Low-dose interleukin-8 induces the adhesion, migration and invasion of the gastric cancer SGC-7901 cell line

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Abstract. Interleukin-8 (IL-8), an important inflammatory cytokine, is strongly associated with gastric cancer development and metastasis. High-dose (>1 ng/ml) IL-8 has been revealed to promote the adhesion, migration and invasion of human gastric cancer SGC-7901 cells in a dose-dependent manner. However, the IL-8 level produced by gastric cells is marginal, at even less than 1 ng/ml. It is unclear whether low-dose IL-8 also induces these capacities. In the present study, the effect of low-dose IL-8 on the adhesion, migration and invasion of the SGC-7901 cell line and the underlying molecular mechanism with regard to cluster of differentiation 44 (CD44) were investigated. The SGC-7901 cells were exposed to various concentrations of IL-8 (0, 0.2, 0.5, 0.8 and 1 ng/ml) *in vitro*. The adhesion of the SGC-7901 cells to fibronectin, an extracellular matrix component, was then detected by cell counting kit 8 assay. Migration and invasion abilities were evaluated by wound scratch and Transwell chamber assays. In addition, protein and mRNA levels of CD44 were measured using immunofluorescence and western blotting, and quantitative polymerase chain reaction, respectively, in cells cultured for 72 h. Following the exposure of the SGC-7901 cells to the various low doses of IL-8, the cell adhesion, migration and invasion capacities were promoted by IL-8, but not in a significant dose-dependent manner. Low-dose IL-8 upregulated the protein and mRNA expression of CD44. In conclusion, low-dose IL-8 potently induces the adhesion, migration and invasion of SGC-7901 cells, and the regulation of CD44 expression is one of the potential molecular mechanisms involved.

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Introduction

The incidence of gastric cancer is gradually declining throughout the world, but it remains the second most common type of fatal malignancy worldwide (1). Gastric cancer is one of the most aggressive tumors due to its important pathological features of easy invasion and metastasis. The key steps of invasion and metastasis include dissociation from the primary lesions, degradation and permeation about the extracellular matrix (ECM), migration in the blood or lymph stream, and adhesion and growth within secondary organs (2). A number of pathways and genes have been indicated to be involved in the metastasis of gastric cancer. Previous studies have demonstrated that chemokines and their receptors function as essential regulators of metastatic cancers, including gastric cancer, and are involved in a number of neoplastic processes (3,4).

Interleukin-8 (IL-8), a member of the neutrophil-specific CXC subfamily of chemokines with a Glu-Leu-Arg motif, is important in leukocyte chemotaxis, inflammatory responses and infectious diseases (5), as well as in the migration, invasion and proliferation of endothelial cells via their receptors, and in angiogenesis *in vivo* (6,7). Previous studies have suggested that cancer cells, including those of pancreatic, colon, ovarian and lung cancer, produce IL-8 (8-15). IL-8, an autocrine growth factor, is associated with tumor growth, angiogenesis, invasion and metastasis (8-15). In our previous study, IL-8 ranging from 0 to 100 ng/ml was shown to interfere with human gastric cancer SGC-7901 cells *in vitro*; it was found that high-dose IL-8 promoted cell adhesion to endothelial cell and ECM components, while also inducing the migration and invasion capacities of the SGC-7901 cells (16). However, the IL-8 level produced by gastric cells is marginal, at even less than 1 ng/ml (17). It remains unclear whether low-dose IL-8 treatment also induces these capacities in cells.

The purpose of the present study was to provide direct information with regard to the role of low-dose IL-8 in determining the metastasis of gastric cancer. In the current study, IL-8 ranging from 0 to 1 ng/ml was used to interfere with the SGC-7901 cells *in vitro* to investigate the effect of low-dose IL-8 on the adhesion, migration and invasion capacities of the cells, and the correlated molecular mechanism of cluster of differentiation 44 (CD44). The present study was approved by

the ethics committee of the Second Military Medical University (Shanghai, China).

Materials and methods

Cell culture. The human gastric cancer SGC-7901 cell line was purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The SGC-7901 cells were cultured in RPMI-1640 medium (Wisent, St. Bruno, Quebec, Canada) supplemented with 10% fetal bovine serum (Zhejiang Tianhang Biological Technology Co., Ltd., Hangzhou, China), 1% penicillin/streptomycin and 1% L-glutamine, and maintained at 37˚C in a humidified chamber containing 5% CO_2.

Adhesion assay. Fibronectin is an ECM component. To analyze the attachment of the SGC-7901 cells to fibronectin, 96-well plates were coated with 100 μg fibronectin (Sigma-Aldrich, St. Louis, MO, USA) overnight at 4˚C. Subsequent to being washed three times with phosphate-buffered saline (PBS) solution containing 1% bovine serum albumin (Sigma-Aldrich) to block non-specific cell adhesion, IL-8 (0, 0.2, 0.5, 0.8 and 1.0 ng/ml; Sigma-Aldrich) was added to $1x10^5$ cells/well for 2 h. Thereafter, the non-adherent cells were washed off with PBS. Cell adhesion was assessed by a cell counting kit 8 (Dojindo, Kunamoto, Japan) assay, using cellular DNA labeled with fluorescence reagent. The cells were cultured for an additional 4 h. Colorimetric absorbance was measured by a microplate reader (Multiskan MK3; Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm to obtain an optical density (OD) value. OD ultimate value = OD measured value - OD blank value.

Wound scratch assay. Cell migration was monitored in a wound scratch assay. Briefly, the SGC-7901 cells were seeded on a 6-well plate at a density of $2x10^5$ cells/well. A scratch was made with a sterile 10-μl pipette tip in a confluent cell monolayer. Subsequent to washing twice, IL-8 (dose identical to adhesion assay) was added in serum-free medium. Images of the wells were captured at the beginning of the experiment and after 12 and 24 h on an inverted microscope (CK40-F200; Olympus, Tokyo, Japan). Digital images were obtained with an Optronics MicroFire digital camera (Optronics, Goleta, CA, USA) driven by the Picture Frame imaging software (Optronics). All experiments were repeated three times.

Transwell chamber invasion assay. Transwell chambers (Corning, Tewksbury, MA, USA) were used to examine the invasion ability of the SGC-7901 cells, according to the manufacturer's instructions. Briefly, the SGC-7901 cells ($8x10^4$) were seeded in the upper chamber consisting of a thin layer of Matrigel basement membrane matrix (BD Bioscience, San Jose, CA, USA). Next, 600 μl culture medium and IL-8 (dose identical to adhesion assay) were added to the lower chamber. Subsequent to 24 h of incubation, the cells on the upper surface of the membrane were removed with a cotton swab, and the cells that had migrated through and attached to the lower surface of the membrane were fixed with 4% paraformaldehyde for 15 min. Thereafter, the cells were stained using the crystal violet cell colony staining kit (Shanghai Genmed Biological Technology Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The results are expressed as the mean number of cells invading in four random microscopic fields (magnification, x10).

Immunofluorescence staining. A total of $2x10^5$ SGC-7901 cells were seeded on 6-well plates and cultured with IL-8 (dose identical to adhesion assay) for 72 h. Subsequently, $7x10^4$ cells were placed on coverslips and fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 (Shanghai Sangon Biotech Co., Ltd., Shanghai, China) for 10 min, incubated in blocking buffer, and then incubated with CD44 rabbit anti-human monoclonal antibody (1:80; Epitomics, Burlingame, CA, USA) at 4˚C overnight. Cy3-conjugated Affinipure goat polyclonal anti-rabbit immunoglobulin (Ig)G (H+L; 1:1,000 dilution; Proteintech Group, Wuhan, Hubei, China) was added for an additional 1-h incubation. The cell nuclei were labeled with DAPI (Thermo Fisher Scientific). The coverslips were then mounted on a glass slide and visualized under a laser confocal scanning microscope (LSM710; Zeiss, Oberkochen, Germany).

Western blot analyses. A total of $2x10^5$ SGC-7901 cells were seeded in each well of 6-well plates and incubated for 72 h with IL-8 (dose identical to adhesion assay). Following stimulation, the cells were collected and denatured by 150 μl loading buffer. Proteins in the total cell lysate were separated by SDS-PAGE (10% separation gel and 5% spacer gel) and electrotransferred to polyvinylidene difluoride films (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Blotted films were placed in blocking solution for 1 h at room temperature. CD44 rabbit anti-human monoclonal antibody (1:250, Epitomics) was used to probe the blots overnight at 4˚C. The film was washed thoroughly, incubated with goat polyclonal anti-rabbit IgG horse radish peroxidase secondary antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h, and then visualized using the enhanced chemiluminescence method (Perkin Elmer Inc., Waltham, MA, USA). Blots were exposed to plain X-ray film in a darkroom. Grayscale reconstruction was performed using Image J software 1.48 (http://rsb.info.nih.gov./ij/), and the expression rate of CD44 versus that of GAPDH protein, which served as an internal control protein, was calculated. All experiments were repeated three times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The SGC-7901 cells ($1x10^5$) were collected after 72 h of incubation with IL-8 (dose identical to adhesion assay). Briefly, the total RNA of the cells was extracted using TRIzol reagent (Takara, Shiga, Japan) according to the manufacturer's instructions, and reverse transcribed. RT-qPCR was performed with SYBR Green in a Bio-Rad iQ5 Real-Time PCR system (Bio-Rad Laboratories Inc.). Cycling conditions consisted of one cycle of 95˚C for 2 min, 95˚C for 15 sec, 60˚C for 20 sec and 72˚C for 20 sec, and then 71 cycles of 60-95˚C for 30 sec (increasing by 0.5˚C every other cycles). The primer sequences used for amplification are shown in Table I. Based on the $2^{-\Delta\Delta Ct}$ value, the relative levels of CD44 mRNA expression were

Table I. Primer sequences used for quantitative polymerase chain reaction.

mRNA	Sense primer sequence	Size, bp
hGAPDH-F	5'-GGGTGTGAACCATGAGAAGTATG-3'	145
hGAPDH-R	5'-GATGGCATGGACTGTGGTCAT-3'	
CD44-F	5'-ATGGACAAGTTTTGGTGGCA-3'	230
CD44-R	5'-CAGGTCTCAAATCCGATGCTC-3'	

F, forward; R, reverse; CD44, cluster of differentiation 44.

Table II. Effect of IL-8 on gastric cancer SGC-7901 cell adhesion and invasion.

Concentration, ng/ml	Adhesion (OD value)	Invasion (invasive cell number)
0	0.186±0.012	785.00±48.13
0.2	0.192±0.018	758.00±18.04
0.5	0.208±0.009[a]	1115.00±81.85[b]
0.8	0.220±0.001[b,c]	1041.00±136.59[b]
1.0	0.213±0.003[a,d]	1103.33±134.52[b]

[a]P<0.05 vs. 0 ng/ml; [b]P<0.01 vs. 0 ng/ml; [c]P<0.01 vs. 0.2 ng/ml; [d]P<0.05 vs. 0.2 ng/ml. IL-8, interleukin 8; OD, optical density.

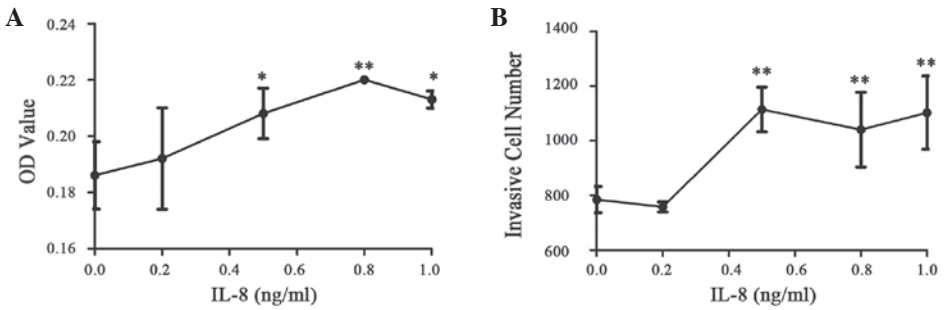

Figure 1. Effect of interleukin (IL)-8 on gastric cancer SGC-7901 cell adhesion and invasion. (A) Cell adhesion was significantly different at various IL-8 concentrations (P=0.012). Meanwhile, 0.5, 0.8 and 1.0 ng/ml IL-8 all significantly promoted SGC-7901 cell adhesion compared with 0 ng/ml IL-8 (P=0.028, P=0.002 and P=0.010, respectively). The most significant adhesion was found following addition of 0.8 ng/ml IL-8. However, this effect did not occur in a dose-dependent manner (all P>0.05 between the three groups). (B) Similar to the adhesion assay, cell invasion was significantly different at various IL-8 concentrations (P<0.001). Moreover, 0.5, 0.8 and 1.0 ng/ml IL-8 all significantly promoted SGC-7901 cell invasion compared with 0 ng/ml IL-8 (P<0.001, P=0.002 and P<0.001, respectively), but not in a dose-dependent manner (all P>0.05 between the three groups). *P<0.05 and **P<0.01 vs. 0 ng/ml IL-8 group. OD, optical density.

calculated. The data were normalized using GADPH as an internal control.

Statistical methods. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). All results are presented as the mean ± standard deviation. A one-way analysis of variance was used to assess invasion, adhesion, and protein and mRNA expression levels. The least significant difference method was used to analyze multiple post hoc comparisons. P≤0.05 was considered to indicate a statistically significant difference.

Results

IL-8 promotes SGC-7901 cell adhesion. The present study investigated the effect of low-dose IL-8 on the adhesion capacity of SGC-7901 cells to fibronectin. The data showed that IL-8 at concentrations of ≥0.5 ng/ml significantly promoted cell adhesion (P=0.028, P=0.002 and P=0.010 for 0.5, 0.8 and 1.0 ng/ml, respectively). The increase in adhesion peaked at a concentration of 0.8 ng/ml. Notably, this effect was not in a significant dose-dependent manner. No significant differences were identified in adhesion between the groups treated with 0.5, 0.8 and 1 ng/ml IL-8 (P>0.05 between all three groups; Table II; Fig. 1A).

IL-8 promotes SGC-7901 cell invasion. The effect of low-dose IL-8 on SGC-7901 cell invasion was also investigated. Similar to the adhesion assay, the invasion of the SGC-7901 cell line was promoted by IL-8 at concentrations ≥0.5 ng/ml (P<0.001, P=0.002 and P<0.001 for 0.5, 0.8 and 1.0 ng/ml, respectively) and this effect was not in a significant dose-dependent manner.

Table III. Effect of IL-8 on expression of CD44 protein and mRNA.

Concentration, ng/ml	CD44 protein	CD44 mRNA
0	0.899±0.006	1.00±0.06
0.2	0.894±0.001	1.10±0.11
0.5	0.941±0.009[a,b]	1.33±0.12[c]
0.8	0.964±0.005[a,b,d]	1.40±0.22[a,e]
1.0	0.911±0.001[b-d,f]	1.37±0.18[c,e]

[a]P<0.01 vs. 0 ng/ml; [b]P<0.01 vs. 0.2 ng/ml; [c]P<0.05 vs. 0 ng/ml; [d]P<0.01 vs. 0.5 ng/ml; [e]P<0.05 vs. 0.2 ng/ml; [f]P<0.01 vs. 0.8 ng/ml. IL-8, interleukin 8; OD, optical density; CD44, cluster of differentiation 44.

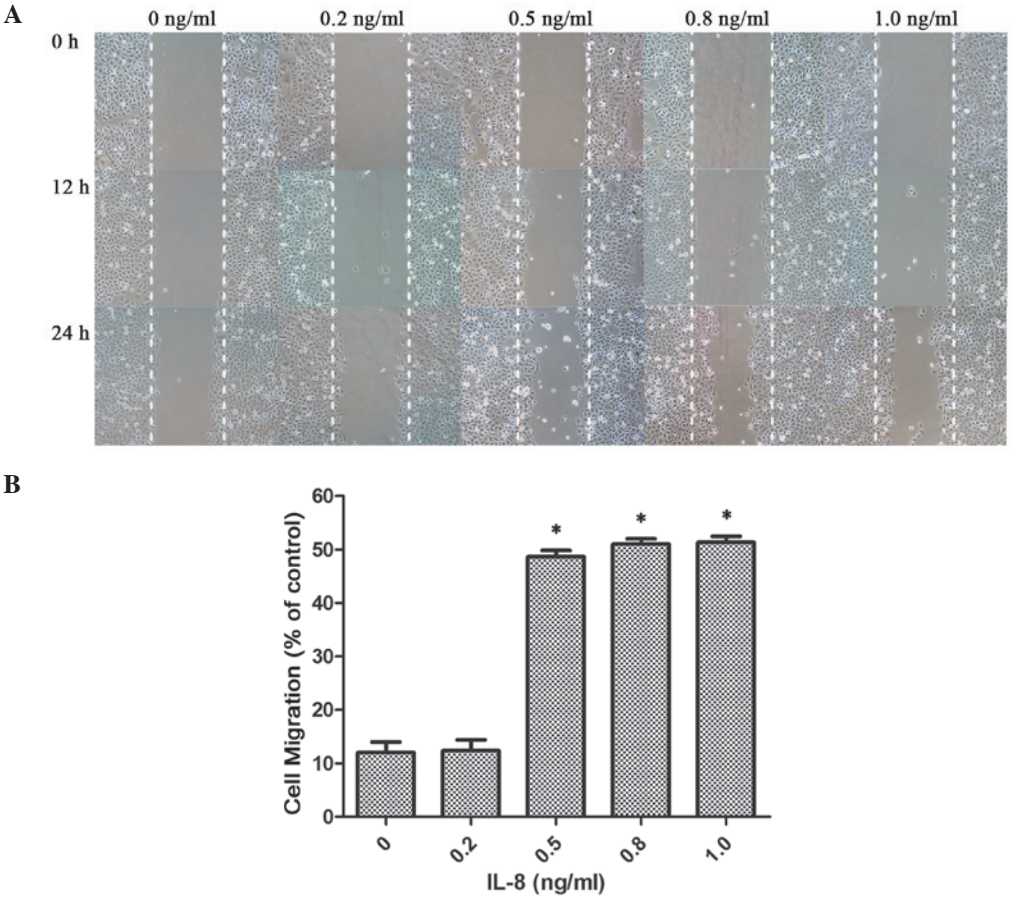

Figure 2. Effect of interleukin (IL-8) on gastric cancer SGC-7901 cell migration (wound scratch assay). (A) Monolayers of SGC-7901 cells exposed to low-dose IL-8 were wounded with pipette tips. The images obtained after 24 h demonstrated that low-dose IL-8 enhanced the migration capability of the SGC-7901 cells. (B) Quantification of wound healing assay. Treatment with 0.5, 0.8 and 1.0 ng/ml IL-8 significantly promoted SGC-7901 cell migration compared with the 0 ng/ml IL-8 treatment group (P<0.001 between all three groups), however this effect was not in a significant dose-dependent manner (P>0.05 between all three groups). *P<0.001 vs. 0 ng/ml IL-8 group.

No significant differences were identified between the groups treated with 0.5, 0.8 and 1 ng/ml IL-8 (P>0.05 between all three groups) (Table II; Fig. 1B).

IL-8 promotes SGC-7901 cell migration. The scratch assay showed IL-8-induced activation of SGC-7901 cell migration. After 24 h, the scratched area recovered more rapidly in IL-8-treated cells compared with untreated cells (P<0.001; Fig. 2). IL-8 significantly promoted the migration of the SGC-7901 cells, however this effect was not in a significant dose-dependent manner. No significant differences were identified between the groups treated with 0.5, 0.8 and 1 ng/ml IL-8 (P>0.05 between all three groups; Fig. 2).

IL-8 promotes CD44 protein and mRNA levels in SGC-7901 cells. To investigate the possible mechanism of the IL-8-induced adhesion and invasion of gastric cancer cell, the protein and mRNA expression levels of CD44 in SGC-7901 cells exposed to the various low doses of IL-8 were detected. IL-8 at concentrations of ≥0.5 ng/ml (0.5, 0.8 and 1.0 ng/ml)

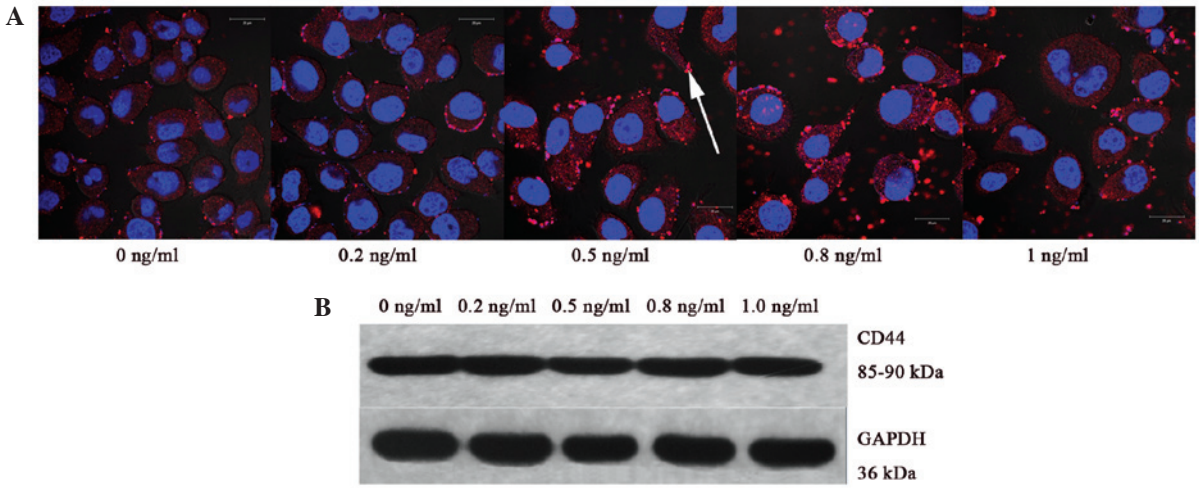

Figure 3. Interleukin (IL)-8 promotes the expression of cluster of differentiation (CD)44 protein in SGC-7901 cells, as determined by immunofluorescence staining and western blotting. (A) Nuclei were marked with DAPI (blue). CD44 immunostaining was restricted to the cell membrane. The white arrowhead points to CD44 counterstained with Cy3 (Red). (B) CD44 protein expression was determined by western blotting. CD44 protein expression was significantly different at various IL-8 concentrations (P<0.001). Furthermore, 0.5, 0.8 and 1.0 ng/ml IL-8 all significantly upregulated CD44 protein expression compared with 0 ng/ml IL-8 (P<0.001, P<0.001 and P=0.018, respectively) in a dose-dependent manner. The CD44 protein expression level peaked when the cells were exposed to 0.8 ng/ml IL-8.

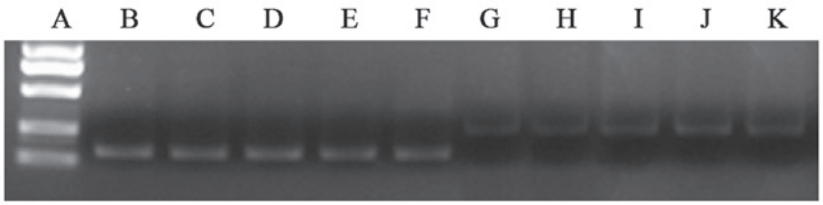

Figure 4. Effect of interleukin (IL)-8 on cluster of differentiation (CD)44 mRNA expression levels in SGC-7901 cells, as determined by reverse transcription-quantitative polymerase chain reaction. (A) Marker DL2000 (from top to bottom: 1000, 750, 500, 250 and 100 bp). (B-F) hGAPDH at (B) 0, (C) 0.2, (D) 0.5, (E) 0.8 and (F) 1.0 ng/ml (145 bp). (G-K) CD44 at (G) 0, (H) 0.2, (I) 0.5, (J) 0.8 and (K) 1.0 ng/ml (230 bp). Similar to protein expression, CD44 mRNA expression was significantly different at various IL-8 concentrations (P=0.026). Moreover, 0.5, 0.8 and 1.0 ng/ml IL-8 all significantly upregulated CD44 mRNA expression compared with 0 ng/ml IL-8 (P=0.021, P=0.008 and P=0.012, respectively), but not in a dose-dependent manner (all P>0.05 between the three groups). The mRNA expression level peaked when the cells were exposed to 0.8 ng/ml IL-8.

significantly upregulated CD44 protein (P<0.001, P<0.001 and P=0.018) and mRNA expression (P=0.021, P=0.008 and P=0.012). Corresponding to the adhesion assay, CD44 protein and mRNA expression levels peaked following exposure to 0.8 ng/ml IL-8. A dose-dependent effect was found in CD44 protein expression between the 0.5, 0.8 and 1.0 ng/ml groups, but not in mRNA expression (P>0.05 between all three groups; Table III; Figs. 3 and 4).

Discussion

Metastasis is one of the most fatal characteristics of malignancies, accounting for >90% of tumor-related mortalities worldwide (18). Although the incidence of gastric cancer has declined, gastric cancer remains one of the most common malignancies worldwide and frequently develops lymph node, peritoneal and liver metastases (19,20). Distant metastasis is an important sign of a poor prognosis in gastric cancer patients. The metastasis of tumor cells is a complex, multi-staged process that involves tumor cell transformation, growth, angiogenesis, invasion, dissemination and survival in the circulation, followed by adhesion and colonization of a secondary organ or tissue (18,21). Therefore, it has been widely accepted that the capacity of cancer cell adhesion, migration and invasion is one of the most important prerequisites for cancer metastasis (22). IL-8, a multifunctional pro-inflammatory cytokine, has been shown to be associated with infection, inflammation and other disease states, including tumorigenesis (5,6). As an important regulatory autocrine factor within the tumor microenvironment (23), IL-8 is considered to be produced by a range of human cancer cell types, including human melanoma (24), squamous cell carcinoma (25), cervical cancer (26), ovarian cancer (27), non-small cell lung cancer (28) and colon cancer (12), and it mediates potential mitogenic, motogenic and angiogenic effects (29,30). It has been widely accepted that IL-8 plays a significant role in the development and metastasis of cancer (12).

Several studies have found an association between IL-8 and gastric cancer. In one *in vitro* study, gastric cancer cells produced IL-8 in response to exposure to the cytotoxic strain of *Helicobacter pylori*, which was classified by WHO as a group I carcinogen (31). IL-8 is involved in the progressive growth of gastric cancer through autocrine or paracrine mechanisms (32). *In vivo*, IL-8 has been shown to be the most markedly upregulated

gene in gastric cancer and may regulate the neovascularization of human gastric cancer (33). A significant correlation has bee found between IL-8 levels and the depth of invasion, venous invasion and lymphatic invasion, and IL-8 may be an independent prognostic factor in human gastric carcinoma (34). In a previous study, using treatment of IL-8 at a concentration of >1 ng/ml, we reported that IL-8 could enhance the adhesion, migration and invasion of the human gastric cancer SGC-7901 cell line in a dose-dependent manner (16). This result was supported by another similar study by Kuai *et al* using cDNA and small interfering RNA transfectants (35). However, IL-8 production by gastric cells is marginal, at even less than 1 ng/ml. For example, the highest level of IL-8 has been recorded at only 0.17 ng/ml in IM95 gastric cancer cells cultured for 3 days *in vitro* (17). It remains unclear whether low-dose IL-8 also induces the adhesion, migration and invasion of gastric cancer cells in a dose-dependent manner. In the current study, IL-8 ranging from 0.5 to 1 ng/ml promoted the adhesion, migration and invasion of SGC-7901 gastric cancer cells. Nevertheless, a dose-dependent effect was not found. These results suggested that IL-8 may have potential pro-metastatic effects, even at low doses.

CD44, as a polymorphic integral membrane glycoprotein expressed by a number of cell types, plays a significant role in lymph node homing (36), matrix adhesion (37), and T lymphocyte activation (38). Serving as the main transmembrane hyaluronate receptor, CD44 is regarded not only as a cell adhesion molecule, but also as a determinant of metastatic and invasive behavior in a variety of malignancies, including malignant melanoma, lung carcinoma, breast cancer, leukemia and gastrointestinal carcinomas (39-42).

Metastasis is characterized by a loss of adhesion, and is a significant event in human cancer development that allows cancer cells to leave their original site and subsequently invade and adhere to other sites, for example, the lymph nodes, liver or peritoneum (43). In gastric adenocarcinoma, CD44 is highly expressed and is correlated with a poor prognosis in patients with the intestinal disease type (44). In a previous study, we reported that the regulation of matrix metalloproteinase-9, intercellular adhesion medlecule-1 and E-Cadherin expression may be one of the potential molecule mechanisms for IL-8-induced adhesion, migration and invasion in gastric cancer (16). In the current study, the role of CD44 was further investigated in IL-8-induced adhesion, migration and invasion as another potential molecule mechanism. Similar to the results of the adhesion assay, it was found that CD44 protein and mRNA expression were promoted by low-dose IL-8. An increase in the CD44 protein and mRNA expression level was induced most significantly by 0.8 ng/ml IL-8. This result suggests that the regulation of CD44 expression may be another potential molecule mechanism involved in IL-8-induced adhesion, migration and invasion in gastric cancer.

In conclusion, low-dose IL-8 may promote the adhesion, migration and invasion of the gastric cancer SGC-7901 cell line, but not in a dose-dependent manner, and that this is correlated with the regulation of CD44 expression.

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