Cisplatin at Sub-toxic Levels Mediates Integrin Switch in Lung Cancer Cells

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Abstract. *Background: Resistance to chemotherapeutic agents, as well as enhanced metastasis, have been frequently reported in lung cancer. Materials and Methods: Cytotoxicity and proliferative effects of cisplatin on H460 lung cancer cells were evaluated by the MTT assay. Migration capacity was evaluated by the wound healing assay. The number of filopodia per cell were detected by rhodamine-phalloidin staining assay. The changes of protein levels of integrins, and migration-related proteins in response to cisplatin at sub-toxic concentrations were determined by western blotting. Results: Herein we demonstrate for the first time that exposure to low concentrations of cisplatin results in increase of cell motility with the alteration of integrin expression. Cisplatin-treated cells exhibited a significant increase in the number of filopodia per cell in correlation with enhanced migration. Migration regulatory proteins, namely activated forms of focal-adhesion kinase (FAK) and ATP-dependent tyrosine kinase (AKT), were found to significantly be up-regulated in cisplatin-treated cells in comparison to those of the non-treated control. Active Rho A-GTP and Rac-GTP were found to be increased in accordance with activation of FAK/AKT signals. Furthermore, we found that such migration enhancement may be in part due to the integrin switch mediated by cisplatin treatment. Cisplatin induced a dramatic alteration in the integrin expression pattern by up-regulating integrin α_4, α_v, β_1, and β_5 which were previously reported to increase cell motility, while it had no effect on integrin α_5, and β_3. Conclusion: As the integrin switch is a hallmark of highly aggressive cancer, these findings may provide insights for better understanding of cancer cell adaptation after exposure to cisplatin.*

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The incidence as well as mortality rates of patients with lung cancer have increased annually. The main causes of cancer-related death in lung cancer are chemotherapeutic resistance and metastasis (1). Evidence suggests that certain cancer cells receiving chemotherapeutic agents alter their behaviors towards a more aggressive phenotype (2, 3). Moreover, studies indicate that chemotherapeutic drugs enhance the metastatic capacity of cancer cells (4, 5). Among well-known chemotherapeutic agents, cisplatin, a cis-diamminechloroplatinum (II) is a potent cytotoxic compound for the treatment of many solid tumors, including lung cancer (6). Although this drug has been shown to be highly effective in certain patients, cancer relapse and metastasis are frequently observed (1). A better understanding of the molecular basis of these phenomena, as well as of the possible effects of cisplatin on cisplatin-resistant lung cancer cells, may help improve strategic uses of cisplatin.

Interestingly, numerous studies have revealed that the metastatic potential of cancer cells can be potentiated by the distinct expression pattern of adhesion molecules named integrins (7-9). An integrin molecule is composed of two non-covalently transmembrane glycoprotein subunits termed α and β (8). Integrins are important biological molecules that help maintain firm adhesion of the cell to extracellular matrix and such adhesion provides fundamental survival signals to the cells (10). It is widely accepted that an increase of certain integrins, especially integrin α_4, α_5, α_v, and β_1, enhances motility of cancer cells.

So far, the effect of low concentrations of cisplatin on integrin switch in lung cancer cells is largely unknown. We, thus, investigated the effect of this widely prescribed chemotherapeutic agent on integrin expression pattern in non-small cell lung cancer cells. The knowledge gained from the present study may benefit better understanding of cancer cell biology and adaptive response of the cell during chemotherapy.

Materials and Methods

Cells and reagents. Human lung cancer epithelial H460 cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and culture in RPMI-1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine and 100 units/ml of penicillin/streptomycin (Gibco, Gaithersburg, MA, USA) at 37˚C with 5% CO_2 in a humidified incubator. Cisplatin, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), Hoechst 33342, propidium iodide (PI), phalloidin-tetramethylrhodamine B isothiocyanate, and bovine serum albumin (BSA) were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Antibodies for integrins α_4, α_5, α_v, β_1, β_3 and β_5, ATP-dependent tyrosine kinase (AKT), phospolylated ATP-dependent tyrosine kinase (p-AKT) (S473), focal adhesion kinase (FAK), phospholylated focal adhesion kinase (p-FAK) (Y397), β-actin and peroxidase-labeled secondary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA) and Rho-GTP, Rac-GTP were obtained from NewEast Bioscience, (King of Prussia, PA, USA).

Cell viability assay. Cell viability was determined by MTT colorimetric assay. Initially, cells were seeded at a density of 10^4 cell/well in 96-well overnight. After that, cells were treated with different concentrations of cisplatin for 24 h. The medium was replaced with 500 μg/ml of MTT for 4 h at 37˚C. The supernatant was then removed and 100 μl DMSO was added to solubilize the formazan product and the intensity of formazan product was measured by spectrophotometry at 570 nm using an ELISA reader (Anthros, Durham, NC, USA). The percentage cell viability was calculated as the absorbance of cisplatin-treated cells relative to control cells.

Apoptosis assay. Cells were seeded at a density of 10^4 cells/well onto 96-well plate and incubated overnight for cell attachment. After the indicated treatments of cisplatin (0.25-10 μM), cell were washed and incubated with 10 μg/ml Hoechst33342 and 5 μg/ml propidium iodide (PI) for 30 min. Nuclear condensation and DNA fragmentation of apoptotic cells and PI-positive necrotic cells were visualized by fluorescence microscopy (Olympus IX51 with a DP70 digital camera system, Tokyo, Japan).

Proliferation assay. Cells were exposed to cisplatin at indicated concentrations (0.25-1 μM) and were subjected to the cell proliferation assay after 0, 24, 48, and 72 h. Cells were seeded at a density of 2×10^3 cell/well in a 96-well plate. Cell proliferation was determined through incubation with 500 μg/ml MTT for 4 h, and the absorbance of formazan product which was dissolved by DMSO was measured by spectrophotometry at 570 nm using an enzyme-linked immunosorbent assay reader (Anthros, Durham, NC,USA).

Migration determination. Migration was determined by wound-healing assays. For the wound-healing assay, a monolayer of cells was cultured in a 96-well plate, and a wound was made with a 1-mm-wide tip. After rinsing with PBS, the cell monolayers were treated with cisplatin (0.25-1 μM) and allowed to migrate for 24 h. Micrographs were taken under a phase contrast microscope (Olympus IX51 with a DP70 digital camera system, Tokyo, Japan), and the wound spaces were measured using Olympus DP controller software. Quantitative analysis of cell migration was performed using an average wound space from random fields of view, and the percentage of change in the wound space was calculated using the following formula: % change=(average space at time 0 h)–(average space at time 24 h)/(average space at time 0 h)×100. Relative cell migration was calculated by dividing the percentage change in the wound space of treated cells by that of the control cells in each experiment.

Morphological characteristics of cancer cells. Cell morphology was investigated using phalloidin-rhodamine assay. After treated with cisplatin at indicated concentration, the cells were fixed with 4% paraformaldehyde in PBS for 10 min at 37˚C, permeabilized with 0.1% Triton-X100 in PBS for 5 min, rinsed with PBS and then blocked for unspecific binding by incubation with 0.2% BSA in PBS for 30 min. The fixed cells were then incubated with a 1:100 dilution of phalloidin-rhodamine in PBS for 20 min, then rinsed with PBS three times and mounted with 50% glycerol in PBS. The cell morphology was captured using a Nikon eclipse Ti-U fluorescence microscope, Nikon Corporation, Tokio, Japan.

Western blot analysis. After specific treatments, cells were incubated in lysis buffer containing 20 mMTris-HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) for 40 min on ice. The cell lysates were collected, and the protein content was determined using the Bradford method (Bio-Rad Laboratories). Equal amounts of proteins from each sample were denatured by heating at 95˚C for 5 min with Laemmli loading buffer and subsequently loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After separation, proteins were transferred onto 0.45 μM nitrocellulose membranes (Bio-Rad). The transferred membranes were blocked for 1 h in 5% nonfat dry milk in TBST (25 mMTris-HCl pH 7.5, 125 mM NaCl, and 0.05% Tween 20) and incubated with the appropriate primary antibodies (anti-integrin α_4, α_5, α_v, β_1, β_3 and β_5, AKT, p-AKT (S473), FAK, p-FAK (Y397), Rho-GTP, Rac-GTP and β-actin) at 4˚C overnight. The membranes were washed twice with TBST for 10 min and incubated with horseradish peroxidase-labeled isotype-specific secondary antibodies for 2 h at room temperature. The immune complexes were detected by enhancement with chemiluminescence substrate (Supersignal West Pico; Pierce) and quantified using analys.t/PC densitometry software (Bio-Rad).

Statistical analysis. All data are expressed as the means±S.E.M. from three or more independent experiments. Multiple comparisons were examined for significant differences of multiple groups, using analysis of variance (ANOVA), followed by individual comparisons with the Scheffe's *post-hoc* test. Statistical significance was set at $p<0.05$.

Results

Effect of cisplatin on the viability of the H460 human lung cancer cell line. We first determined the cytotoxic effect of cisplatin on H460 human lung cancer cell line. The cells were cultured in the presence or absence of cisplatin (0-50 μM) for 24 h, and cell viability was determined by the MTT assay. Figure 1A shows that when cells were treated with cisplatin at concentrations ranging 0.1-2 μM, neither cytotoxicity nor proliferative effects were observed. A significant decrease in

terms of cell viability was first detected in cells treated with 5 μM, with approximately 90% of the cells remaining viable.

Consistent with the above findings, apoptosis and necrotic cell death determined by Hoechst33342/PI assay were not found in response to 0-1 μM cisplatin treatment. Significant apoptotic nuclei exhibiting condensed/fragmented nuclei were detected in cells treated with 10 μM (Figure 1 C-E). To investigate the effect of cisplatin on cell proliferation, H460 cells were treated with sub-toxic concentrations of cisplatin (0.25-1 μM) for 24 h, then the treated cells were seeded in 96-well plate and were determined for proliferation at 0, 24, 48, and 72 h (Figure 1B). A significant decrease in cell proliferative capacity was detected at 48, and 72 h in cells treated with 1 μM of cisplatin.

Effect of cisplatin on H460 migration. To investigate the effect of cisplatin on cell migration, we performed scratch would-healing assays. Cells were exposed to non-toxic concentrations of cisplatin (0.25-1 μM) for 24 h and were subjected to migration assay for a further 24 and 48 h. Figure 2A shows that treatment with cisplatin enhanced migration of the cells in a dose-dependent manner compared to the H460 control cells. Treatment with 0.5 and 1 μM cisplatin significantly potentiated the migration of the cells at 24 h, while treated with 0.25 μM cisplatin significantly enhanced motility of cell at 48 h of the assay (Figure 2B). Treatment with cisplatin 1 μM increased the migration of cells by approximately 1.5- and 2.5-fold compared to that of the non-treated cells in the 24 and 48 h assays, respectively. These results indicate that sub-toxic concentrations of cisplatin enhance migration behavior of these cells.

Cisplatin enhanced filopodia formation in H460 lung cancer cells. Filopodia, cellular protrusions at the edge of motile cells, are produced by actin polymerization and rearrangement of actin filaments, and the formation of filopodia has been shown to play an essential role in cell movement (11). To confirm the enhancement effect of cisplatin treatment on cell motility, cells were treated with non-toxic concentrations of cisplatin for 24 h as previously described, and the filopodia were determined using a phalloidin-rhodamine staining assay. Figure 3 shows that cisplatin-treated cells exhibited a significant increase in filopodia protrusions accumulating at the cellular edge in a dose-dependent manner as compared with the H460 control cells.

Cisplatin mediates integrin switch and activates migration signaling pathways. The switching in the expression pattern of integrins towards increasing the level of integrins α_4, α_5, α_v, and β_1 was shown to enhance motility in cancer cells. Together with the above observations demonstrating the potentiating effect of cisplatin exposure on lung cancer cell movement, we next examined the underlying mechanisms by evaluating migration regulatory proteins as well as integrins. Cells were treated with cisplatin for 24 h, and the expression of proteins including phosphorylated FAK (Tyr 397), phosphorylated AKT (Ser 473), RhoA-GTP, Rac-GTP, and integrins α_4, α_5, α_v, β_1, β_3 and β_5 were evaluated by western blot analysis. Figure 4 shows that treatment with cisplatin caused a significant increase of activated FAK, RhoA-GTP, Rac-GTP, and activated AKT in dose-dependent manner as compared to those of non-treated control cells.

Figure 5 shows that cisplatin caused substantial up-regulation of integrins α_4, β_1 and β_5. Importantly, the expression of integrin α_v was found to be dramatically increased in response to cisplatin treatment. However, such cisplatin exposure had no effect on integrins α_5, and β_3. Our results not only confirm the previous findings indicating that the up-regulation of these integrins α_4, α_v and β_1 enhance motility of cancer cells, but also provide evidence indicating that treatment with cisplatin can mediate such an integrin switch, leading to increased migratory activity of lung cancer cells.

Discussion

Most lung cancer deaths are attributed to cancer metastasis and resistance to chemotherapeutic agents (12). Increassing evidence has suggested that among the many steps of cancer metastasis, movement of the cancer cells from their original tumors and their motility during the process of cancer dissemination are an important hallmark for the successful cancer spread. However, information regarding the possible inducing factors and mediators that potentiate the migratory activities of cancer cells remains largely unknown. Certain cancer cells develop mechanisms to overcome the death signals provided by anticancer agents and such a population remains after chemotherapy (13). These chemoresistant cancer cells have frequently been shown to be a key player in metastasis as well as in cancer relapse (14-16). This concept led us to the hypothesis of whether chemotherapeutic agents such as cisplatin could increase the metastatic potential of lung cancer cells. Previously, we demonstrated that sub-toxic concentrations of cisplatin conferred resistance to anoikis in human lung cancer cells (5). Herein, we provide further information regarding the effect of such sub-toxic concentrations of cisplatin on migration of lung cancer cells.

Integrins are a family of transmembrane glycoprotein receptors that mediate cell–matrix and cell–cell interactions which play a pivotal role in cell behavior, such as cellular adhesion and cellular movement. Integrins consist of α and β subunits; there are 18 α and eight β subunits, which provides heterodimerization at least distinct 24 subtypes (17). In cancer biology, an increase in the expression of integrin α_4, α_5, α_v and β_1 are correlated with more aggressiveness

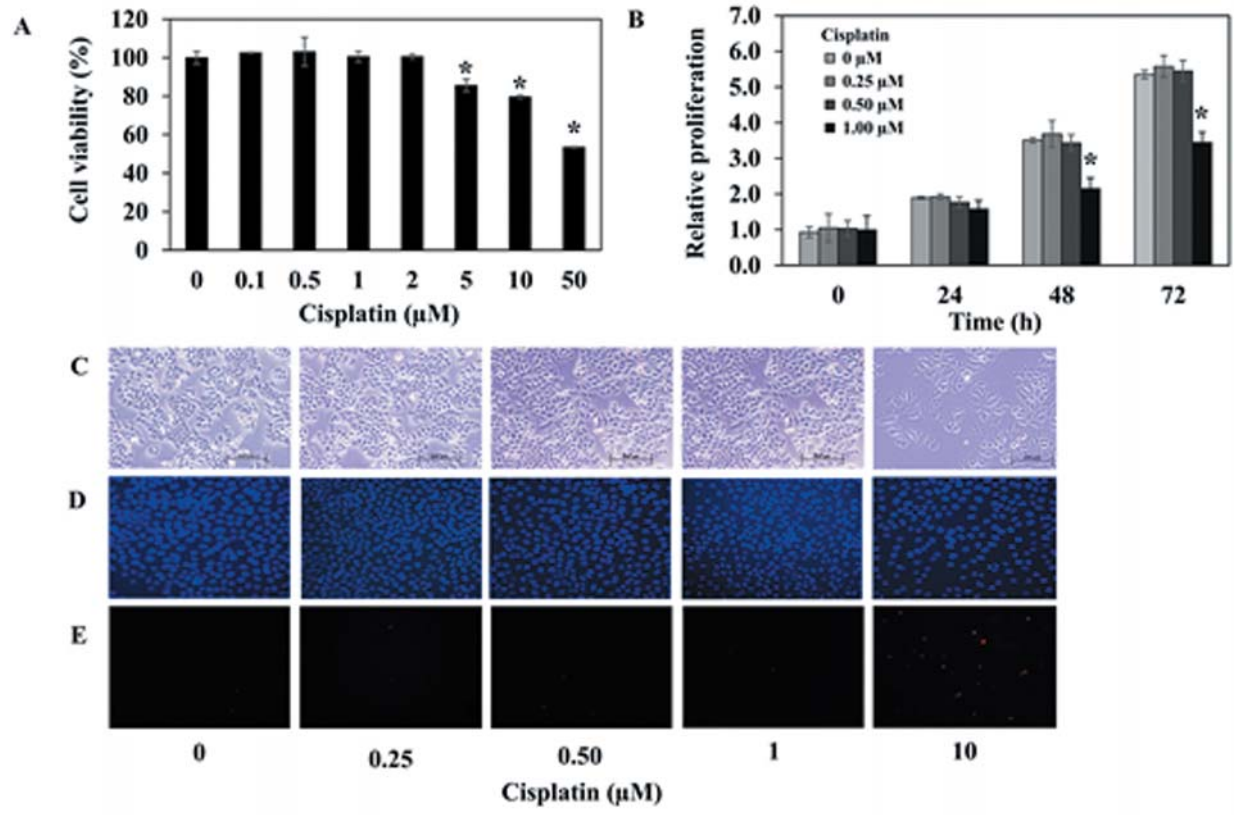

Figure 1. *Cytotoxicity of cisplatin on H460 human lung cancer cell line. A: Cells were treated with different concentrations of cisplatin (0-50 μM) for 24 h. B: Proliferation of the cells treated with cisplatin (0-1 μM) for 24 h determined at 0, 24, 48, and 72 h. C: After the indicated treatment for 24 h, phase-contrast images were captured. D: After indicated treatment for 24 h, apoptosis cell death was examined by Hoechst33342 staining assay, E: After indicated treatment for 24 h, necrosis cell death was examined by PI stained assay. Data represent the mean±SD (n=3). *p<0.05 versus non-treated control cells.*

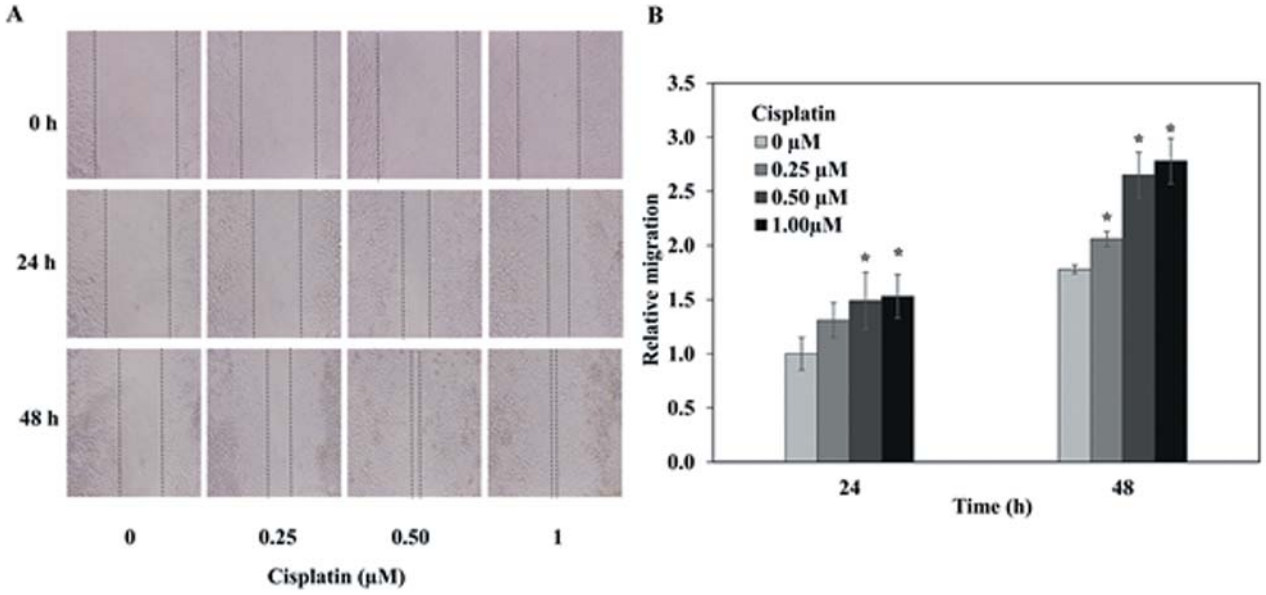

Figure 2. *Effects of cisplatin on H460 cell migration. A: After treatment with cisplatin (0-1 μM) for 24 h, the confluent monolayer of H460 was wounded using a 1-mm width tip and cultured with complete medium for the indicated times. B: Wound space was analyzed and presented as relative migration levels. Data represent the mean±SD (n=3). *p<0.05 versus non-treated control cells.*

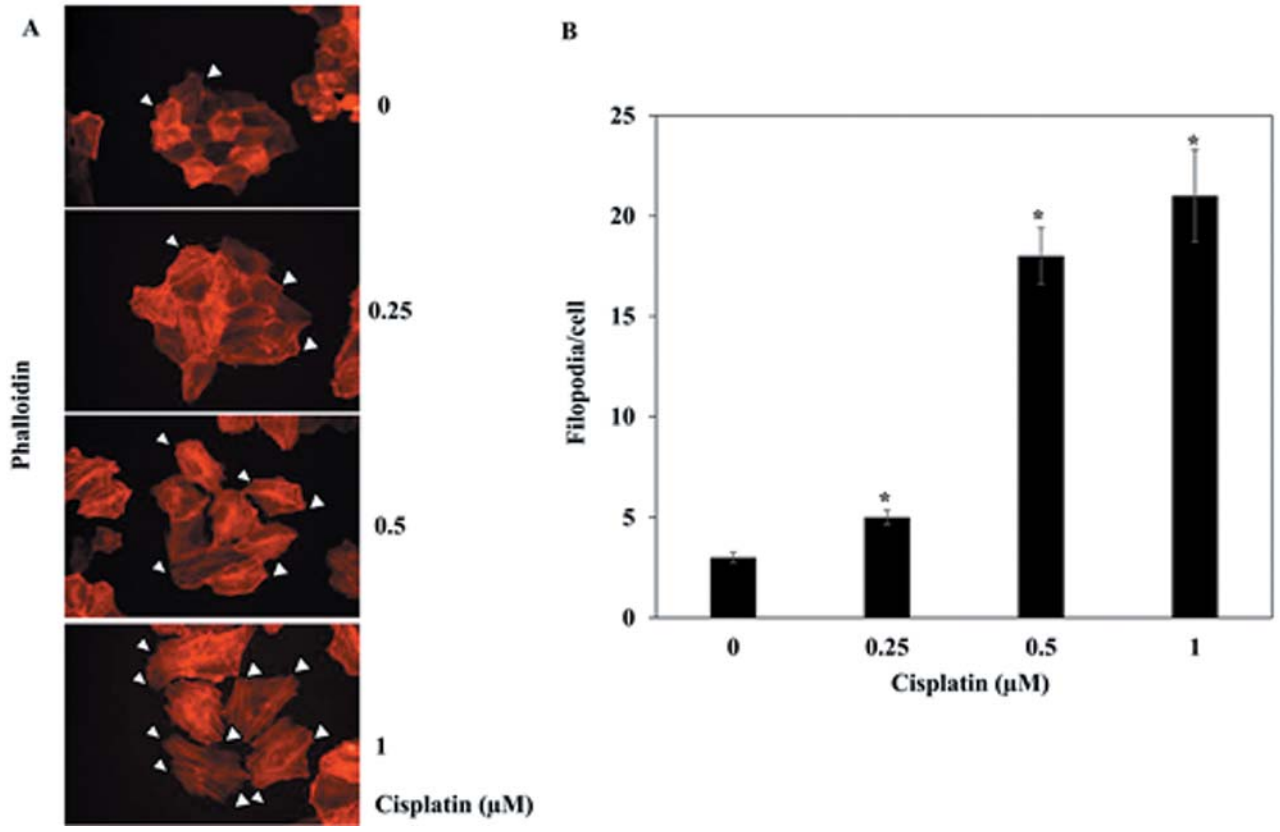

Figure 3. *Filopodia formation in H460 cells treated with cisplatin. A: Cells were treated with 0-1 μM cisplatin for 24 h. The cells were stained with phalloidin-rhodamine and visualized under fluorescence microscopy. Filopodia are indicated by arrowheads. B: The number of filopodia per cisplatin-treated cell was analyzed and compared to that of non-treated control cells. Data represent the mean±SD (n=3). *p<0.05 versus non-treated control cells.*

behavior of cancer cells (18-22). Studies of human cancer pathogenesis revealed that integrin subtype was altered with tumor stage (23) and switches in integrin expression frequently enhance the aggressive behavior of cancer cells (24, 25).

Evidence has indicated that molecules such as FAK and AKT play important roles in triggering cell movement (26, 27). In motile cells, FAK at the edge of cells incorporating integrin is activated by phosphorylation at Tyr 397 (26, 28). The active form of FAK in turn activates AKT, leading to actin polymerization and filopodia formation. Accordingly, we found that treatment with cisplatin enhanced activation of FAK–AKT signals (Figure 4) in correlation with increased filopodia in cells (Figure 3). For downstream regulation of cell migration, the role of small GTPase Rac and RhoA on actin rearrangements have been demonstrated in a number of studies (29-32). The active form of Rac (Rac-GTP) induces plasma membrane protrusion and regulates lamellipodia formation (33). In addition, active RhoA (RhoA-GTP) promotes stress fiber accumulation and induces new focal adhesions (34). We also observed the involvement of theses downstream signals in cisplatin-treated cells. Both active forms of Rac and Rho A were found to be significantly increased in cisplatin-treated cells in a dose-dependent manner (Figure 4).

Collectively, the present study reveals the possible adaptive mechanisms of cancer cells exposed to low or insufficient doses of cisplatin. The cisplatin-treated cancer cells adapted themselves toward increased expression of migratory-related integrins, namely integrins α_4, α_v, β_1, β_5 and in combination with activation of the migratory proteins FAK, AKT, Rho A, and Rac. The information gained from this study raises concerns regarding the awareness of such adaptation to inadequate cisplatin concentrations, as well as the need for better strategic use for chemotherapy for highly metastatic cancer.

Conflicts of Interest

The Authors declare that there are no conflicts of interest regarding this research.

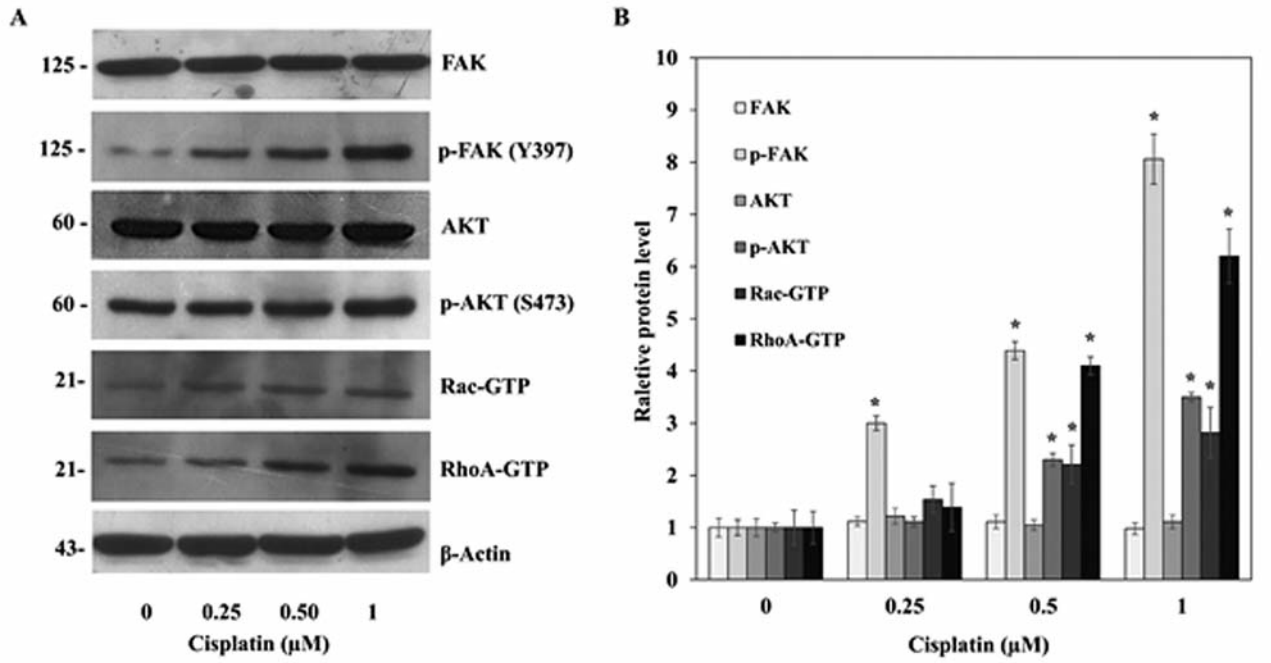

Figure 4. *Cisplatin exposure activates the focal-adhesion kinase/ATP-dependent tyrosine kinase (FAK/AKT)-associated migration pathway. A: After treating H460 cells with cisplatin for 24 h, the expression levels of phosphorylated FAK, phosphorylated AKT, Rac-GTP, and RhoA-GTP were determined by western blot analysis. To confirm equal loading of the protein samples, the blots were re-probed with β-actin antibody. B: The immunoblot signals were quantified by densitometry. Data represent the mean±SD (n=3). *p<0.05 versus non-treated control cells.*

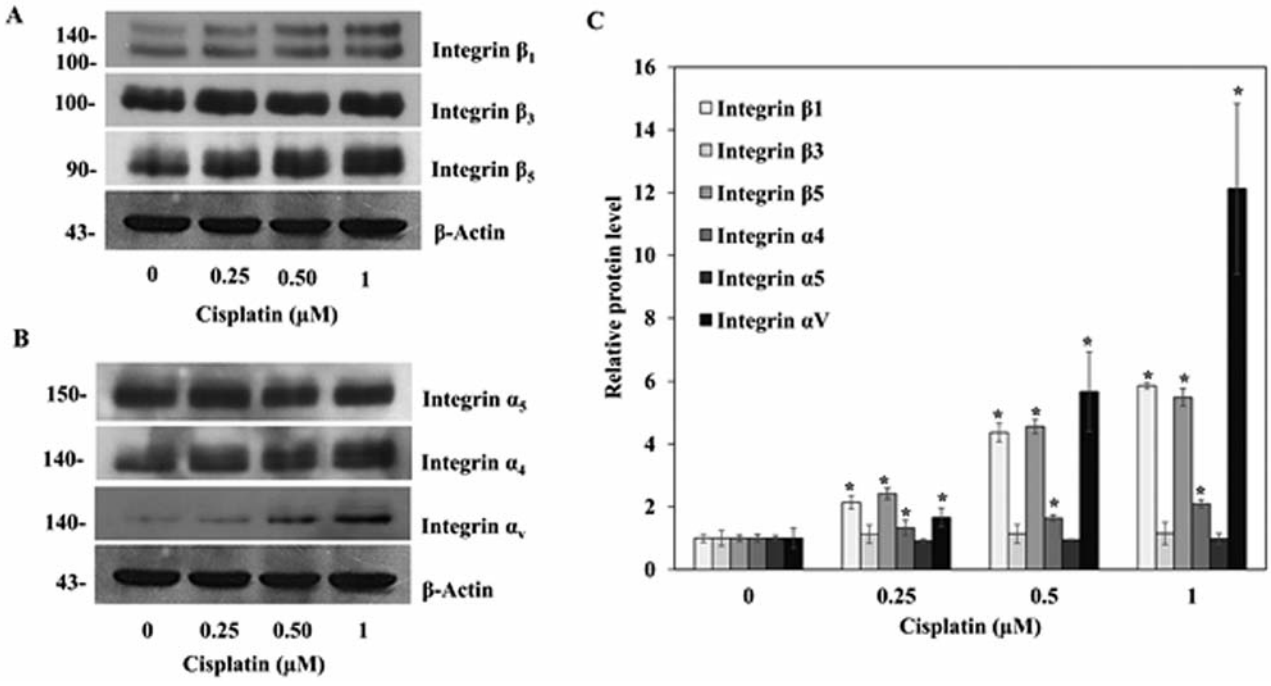

Figure 5. *Effects of cisplatin on integrin expression. A and B: After treating cells with cisplatin for 24 h, the expression levels of integrins α_4, α_5, α_v, β_1, β_3 and β_5 were determined by western blotting. β-Actin was used as loading control to confirm equal loading of the samples. C: The protein signals were quantified by densitometry. Data represent the mean±SD (n=3). *p<0.05 versus non-treated control cells.*

Acknowledgements

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