Overexpression of IL-6 by Gene Transfer Stimulates IL-8-mediated Invasiveness of KYSE170 Esophageal Carcinoma Cells

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Abstract. Interleukin-6 (IL-6) has been associated with disease progression and poor prognosis in esophageal carcinoma. The aim of this study was to investigate the possible influence of IL-6 on the biological activities of esophageal carcinoma cells in terms of invasiveness. The human esophageal carcinoma cell line, KYSE170, was transfected with a plasmid vector expressing IL-6, and a stable transfectant overexpressing IL-6 was established. Invasiveness was evaluated by an invasion assay and compared between IL-6 and control transfectants. The invasiveness of the IL-6 transfectant was significantly higher than that of the control transfectant, and was significantly reduced by IL-6-specific siRNA. In reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, IL-8 expression was significantly higher in the IL-6 transfectant than in the control transfectant, whereas the expression of Hepatocyte growth factor (HGF) and Vascular endothelial growth factor (VEGF) was not different. IL-8 expression in the IL-6 transfectant was significantly inhibited by IL-8-specific siRNA, whereas IL-6 expression was not. In addition, the invasiveness of the IL-6 transfectant was significantly reduced by IL-8-specific siRNA. These results indicate that the overexpression of IL-6 increases the invasiveness of KYSE170 esophageal carcinoma cells and IL-6-induced IL-8 plays a predominant role in increasing invasiveness.

Esophageal carcinoma is one of the most malignant tumor types due to its aggressive behavior. Patients with esophageal carcinoma frequently have an advanced tumor stage and a high incidence of recurrence even if curative resection is performed (1). Multimodal treatment consisting of preoperative chemotherapy or chemoradiotherapy has recently been shown to significantly improve survival of patients with advanced esophageal carcinoma; however, the 5-year survival rate is still not satisfactory. A subgroup of tumors is resistant to these treatments (2, 3).

Interleukin-6 (IL-6), a potent pro-inflammatory cytokine, has been associated with disease progression in various malignancies (4). Serum IL-6 levels have been shown to be higher in patients with esophageal carcinoma than in healthy controls, and have been correlated with disease progression and poor prognosis (5, 6). IL-6 levels have also been shown to be higher in tumor tissues than in adjacent normal esophageal tissues (6-8). This indicates that IL-6 in tumor tissues plays a pivotal role in the pathological behavior of esophageal carcinoma. In this regard, we reported that elevated serum IL-6 levels following the completion of chemoradiotherapy, but not before chemoradiotherapy, were associated with a poor prognosis in patients with advanced esophageal squamous cell carcinoma who underwent induction chemoradiotherapy followed by esophagectomy, and that IL-6 expression induced by chemoradiotherapy in tumor cells or within tumor tissues may contribute to resistance to chemoradiotherapy (9). This also suggests that the overexpression of IL-6 may modulate the malignant potential of esophageal carcinoma cells.

The aim of the present study was to investigate the possible influence of IL-6 on the malignant behaviors of esophageal carcinoma, specifically its invasiveness, using esophageal carcinoma cells overexpressing IL-6 by gene transfection.
Materials and Methods

Cells and cell culture. The human esophageal squamous cell carcinoma cell line, KYSE170, was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). Cells were maintained in RPMI-1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, and 4 mM L-glutamine (culture medium: CM) at 37°C in 5% CO₂. IL-6-overexpressing KYSE170 cells were established as described previously (10); briefly, 24 h after plating KYSE170 cells (1×10⁵ cells/well) on 24-well plates in CM, cells were transfected with pBapo-CMV-Neo or pBapo-CMV-hIL-6, using lipofectamine (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. After 24 h, the transfected cells were trypsinized from the plate and replated onto a 10-cm culture dish in CM containing a final concentration of 1 mg/ml G418 (Geneticin; Invitrogen). G418-resistant clones were selected and expanded. In the present study, one of the clones expressing the highest level of IL-6 was used designated as KYSE-IL6. Cells transfected with pBapo-CMV-Neo were used as the control (KYSE-Neo).

Enzyme-linked immunosorbent assay (ELISA). ELISA for IL-6 and IL-8 was performed with the cell culture supernatants of KYSE-IL6 and KYSE-Neo using the Human IL-6 and IL-8 ELISA kit (Endogen, Rockford, IL, USA), according to the manufacturer’s instructions. Briefly, cells were treated under appropriate conditions, and culture supernatants were collected, centrifuged, and filtered through a 0.45-μm Steriflip Filter Unit (Millipore, Bedford, MA, USA). The absorbance (540 nm) for each sample was analyzed by an ELISA reader and interpolated with a standard curve.

Inhibition by siRNAs. Two different types of siRNA (028 and 029) specific for hIL-6 (GenBank accession no. NM_000660) were prepared for IL-6 inhibition. The siRNAs specific for hIL-6 and siRNA-random for the control were purchased from Takara Bio Incorporation (Otsu, Japan). The sense and anti-sense strands of IL-6-specific and control siRNAs were described in our previous study (10). In the present study, siRNA was used for IL-6 inhibition experiments. All of these sequences were shown by a BLAST search to not share sequence homology with any known human mRNA. The siRNA specific for hIL-8 (sc-39631) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Transient transfection of these siRNAs into KYSE-IL6 was performed using Trans IT-TKO (Invitrogen) and the effects of siRNA inhibition were assessed by ELISA for IL-6 and IL-8 (Endogen). Briefly, KYSE-IL6 cells (1×10⁵ cells/well) were seeded on a 6-well plate in 2 μl CM. After 24 h incubation, IL-6 or IL-8-specific siRNA was then transiently transfected into the cells using Trans IT-TKO (Invitrogen). After 48 h, the culture supernatant was collected and the concentration of IL-6 and IL-8 in the supernatant was determined by ELISA (Endogen).

Invasion assay. The invasiveness of KYSE-IL6 and KYSE-Neo cells was evaluated using a Boyden chamber with filter inserts (pore size, 8 μm) coated with Matrigel in 24-well dishes (BD Biosciences, Bedford, MA, USA). Cells (1×10⁵ cells/well) were seeded in the upper chamber in serum-free medium. The lower chamber contained CM. The chambers were incubated for 48 h at 37°C in 5% CO₂, and non-invaded cells were removed from the top surface of the insert by scrubbing with cotton swabs. Invaded cells were fixed on the membrane and stained with Diff-Quick staining reagents (Sysmex, Kobe, Japan). The number of cancer cells on the underside of the filters was counted in three independent fields of view at ×200 magnification for each insert. In siRNA experiments, KYSE-IL6 cells were pre-treated with siRNA as follows: briefly, cells were seeded in 12-well plates (1×10⁵ cells/well) and incubated for 24 h. IL-6 or IL-8-specific siRNA was then transiently transfected into the cells using Trans IT-TKO (Invitrogen). After 48 h, collected cells were subjected to the invasion assay.

Real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from KYSE-IL6 and KYSE-Neo with an RNeasy Mini kit (Qiagen, Hilden, Germany). PCR primers were designed according to the IL-6 sequence: 5’-TCAATGAGGAGACTTGCCTG-3’, and 5’-GAITGATTGTCACTGTCCTGC-3’. The primers for IL-8, Hepatocyte growth factor (HGF), and Vascular endothelial growth factor C (VEGF-C) were designed as follows IL-8: 5’-CAATCCTAGTTGTGACTCTCC-3’, and 5’-AATTACTAAATATGGTCTGGAG-3’; HGF: 5’-CCCTATTCTCTGTGTGAAGTG-3’, and 5’-TGTTTCTGTTTTGGCAAAAGA-3’; VEGF-C: 5’-CACGACGAAGGACCTCCAGCAAG-3’, and 5’-TTAGACATGACATCGGCCAGGA-3’. The primers for GAPDH were designed as follows: 5’-TGTTATCGTGGAAGGACTCATGAC-3’, and 5’-ATGCCCCAGTGGTCCCGTTCAGC-3’, and were used as an internal reference gene for analysis. Quantitative real-time one-step RT-PCR was performed by monitoring the increase in fluorescence of SYBR Green I dye with a Quantitect SYBR Green RT-PCR kit (Qiagen) and a Light Cycler 1.5 instrument (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s protocol.

Statistical analysis. All values are presented as the mean standard deviation. The Student’s t-test was used for statistical comparisons. Differences were considered significant if a p-value less than 0.05 was obtained.

Results

Invasiveness of the IL-6 transfectant KYSE-IL6. To assess the effects of IL-6 overexpression by gene transfection on IL-6 production and invasiveness, an ELISA for IL-6 and an invasion assay were performed comparing between the IL-6 transfectant (KYSE-IL6) and control transfectant (KYSE-Neo). In the ELISA, the level of IL-6 produced by KYSE-IL6 was 3.3-times higher than that by KYSE-Neo (p<0.01) (Figure 1A). In the invasion assay, the invasiveness of KYSE-IL6 was significantly higher than that of KYSE-Neo (p<0.01) (Figure 1B and C).

Effect of IL-6 inhibition on the invasiveness of the IL-6 transfectant. The effects of siRNA-mediated IL-6 inhibition on KYSE-IL6 were assessed by ELISA for IL-6 and an invasion assay. IL-6 production (p<0.01) by and the invasiveness (p<0.01) of KYSE-IL6 were significantly lower with IL-6-specific siRNA than with control siRNA (Figure 2).
Expression of IL-8, HGF, and VEGF in the IL-6 transfectant. To assess the underlying mechanisms for the IL-6-increased invasiveness of KYSE-IL6, the expression of IL-8, HGF, and VEGF was investigated by RT-PCR and compared between KYSE-IL6 and KYSE-NEO. The expression of HGF and VEGF was not different between KYSE-IL6 and KYSE-NEO, whereas IL-8 expression was significantly higher in KYSE-IL6 than in KYSE-NEO \( (p<0.01) \) (Figure 3).

Effects of IL-8 inhibition on the expression of IL-6 and IL-8 in the IL-6 transfectant. The effects of IL-8-specific siRNA on the expression of IL-6 and IL-8 in KYSE-IL6 were investigated by RT-PCR. IL-8 expression was significantly inhibited by IL-8-specific siRNA \( (p<0.01) \), whereas IL-6 expression was not affected by IL-8 inhibition (Figure 4).

Effects of IL-8 inhibition on the invasiveness of the IL-6 transfectant. The effects of siRNA-mediated IL-8 inhibition on KYSE-IL6 were assessed by ELISA for IL-8 and an invasion assay. IL-8 production \( (p<0.01) \) by and the invasiveness \( (p<0.01) \) of KYSE-IL6 were significantly lower with IL-8-specific siRNA compared to that of control siRNA (Figure 5).
Discussion

The elevated expression of pro-inflammatory cytokines within tumor tissues or in the systemic circulation has been associated with disease progression and poor prognosis in various types of cancers including esophageal carcinoma (11). In addition, treatment-induced overexpression of pro-inflammatory cytokines within tumor tissues or in tumor cells may influence treatment outcomes or modulate the malignant potential of tumor cells (9, 12). The present study demonstrated that the intracellular overexpression of IL-6 by gene transfection increased the invasiveness of KYSE170 esophageal carcinoma.
Figure 4. The effects of Interleukin-8 (IL-8) inhibition on the expression of IL-6 and IL-8 in the IL-6-overexpressing transfectant KYSE-IL6. IL-6 and IL-8 expression in KYSE-IL6 cells pre-treated with IL-8-specific siRNA or control siRNA was assessed by Real-time reverse transcription-polymerase chain reaction (RT-PCR). Data represent the mean±standard deviation and were statistically significantly at p<0.01.

Figure 5. The effects of Interleukin-8 (IL-8) inhibition on the invasiveness of the IL-6-overexpressing transfectant. IL-8 concentrations in the culture supernatant of KYSE-IL6 cells pretreated with IL-8-specific siRNA or control siRNA were determined by IL-8 ELISA (A), and their invasiveness was assessed by invasion assay (B). Data represent the mean±standard deviation and were statistically significantly at p<0.01.
cells and IL-6-induced IL-8 expression played a predominant role in this increased invasiveness.

IL-6 has diverse actions on tumor cells; although IL-6 inhibits tumor cell proliferation (13-15), it can also stimulate tumor cell proliferation, act as an anti-apoptotic factor, and confer resistance to chemotherapeutic agents or radiation (4). In our previous study by using the same esophageal carcinoma cells as in the present study, IL-6 overexpression by gene transfection rendered esophageal carcinoma cells resistant to cisplatin-induced apoptosis, but had no influence on tumor cell proliferation (10). In the present study, overexpression by gene transfection and siRNA-mediated inhibition of IL-6 clearly showed that IL-6 was responsible for the increased invasiveness of KYSE170 cells. In previous reports, IL-6 has been shown to promote the invasiveness of head and neck squamous cell carcinoma cells (13) and breast carcinoma cells (16). These studies focused on the possible influence of exogenous IL-6 on tumor cells within the tumor microenvironment, whereas the present study used a transfection approach based on possible treatment-induced intracellular overexpression of IL-6 in tumor cells.

The overexpression of a pro-inflammatory cytokine is frequently accompanied by the co-expression of and elevation of expression of other cytokines or growth factors in tumor cells or tumor tissues. The overexpression of angiogenic factors such as IL-8, HGF, and VEGF has been shown in relation to tumor progression in esophageal carcinoma (12, 17-18). In the present study, the overexpression of IL-6 led to a significant increase in IL-8 expression, and siRNA-mediated inhibition of IL-8 reduced IL-6-promoted invasiveness of KYSE170 cells. These findings clearly indicate that IL-6 promoted invasiveness is largely mediated by IL-8.

IL-8 is a pro-inflammatory and pro-angiogenic cytokine, and similarly to IL-6, its overexpression has been shown in tumor cells and stromal cells such as endothelial cells, neutrophils, and tumor-associated macrophages, within tumor tissues in a variety of cancer types. IL-8 also plays a significant role in mediating angiogenesis, invasiveness, and metastasis through tumor–host or tumor–stromal interactions within the tumor microenvironment (19). In addition, elevated levels of serum IL-8 have been correlated with disease progression, especially with the most advanced stages of disease (20-23). In esophageal squamous cell carcinoma, elevations in serum IL-8 levels have been correlated with lymph node and distant metastases, i.e. the ability of tumor cells to spread (24). Regarding its association with invasiveness, IL-8 has been reported to promote the invasiveness of breast carcinoma cells (25) and pancreatic carcinoma cells (26). On the other hand, the underlying mechanisms for the IL-6-promoted invasiveness of tumor cells have been studied in association with epithelial–mesenchymal transition and mainly in breast carcinoma (27). The present study demonstrated for the first time that IL-8 regulated tumor cell invasiveness by acting as a downstream effector of IL-6 in esophageal carcinoma cells.

Regarding the cytokine cross-talk associated with invasiveness, Lederle et al. reported that IL-6 promoted the invasiveness of skin squamous cell carcinoma cells by inducing a reciprocally-regulated cytokine network in these tumor cells (28). IL-6 induced the production of IL-8, Granulocyte-macrophage colony-stimulating factor (GM-CSF), and VEGF, whereas IL-8 and VEGF, induced GM-CSF, but not IL-6. This IL-6 to IL-8 one-directional action seems to be in line with our finding that gene transfection of IL-6 induced IL-8 expression, whereas siRNA-mediated inhibition of IL-8 had no influence on IL-6 expression.

In conclusion, the present study demonstrated that the overexpression of IL-6 by gene transfection stimulated KYSE170 esophageal carcinoma cell invasiveness and that the intracellular IL-6 to IL-8 one-directional signaling pathway plays a predominant role in the increased invasiveness. A complex, reciprocally regulated cytokine network within the tumor microenvironment may regulate the clinical behavior of cancer cells, such as invasiveness, metastasis, or treatment resistance. Accordingly, specific inhibition of a key signaling pathway that plays a central role in the cytokine network may have significant therapeutic potential for modulating disease progression.

References


