Overexpression of Interleukin-6 Suppresses Cisplatin-induced Cytotoxicity in Esophageal Squamous Cell Carcinoma Cells

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Abstract. Interleukin-6 (IL-6) expression at local tumor sites or in systemic circulation is associated with disease progression and poor prognosis of esophageal cancer. The aim of this study was to investigate the possible influence of IL-6 on biological activities of esophageal cancer cells in terms of chemosensitivity. Human esophageal cancer cell lines TE13 and KYSE170 were transfected with a plasmid vector expressing IL-6 and stable transfectants overexpressing IL-6 were thus established. The sensitivity of IL-6 transfectants to cisplatin was evaluated using a WST-8 assay and cell-cycle analysis. In addition, the inhibitory effects of IL-6-specific siRNAs were investigated. IL-6 transfectants showed significantly reduced sensitivity to cisplatin compared to control transfectants. In addition, the reduced cisplatin sensitivity of IL-6 transfectants was restored by pretreatment with IL-6-specific siRNA. These results suggest that intracellular IL-6 expression in tumor cells may act as a resistance factor against cisplatin-based treatments for esophageal cancer.

Esophageal carcinoma is one of the most malignant tumors in terms of its aggressive behavior and poor prognosis. At the time of diagnosis, patients with tumors are often found to have an advanced stage of the disease with a potentially systemic spread and there is a high incidence of recurrence even if curative resection is performed (1-3). Chemotherapy using 5-fluorouracil and cisplatin in combination with radiotherapy is considered the standard treatment for advanced esophageal cancer (4-6).

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Serum IL-6 levels have been shown to be elevated in patients with esophageal carcinoma in comparison to healthy controls (22). Elevated serum IL-6 levels are correlated with disease progression and poor prognosis (22, 23). IL-6 expression has been shown to be elevated in tumor tissues compared with adjacent normal esophageal tissues (23-25). Moreover, IL-6 and its receptor are expressed in esophageal carcinoma cells in resected tumor specimens (23) and esophageal carcinoma cell lines (25, 26). These findings suggest that IL-6 may modulate the biological activities of esophageal carcinoma cells.

The present study aimed to elucidate the functional role of IL-6 expression in esophageal carcinoma cells, specifically in terms of chemosensitivity. The IL-6 gene was transfected into two different esophageal carcinoma cell lines. IL-6-overexpressing clones were established from each cell line and their chemosensitivity to cisplatin was examined. The effect of IL-6 suppression by the transient transfection of IL-6-selective siRNA was also examined.

Materials and Methods

Cells and cell culture. The human esophageal squamous cell carcinoma cell lines, KYSE170 and TE13, were obtained from the Cell Resource Center for the Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). The cells were maintained in a culture medium (CM) consisting of 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 at 37°C in 5% CO2.

Establishment of IL-6 transfectants. Human IL-6 cDNA (639 bp) was synthesized using the human IL-6 sequence registered in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/) with Accession No. NM_000600 and then subcloned into the mammalian expression plasmid vector, pBapo-CMV-Neo (Takara Bio, Otsu, Japan) to construct the hIL-6-expressing plasmid vector, pBapo-CMV-hIL-6.

Twenty-four hours after plating KYSE170 or TE13 cells (1×10⁵ cells/well) on 24-well plates in CM, the 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 placed in a 10 cm culture dish in CM containing a final concentration of 1 and 0.25 mg/ml G418 (Geneticin; Invitrogen) for KYSE170 and TE13 cells, respectively. The G418-resistant clones were then selected and expanded.

IL-6-specific inhibition by siRNAs. Two different types of siRNA (028 and 029) specific for hIL-6 (GenBank Accession No. NM_000600) were prepared for IL-6 inhibition. These siRNAs and random siRNAs for control were obtained from Takara Bio Incorporated (Otsu, Japan). The sense and anti-sense strands of siRNAs were as follows: (sequence 028), 5'-GCACAGAACUAUGUGUGUTC-3' (sense); 5'-AACAACAUAGUGUGUGCTT-3' (antisense); (sequence 029), 5'-GUAGGCUACCAAAAUAATT-3' (sense); 5'-GUAUUAUGUGGUAAAGGCUAAGGCTTT-3' (antisense); siRNA-random, 5'-UCUUAUUAUGGUUGACCGUUAAGATT-3' (antisense). All of these sequences were determined through a BLAST search (http://blast.ncbi.nlm.nih.gov/) to avoid sharing sequence homology with any known human mRNA. Transient transfection into IL-6-overexpressing cells was performed using Trans IT-TKO (Invitrogen).

Enzyme-linked immunosorbent assay (ELISA) for IL-6. Cells were seeded in 12-well plates (1×10⁵ cells/well) and incubated for 24 h. Culture supernatant was collected and the IL-6 concentration in the supernatant was determined using an IL-6 ELISA kit (Endogen, Rockford, CT, USA) according to the manufacturer’s instructions.

In the IL-6 inhibition experiment using siRNAs, the transfectants (1×10⁵ cells/well) were seeded on a 96-well plate in 100 μl CM. After 24 h incubation, siRNA was transiently transfected as described above. After 48 h, the IL-6 concentration in the supernatant was determined as described above.

Cell proliferation assay. Cell proliferation ability was evaluated by WST-8 colorimetric assay. IL-6 transfectants and control cells (1×10⁵ cells/well) were seeded into 96-well plates in 100 μl CM. After 96 h incubation, 10 μl of WST-8 reagent solution (Cell Counting Kit; Dojindo Laboratories, Kumamoto, Japan) was added and incubated for 1.5 h. Cell viability was determined by colorimetric comparison in which optical density values were read from a microplate reader at an absorption wavelength of 450 nm (A450). In addition, cell proliferation ability was evaluated by counting the number of cells. IL-6 transfectants and control cells (1×10⁵ cells/well) were seeded into 6-well plates in 2,000 μl CM. After incubating for one, three and five days, the number of cells was counted.

Chemosensitivity testing. Cisplatin, obtained from Sigma Aldrich (St. Louis, MO, USA) was dissolved in sterile 0.9% saline as a stock solution and stored at −20°C. Cells were plated at a density of 1×10⁵ cells/ml in 96-well plates in a volume of 100 μl and incubated for 24 h. The medium was discarded and cells were incubated in the presence of graded concentrations of cisplatin (0, 0.1, 1, 10 and 100 μg/ml) for 72 h. Subsequently, cell sensitivity to cisplatin was evaluated by WST-8 assay as described above. The data represented the average A450 absorbance of six wells in one experiment. The percentage of surviving cells was estimated by dividing the A450 of treated cells by the A450 of control cells.

In the IL-6 inhibition experiment using siRNAs, the transfectants (1×10⁴ cells/well) were seeded on a 96-well plate in 100 μl CM and siRNA was transiently transfected as described above. After 24 h, the medium was discarded. The cells were treated with the above mentioned graded concentrations of cisplatin for 48 h. Cell viability was determined by WST-8 assay.

Flow cytometry for cell-cycle analysis. Cells (1×10⁵ cells/well) were seeded into 6-well plates in 2 ml CM for 24 h before drug exposure, and graded concentrations of cisplatin (0.1 and 1.0 μg/ml) were added to each well. Control cultures received CM only. After 48 h, the cells were collected and resuspended in a propidium iodide solution containing 0.1 M propidium iodide (Sigma, Welwyn, UK), 0.1% v/v Triton X-100 (Nakalai Tesque, Kyoto, Japan), and 20% RNase A (Qiagen, Valencia, CA, USA) in phosphate-buffered saline. After incubation on ice in the dark for 2 h, the samples were analyzed by flow cytometry. A total of 10,000 events were recorded and the proportion of cells in various phases of the cell cycle was...
analyzed using the ModFitLT DNA analysis program (Becton Dickinson, San Jose, CA, USA).

In the IL-6 inhibition experiment using siRNAs, the transfecants (1×10⁶ cells/well) were seeded on a 6-well plate in 2,000 μl CM. After 24 h incubation, siRNA was transiently transfected as described above. After 24 h, the medium was discarded. Cells were treated with graded concentrations of cisplatin for 48 h. The cell cycle was then analyzed as described above.

Statistical analysis. For comparison of the data obtained for IL-6-transfected cells with those obtained for the control cells, the SPSS 12.0.1 program (SPSS Inc., Chicago, IL, USA) was used. A comparison of means was carried out using one-way analysis of variance or the equality of means test, as appropriate. Differences were considered significant if a p-value less than 0.05 was obtained. Data are reported as the mean±standard deviation of triplicate experiments.

Results

Establishment and characterization of IL-6 transfectants (Figure 1). KYSE170 and TE13 cells were transfected with the IL-6 gene and clones selected producing the highest levels of IL-6. Two clones were established from each cell line and
were designated KYSE170 IL-6A, KYSE170 IL-6B and TE13 IL-6A, TE13 IL-6B, respectively. In KYSE170 cells, the control cells transfected with the NeoR gene (KYSE170-Neo) produced IL-6 (110.5±6.4 pg/ml/1×10⁵ cells/24 h) whereas IL-6 gene-transfected clones produced 5- to 6-fold higher levels of IL-6 compared to KYSE170-Neo (KYSE170 IL-6A: 532.5±25.9 pg/ml, KYSE170 IL-6B: 639.6±22.4 pg/ml). In TE13 cells, control cells (TE13-Neo) produced IL-6 (37.4±3.9 pg/ml/1×10⁵ cells/24 h) whereas IL-6 gene-transfected clones produced 16- to 17-fold-higher levels of IL-6 compared to TE13-Neo (TE13 IL-6A: 512.4±25.8 pg/ml, TE13 IL-6B: 558.9±31.3 pg/ml). In addition, no significant differences in growth potential were observed between control cells and IL-6 transfectants in both cell lines when evaluated by WST-8 assay and by counting the number of cells.

**Effect of IL-6 overexpression on the sensitivity to cisplatin (Figure 2).** In KYSE170, KYSE170 IL-6A and KYSE170, IL-6B significantly reduced sensitivity to cisplatin compared to the control NeoR-transfectant at concentrations of 0.1 μg/ml (KYSE170 IL-6A: 105.0±6.0% vs. control: 52.4±3.2%, p<0.01; KYSE170 IL-6B: 86.7±12.8%, p<0.01) and 1 μg/ml (KYSE170 IL-6A: 35.6±3.2% vs. control: 14.9±2.4%, p<0.01; KYSE170 IL-6B: 29.3±2.6%, p<0.01). Similarly, in TE13, TE13 IL-6A and TE13 cells, IL-6B significantly reduced sensitivity to cisplatin at concentrations of 0.1 μg/ml (TE13 IL-6A: 96.6±5.3% vs. control: 75.8±3.8%, p<0.01; TE13 IL-6B: 98.3±4.9%, p<0.01) and 1.0 μg/ml (TE13 IL-6A: 55.0±4.5% vs. control: 35.0±2.5%, p<0.01; TE13 IL-6B: 45.1±3.6%, p<0.01).

**Effect of IL-6 overexpression on cell cycle in the presence of cisplatin (Figure 3).** In KYSE170, in control transfectants, G2-M and sub-G1 populations in the DNA histogram increased markedly with mild increase of the S-phase population in accordance with the increase of cisplatin dose. In contrast, the G0-G1 population decreased markedly. The increase in G2-M and sub-G1 populations reflects the inhibition of mitosis (G2-M arrest) and the induction of apoptosis by cisplatin, respectively. In contrast, in both IL-6 transfectants, the increase of G2-M and sub-G1 populations induced by cisplatin was markedly inhibited. The presence of apoptotic cells induced by cisplatin was confirmed not only by flow cytometry as the sub-G1 population, but also by fluorescence microscopy as nuclear condensation (data not shown). In TE13, a remarkable difference between the control and IL-6 transfectants in the DNA histogram was observed at a cisplatin concentration of 0.1 μg/ml. The proportion of each population, except for the sub-G1 population, was not evaluated in either the control or IL-6 transfectants due to the potent apoptosis induction at the cisplatin concentration of 1.0 μg/ml. At a cisplatin concentration of 0.1 μg/ml, the increase of the G2-M population induced by cisplatin in the control transfectants was remarkable compared to KYSE170 cells and was markedly inhibited in both IL-6 transfectants. No difference in the sub-G1 population was observed between the control and IL-6 transfectants.

**Effects of IL-6 inhibition by siRNAs on IL-6 production (Figure 4).** IL-6-transfected cells treated with IL-6-selective
siRNA produced almost half the level of IL-6 compared with those treated with control siRNA. In both KYSE170 and TE13 cells, the IL-6-transfected cells treated with control siRNA produced IL-6 (KYSE170 IL-6A: 416.6±22.7 pg/ml/1×10^5 cells/24 h; KYSE170 IL-6B: 478.9±25.3 pg/ml; TE13 IL-6A: 435.8±20.4 pg/ml; TE13 IL-6B: 396.7±26.6 pg/ml), whereas the cells treated with IL-6-selective siRNA (028) or (029) produced almost half the level of IL-6 (KYSE170 IL-6A: 229.8±14.3 pg/ml, KYSE170 IL-6B: 287.1±16.9 pg/ml; TE13 IL-6A: 207.8±18.4 pg/ml, TE13 IL-6B: 238.3±22.5 pg/ml) or (KYSE170 IL-6A: 213.9±11.8 pg/ml, KYSE170 IL-6B: 257.7±13.2 pg/ml, TE13 IL-6A: 196.4±13.9 pg/ml, TE13 IL-6B: 204.3±14.9 pg/ml), respectively.

Effect of IL-6 inhibition by siRNAs on cisplatin sensitivity (Figure 5). IL-6-transfected cells treated with IL-6-selective siRNA showed higher sensitivity to cisplatin compared with those treated with control siRNA.

In KYSE170 cells, at a concentration of 0.1 μg/ml of cisplatin, the viability of the cells treated with control siRNA was 90.2±3.0% (KYSE170 IL-6A) and 94.3±19.7% (KYSE170 IL-6B), whereas that of the cells treated with IL-6-selective siRNA (028) was 54.1±6.9% (KYSE170 IL-6A, p<0.01) and 76.8±11.5% (KYSE170 IL-6B, p<0.01); the viability of the cells treated with siRNA (029) was 60.3±3.6% (KYSE170 IL-6A, p<0.01) and 75.1±10.6% (KYSE170 IL-6B, p<0.01). At a concentration of 1.0 μg/ml of cisplatin, the viability of the cells treated with control siRNA was 50.0±4.4% (KYSE170 IL-6A) and 63.3±12.1% (KYSE170 IL-6B) whereas that of the cells treated with siRNA (028) was 31.0±8.4% (KYSE170 IL-6A, p<0.01) and 45.2±6.7% (KYSE170 IL-6B, p<0.05); the viability of the cells treated with siRNA (029) was 24.9±4.5% (KYSE170 IL-6A, p<0.01) and 32.4±3.3% (KYSE170 IL-6B, p<0.01).

In TE13 cells, at a concentration of 0.1 μg/ml of cisplatin, the viability of the cells treated with control siRNA was 98.8±16.5% (TE13 IL-6A) and 91.4±4.2% (TE13 IL-6B), whereas that of the cells treated with siRNA (028) was 87.4±16.5% (TE13 IL-6A, p>0.05, not significant [NS]) and 81.5±4.5% (TE13 IL-6B, p<0.05); that treated with siRNA (029) was 92.1±14.0% (TE13 IL-6A, NS) and 59.5±6.9% (TE13 IL-6B, p<0.05). At a concentration of 1.0 μg/ml of cisplatin, that treated with control siRNA was 36.2±9.2% (TE13 IL-6A) and 35.9±8.1% (TE13 IL-6B) whereas that treated with siRNA (028) was 34.1±10.1% (TE13 IL-6A, NS) and 19.3±5.5% (TE13 IL-6B, p<0.01); that treated with siRNA (029) was 19.5±11.4% (TE13 IL-6A, p<0.05) and 7.4±2.0% (TE13 IL-6B, p<0.01).

Effect of IL-6 inhibition by siRNAs on cell cycle (Tables I and II). To evaluate the influence of IL-6 inhibition by siRNAs on cell cycle, the combination of KYSE170 IL-6A and siRNA (029) and TE13 IL-6B and siRNA (029) was used (Figure 5).
In KYSE170 IL-6, an increase in sub-G1 and G2-M populations was observed by siRNA treatment although the extent was small compared to that expected from the cisplatin sensitivity result. In TE13 IL-6, the siRNA treatment induced a remarkable increase in sub-G1 and G2-M populations.

Discussion

Although IL-6 has been detected in human esophageal carcinoma cells, its biological functions are not yet fully understood. This study demonstrated a pathophysiological role of IL-6 expressed in esophageal carcinoma cells in terms of chemosensitivity. Overexpression of IL-6 by transfection of IL-6 cDNA into esophageal squamous cell carcinoma cell lines, KYSE170 and TE13, reduced the sensitivity to cisplatin. Pretreatment of IL-6-overexpressing cells with siRNA selective for IL-6 restored the sensitivity of these cells to cisplatin.

Cisplatin and 5-fluorouracil are the most effective and widely used anticancer agents in the treatment of esophageal carcinoma (4-6). Nevertheless, no difference in 5-fluorouracil sensitivity was observed between parental cells and IL-6-transfectants in both cell lines (data not shown). These results strongly suggested that IL-6 expressed in esophageal carcinoma cells may act as a resistance factor against cisplatin-based chemotherapy or chemoradiotherapy. In this

Figure 4. Effect of IL-6-selective siRNA on IL-6 production by IL-6 gene-transfected esophageal carcinoma cells. A: KYSE170 and B: TE13. IL-6 concentration in the culture supernatant was determined using ELISA. Data are shown as the mean±standard deviation of triplicate experiments.
A recent study examined the relationship between IL-6 expression in serum and tumor tissues and the response to chemoradiotherapy using 5-fluorouracil and cisplatin in patients with advanced esophageal squamous cell carcinoma who underwent preoperative chemoradiotherapy followed by surgery (27). It was found that elevated serum IL-6 levels after chemoradiotherapy were associated with poor treatment response and size of residual tumor where IL-6 expression was detected by immunohistochemistry (27). This suggests that IL-6 may be a possible target associated with chemotherapy or radiotherapy resistance.

The KYSE170 and TE13 cells used in the present study expressed IL-6 and produced a small amount of IL-6 that was identified by ELISA. They also expressed IL-6 receptor (IL-6R) mRNA (α subunit and gp130) that was identified by reverse transcription polymerase chain reaction (data not shown). Several malignant tumors such as multiple myeloma (12), prostate cancer (14) and ovarian cancer (19) express IL-6 and IL-6R; IL-6 acts as an autocrine growth factor in these tumors. In esophageal carcinoma, IL-6 and IL-6R were detected in both esophageal carcinoma cells in resected tumor tissues (23) and esophageal carcinoma cell lines (25, 26). However, exogenous IL-6 showed no substantial growth stimulation on IL6R-expressing esophageal carcinoma cell lines (26). This is consistent with the present finding shown in the proliferation assay evaluated by cell count and WST-8 assay using KYSE170 and TE13 cells modified to overexpress endogenous IL-6 by gene transfection.
IL-6 has, however, been shown to act as an antiapoptotic factor for various agents or stimuli, including anticancer agents, in various malignant tumors (14, 16-21, 25). In esophageal carcinoma, Leu et al. reported that exogenous IL-6 protected esophageal carcinoma cells from apoptosis induced by staurosporine, a protein kinase inhibitor, through the activation of both STAT3 and mitogen-activated protein kinase pathways (25). For cisplatin, IL-6 is reported to reduce cisplatin-induced cytotoxicity in several types of malignant tumors such as prostate cancer (14), renal cell carcinoma (21), ovarian carcinoma (19) and uterine cervical carcinoma (20). The present study demonstrated for the first time that IL-6 acts as a resistance factor against cisplatin-induced cytotoxicity in esophageal carcinoma.

Cisplatin exerts cytotoxicity by its interaction with DNA to form DNA adducts, primarily intrastrand crosslink adducts, which activate several signal transduction pathways that result in the activation of apoptosis (28). The present study examined the influence of endogenous IL-6 on cisplatin-induced cytotoxicity, specifically focusing on cell-cycle analysis using flow cytometry. Cisplatin-induced DNA damage results in G2/M arrest and induction of apoptosis (28), which are indicated as an increase in the G2-M population and an appearance of a sub-G1 population (29), respectively, in cell-cycle analysis using flow cytometry. Indeed, IL-6-induced cisplatin resistance was closely associated with suppression of the increase in the G2-M and sub-G1 populations in IL-6-transfectants. Conversely, pretreatment of IL-6-transfectants by siRNA for IL-6 restored cisplatin sensitivity with an increase in G2-M and sub-G1 populations in the cell cycle.

IL-6-induced resistance to cisplatin seems to be determined by the crosstalk among multiple factors or signaling pathways and differs among cell types. The increased expression of multidrug-resistant-related proteins such as MDR1 (19) and GSTpi (21), anti-apoptotic Bcl-2 family proteins such as Bcl-2/Bcl-xL (19) and Mcl-1 (20), or activation of Ras/MEK/ERK (14, 19) or PI3K/Akt (19) signaling, have been reported to be associated with IL-6-induced resistance to cisplatin. In esophageal carcinoma, transcription factors such as NF-κB (30) and STAT3 (25) may also contribute to cisplatin resistance caused by IL-6. The induction of manganese superoxide dismutase caused by endogenous cytokine expression to scavenge reactive oxygen molecules may be a possible target for cisplatin resistance (31).

Clinically, IL-6 is secreted either by esophageal carcinoma cells or by stromal cells, including the inflammatory cells recruited to a local tumor site in response to both treatment stimuli and tumor progression. This suggests that exogenous IL-6, in addition to endogenous IL-6, may contribute to the cisplatin resistance of esophageal carcinoma cells.

In conclusion, this study demonstrated that IL-6 acts as a resistance factor to cisplatin in two esophageal carcinoma cell lines, KYSE170 and TE13, by modulating IL-6 expression levels by transfection of IL-6 cDNA and siRNA selective for IL-6. Further investigation should be directed toward clarifying the molecular mechanisms underlying the IL-6-induced cisplatin resistance of esophageal carcinoma cells.

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