended in medium and their invasive behavior was examined in an invasion chamber (Becton Dickinson, Bedford, MA). Cell viability was evaluated with the 0.1% trypan blue exclusion method. Transwell cell culture chambers with 8-mm diameter filters were used for this assay. The Matrigel-coated filters were placed on Boyden chambers, and the cells (5 × 10⁵) suspended in RPMI medium were placed in the upper chamber. RPMI containing recombinant IL-8 was placed in the lower compartment of the Boyden chambers. The chambers were incubated in 5% CO₂ at 37°C, and the cells in the lower compartment were counted hourly for 24 h. The assays were performed in triplicate.

Statistical Analysis. The significance of the differences in in vitro data were analyzed by the unpaired Student’s t test (two-tailed).

RESULTS
Expression of IL-8 Receptors by Human Gastric Carcinoma Cell Lines and Gastric Carcinoma Tissues. The expression of IL-8 receptor mRNA by gastric carcinoma cell
Table 1  Effect of IL-8 on the expression of the disease-progression genes in human gastric carcinomas

The numbers shown are the densitometric quantitation of the ratios of the area between the specific transcripts and the β-actin transcript after treatment with IL-8, compared in each case with the respective control (the value for untreated cells with IL-8) defined as 1.0.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IL-8&lt;sup&gt;a&lt;/sup&gt;</th>
<th>VEGF&lt;sup&gt;b&lt;/sup&gt;</th>
<th>EGFR&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MMP-9&lt;sup&gt;b&lt;/sup&gt;</th>
<th>E-cadherin&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>MKN-1</td>
<td>7.7</td>
<td>3.1</td>
<td>2.7</td>
<td>3.5</td>
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</tr>
<tr>
<td>MKN-7</td>
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<td>1.2</td>
<td>1.6</td>
<td>0.8</td>
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<td>MKN-28</td>
<td>3.4</td>
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<td>1.6</td>
<td>0.8</td>
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<tr>
<td>MKN-45</td>
<td>5.5</td>
<td>1.6</td>
<td>1.3</td>
<td>1.0</td>
<td>1.0</td>
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<tr>
<td>MKN-74</td>
<td>4.6</td>
<td>1.1</td>
<td>1.5</td>
<td>1.6</td>
<td>0.9</td>
</tr>
<tr>
<td>TMK-1</td>
<td>6.2</td>
<td>1.1</td>
<td>1.0</td>
<td>1.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Relative mRNA expression was evaluated 3 h after IL-8 treatment.

<sup>b</sup> Relative mRNA expression was evaluated 24 h after IL-8 treatment.

<sup>c</sup> No mRNA transcripts were detected in control or IL-8-treated cells.

Constitutively expressed by MKN-1 and MKN-7 cells and was increased by treatment with IL-8. On the other hand, 3 h after exposure to exogenous IL-8, the expression of E-cadherin mRNA in MKN-1 cells was transiently decreased (Fig. 3A). No expression of basic fibroblast growth factor and 72-kDa type IV collagenase (MMP-2) mRNA was detected in control or IL-8-treated cells (data not shown).

**Effect of IL-8 on Invasion of Gastric Carcinoma Cells.**

We next analyzed whether the increased expression of MMP-9 mRNA by IL-8 treatment correlated with collagenase type IV activity and an increase in penetration through reconstituted basement membranes. We used the two representative cell lines, MKN-1 and TMK-1, for this experiment because enhanced expression of MMP-9 mRNA by IL-8 was observed in MKN-1 but not TMK-1. These cell lines were assayed for their potential to penetrate filters coated with Matrigel (Table 2). Treatment with IL-8 did not affect invasion of TMK-1 cells. In contrast, treatment with IL-8 significantly increased collagenase type IV activity (Fig. 3B) and the number of invading MKN-1 cells, agreeing with the above data that treatment with exogenous IL-8 increased collagenase type IV mRNA expression in MKN-1 but not TMK-1 cells (Fig. 3A).

**DISCUSSION**

In the present study, we examined the expression of IL-8 receptors in human gastric carcinomas. Both IL-8RA and IL-8RB were constitutively expressed by all human gastric cancer cell lines examined. IHC analysis of surgical specimens of human gastric carcinomas showed that IL-8 receptors were expressed by tumor cells, microvascular endothelial cells, and inflammatory cells. IL-8 is expressed by a variety of malignant tumor cells, including pancreas (26), lung (27), prostate (28), bladder (29), head and neck squamous carcinomas (30), and melanoma (5). IL-8 produced by lung carcinoma cells has actually been shown to stimulate angiogenesis (27, 31). More recently, IL-8 has been shown to act as an autocrine growth stimulator for lung (32), liver, and pancreatic carcinomas (26), and melanoma cells (5, 33). Our recent data have revealed that IL-8 mRNA levels in gastric carcinoma tissues directly correlated with tumor vascularity (11), and transfection of the IL-8 gene into gastric carcinoma cells enhanced tumorigenicity and angiogenesis after implantation of these cells into the gastric wall (orthotopic site) of nude mice (12). Collectively, these data suggest that IL-8 is an important regulator of angiogenesis in gastric carcinoma.

The IL-8 receptors IL-8RA and IL-8RB bind IL-8 with high affinity and act via G proteins through the phospholipase C pathway, which induces the release of intracellular calcium and the activation of protein kinase C (13). The affinity of IL-8 for the IL-8RB (kDa = 0.031–0.133 nM) is two to five times greater than the affinity for IL-8RA (kDa = 0.096–0.168; Ref. 16). MicrovesSEL endothelial cells in human head and neck squamous cell carcinomas have been shown to express IL-8RA and IL-8RB (17), as do inflammatory cells, keratinocytes, smooth muscle cells, and fibroblasts (24). Differential expression of the two receptors on SVECs and LVECs was reported for human breast carcinoma tissues (18). Whereas IL-8RB was expressed prominently on both SVECs and LVECs, IL-8RA was present in only

**Table 1**

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<td>1.6</td>
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<td>MKN-28</td>
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<tr>
<td>MKN-45</td>
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<td>1.3</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>MKN-74</td>
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25% of the SVECs and 14% of the LVECs (18). Our demonstration that microvessel endothelial cells in human gastric carcinomas express both IL-8 receptors expands these observations and suggests that IL-8, indeed, plays a role in angiogenesis of these cancers.

The metastatic potential of neoplasms has been correlated with the expression level of several independent genes that regulate, among others, cell growth, angiogenesis, invasion, motility, and adhesion (34). Our study revealed that treatment of MKN-1 cells with IL-8 enhanced the expression of EGFR (proliferation), IL-8, and VEGF (angiogenesis), and collagenase type IV (invasion), whereas the expression of E-cadherin (cohesion) was decreased (Fig. 3A). Furthermore, IL-8 increased MMP-9 activity (Fig. 3B) and invasion through Matrigel of some, but not all, gastric carcinoma cells (Table 2).

The growth and spread of tumor cells depends on adequate vasculature (35–37). Invasion of the host stroma and degradation of the blood vessel basement membrane are necessary for metastasis (38, 39). The expression of E-cadherin, which is directly related to cell-to-cell cohesion, is inversely correlated with progression and metastasis (40). Thus, a simultaneous increase in the expression of EGFR (growth), VEGF (angiogenesis), and type IV collagenase (invasion) and decrease in the expression of E-cadherin (adhesion) should enhance metastatic potential of cancer cells. IL-8RA and IL-8RB expression was similar on the gastric carcinoma cell lines examined, however, response to IL-8 was different among these cell lines. In contrast to MKN-1 cells, the expression of disease-progression genes (except IL-8) by TMK-1 cells was not affected by treatment with IL-8 (Fig. 3). To elucidate the mechanism of differential response to IL-8 by these two cell lines and which receptor is more responsible for the behavior, additional studies concerning signal transduction of IL-8 receptors using several cell lines are necessary.

In summary, gastric carcinoma cells express both IL-8 and its receptors. The IL-8 receptor system regulates expression of disease-progression genes in human gastric cancer cells and may be involved in cancer metastasis and, hence, should be a target for therapy.

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