Involvement of oligosaccharide changes in α5β1 integrin in a cisplatin-resistant human squamous cell carcinoma cell line

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Abstract

Multiple mechanisms are involved in the resistance of cancer cells to cisplatin, including the expression of multidrug resistance-associated protein (MRP) and enhanced DNA repair. Here, we report findings to show that oligosaccharide changes in α5β1 integrin are associated with cisplatin resistance in a head and neck squamous cell carcinoma cell line, HSC-2. Cisplatin-resistant HSC-2 (HSC-2/CR) cells were established by stepwise treatment with various concentrations of cisplatin. The oligosaccharides containing β1, 6-*N*-acetylglucosamine (β1-6GlcNAc) branching, detected by leukoagglutinating phytohemagglutinin (L_4-PHA) lectin blot, were found to be dramatically decreased in α5β1 integrin immunoprecipitated from HSC-2/CR cells. To better understand the mechanisms underlying cisplatin resistance and oligosaccharide alteration, we analyzed the downstream signaling of α5β1 integrin, one of the target glycoproteins of β1-6GlcNAc transferase [UDP-GlcNAc:α-D-mannoside β1, 6-*N*-acetylglucosaminyltransferase (GnT-V)]. Cell adhesion to fibronectin and phosphorylation of focal adhesion kinase (FAK), which are associated with α5β1 integrin and involved in a cell survival signaling, were found to be increased in the cisplatin-resistant cells. Enhancement of the inhibition of cell adhesion and FAK phosphorylation also support the above data in GnT-V transfectants of HSC-2 cells. Interestingly, the differences in sensitivity to cisplatin and FAK phosphorylation between cisplatin-sensitive and -resistant cells were completely abolished by treatment with a neutral antibody of α5β1 integrin. These results suggest that modification of oligosaccharides of α5β1 integrin represents one of the possible mechanisms of drug resistance in head and neck cancer cells. (Mol Cancer Ther. 2003;2:1207–1214)

Introduction

Cisplatin is a neutral, square planar platinum (II) complex containing two chloride ligands oriented in a *cis* configuration (1), which enters the cell by passive diffusion. Treatment with this drug inhibits DNA replication and RNA transcription resulting in cell arrest at the G_2 phase of the cell cycle and/or apoptosis (2). Cisplatin is one of the most potent and useful antitumor agents for the treatment of malignant solid tumors, including squamous cell carcinoma of the head and neck. However, there is a problem in that many tumors show an intrinsic resistance to cisplatin or develop resistance after initially responding to treatment with anti-cancer drugs (3). In attempts to understand the mechanism underlying cisplatin resistance in tumor cells, numerous studies concerning the establishment of cisplatin-resistant sublines have been reported. One of the well-studied examples concerns the expression of ATP binding cassette transmembrane transporters, multidrug resistance-associated protein (MRP) and canalicular multispecific organic anion transporter (cMOAT), which is overexpressed in multidrug-resistant cells and functions as a drug efflux pump (4, 5). Other factors include an increased inactivation of cisplatin by intracellular proteins such as glutathione and metallothionein, enhanced repair of damaged DNA, and alterations in signal transduction pathways (2). These studies of cisplatin-resistant cells demonstrate that the mechanisms of cisplatin resistance are very likely quite complicated.

Modification of cell surface glycoproteins is one of the critical steps involved in cellular transformation and is thought to play a critical role in a variety of specific biological interactions (6). A few studies have focused on the relationship between glycosylation and drug resistance. For example, treatment with tunicamycin, which blocks the initial step in the synthesis of N-linked oligosaccharides associated with glycoproteins, led to an enhanced sensitivity to cisplatin *in vitro* and *in vivo* (7). It has been reported that α1, 2-fucosyltransferase and histo-blood group antigen H type 2 were involved in cellular resistance to 5-fluorouracil (8). These studies suggest that oligosaccharide changes in some glycoproteins such as adhesion molecules and/or receptors might also be involved in cisplatin resistance.

Integrins comprise a large family of heterodimeric cell surface receptors that govern cell-extracellular matrix (ECM) interactions and mediate cellular adhesion and migration, as well as intracellular signal transduction (9). Because integrins contain approximately 20–30 consensus N-glycosylation sites, modifications of these N-glycans on

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integrins might change their functions. α5β1 integrin is one of the integrin family members and could play a role in cell survival. It has been reported that adhesion, mediated by the α5β1 integrin, protects intestinal epithelial cells (10) and HT29 colon carcinoma cells (11) from apoptosis. In particular, β1 integrin-stimulated tyrosine kinase activation has been reported to suppress chemotherapy-induced apoptosis in small cell lung cancer (12). Furthermore, oligosaccharide structures of α5β1 integrin can be changed by the up/down-regulation of glycosyltransferases (13, 14).

Some of the downstream molecules involved in α5β1 integrin-mediated cell survival have been identified (10). Signaling originates in the region of the integrin cytoplasmic domain and the focal adhesion complex. Focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase of M_r 125,000, is located in the focal adhesion complex and is a major protein phosphorylated after integrin activation (15). FAK plays a pivotal role in integrin-mediated signal transduction, including the regulation of cell survival (16). The Tyr397 residue of FAK constitutes a major autophosphorylation site and plays a major role in FAK-mediated survival signals (17). This phosphorylation could activate the phophatidylinositide 3′-OH-kinase-Akt (PI3K/Akt) survival pathway, which has also been reported to be involved in FAK-induced resistance to apoptosis (16, 18).

In the present study, we established a head and neck squamous cell carcinoma cell line that is resistant to cisplatin by continuous stepwise treatment with this agent, and investigated the mechanisms involved in this resistance in terms of oligosaccharide alterations. The findings show that a decrease in β1, 6-*N*-acetylglucosamine (β1-6GlcNAc) branching in oligosaccharides of α5β1 integrin followed by the up-regulation of FAK phosphorylation is a key factor in cisplatin resistance.

Materials and Methods

Cell Culture and Transfection

An HSC-2 cell line, derived from human squamous carcinoma cells of the mouth, was kindly provided from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University (Sendai, Japan). HSC-2 cells were maintained in RPMI-1640 (Sigma, St. Louis, MO) containing 10% fetal bovine serum (Sigma) and antibiotics under 5% CO_2 at 37°C. Cisplatin-resistant HSC-2/CR cells were established by the stepwise exposure of parental HSC-2 cells to increasing concentrations of cisplatin (Nippon Kayaku Corp., Tokyo, Japan) over a 12-month period, as reported previously (19). Briefly, the cells were initially treated with a 2-μM solution of cisplatin for 6 months, and the cisplatin concentration was then increased to 3 μM for 3 months, and final concentration of 5 μM for an additional 3 months.

A human UDP-GlcNAc:α-D-mannoside β1, 6-*N*-acetylglucosaminyltransferase (GnT-V) cDNA (20) was inserted into the mammalian expression vector pCXN2 which is regulated by the β-actin promoter (21), and 2 μg of the GnT-V expression vector or only vector were transfected into HSC-2 cells and HSC-2/CR cells by means of Effectene (QIAGEN, Tokyo, Japan), respectively. After culturing the cells for 48 h, they were selected by the addition of 500μg/ml of G418 (Nacalai Tesque, Inc., Kyoto, Japan).

Cell Survival Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess the sensitivity of the cells to the agents *in vitro*. Approximately 1×10^4 cells in 200 μl medium were seeded in each well of a 48-well plate in triplicate and then cultured. After 12 h, the cells were treated with various concentrations of cisplatin. After 24–48 h of cisplatin treatment, 20 μl of MTT solution (5 mg/ml) (Dojindo, Kumamoto, Japan) were administered and the cells were incubated at 37°C for 4 h. Eight hundred microliters of 2-propanol were added and each well was measured at an absorbance of 560 nm ($A_{560\ nm}$) with a spectrometer (Nihon Inter Med Corp., Tokyo, Japan). The effect of the drugs on cell survival was expressed as the percent viability. The percent viability was calculated using the following equation: ($A_{560\ nm}$ (Cisplatin-treated)/$A_{560\ nm}$ (Cisplatin-free)) × 100. The test was performed independently 3 times, and 50% inhibitory concentration (IC_{50}) was defined as the concentration of cisplatin required to kill 50% of the cells.

Lectin Blot Analysis

Cells were homogenized in TNE buffer, which contained 10 mM Tris-HCl (pH 7.8), 1% NP40, 0.15 M NaCl, 1 mM EDTA, and a mixture of protease inhibitors (Wako, Osaka, Japan) for 20 min on ice, and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant fraction was saved as a postnuclear supernatant and protein concentrations were determined with a BCA kit (Pierce, Rockford, IL) using BSA as a standard. Twenty micrograms of proteins extracted from the cells were electrophoresed on a 10% polyacrylamide gel and then transferred onto a nitrocellulose membrane. After blocking, the membrane was incubated with 1 μg/ml of biotinylated various lectins [Con A, SSA, MAM, leukoagglutinating phytohemagglutinin (L_4-PHA), E_4-PHA, LCA] (Seikagaku Corp., Tokyo, Japan) for 3 h. The washing and developing procedures have been described previously (22). Reactive glycoprotein bands were visualized by chemiluminescence using an ECL system (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, United Kingdom).

Western Blot Analysis and Assay of GnT-V Activity

For Western blotting of GnT-V, 20 μg of proteins were electrophoresed on an 8% polyacrylamide gel and the transferred membrane was incubated with anti-human GnT-V monoclonal antibody (mAb) 24D11 (provided from Fujirebio, Hachiohji, Japan).

GnT-V activity was analyzed by a fluorescent assay method with slight modification (23). The extracted proteins (20–50 μg) were incubated at 37°C for 8 h with 5 μM pyridylaminated agalacto bi-antennary oligosaccharide as an acceptor and 40 mM UDP-GlcNAc as a donor.

Lectin Blot Analysis on Immunoprecipitated α5β1 Integrin

For immunoprecipitation, 15 μl of protein G-Sepharose 4EF beads (Amersham Pharmacia Biotech AB, Uppsala,

Sweden) were added to 600 μg of proteins that had been extracted from the cells followed by incubation for 3 h at 4°C. Protein G-Sepharose 4EF beads were precleared by centrifugation at 3000 rpm for 5 min at 4°C. After incubation with 600 ng/ml of anti-human α5β1 integrin antibody P1D6 (Dako, Carpinteria, CA) for 2 h at 4°C, immune complexes were collected with 15 μl of protein G-Sepharose 4EF beads. The complexes were released by boiling in Laemmli's sampling buffer without a detergent, separated by 8% SDS-PAGE. The membrane filter was analyzed by L_4-PHA lectin blot, as described above. After deprobing and blocking, it was subjected to Western blot analysis using anti-α5 integrin mAb (BD Transduction Laboratories, San Diego, CA) and anti-β1 integrin mAb 4B7R (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as described above to verify that the α5β1 integrin had been equally loaded.

Cell Adhesion Assay

Cell adhesion was assayed by crystal violet staining, as described previously (24). Briefly, 96-well culture plates were precoated with 50 μl of a serially diluted fibronectin and air-dried overnight followed by blocking with 3% BSA in RPMI-1640 at 37°C for 1 h. The cells (1×10^5) were allowed to attach to each matrix at 37°C for 30 min. After washing with 200 μl of PBS, the cells were stained with 0.04% crystal violet for 10 min, followed by washing and lysing the cells. The absorbance at 560 nm ($A_{560\ nm}$) was measured spectrophotometrically. The rate of cell adherence was calculated as follows: ($A_{560\ nm}$ (matrix) − $A_{560\ nm}$ (no matrix))/$A_{560\ nm}$ (no matrix).

Detection of Tyrosine Phosphorylation of FAK

Cells were plated at a density of 5×10^5 cells on 6-cm dishes. After incubating for 12 h, these cells were treated with 10–30 μM cisplatin, and then incubated for an additional 24 h. The cells were lysed in an ice-cold lysis buffer (5 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.5% Triton X-100, protease inhibitor mix), and 20 μg of proteins that had been extracted from the cells were subjected to Western blot analysis using the anti-FAK (pY397) phosphospecific antibody (BioSource International, Inc., Camarillo, CA). After deprobing and blocking, it was subjected to Western blot analysis using the anti-FAK mAb (BD Transduction Laboratories). To assess the viability of cisplatin-exposed cells, an MTT assay was performed at 24 h after cisplatin treatment, as described above.

Effect of Anti-α5β1 Integrin Neutral Antibody on the Survival of HSC-2/CR Cells

HSC-2/CR cells were plated at a density of 5×10^5 cells on a six-well culture plate with or without 1 μg/ml solution of α5β1 integrin neutral antibody. After incubation for 12 h, the cells were treated with 30 μM cisplatin, and then incubated for an additional 12 h. These cells were subjected to Western blot analysis as described above, to detect FAK phosphorylation. For cell survival assay, 2×10^3 HSC-2/CR cells in 50 μl of medium with or without 1 μg/ml α5β1 integrin neutral antibody were seeded in each well of a 96-well plate in triplicate and then cultured. After 12 h, 50 μl of medium containing various concentrations of cisplatin were added, and the cells were cultured for an additional 48 h. At that stage, an MTT assay was performed as described above.

Results

Establishment of Cisplatin-Resistant Cell Line from Human Squamous Cell Carcinoma HSC-2 Cells

By the stepwise exposure of human mouth squamous cell carcinoma HSC-2 cells to increasing concentrations of cisplatin over 12 months, we were able to establish a highly cisplatin-resistant cell line HSC-2/CR. The killing curves as shown in Fig. 1A indicate the level of cisplatin resistance of the HSC-2 and HSC-2/CR. The IC_{50} value of HSC-2 and HSC-2/CR was 4.0 and 14.6 μM, respectively. The relative resistance level for HSC-2/CR was 3.65-fold higher than for its parental cell line HSC-2. This result is similar to other established cisplatin-resistant cells reported in other various studies.

Lectin Blot Analysis and Expression of GnT-V

To better understand the relationship between drug resistance and oligosaccharide structures, the patterns of glycosylation of a number of glycoproteins were investigated by a variety of lectin blot analysis. ConA, SSA, MAM, E_4-PHA, and LCA lectin blot analyses showed only slight changes in oligosaccharides (data not shown). However, L_4-PHA lectin blot analysis, which preferentially recognizes the GlcNAc residues on β1-6GlcNAc branches of tri- or tetra-antennary sugar chains (25), showed that the extent of β1-6GlcNAc branching of

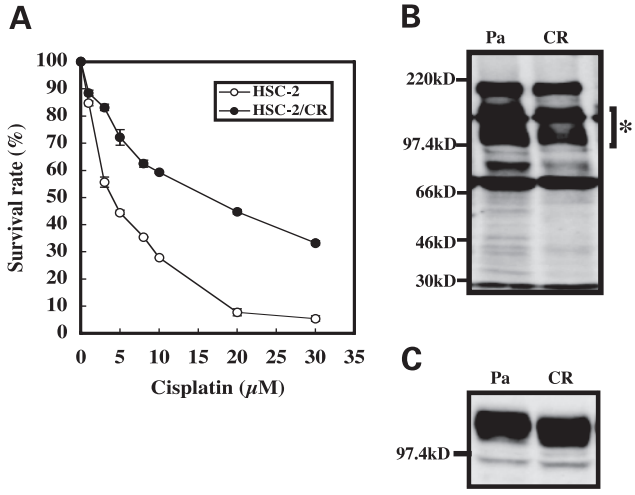


Figure 1. Analysis of sensitivity to cisplatin, oligosaccharide structure, and GnT-V expression of HSC-2 and HSC-2/CR cells. **A,** both HSC-2 and HSC-2/CR cells were exposed to various concentrations of cisplatin. The effect of the agents was examined by means of an MTT assay. *Points,* means of triplicate determinations; *bars*, SD. **B,** 20 μg of proteins extracted from HSC-2 cells (*Pa*) and HSC-2/CR cells (*CR*) were subjected to L_4-PHA lectin blot analysis. An *asterisk* indicated major glycoproteins, the β1-6GlcNAc branchings of which were decreased. **C,** 20 μg of total cellular proteins from HSC-2 cells (*Pa*) and HSC-2/CR cells (*CR*) were subjected to Western blot analysis using anti-GnT-V mAb. These results show typical examples of five independent experiments, respectively.

glycoproteins of M_r 90,000–150,000 was significantly decreased in HSC-2/CR cells compared to HSC-2 cells (Fig. 1B). It is known that β1-6GlcNAc branching is catalyzed by the action of GnT-V (20). Therefore, we investigated expression of GnT-V and its enzymatic activity in HSC-2 and HSC-2/CR cells. While the expression of GnT-V proteins was almost the same in HSC-2 and HSC-2/CR cells (Fig. 1C), the activity of GnT-V was slightly decreased in HSC-2/CR cells compared to HSC-2 cells but the value was not significant by the Student's t test (Table 1).

GnT-V-Transfected HSC-2 Cells Were More Sensitive to Cisplatin Compared to Mock Cells

To investigate the issue of whether increases in β1-6GlcNAc branching are correlated with cisplatin resistance in HSC-2 cells, we transfected a mammalian expression vector of human GnT-V into HSC-2 cells and analyzed cisplatin resistance. Increases in GnT-V activities and β1-6GlcNAc branching were observed in GnT-V transfectants as compared to mock cells (Fig. 2, A and B, Table 1). We also assessed the sensitivity of GnT-V transfectants to cisplatin by means of an MTT assay. As expected, the GnT-V transfectants of HSC-2 cells were more sensitive to cisplatin compared to mock cells (Fig. 2C). These results suggest that increases in β1-6GlcNAc branching are key factors in the decreased resistance of HSC-2 cells to cisplatin.

Lectin Blot Analysis on Immunoprecipitated α5β1 Integrin

L_4-PHA lectin blot analysis of HSC-2/CR cells showed dramatic changes in the levels of glycoproteins of M_r 90,000–150,000 (Fig. 1B), including the molecular weight of integrins. It has been reported that cisplatin is able to induce apoptosis and that α5β1 integrin protects intestinal epithelial cells and colon cancer cells from apoptosis (10, 11). Therefore, we hypothesize that α5β1 integrin could be the key molecule responsible for cisplatin resistance in HSC-2/CR cells. To investigate the glycosylation state of α5β1 integrin, an L_4-PHA lectin blot analysis of immunoprecipitated α5β1 integrin was performed. Interestingly, β1 integrin, which had been immunoprecipitated from HSC-2/CR cells, contained significantly decreased levels of β1-6GlcNAc branching compared to HSC-2 parental cells, but no expression of β1-6GlcNAc structure on α5 integrin was detected in these cells (Fig. 3A, *upper panel*), a phenomenon that was also observed in α5β1 integrin purified from HT1080 cells (26). The molecular mass of β1 integrin in HSC-2/CR cells was slightly lower than that in HSC-2 cells because of decreased glycosylation (Fig. 3A, *lower panel*). Furthermore, the levels of β1-6GlcNAc branching on both α5 integrin and β1 integrin, which had been immunoprecipitated from GnT-V transfectants of HSC-2 cells, were significantly increased (Fig. 3A, *upper panel*). These results suggest that the level of β1-6GlcNAc branching on α5β1 integrin, particularly β1 integrin, is related to decreased cisplatin resistance.

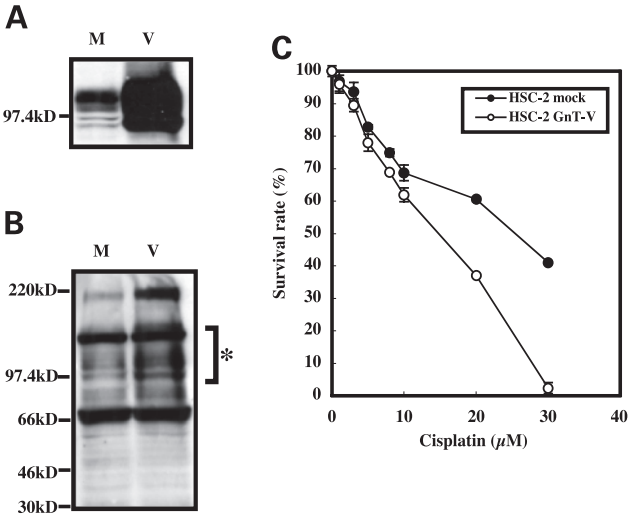


Figure 2. GnT-V transfectants of HSC-2 cells were more sensitive to cisplatin compared to mock cells. **A,** Western blot analysis of GnT-V. After the membrane filter had been deprobed, L_4-PHA lectin blot analysis (**B**) was performed to confirm the amounts of β1-6GlcNAc branching. Both results showed typical examples of three independent examples. (*M,* mock transfectants of HSC-2 cells; *V,* GnT-V transfectants of HSC-2 cells.) **C,** the levels of sensitivity at 36 h after cisplatin treatment were examined using an MTT assay. *Points,* means of triplicate determinations; *bars*, SD.

Table 1. Enzymatic activity of GnT-V[a]

Cells	GnT-V Activity (pmol/mg/h)
HSC-2	83.6 ± 5.7
HSC-2/CR	67.4 ± 3.9[b]
HSC-2 mock	83.7 ± 3.3
HSC-2 GnT-V	1169 ± 54
HSC-2/CR mock	66.6 ± 2.8
HSC-2/CR GnT-V	656 ± 31

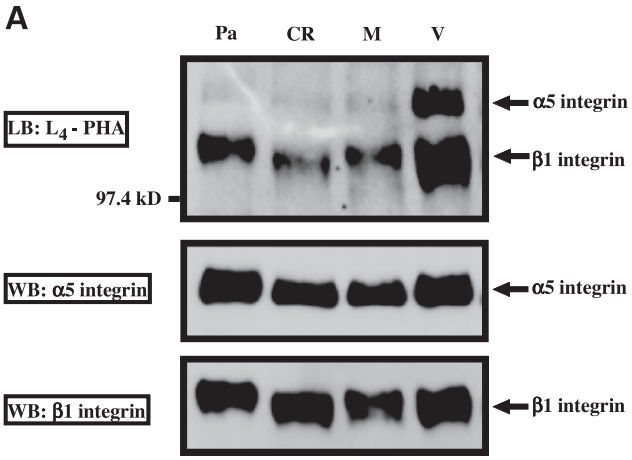
[a] Data represent the mean ± SD from two or three independent experiments.
[b] Not significant, compared with HSC-2 cells, by Student's t test.

Cell Adhesion to the Extracellular Matrix

It was reported that increases in β1-6GlcNAc branching on α5β1 integrin diminished attachment with the extracellular matrix (13). To confirm that changes in oligosaccharides of α5β1 integrin play a role as an adhesion molecule, a cell adhesion assay using fibronectin was performed. As shown in Fig. 3B, cell attachment to fibronectin was dramatically increased in HSC-2/CR cells compared to HSC-2 cells, but the attachment to laminin and collagen type I was not significantly changed among these cells (data not shown).

GnT-V-Transfected HSC-2/CR Cells Did Not Change Its Sensitivity to Cisplatin Compared to Mock Cells

To investigate whether the overexpression of GnT-V in HSC-2/CR cells could influence its sensitivity to cisplatin, we transfected human GnT-V into HSC-2/CR cells and analyzed cisplatin resistance in these cells. The expression of GnT-V protein and its enzymatic activity was significantly increased in GnT-V-transfected HSC-2/CR cells compared to the mock cells (Fig. 4A, Table 1). Interestingly,



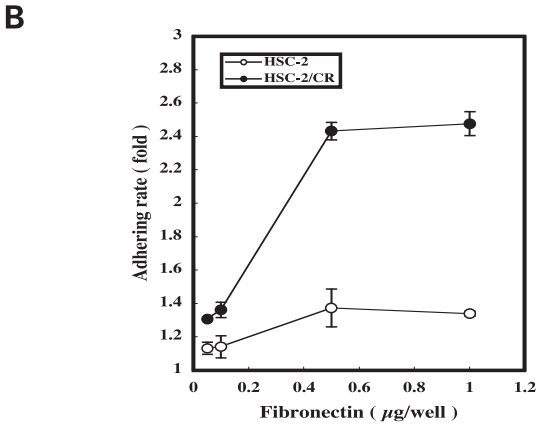


Figure 3. β1-6GlcNAc branching of α5β1 integrin in HSC-2 cells and HSC-2/CR cells and cell adhesion assay to the extracellular matrix. **A,** α5β1 integrin was immunoprecipitated from HSC-2 cells, HSC-2/CR cells, and transfected HSC-2 cells. The amounts of β1-6GlcNAc branching of α5 integrin and β1 integrin were analyzed by means of an L_4-PHA lectin blot (*upper panel*). The membrane was reprobed with a specific mAb to α5 integrin (*middle panel*) and β1 integrin (*lower panel*), respectively, to verify that equal amounts of immunoprecipitated proteins were obtained. These results showed typical examples of three independent experiments. (*Pa,* HSC-2 cells; *CR,* HSC-2/CR cells; *M,* mock transfectants of HSC-2 cells; *V,* GnT-V transfectants of HSC-2 cells.) **B,** 1×10^5 cells were cultured on a 96-well plate coated with fibronectin. Adhesion rate was calculated as described in ''Materials and Methods.'' *Points,* means of triplicate determinations; *bars*, SD.

while increases in β1-6GlcNAc branching on the GnT-V transfectants of HSC-2 cells were significant compared to mock cells (Fig. 2B), it was not observed in the cases of GnT-V transfectants of HSC-2/CR cells (Fig. 4B). Moreover, we performed immunoprecipitation of α5β1 integrin followed by L_4-PHA lectin blot analysis, but the amount of β1-6GlcNAc branching on α5 integrin and β1 integrin was not different between GnT-V transfectants of HSC-2/CR cells and the mock cells (data not shown). Therefore, these results suggest that the expression levels of GnT-V did not determine β1-6GlcNAc branching on glycoproteins in HSC-2/CR cells. We also assessed the sensitivity of GnT-V transfectants of HSC-2/CR cells to cisplatin via an MTT assay. Expectedly, the killing curve of the GnT-V transfectants of HSC-2/CR cells was similar to that of the mock cells (Fig. 4C). These results suggest that the difference in cisplatin sensitivity between HSC-2 cells and HSC-2/CR cells would be correlated with the amount of β1-6GlcNAc branching but not the levels of GnT-V.

Phosphorylation of the Tyr397 FAK before and after Cisplatin Treatment

FAK is a major substrate of tyrosine phosphorylation after integrin activation (15). Because the phosphorylation of Tyr397 FAK is essential for the production of FAK-mediated survival signals (16–18, 27), HSC-2, HSC-2/CR, mock- and GnT-V-transfected HSC-2 cells were treated with cisplatin and the levels of phosphorylation of FAK were investigated. As shown in Fig. 5A (*upper panel*), a dramatic difference in phosphorylation of the Tyr397 FAK was observed between HSC-2 and HSC-2/CR cells. In HSC-2/CR cells, the phosphorylation of the Tyr397 FAK was decreased compared to the other cells before cisplatin treatment but this phosphorylation was dramatically increased at 24 h after treatment with 30 μM cisplatin. In contrast, in the case of HSC-2 parental cells, the level of phosphorylation of the Tyr397 FAK was decreased after treatment with 30 μM cisplatin. Interestingly, FAK was degraded in cisplatin-treated HSC-2 cells, but not in cisplatin-treated HSC-2/CR cells (Fig. 5A, *middle panel*). Moreover, this degradation of FAK as well as the low level of phosphorylation of the Tyr397 FAK was more prominent in GnT-V transfectants of HSC-2 cells treated with cisplatin. Additionally, the degradation of FAK except for HSC-2/CR cells also occurred at 24 h after 20 μM cisplatin treatment (data not shown). It has been reported that the degradation of FAK occurs in mannitol-induced (18) or retinoic acid-induced

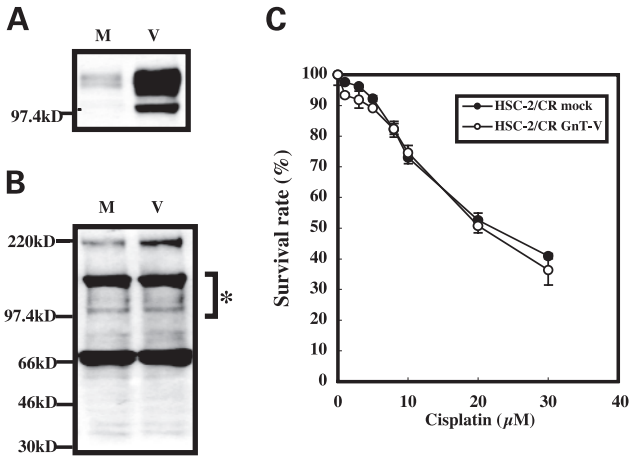


Figure 4. Relationship between GnT-V and β1-6GlcNAc branching in HSC-2/CR cells. Twenty mircrograms of total cellular proteins were subjected to Western blot analysis of GnT-V (**A**). After the membrane filter was deprobed, L_4-PHA lectin blot analysis was performed to confirm the amounts of β1-6GlcNAc branching (**B**). Both results show typical examples of three independent experiments. (*M,* mock transfectants of HSC-2/CR cells; *V,* GnT-V transfectants of HSC-2/CR cells.) **C,** the levels of sensitivity at 48 h after cisplatin treatment were examined using an MTT assay. *Points,* means of triplicate determinations; *bars*, SD.

apoptosis (28). Therefore, the status of the cells, whether cell apoptosis would occur or not, was assessed by an MTT assay. As shown in Fig. 5B, cell viability at 24 h after cisplatin treatment was relatively good even at 30 μM cisplatin except for GnT-V-transfected HSC-2 cells. These results suggest that decreases in β1-6GlcNAc branching on α5β1 integrin prevent the degradation of FAK and the phosphorylation of the Tyr397 FAK results in inhibition of cisplatin-induced apoptosis in HSC-2/CR cells.

Inhibition of Phosphorylation of FAK with α5β1 Integrin Neutral Antibody on Cisplatin Resistance in HSC-2/CR Cells

Because the α5β1 integrin neutral antibody inhibited the function of α5β1 integrin, we investigated the issue of whether or not the phosphorylation of the Tyr397 FAK is also inhibited by this neutral antibody. As shown in Fig. 6A, the phosphorylation of Tyr397 FAK was completely diminished before and after cisplatin treatment in HSC-2/CR cells after treatment with 1 μg/ml of anti-α5β1 integrin neutral antibody. Furthermore, to investigate the effect of α5β1 integrin on cell survival, we performed an MTT assay with or without this neutral antibody. However, the cisplatin sensitivity to HSC-2/CR cells was completely reversed to the same level as that of HSC-2 parental cells after treatment with 1 μg/ml of α5β1 integrin antibody (Fig. 6B). These results suggest that the function of α5β1 integrin could be critical for cisplatin resistance in HSC-2/CR cells.

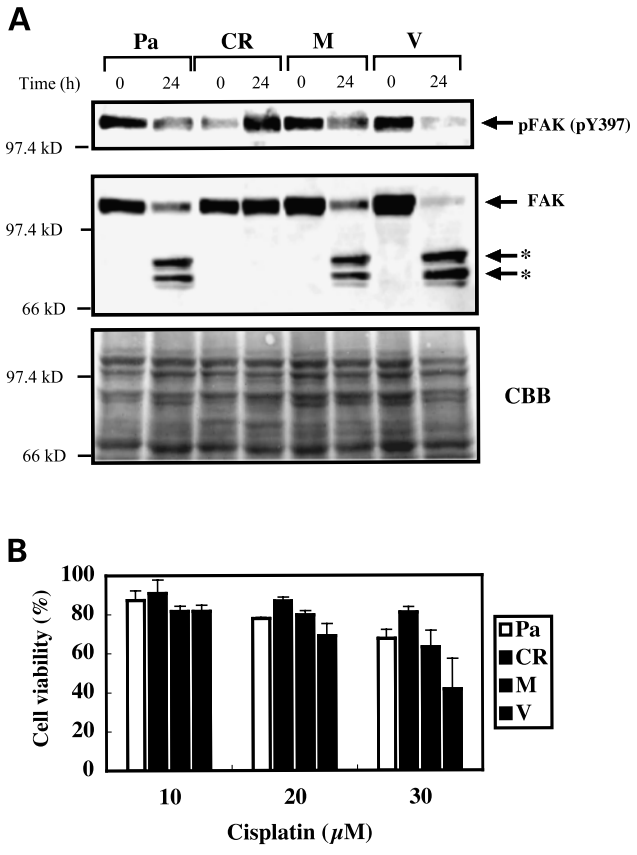


Figure 5. Phosphorylation of FAK in HSC-2 cells, HSC-2/CR cells, and transfected HSC-2 cells. **A,** after cisplatin (30 μM) was added, cells were collected and analyzed at the indicated times after cisplatin treatment. The phosphorylation levels of Tyr397 FAK were analyzed by Western blot analysis using an anti-FAK (pY397) phosphospecific antibody (*upper panel*). The membrane was reprobed with a specific mAb to FAK (*middle panel*). The bands marked with an *asterisk* indicate the degradation of FAK. The amount of protein applied in the SDS-PAGE was evaluated by Coomassie Brilliant Blue (*CBB*) staining (*lower panel*). These results show typical examples of three independent experiments. (*Pa,* HSC-2 cells; *CR,* HSC-2/CR cells; *M,* mock transfectants of HSC-2 cells; *V,* GnT-V transfectants of HSC-2 cells.) **B,** after cisplatin (10 – 30 μM) was added, cell viability was assessed by an MTT assay at 24 h after cisplatin treatment. Cell viability was calculated as described in ''Materials and Methods.'' *Columns,* means of triplicate determinations; *bars*, SD.

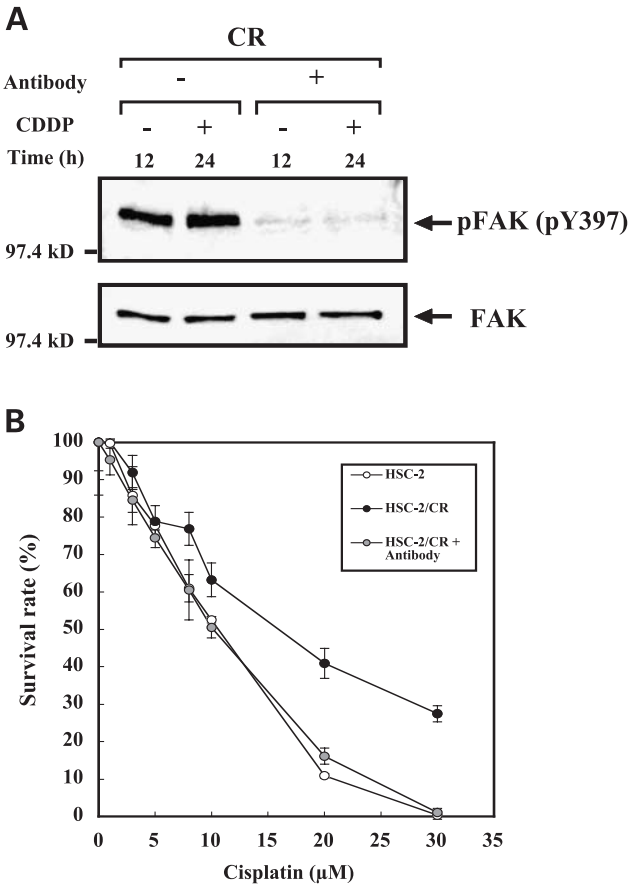


Figure 6. Inhibition of FAK phosphorylation by an α5β1 integrin neutral antibody and the reversal of cisplatin resistance by a neutral antibody in HSC-2/CR cells. **A,** HSC-2/CR cells were plated at a density of 5×10^5 cells/6-cm dish with or without pretreatment of α5β1 integrin neutral antibody (1 μg/ml) and cultured for 12 h. After cisplatin (30 μM) was added, the cells were analyzed at 24 h after neutral antibody treatment. These results show typical examples of two independent experiments. (*CR,* HSC-2/CR cells; *CDDP,* 30 μM cisplatin; *Antibody,* 1 μg/ml of α5β1 integrin neutral antibody.) **B,** HSC-2/CR cells were exposed to various concentrations of cisplatin after pretreatment with anti-α5β1 integrin antibody (1 μg/ml) for 12 h. The sensitivity to cisplatin was examined using an MTT assay. *Points,* means of triplicate determinations; *bars*, SD.

Discussion

The present study clearly demonstrates that functional changes in α5β1 integrin, which could be due to its aberrant

glycosylation, are involved in the cisplatin resistance of a head and neck squamous cell carcinoma cell line. Oligosaccharide modification of α5β1 integrin was observed in β1 integrin, but not in α5 integrin. While decreases in L_4-PHA lectin binding to many glycoproteins were observed in HSC-2/CR cells, decreased levels of β1-6GlcNAc branching on α5β1 integrin were directly associated with cisplatin resistance, since a neutral antibody of α5β1 integrin completely abolished cisplatin resistance. α5β1 integrin, a specific receptor for fibronectin, plays a unique role in survival regulation (10, 29) or *bcl-2* transcription (30). The present study documents another key function of α5β1 integrin as a cell survival factor. Cell surface expression of α5β1 integrin between HSC-2 and HSC-2/CR cells remained unchanged as evidenced by FACS analysis (data not shown). Moreover, cell viability except for HSC-2/CR cells at 24 h after 20 μM cisplatin treatment, in which the degradation of FAK occurred, was more than 70% and was not quite different compared to HSC-2/CR cells (Fig. 5B). Therefore, these results support that decreases in β1-6GlcNAc branching on α5β1 integrin prevented the degradation of FAK and stimulated the phosphorylation of FAK which interacts with several different signaling proteins (27). In general, FAK is mainly phosphorylated by the activation of certain integrin families including α5β1 integrin or syndecans (31). These factors might be involved in lower phosphorylation level in HSC-2/CR cells under no cisplatin treatment (Fig. 5A). However, when a neutral antibody of α5β1 integrin was added to cisplatin-resistant cells, the phosphorylation of Tyr397 FAK almost disappeared, suggesting that this phosphorylation of FAK in the resistant cells was mainly dependent on signaling via α5β1 integrin. Subsequently, when cisplatin was added to the cisplatin-resistant cells with the neutral antibody to α5β1 integrin, the killing curve reversed to the same level of parental cells. Therefore, α5β1 integrin could play a critical role for surviving from cisplatin-induced apoptosis in this cell line.

Although the reason for the decrease in β1-6GlcNAc branching on glycoproteins during cisplatin exposure remains unclear, two possibilities can be proposed as follows. Firstly, in this experiment, cisplatin-resistant cells were established by repeating the passage of all the cells in one dish after the cells were confluent. Only much stronger cells that could be alive had lower levels of β1-6GlcNAc branching on glycoproteins than those in parental cells before cisplatin exposure. This suggests that β1-6GlcNAc branching might be involved in a death signal for cisplatin-treated cells. The second hypothesis is that the long exposure of cisplatin affected the synthetic pathway of oligosaccharides through the down-regulation of glycosyltransferases and/or donor nucleotide sugar substrate UDP-GlcNAc.

While the expression of GnT-V was not changed between HSC-2 and HSC-2/CR cells, the enzymatic activity of GnT-V was slightly lower in HSC-2/CR cells. This decrease might be due to changes in function of GnT-V with its oligosaccharide modification because the molecular weight of GnT-V in HSC-2/CR cells was slightly smaller than that of HSC-2 cells (Fig. 1C). Decreases in β1-6GlcNAc branching in HSC-2/CR cells would be due to changes in down-regulation and/or localization of GnT-V or decreases in levels of the substrate of GnT-V, UDP-GlcNAc, because the K_m value of UDP-GlcNAc was quite high in the reaction of GnT-V (32).

In conclusion, we report on the relationship between cisplatin resistance and α5β1 integrin with β1-6GlcNAc branching using a newly established cisplatin-resistant head and neck carcinoma cell line. The modification of oligosaccharides on α5β1 integrin represents a possible mechanism for cisplatin resistance in head and neck cancer cells. Therefore, the modification of α5β1 integrin by glycosylation might be a useful therapeutic strategy for successful chemotherapy against cisplatin-resistant tumor cells.

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