Abstract. Esophageal squamous cell carcinoma (ESCC) is a common and highly fatal cancer in Japan. Systemic chemotherapy is used, but some tumors show resistance to it. The mechanisms of tumor resistance to chemotherapy remain largely unknown. We determined the chemosensitivity of 15 ESCC cell lines (TE-1-5, TE-8-15, KYSE140 and KYSE150) to docetaxel by clonogenic and MTT assays. We used cDNA microarray analysis and quantitative RT-PCR to determine which genes might determine resistance to docetaxel. Small interfering RNA (siRNA) was used to suppress gene expression and its effect on the chemosensitivity of the cell was determined. The cell line with the most resistance to docetaxel was TE-2. Using microarray analysis, we identified ß1 integrin (ITGB1) to be overexpressed in this cell line. Higher expression of ITGB1 mRNA was significantly associated with docetaxel resistance (n=15, r²=0.66, P=0.0110). Suppression of ITGB1 expression using siRNA sensitized the TE-2 cells to docetaxel. These data suggest that overexpression of ITGB1 may be related to resistance to chemotherapy and that targeting ITGB1, particularly in patients on docetaxel therapy, may enhance the effect of chemotherapy in patients with ESCC.

Introduction

In Japan, >11,000 patients die of esophageal cancer every year, accounting for ~3% of Japanese cancer deaths; esophageal cancer is the sixth highest cause of cancer death in Japanese males. The treatment of advanced esophageal squamous cell carcinoma (ESCC) includes surgery, radiotherapy, chemotherapy, or more commonly a combined treatment regimen. Compared with radiotherapy alone, cisplatin-based chemoradiotherapy has been shown to improve the survival of patients with ESCC. Systemic chemotherapy has significantly reduced the incidence of distant metastasis and local recurrence, but its effect on overall survival has been less than satisfactory and newer drugs are currently being tested.

Docetaxel is a taxane that shows significant antitumor effects. It has shown clinical activity in a wide spectrum of solid tumors, including those of the breast, lung, ovary and prostate (1,2). Data concerning the biological effects and mechanisms of docetaxel on ESCC cells in vitro and in vivo are limited.

Herein, we have used microarray analysis to identify that the gene ITGB1, which encodes ITGB1, was specifically overexpressed in the cell lines that showed resistance to docetaxel. Integrins are cell surface adhesion receptors composed of α and ß subunits; they mediate cell-extracellular matrix and cell-cell interactions (3). ITGB1 transduces biochemical signals from the extracellular environment, particularly those involved in growth, differentiation, invasiveness and metastatic potential of malignant cells (4,5). Altered expression of integrins has been reported to be involved in tumor suppression and progression (6-8). Here, we report that ITGB1 expression might be related to tumor cell resistance to chemotherapy; thus, ITGB1 might provide a therapeutic target, particularly in patients on docetaxel.

Materials and methods

Docetaxel and cell lines. Docetaxel (Sanofi-Aventis) was suspended in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS). Serial dilutions were prepared to achieve final docetaxel concentrations of 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25 and 50 ng/ml. The ESCC cell lines TE-1-5, TE-8-15, KYSE140 and KYSE150 were grown in RPMI-1640 containing 10% FBS. Giemsa staining, clonogenicity assays and small interfering RNA (siRNA) transfection were performed at 70-80% confluence.

Chemosensitivity. Cells were seeded into 96-well microtiter plates containing 100 µl culture medium at a concentration of
performed in triplicate. IC50 values were calculated from absorption measured in the docetaxel-treated cells (A) to that reader. Relative proliferation was defined as the ratio of proliferation reagent WST-1 (Roche Applied Sciences, Mannheim, Germany) was added to 200 μl of the cell culture medium and held for 3 h. The absorbance of the samples was measured at 450 and 690 nm using a bichromatic ELISA reader. Relative proliferation was defined as the ratio of absorption measured in the docetaxel-treated cells (A) to that in the untreated control cells (A0). All experiments were performed in triplicate. IC50 values were calculated from plots of drug concentration vs. the proportion of cells that survived.

Oligonucleotide microarray. In order to reduce experimental errors, a set of oligonucleotide microarray slides containing a duplicate set of 34,594 spots was used to analyze each expression profile. Total RNA was isolated using the RNaseasy mini kit (Qiagen, Chatsworth, CA) and digested by RNase-free DNase I (Nippon Gene Co., Tokyo, Japan) according to the recommendations of the manufacturers. We then performed T7-based RNA amplifications and preparations of cDNA probes using 5 μg of the total RNA, as described elsewhere (9,10). Amplified RNA (2.5 μg) from each cell line was labeled with Cy5-dCTP (Amersham Pharmacia Biotech, Upsala, Sweden) and an equal amount of amplified RNA from a pool of the total RNA of the normal human esophageal mucosa cell line Het-1A (Invitrogen, Carlsbad, CA) was labeled with Cy3-dCTP (Amersham Pharmacia Biotech). Hybridization, washing and scanning were performed as described elsewhere (11,12). The intensity of each duplicated signal was evaluated photometrically using the ArrayVision computer program (Imaging Research, Inc., St. Catharines, Ontario, Canada). To normalize the mRNA in the cell lines, the Cy5: Cy3 ratio of each expressed gene was adjusted such that the mean Cy5: Cy3 ratio of Het-1A was 1.0. Subsequently, the duplicated spots on each slide were averaged (9,11,12). In addition, a cut-off value for each expression level was automatically calculated by variance analysis and data with low signal intensities were excluded from further investigation.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA (2 μg) was converted to cDNA using the GeneAmp RNA PCR core kit (Applied Biosystems, Tokyo, Japan), according to the manufacturer’s instructions. qRT-PCR amplification of the cDNA template corresponding to 20 ng of total RNA was performed using Taq Man PCR Universal PCR Master Mix (Applied Biosystems, Foster City, CA) in an ABI PRISM 7500 sequence detection PCR system (Applied Biosystems). The PCR conditions were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles each of 95°C for 15 sec and 60°C for 1 min. ITGB1 Taq Man probes were synthesized from sequences in exons 10, 11 and 12 (Assays-on-Demand Gene Expression System, ITGB1 assay ID: Hs00236976_m1; Applied Biosystems). Expression levels were normalized against glyceraldehyde-3-phosphate dehydrogenase (Assays-on-Demand Gene Expression Systems, assay ID: Hs99999905_m1; Applied Biosystems).

Western blotting. Cells propagated in three-dimensional IrECM were first isolated with ice-cold phosphate-buffered saline/EDTA [0.01 mol/l sodium phosphate (pH 7.2) containing 138 mmol/l sodium chloride and 5 mmol/EDTA]; then lysed in a radioimmunoprecipitation assay buffer. Equal amounts of protein were loaded onto reducing SDS gels. After transfer onto a nitrocellulose membrane (Invitrogen), blots were blocked with 5% non-fat milk and probed. Primary antibodies used include ITGB1, clone 18 (1:2500; BD Transduction Laboratories, Lexington, KY), α-PCNA (1:100; Santa Cruz Biotech, Santa Cruz, CA). Blots were washed, incubated with secondary antibody (Mouse IgG1) and exposed to X-rays.

siRNA transfection and cell proliferation assay. ITGB1 siRNA (M-004506-00 Dharmacon, Lafayette, Co.) and control (siControl™ Non-targeting pool) transfections were performed using human T-cell nucleofector kits (Amaxa, Cologne, Germany) according to the manufacturer’s instructions. The cells were seeded into 96-well plates in complete culture medium at a density of 5x10^4 cells/well. After 24 h, medium was added with or without docetaxel (0.05, 5, 1, 2.5, 5, 10, 25 and 50 ng/ml). After 72 h, cell proliferation was assessed using an MTT assay (CellTiter 96; Promega, Madison, WI). Cell proliferation was determined as the ratio of the absorbance of docetaxel-treated cells to that of the control cells seeded with non-target siRNA. Each assay was performed in triplicate.

Statistical methods. The Graphcel software program was used to determine IC50 values. Statistical analyses were performed using the Mann-Whitney U test for unpaired samples and Wilcoxon’s signed rank test for paired samples. Linear relationships between variables were determined by...
means of a simple linear regression. The StatView software package (Abacus Concepts Inc., Berkeley, CA) was used for all analyses and P<0.05 was considered significant.

**Results**

**Chemosensitivity of esophageal cancer cell lines to docetaxel.** We used an MTT assay to determine the sensitivity (IC50) of 15 esophageal cancer cell lines to docetaxel (Fig. 1). TE-2 was the most resistant cell line, followed by TE-4, TE-10, TE-12 and TE-1. Other cell lines showed moderate to high sensitivity (IC50 <5 ng/ml). The clonogenic potential of each cell line after docetaxel treatment varied and Fig. 2A illustrates two representative cell lines, the sensitive KYSE150 and the resistant TE-2. Fig. 2B shows the response of eight representative cell lines to varying concentrations of docetaxel measured in three separate experiments, each time in triplicate. Of the cell lines shown, three were sensitive (TE-3, KYSE140 and TE-2 was the most resistant cell line, followed by TE-4, TE-10, TE-12 and TE-1. Other cell lines showed moderate to high sensitivity (IC50 <5 ng/ml). The clonogenic potential of each cell line after docetaxel treatment varied and Fig. 2A illustrates two representative cell lines, the sensitive KYSE150 and the resistant TE-2. Fig. 2B shows the response of eight representative cell lines to varying concentrations of docetaxel measured in three separate experiments, each time in triplicate. Of the cell lines shown, three were sensitive (TE-3, KYSE140 and
KYSE150), four were resistant (TE-2, TE-4, TE-10 and TE-12) and the remaining eight cell lines exhibited intermediate sensitivity to docetaxel (Fig. 2B). TE-2 was the most resistant to docetaxel (IC50 >20 ng/ml).

Microarray analysis of genes differentially expressed in cell lines sensitive or resistant to docetaxel. Next, a microarray analysis was performed in order to identify the genes whose expression may confer resistance to docetaxel. Total RNA was extracted from each cell line and the reverse transcriptase reaction was carried out. Table I shows the genes whose expression was high in the docetaxel-resistant TE-2 cell line and low in the other cell lines. We focused on ITGB1 for further analysis.

Expression of ITGB1 in esophageal cancer cell lines and its relationship with docetaxel sensitivity. We examined ITGB1 mRNA expression in the 15 cell lines and in the normal human esophageal mucosa cell line Het-1A by quantitative PCR. ITGB1 expression was found to be higher in many esophageal cancer cell lines than in Het-1A. ITGB1 mRNA expression was very high in TE-1, TE-2 and TE-4 (Fig. 3A).
Figure 4. Expression of ITGB1 mRNA in TE-2 was down-regulated by a siRNA. (A) Expression levels were measured by real-time RT-PCR. The mean and standard error of the triplicate assay are shown. Western blot analysis of ITGB1 protein expression in the siRNA-treated TE-2 cells. (B) siRNA of ITGB1 down-regulated the expression of ITGB1 protein in TE-2; however, it had little effect on α-PCNA. Non-specific siRNA (non-target siRNA) or mock transfection (mock siRNA) had no effect. Sensitivity to docetaxel of TE-2 cells treated with siRNA of ITGB1. (C) Cells that were treated with siRNA of ITGB1 or seeded with non-target siRNA were incubated with varying concentrations of docetaxel for 72 h and each well was stained with Giemsa. Sensitivity to docetaxel of TE-2 cells treated with siRNA of ITGB1. (D) Cells treated with or without the siRNA of ITGB1 or non-target siRNA were incubated with varying concentrations of docetaxel for 72 h and cell viability was determined using an MTT assay. The data shown are the mean of triplicate cultures. The standard deviations were <10% of the mean. (E) Docetaxel IC50s of ESCC cell lines, calculated using the data shown in (D). Cells were exposed to varying concentrations of docetaxel. Cell survival was determined using the MTT assay.
It was highest in the TE-2 cell line, which was the most resistant to docetaxel. Regression analysis revealed a strong positive correlation between expression of \textit{ITGB1} mRNA and docetaxel IC\textsubscript{50} in ESCC cell lines. The coefficient of correlation was 0.813 (Fig. 3B).

**Inhibition of \textit{ITGB1} restores chemosensitivity of esophageal cancer cells to docetaxel.** We used the siRNA technique to down-regulate \textit{ITGB1} mRNA in the TE-2 cell line (Fig. 4A), which was chosen because it was the most resistant to docetaxel and had the highest expression of \textit{ITGB1}. Western blotting proved that \textit{ITGB1} protein level was also reduced by siRNA (Fig. 4B). The down-regulation of \textit{ITGB1} had a pronounced effect on the sensitivity of the cell to docetaxel. At the same concentration of docetaxel, cells treated with \textit{ITGB1} siRNA had fewer colonies than cells treated with non-target siRNA (Fig. 4C). This enhancement of sensitivity by siRNA of \textit{ITGB1} was further confirmed using the quantitative MTT assay (Fig. 4D). Fig. 4E shows that the IC\textsubscript{50} of TE-2 cells treated with \textit{ITGB1} siRNA was significantly lower than untreated cells, as calculated from Fig. 4D.

**Discussion**

Despite the fact that many different chemotherapeutic agents and regimens have been developed to treat advanced ESCC, there has been no significant increase in patient survival. Therefore, novel chemotherapeutic strategies are necessary. Docetaxel is an antimicrotubulin agent that has antiproliferative and suppressive effects on cancer cells. It is known that in the basic mechanism, it binds to tubulin and disturbs the equilibrium between microtubule assembly and disassembly during mitosis (13). Stabilization of microtubules by docetaxel impairs mitosis and exerts an anticancer effect in tumors (13). In addition to its effects on microtubules, docetaxel also induces apoptosis with down-regulation of bcl-xL and bcl-2 and up-regulation of p21WAF1 and p53 (14,15). Integrins, heterodimeric transmembrane receptors composed of \(\alpha\) and \(\beta\) subunits, can be found in focal adhesions. In ESCC patients, no clear correlation has been demonstrated between tumor formation, invasion and metastasis has in cell adhesion. A complete understanding of the molecular events that mediate integrin-dependent survival of cancer cells may lead to new therapeutic tools for cancer treatment.

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**References**


