Expression of Interleukin 8 and Its Receptors in Human Colon Carcinoma Cells with Different Metastatic Potentials

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ABSTRACT

Purpose: In the present study, we examined the expression of interleukin 8 (IL-8), and its receptors, CXCR1 and CXCR2, in human colon carcinoma cells with different metastatic potentials and determined their role in modulating phenotypes associated with metastasis.

Experimental Design: IL-8, CXCR1, and CXCR2 protein and mRNA expression were examined using ELISA, immunocytochemistry, and reverse transcription-PCR in human colon carcinoma cells with different metastatic potentials. IL-8-mediated proliferation, migration, and tumor-endothelial cell interaction were analyzed.

Results: IL-8 mRNA and protein expression was very low in Caco2 cells but elevated in KM12C cells and very high in KM12L4 cells, suggesting an association between the IL-8 production and metastatic potential. Similarly, CXCR1 and CXCR2 expression was lower in Caco2 cells than in low and high metastatic KM12C and KM12L4 cells. The recombinant human IL-8 enhanced the proliferation of colon carcinoma cells. Furthermore, proliferation of low and high metastatic cells expressing different levels of IL-8 was inhibited by neutralizing antibodies to IL-8, CXCR1, and CXCR2. We observed significant differences in the invasive potential of colon carcinoma cells expressing different levels of IL-8. In addition, we observed that IL-8 modulates adhesion of tumor cells to endothelial cells in an autocrine and paracrine manner.

Conclusion: Our present data suggest an association between constitutive expression of IL-8 and aggressiveness in human colon carcinoma cells and the possible role of IL-8 in modulating different metastatic phenotypes associated with progression and metastasis.

INTRODUCTION

IL-8 is a member of the chemokine superfamily of structurally and functionally related inflammatory cytokines that stimulate the migration of distinct sets of cells including neutrophils, monocytes, lymphocytes, and fibroblasts (1–4). CXCR1 and CXCR2, also named IL-8RA and IL-8RB, are receptors for IL-8. CXCR1 and CXCR2 both bind IL-8 with high affinity, but CXCR2 also binds to other CXC chemokines (5–9). Recent studies demonstrated that IL-8 regulates tumor cell growth and metastasis in melanoma (10), carcinoma cells of lung, stomach, pancreas, liver, gall bladder (11, 12), and prostate cancer (13). It also regulates angiogenesis in human bronchogenic carcinoma (14), squamous cell carcinoma (15), and non-small cell lung carcinoma in nude mice (16). Expression of CXCR1 and CXCR2 receptors was found in keratinocytes, fibroblasts, endothelial cells, melanoma, and colon cancer cells (9, 11, 17, 18). These receptors have also been implicated in angiogenic responses to IL-8 and neutrophil and lymphocyte migration (5, 7, 9, 17, 19).

Metastasis is a complex process influenced by genetic, biochemical, immunological, and biological changes and influenced by tumor-host interaction (20). Several growth factors and cancer-associated genes, such as epidermal growth factor, carcinoembryonic antigen, and type IV collagenase, are involved in colon carcinoma metastases (21). We have reported that IL-8 expression correlates with tumor growth and metastatic potential in melanoma cells (10, 22, 23). Recently, several studies report that IL-8 up-regulates inflammatory responses, tumor cell proliferation, and migration in colon epithelial cell lines (17, 24–26). However, whether the expression of IL-8 is related to the metastatic potential in colon carcinoma cells remains unclear.

The purpose of this study was to examine the expression of IL-8 and its receptors in colon carcinoma cells with different metastatic potentials and determine the role of IL-8 in modulating phenotypes associated with tumor progression and metastasis. We examined the expression of IL-8, CXCR1, and CXCR2 and their roles in proliferation of colon carcinoma cells with different metastatic potentials. Our data suggested that constitutive expression of IL-8 in colon carcinoma cells is associated with metastatic potential. Furthermore, these studies demonstrated that IL-8 might act as an autocrine/paracrine growth factor in colon cancer progression and metastasis.

MATERIALS AND METHODS

Cell Lines and Reagents. The human Caco2 cell line isolated from primary colonic tumor (27, 28) was obtained from...
American Type Tissue Culture (ATCC, Manassas, VA). The heterogeneous low metastatic human KM12C cell line was originally established in culture from a Duke’s stage B2 primary tumor surgical specimen (29, 30). The high metastatic KM12L4 cell line is a subclone derived from KM12C in nude mice (29). Human umbilical vein endothelial cells (HUVEC) were obtained from American Type Culture Collection. The cell lines were maintained in culture as an adherent monolayer in MEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, l-glutamine, 2-fold vitamin solution, and gentamicin (Life Technologies, Inc., Gaithersburg, MD). HUVECs were additionally supplemented with bFGF and endothelial cell growth factor. All cultures were free of Mycoplasma and pathogenic murine viruses. Cultures were maintained for no longer than 4 weeks after recovery from frozen stocks.

**ELISA for Human IL-8.** IL-8 levels in culture supernatants were determined using ELISA-paired antibodies purchased from Endogen, Inc. (Woburn, MA). This assay is a quantitative “sandwich” enzyme immunoassay. One hundred μl of the primary monoclonal antibody against IL-8 (2 μg/ml) were coated in Nunc Maxisorp plates in each well. After overnight incubation at 4°C, the plates were washed and blocked for 1 h with blocking buffer (4% BSA in PBS). After the plates were washed four times, 50 μl of culture supernatant or recombinant IL-8 protein at different concentrations (Endogen, Inc.) and 50 μl of biotinylated IL-8 antibody were added to each well. After 2 h of incubation, the plates were washed, and the immunoreactivity was determined using the avidin-HRP-TMB detection system (Dako Corp., Carpinteria, CA). The plates were washed and blocked for 1 h with blocking buffer (4% BSA in PBS). After the plates were washed four times, 50 μl of culture supernatant or recombinant IL-8 protein at different concentrations (Endogen, Inc.) and 50 μl of biotinylated IL-8 antibody were added to each well. After 2 h of incubation, the plates were washed, and the immunoreactivity was determined using the avidin-HRP-TMB detection system (Dako Corp., Carpinteria, CA). The plates were washed and blocked for 1 h with blocking buffer (4% BSA in PBS). After the plates were washed four times, 50 μl of culture supernatant or recombinant IL-8 protein at different concentrations (Endogen, Inc.) and 50 μl of biotinylated IL-8 antibody were added to each well. After 2 h of incubation, the plates were washed, and the immunoreactivity was determined using the avidin-HRP-TMB detection system (Dako Corp., Carpinteria, CA). The plates were washed and blocked for 1 h with blocking buffer (4% BSA in PBS). After the plates were washed four times, 50 μl of culture supernatant or recombinant IL-8 protein at different concentrations (Endogen, Inc.) and 50 μl of biotinylated IL-8 antibody were added to each well. After 2 h of incubation, the plates were washed, and the immunoreactivity was determined using the avidin-HRP-TMB detection system (Dako Corp., Carpinteria, CA). The plates were washed and blocked for 1 h with blocking buffer (4% BSA in PBS). After the plates were washed four times, 50 μl of culture supernatant or recombinant IL-8 protein at different concentrations (Endogen, Inc.) and 50 μl of biotinylated IL-8 antibody were added to each well. After 2 h of incubation, the plates were washed, and the immunoreactivity was determined using the avidin-HRP-TMB detection system (Dako Corp., Carpinteria, CA). The plates were washed and blocked for 1 h with blocking buffer (4% BSA in PBS). After the plates were washed four times, 50 μl of culture supernatant or recombinant IL-8 protein at different concentrations (Endogen, Inc.) and 50 μl of biotinylated IL-8 antibody were added to each well. After 2 h of incubation, the plates were washed, and the immunoreactivity was determined using the avidin-HRP-TMB detection system (Dako Corp., Carpinteria, CA). The plates were washed and blocked for 1 h with blocking buffer (4% BSA in PBS). After the plates were washed four times, 50 μl of culture supernatant or recombinant IL-8 protein at different concentrations (Endogen, Inc.) and 50 μl of biotinylated IL-8 antibody were added to each well. After 2 h of incubation, the plates were washed, and the immunoreactivity was determined using the avidin-HRP-TMB detection system (Dako Corp., Carpinteria, CA). The plates were washed and blocked for 1 h with blocking buffer (4% BSA in PBS). After the plates were washed four times, 50 μl of culture supernatant or recombinant IL-8 protein at different concentrations (Endogen, Inc.) and 50 μl of biotinylated IL-8 antibody were added to each well. After 2 h of incubation, the plates were washed, and the immunoreactivity was determined using the avidin-HRP-TMB detection system (Dako Corp., Carpinteria, CA). The plates were washed and blocked for 1 h with blocking buffer (4% BSA in PBS). After the plates were washed four times, 50 μl of culture supernatant or recombinant IL-8 protein at different concentrations (Endogen, Inc.) and 50 μl of biotinylated IL-8 antibody were added to each well. After 2 h of incubation, the plates were washed, and the immunoreactivity was determined using the avidin-HRP-TMB detection system (Dako Corp., Carpinteria, CA).
radioactivity was analyzed using a gamma counter (Packard, Downers Grove, IL). The percent of binding is: \( \frac{A}{B} \times 100 \), where \( A \) is the cpm of treated cells, and \( B \) is the cpm in untreated control cells.

**Statistical Analysis.** The significance of the data was determined by the Student’s \( t \) test (two-tailed) using SPSS software (SPSS, Inc., Chicago, IL). \( P < 0.05 \) was deemed significant.

**RESULTS**

**Expression of IL-8 in Colon Carcinoma Cells.** In the first set of experiments, we analyzed the production of IL-8 protein in human colon carcinoma cells with different metastatic potentials. Cell-free culture supernatant was harvested from \( 1 \times 10^5 \) cells with 24 h culture, and IL-8 protein level was determined by ELISA. Very low levels of IL-8 protein were detected in Caco2 cells (1.7 pg/ml; Fig. 1). High metastatic KM12L4 cells expressed the highest level of IL-8 protein (758 pg/ml), whereas low metastatic KM12C cells expressed lower levels of IL-8 (137 pg/ml; Fig. 1; \( P < 0.05 \)).

Next, we analyzed the mRNA expression of IL-8 in non-metastatic and low and high metastatic colon carcinoma cell lines by RT-PCR (Fig. 2A). Total cellular RNA was isolated from \( 1 \times 10^6 \) cultured cells. The mRNA expression level of IL-8 was quantitated as an expression index (Fig. 2B). We did not observe IL-8 mRNA expression in Caco2 cells, whereas IL-8 mRNA levels were higher in KM12L4 than in KM12C cells. Different IL-8 protein levels and mRNA expression suggest that constitutive expression of IL-8 in human colon carcinoma cells correlates with metastatic potential.

**Expression of CXCR1 and CXCR2 in Colon Carcinoma Cells.** In the next set of experiments, we analyzed the expression of CXCR1 and CXCR2 by ICC (protein) and RT-PCR (mRNA). Colon carcinoma cells (\( 1 \times 10^5 \) cells/ml) were plated on four-well chamber slides. After 72 h culture, cells were fixed and immunostained with anti-CXCR1 or anti-CXCR2 antibodies. A heterogeneous pattern of CXCR1 as well as CXCR2 immunoreactivity was detected in the three colon carcinoma cell lines with different metastatic potentials (Fig. 3). Less than 10% of Caco2 cells showed CXCR1 or CXCR2 immunoreactivity. CXCR1 immunoreactivity was observed in 40% of KM12C and KM12L4 cells, whereas >60% of KM12C or KM12L4 cells bound to anti-CXCR2. Thus, expression of CXCR1 and CXCR2 was lower in Caco2 cells compared with KM12C and KM12L4 cells. We did not observe a difference in immunostaining intensity between KM12C (low metastatic) and KM12L4 (high metastatic) cells. RT-PCR analysis demonstrated that high metastatic KM12L4 cells expressed high levels of CXCR1 and CXCR2 mRNA compared with low metastatic or nonmetastatic KM12C and Caco2 cells (Fig. 2). These data suggest that colon carcinoma cells with different metastatic potentials differentially express receptors for IL-8.
and KM12C cell proliferation in a concentration-dependent manner, and their response is inversely associated with levels of IL-8 produced by these cells.

**Differential Migratory Potential of Colon Carcinoma Cells.** We determined the putative role of IL-8 expression on the invasive potential of human colon carcinoma cells with different metastatic potentials. KM12C (low IL-8 expression) and KM12L4 (high IL-8 expression) were cultured in Matrigel-coated 8-μm-pore size Transwell chambers, and their migratory potential was determined. The percentage of migration was higher in KM12L4 cells (39.2 ± 6.7) as compared with KM12C cells (19.4 ± 3.5), suggesting an association between IL-8 expression and aggressiveness in colon carcinoma cells.

### Table 1

<table>
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<th>IL-8 (ng/ml)</th>
<th>Caco2</th>
<th>KM12C</th>
<th>KM12L4</th>
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<td>12.09 ± 3.86</td>
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<tr>
<td>100</td>
<td>100.98 ± 13.97</td>
<td>43.66 ± 9.43</td>
<td>7.82 ± 5.77</td>
</tr>
</tbody>
</table>

*Significantly different from KM12L4 cells expressing high levels of IL-8 at the corresponding concentrations of exogenously added recombinant IL-8 (P < 0.05).*

**Fig. 4** Inhibition of IL-8-mediated proliferation of human colon carcinoma cells using neutralizing antibody to IL-8, CXCR1, and CXCR2. KM12C and KM12L4 cells (5 × 10⁴) were cultured with medium alone or medium containing 0.1 μg/ml of control antibody of similar isotype, anti-IL-8, anti-CXCR1, or anti-CXCR2 for 72 h, and inhibition in cell proliferation was determined by MTT. The values represent the percentage of inhibition in cell proliferation of triplicate culture; bars, SE. This is a representative experiment of three done in triplicate.

### Materials and Methods

**Induction of Colon Carcinoma Cell Proliferation by IL-8.** In the next set of experiments, we determined whether the exogenous addition of IL-8 regulates colon carcinoma cell proliferation. Caco2 (non-), KM12C (low), and KM12L4 (high) cells were incubated in medium alone or medium containing neutralizing antibodies to IL-8, CXCR1, or CXCR2. Antibodies of the same isotype were used as a control. Colon carcinoma cell proliferation was inhibited by anti-IL-8, anti-CXCR1, or anti-CXCR2 in both KM12C and KM12L4 cells (Fig. 4). The percentage of inhibition ranged from 15.7 to 27.9% (Fig. 4). Effective inhibition of cell proliferation by antibodies to IL-8 and receptors CXCR1 and CXCR2 suggests that IL-8 might be an autocrine growth factor for human colon cancer cells.

**Inhibition of IL-8-induced Proliferation by Antibody to IL-8, CXCR1, and CXCR2.** To determine the function of the constitutive IL-8 expression in colon carcinoma cells, we examined whether autocrine IL-8 production and/or activity regulates colon cancer cell proliferation. KM12C (low IL-8 producer) and KM12L4 (high IL-8 producer) colon cancer cells (5000/well) were plated into 96-well plates in medium alone or medium containing neutralizing antibodies to IL-8, CXCR1, or CXCR2. Antibodies of the same isotype were used as a control. Colon carcinoma cell proliferation was inhibited by anti-IL-8, anti-CXCR1, or anti-CXCR2 for 72 h, and inhibition in cell proliferation was determined by MTT. The values represent the percentage of inhibition in cell proliferation of triplicate culture; bars, SE. This is a representative experiment of three done in triplicate.

### Table 1

**IL-8 induced stimulation of cell proliferation of human colon carcinoma cells with different metastatic potentials expressing different levels of IL-8**

Caco2, KM12C, and KM12L4 cells expressing different levels of endogenous IL-8 were incubated with different concentrations of recombinant IL-8. The percentage of stimulation in cell proliferation by IL-8 was determined as described in “Materials and Methods.” The values are mean % stimulation ± SE. This is representative of three experiments done in triplicate.

**Fig. 3** Immunocytochemical staining of CXCR1 and CXCR2 in human colon carcinoma cells with different metastatic potentials. A, Caco2 cells with CXCR1 staining. B, Caco2 cells with CXCR2 staining. C, KM12C cells with CXCR1 staining. D, KM12C cells with CXCR2 staining. E, KM12L4 cells with CXCR1 staining. F, KM12L4 cells with CXCR2 staining. A–F, ×400.
Inhibition of Colon Carcinoma Cells Binding to Endothelial Cells. To determine the role of IL-8 in tumor-endothelial cell interaction, adhesion of colon carcinoma cells to HUVECs was examined. 51 Cr-labeled Caco2 and KM12C cells were plated on HUVEC-coated wells in the presence or absence of recombinant human IL-8. Treatment of cells with recombinant IL-8 enhanced colon carcinoma cells binding to HUVECs. The binding stimulation by IL-8 was 50% in low metastatic KM12C cells (Fig. 6A). In another set of experiments, we determined the autocrine role of IL-8 production in colon carcinoma-endothelial cell interaction by using high IL-8-producing KM12L4 cells and neutralizing antibodies against IL-8, CXCR1, and CXCR2 in different combinations. Binding of KM12L4 cells to HUVECs was inhibited by anti-IL-8, anti-CXCR1, and anti-CXCR2 alone or anti-IL-8 in combination with anti-CXCR1 and/or anti-CXCR2 (Fig. 6B). These results suggest a relationship between IL-8 and metastasis in colon carcinoma.

DISCUSSION

We observed a differential expression of IL-8 and its receptors, CXCR1 and CXCR2, in human colon carcinoma cells with different metastatic potentials. IL-8 protein and mRNA expression was significantly lower in nonmetastatic and low metastatic colon carcinoma cells than in high metastatic colon carcinoma cells, suggesting an association with metastatic potential. The expression of CXCR1 and CXCR2 protein and mRNA was lower in nonmetastatic Caco2 cells as compared with metastatic KM12C and KM12L4 cells. Exogenous recombinant IL-8 induced cell proliferation in colon carcinoma cells. Neutralizing anti-IL-8, anti-CXCR1, and anti-CXCR2 showed inhibition of cell proliferation in both KM12C and KM12L4 cells. Studies have reported that IL-8 regulates cell proliferation and angiogenesis in various tumors (10–18). Our data suggest that expression of IL-8 in colon carcinoma cells might play an important role in growth and metastasis. These data also confirm a recent study (23) that reports that IL-8 can be an autocrine factor, playing a role in colon carcinoma growth.

Primary malignant neoplasms consist of cells with different metastatic potentials expressing different levels of genes regulating metastatic phenotype (20, 35). KM12L4 is a high metastatic subclone from low metastatic KM12C cells. In the present study, we observed different IL-8 mRNA and protein expression among the three colon carcinoma cell lines with different metastatic potentials. Our data suggest that colon carcinoma cells with different metastatic potentials, constitutively expressing different levels of IL-8, respond differently to exogenous IL-8. Furthermore, neutralizing antibodies to IL-8 and its receptors inhibited the proliferation of colon carcinoma cells, suggesting that IL-8 may act as an autocrine growth factor and may contribute to metastatic potential in colon carcinoma cells.

IL-8 can bind two receptors, CXCR1 and CXCR2. CXCR1 only binds IL-8, whereas CXCR2 binds IL-8 and several other CXC chemokines (5–9). These receptors have been shown to play an important role in angiogenesis and tumor progression (3, 5, 7, 9, 36). In this study, different CXCR1 and CXCR2 mRNA and protein expression was observed in colon carcinoma cells in accordance with metastatic potential. In addition, antibodies to
CXCR1 and CXCR2 showed significant inhibition of cell proliferation in both KM12C and KM12L4 cells. These data suggest that inhibition of IL-8 production and/or activity can inhibit proliferation of colon carcinoma cells, and IL-8 may act as an autocrine or paracrine growth factor.

Tumor-endothelial cell interaction is important for tumor invasion and metastasis (37–39). A key event in cancer metastasis is transendothelial migration of tumor cells (40). This process involves multiple adhesive interactions between tumor cells and endothelial cells (35, 38). In the present study, we observed that recombinant human IL-8 enhanced adherence of colon carcinoma cells to endothelial cells. In addition, neutralization of IL-8 activity by antibodies to IL-8 and its receptors inhibited tumor cell adherence to endothelial cells in high metastatic KM12L4 cells producing higher levels of IL-8. These studies provide an additional evidence for the role of IL-8 in modulating phenotypes associated with metastasis. However, it is not clear whether IL-8-mediated regulation of adherence of colon carcinoma cells to endothelial cells requires a specific set of adhesion molecules. Studies are in progress to determine the role of adhesion molecules in IL-8-mediated tumor-endothelial cell interaction and transendothelial migration.

Our laboratory and others have demonstrated that IL-8 expression by malignant cells can induce the proliferation of tumor cells in an autocrine manner and induce angiogenesis, migration, and collagenase production, all of which are important steps in tumor growth and metastasis (23, 25, 41, 42). Several studies have reported that IL-8 regulates cell migration (25), proliferation, and angiogenesis in colon carcinoma cells (24, 25). Serum levels of IL-8 in colon cancer patients were significantly increased in patients with metastasis (43). The present study showed that constitutive expression of IL-8 in colon carcinoma cells correlated with different metastatic potentials.

In summary, we demonstrated a correlation between constitutive expression of IL-8 and its receptors in colon carcinoma cells with different metastatic potentials. Our data suggest that IL-8 may act as an autocrine/paracrine growth factor in colon carcinoma cells and modulate phenotypes associated with tumor growth and metastasis.

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