0026-895X/04/6505-1217–1224
MOLECULAR PHARMACOLOGY
U.S. Government work not protected by U.S. copyright
Mol Pharmacol 65:1217–1224, 2004

Vol. 65, No. 5
3156/1146846
Printed in U.S.A.

Down-Regulation and Altered Localization of γ-Catenin in Cisplatin-Resistant Adenocarcinoma Cells

Xing-Jie Liang, Ding-Wu Shen, and Michael M. Gottesman

Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

Received December 12, 2003; accepted February 4, 2004

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Resistance to cisplatin, one of the most widely used anticancer chemotherapeutic agents, is a major clinical problem. There is no effective way to predict development of cisplatin resistance in cancers. As determined by reverse transcription-polymerase chain reaction and Western blotting, the expression of γ-catenin, an adherens junction protein, was decreased in KB-CP20 and 7404-CP20 cells compared with parental-sensitive cells. Short-term treatment with cisplatin of the parental cells resulted in proteolysis of γ-catenin as evaluated in membrane pellet preparations, and the extent of cleavage increased as cisplatin concentration was raised from 1 to 5 μg/ml during 1 h of treatment. Uncleaved cytoplasmic γ-catenin increased under the same conditions. These biochemical results were supported by confocal microscopy, which showed a loss of γ-catenin from adherens plaques after cisplatin treatment. Cleavage of γ-catenin was specific to cisplatin treatment in that cleavage did not occur after treatment with doxorubicin and cytosine arabinoside. Pretreatment of KB and 7404 cells with cisplatin for 1 h resulted in reduced uptake of [^{14}C]carboplatin, suggesting that the biochemical changes induced by cisplatin treatment, including cleavage of γ-catenin, could affect the ability of cells to internalize platinum compounds. Cells transfected with the γ-catenin gene are sensitive to cisplatin compared with cells transfected with a control vector. Our data suggest that proteolysis and altered localization of γ-catenin are early markers for the response of cells to cisplatin, and reduced levels of γ-catenin in resistant cells may indicate an important role for γ-catenin in mediating or modulating the toxicity of cisplatin in cancer cells.

The biological activity of cisplatin [*cis*-diamminedichloroplatinum(II)] was discovered serendipitously more than 40 years ago; since then, research efforts have focused on elucidating its mechanism of action. Cisplatin is a potent anticancer drug that has been used successfully to treat tumors of the head, neck, lungs, and genitourinary tract (Shen et al., 2000). The discovery that the ultimate cellular target of cisplatin is DNA has led to intense investigations into the mechanism of cisplatin resistance. As with other cytotoxic anticancer drugs, tolerance or resistance of tumors to cisplatin represents a major impediment to successful treatment, and many different mechanisms of resistance to cisplatin, ranging from decreased uptake to repair of DNA damage, have been described previously (Siddik, 2003). Inside the cell, cisplatin interacts with many different macromolecules. Protein and peptide binding are thought to play a role in mediating cisplatin resistance through coordination to sulfur donor atoms of the amino acids cysteine and methionine (Boulikas and Vougiouka, 2003). A complete understanding of how tumor cells acquire resistance is pivotal in developing a means of reversing resistance to cisplatin compounds.

A large number of studies have focused on mechanisms of acquired drug resistance after prolonged treatment with cytotoxic drugs. However, increasing evidence supports the role of cell-cell contact in the initial escape of the tumor cells from toxicity of anticancer drugs. This phenomenon has been termed "cell adhesion-mediated drug resistance" (Shain and Dalton, 2001). A number of studies have reported that drug resistance develops as a result of cell-cell contact when cells reach confluence in a monolayer culture. Adhesive cell interactions may play an important role in both intrinsic and acquired multicellular resistance to alkylating agents (Croix et al., 1996b; Hazlehurst et al., 2000). This resistance cannot be explained solely on the basis of known drug resistance mechanisms, such as reduced drug accumulation or increased levels of P-glycoprotein (Gottesman, 2002). It has been postulated that an adhesion-dependent reduction in tumor cell growth may be essential for the development of this type of resistance (Croix et al., 1996a).

Cell-to-cell adhesion is mediated by cadherins, which form

This work was supported by the National Institutes of Health.

ABBREVIATIONS: CP-r, cisplatin-resistant; RT-PCR, reverse transcription-polymerase chain reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

a complex with catenins. In vertebrate cells, the "classic" cadherins, found at adherens junctions, interact through γ-catenin and additional accessory proteins with the cell's microfilament system (Luna and Hitt, 1992). The other "desmosomal" cadherins interact through γ-catenin and a different set of accessory proteins with intermediate filaments (Karnovsky and Klymkowsky, 1995; Chitaev et al., 1996). γ-Catenin is unique in that it interacts with both classic and desmosomal cadherins and is present at both types of cell-cell adherens junctions (Gumbiner, 1996).

To study cisplatin resistance in detail, we isolated two independent cell populations derived from human KB epidermoid adenocarcinoma cells (KB-CP) and human BEL7404 hepatoma cells (7404-CP). These populations were derived in multiple steps by gradual increases in the selecting concentration of cisplatin. Both cell lines show multiple changes in protein expression (Shen et al., 1998). In this study, using PowerBlot (BD Transduction Laboratories, Lexington, KY) and microarray screening technology, we found that the expression of γ-catenin decreased in both KB and BEL7404 cisplatin-resistant (CP-r) cell lines, which was confirmed at the mRNA and protein level by semiquantitative RT-PCR and Western blotting. In addition, γ-catenin on the plasma membrane was specifically cleaved into several bands, and increased γ-catenin was found in the cytosol of parental cells treated with cisplatin. Pretreatment of parental KB and BEL7404 cells with cisplatin resulted in reduced uptake of [^{14}C]carboplatin, suggesting that the biochemical changes induced by cisplatin treatment, including cleavage of γ-catenin, could affect the capability of cells to internalize platinum compounds. Single-step selected KB-CP.5 cells transfected with the γ-catenin gene showed decreased resistance to cisplatin by the MTT cellular proliferation assay. We conclude that the loss of the cellular adhesion protein γ-catenin contributes to the resistance of tumor cells to cisplatin and is a potential probe whose levels and cellular localization might be used to predict the likelihood of sensitivity to cisplatin.

Materials and Methods

Drugs and Chemicals. [^{14}C]carboplatin and [^{3}H]methotrexate (specific activity, 20 Ci/mmol) were purchased from Amersham Biosciences Inc. (Piscataway, NJ). The monoclonal antibody against human γ-catenin was obtained from BD Biosciences Transduction Laboratories (Lexington, KY). Cisplatin, cytosine arabinoside, doxorubicin, and other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cells and Culture Conditions. KB-3-1 was derived from a single clone of human KB epidermoid carcinoma cells (a variant of HeLa) after two subclonings from the parental cells (Akiyama et al., 1985). KB-CP20 is a population of KB-3-1 cells grown in increasing concentrations of cisplatin up to 20 μg/ml cisplatin over a period of 6 months. BEL7404 is a human liver carcinoma cell line. CP-r BEL7404-CP20 populations were selected by stepwise increases up to 20 μg/ml cisplatin over a period of 24 months. Stock cultures of KB-CP20 and BEL7404-CP20 were maintained in medium containing 5 μg/ml cisplatin and grown without cisplatin for at least 3 days before these experiments (Shen et al., 1995). KB-CP.5 cells were isolated from KB-3-1 cells in a single step by continuous selection in 0.5 μg/ml cisplatin (Liang et al., 2003). All cell lines were grown as monolayer cultures at 37°C in 5% carbon dioxide, using Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose (Invitrogen, Carlsbad, CA) supplemented with L-glutamine, penicillin, streptomycin (Quality Biological, Gaithersburg, MD) and 10% fetal bovine serum (Cambrex Bio Science Walkersville, Inc., Walkersville, MD).

Microarray Analysis. The cDNA microarray filter (nylon membrane) contained 7124 cDNA and expressed sequence tags with high and moderate similarity to known genes in human or other species. Labeling and hybridization procedures were conducted as specified by the manufacturer. Verification of cDNA expression was performed with the GeneAmp RNA RT-PCR Kit (Applied Biosystems, Foster City, CA).

PowerBlot Analysis. Sample preparation was based on the protocol of the manufacturer (BD Biosciences Clontech, Palo Alto, CA). Briefly, after washing with PBS three times, the incubated cells were lysed with 1 ml of cell lysis buffer (10 mM Tris, pH 7.4, 1 mM sodium *ortho*-vanadate, and 1% SDS). After SDS gel electrophoresis, the sample proteins were transferred to a polyvinylidene difluoride membrane and evaluated using an inventory of 678 antibodies by Western blotting (BD Biosciences Transduction Laboratories; BD Biosciences PharMingen, San Diego, CA). Electronic images of blots were captured using the Odyssey infrared imaging system. Images were subjected to automatic spot finding and spot matching using PDQuest software (Bio-Rad, Hercules, CA). Experiments were repeated in triplicate with similar results.

Immunoblotting Detection of the γ-Catenin Protein. 1×10^7 cells from each cell line (untreated and treated with 1 μg/ml or 5 μg/ml cisplatin for 1 h) were harvested at log phase and washed twice with ice-cold PBS. The cells were sedimented by centrifugation at 1400*g* for 10 min and suspended in ice-cold hypotonic solution buffer (0.5 mM KH_2PO_4 and 0.1 mM EDTA containing 1% protease inhibitor aprotinin, pH 8.0) for 5 min on ice. Cells were disrupted on ice by a tight Dounce homogenizer with constant homogenizing for 30 strokes. Samples were checked under a phase-contrast microscope, which showed that more than 80% of the cells were broken. The cytosolic fractions were separated by centrifugation at 2000*g* for 10 min at 4°C. The resulting supernatant was further centrifuged at 100,000*g* for 55 min at 4°C. The fractions that sedimented at the bottom were collected as crude membranes. At the same time, whole-cell lysates were also prepared as described previously (Shen et al., 1998). Protein electrophoresis and immunoblotting with antibodies directed to γ-catenin were as follows. The samples (crude membrane and whole-cell lysate) were separated by SDS-polyacrylamide gel electrophoresis on a 4 to 20% gradient gel and transferred onto nitrocellulose membranes. Membranes were subsequently subjected to immunostaining with monoclonal antibodies against human γ-catenin (1:2000, 1 h). Enhanced chemiluminescence reagents were used for developing signals as described by the manufacturer (Pierce Biotechnology, Rockford, IL).

Isolation of RNA and Amplification by RT-PCR. Total cellular RNA was isolated from parental and CP-r KB and BEL7404 cells with TRIzol reagent (Invitrogen). Reverse transcription was performed with random hexamers in accordance with the manufacturer's instructions (GeneAmp RNA RT-PCR Kit; Applied Biosystems), and the resulting complementary DNA was amplified by PCR with the following specific primer pairs: human γ-catenin (forward, GAG AGT GTG CTG AAG ATT CTG; reverse, TGA TGT CGT CCT TGT CAC C). Amplification was performed for 28 cycles (30 s at 95°C, 60 s at 55°C, and 60 s at 72°C) with a Peltier Thermal Cycler (PTC-200; MJ Research, Watertown, MA). After the final cycle, an elongation step of 10 min was performed at 72°C. Amplified DNA fragments were separated on a 1.5% agarose gel and visualized by staining with ethidium bromide. Reactions performed in the absence of reverse transcriptase or mRNA were used as negative controls during amplification.

Indirect Immunofluorescence Microscopy. The cells cultured on 18-mm glass coverslips were fixed at −20°C with 70% ethanol in PBS for 10 min. Cells were subsequently washed with PBS and preblocked in 3% bovine serum albumin/PBS for 30 min, then incubated for 1 h with a primary antibody against γ-catenin (mouse

monoclonal diluted 1:40; BD Biosciences Transduction Laboratories), which was followed by 1 h of incubation with a rhodamine-conjugated secondary antibody (1:50) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) before being mounted on slides with fluorescence mounting medium. Controls with nonimmune IgG were negative. Cells were processed at room temperature and photographed by immunofluorescence under a laser scanning confocal microscope (MRC-1024 confocal scan head; Bio-Rad) at 600× magnification. Background fluorescence was determined by applying the second antibody, a rhodamine-conjugated AffiniPure goat anti-mouse IgG.

In Vivo Tumorigenicity Assay. An in vivo tumorigenicity assay was designed to test tumorigenicity of CP-r tumor cells and parental cisplatin-sensitive cells. Cells were grown in complete DMEM without cisplatin for 2 days and rinsed with PBS, trypsinized, and 1×10^6 cells were injected subcutaneously into syngeneic nude mice. Tumor volume (V) was calculated by the formula $V = d^2D/2$, where d = the smallest tumor diameter (millimeters) and D = the largest tumor diameter (millimeters).

Accumulation of [^{14}C]Carboplatin and [^{3}H]Methotrexate. Cells were seeded at 1×10^5 cells/ml medium in each well of a 24-well culture dish (Costar, Cambridge, MA). After 48 h, cells were treated with 1 μg/ml or 5 μg/ml cisplatin for 1 h. Cells were washed twice with prewarmed DMEM. [^{14}C]carboplatin (1 μCi) or [^{3}H]methotrexate (0.5 μCi) was added in 0.3 ml of medium per well of cells. Cells were reincubated immediately at 37°C for 30 min. To terminate the incubation, cells were washed with ice-cold PBS three times, then harvested by trypsinization. The cell suspensions in 200 μl of PBS were transferred from each well into counting vials with mixture Formula 989 (PerkinElmer Life and Analytical Sciences, Boston, MA). The radioactivity of the sample was measured in a Beckman LS3801 liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Triplicates were made from each well for cell counting at the same time.

Cell Proliferation Measurement and in Vitro Colony Formation Assay. A Vybrant MTT cell proliferation assay kit (Molecular Probes, Eugene, OR) was used for cell number determination using standard microplate absorbance readers based on the manufacturer's protocol. To allow maximal and uniform drug exposure, six individual clones growing in a monolayer culture were rinsed, trypsinized, and replated in three-dimensional culture. In the case of the cisplatin experiments, each clone was grown in the absence or presence of various concentrations (1–20 μg/ml) of cisplatin for 2 h, after which cells were incubated for 3 days. The IC_{50} of KB-CP.5 cells transfected with the γ-catenin gene or the pcDNA3.1 vector alone was calculated based on the MTT growth curves. These experiments were repeated at least three times with similar results. The data were calculated as the average of the six individual clones.

An in vitro colony-forming assay was also used to obtain IC_{50} values for cisplatin. Individual cell lines transfected with the γ-catenin gene and the control vector were seeded at 300 cells per 60-mm dish. At the time of seeding, different concentrations of individual drugs were added to the dishes for 2 h. After incubation for about 15 days, the colonies formed at each concentration of drug were stained with 0.5% methylene blue in 50% methanol and counted. As before, the IC_{50} for each cell line was calculated based on the drug concentration that reduces the number of colonies to 50% of those in the control, drug-free medium.

Assay for Growth Doubling Time. KB-3-1, KB-CP.5, and KB-CP.5 transfectants were seeded at 2×10^4 cells/ml into each well of a 24-well plate for a 7-day growth curve assay. Parental cell lines served as controls. Cells were trypsinized daily from triplicate wells and counted with a Coulter Particle Counter (Beckman Coulter, Inc.). Calculation of proliferation was based on untreated cells as 100%.

Results

Expression of γ-Catenin is Reduced in CP-Resistant Cells. Cisplatin resistance is associated with many different changes in resistant cells. In our studies, to improve our ability to detect changes that are critical for cisplatin resistance, we developed two different cell lines, KB (human epidermoid adenocarcinoma cells) and BEL7404 (human hepatoma cells), both of which were selected to be highly resistant to cisplatin (Shen et al., 1986). To search for common changes associated with cisplatin resistance, we compared the cDNA global expression profiles of KB and KB-CP20 as well as BEL7404 and BEL7404-CP20 cells using two-color fluorescence hybridization with a cDNA microarray. PowerBlot analysis was also used for protein expression profiles. Alterations in the expression of a large number of genes were observed in both cell lines among more than 7000 genes examined by microarray analysis. The expression of the γ-catenin gene was found to be significantly down-regulated in the CP-resistant cells (decreased 8.8-fold in KB-CP20 and 2.3-fold in BEL7404-CP20 cells). In parallel with the cDNA microarray analysis, we used the PowerBlot analysis on protein expression levels to confirm that CP-r cells had reduced amounts of γ-catenin protein by 10.6-fold in KB-CP 20 cells (Fig. 1).

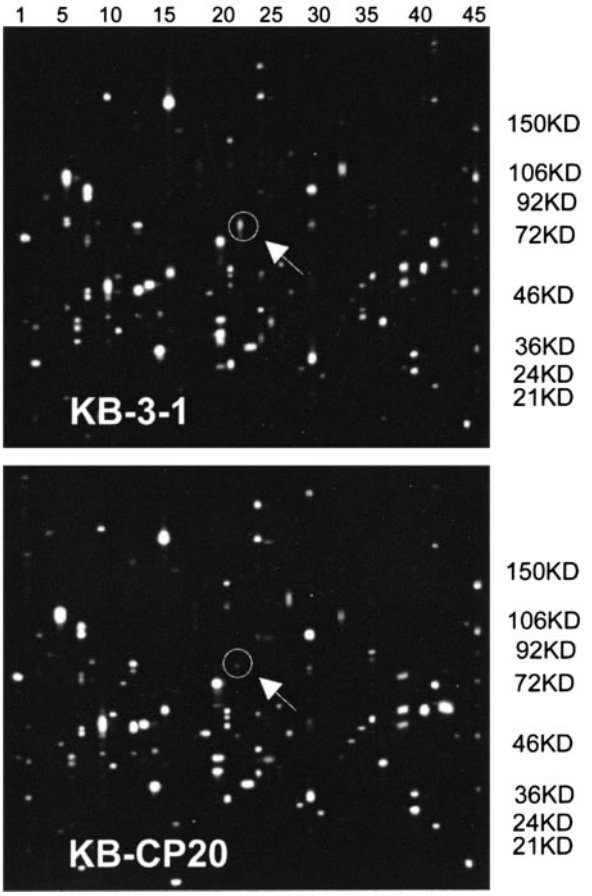

Fig. 1. Reduced expression of γ-catenin in human CP-r cell lines monitored by a protein power-blotting array. The samples were evaluated using an inventory of 698 antibodies from BD Biosciences Transduction Laboratories. The circled spot represents the expression of γ-catenin. The blot images were analyzed by automatic spot finding and spot matching using PDQuest software (Bio-Rad). Experiments were done in triplicate.

To confirm the analysis by microarray and PowerBlot assays, an equal amount of total cell extract and crude membranes from KB and BEL7404 parental and CP-r cell lines were analyzed for γ-catenin expression by immunoblotting. This analysis showed that the expression of γ-catenin was reduced in both CP-r cell lines examined by whole-cell lysis and purified crude plasma membrane (Fig. 2A). Quantitation of the immunoblots showed that there was 10-fold more γ-catenin protein in the CP-sensitive KB and BEL7404 parental cells compared with the CP-r cells (data not shown). Reduced γ-catenin expression was also confirmed in the two different CP-r KB and BEL7404 cell lines by semiquantitative RT-PCR (Fig. 2B).

In addition, when KB and BEL7404 parental and CP-r cells were directly labeled with a specific anti-γ-catenin monoclonal antibody, γ-catenin expression was clearly reduced on the plasma membrane in the CP-r KB and BEL7404 cells as determined by immunofluorescence confocal microscopy (Fig. 2C). KB and BEL7404 cells each have typical morphology and growth properties. The CP-r cells under phase contrast microscopy have somewhat altered morphology and a slower growth rate (Table 1).

Collapse of the γ-Catenin Network Is an Early Effect of Cisplatin Treatment. γ-Catenin can directly associate with cytoskeletal proteins in vitro and in tissue culture (Kowalczyk et al., 1997; Gallicano et al., 1998). To determine whether the γ-catenin network might be affected in parental cells after treatment with cisplatin, we detected γ-catenin in crude plasma membrane pellets and cytosol supernatants of parental-sensitive KB (Fig. 3A) and BEL7404 (Fig. 3B) cells by Western blot and also analyzed γ-catenin localization by confocal imaging (Fig. 3*C*). As seen by comparing Fig. 3 with Fig. 4, γ-catenin in the crude membrane preparation was specifically cleaved in cells treated with cisplatin (Fig. 3) compared with cells treated with two other anticancer drugs, cytosine arabinoside (Fig. 4A) and doxorubicin (Fig. 4B), which also target and damage DNA and induce apoptosis. Interestingly, increased amounts of γ-catenin are found in the cytosol of parental KB and BEL7404 cells after treatment with 1 μg/ml and 5 μg/ml cisplatin (Fig. 3, A and B). Confocal microscopy (Fig. 3C) also showed reduced plasma membrane-associated γ-catenin, which is consistent with the morphological change observed under phase contrast microscopy and the abnormalities that cells in the presence of cisplatin develop on their cell surfaces (Machwe et al., 2001), and with an alteration in the cytoskeleton (D. W. Shen, X. J. Liang, M. M. Gottesman, unpublished data).

Treatment of Cells with Cisplatin Also Results in Reduced Uptake of [^{14}C]Carboplatin and [^{3}H]Methotrexate. To determine whether cleavage and relocalization of γ-catenin might be related to cisplatin resistance in KB and BEL7404 cells, we measured the uptake of [^{14}C]carboplatin and [^{3}H]methotrexate in cisplatin-treated KB and BEL7404 cells. [^{3}H]Methotrexate was also studied because CP-r cells show cross-resistance to methotrexate that is also associated with decreased accumulation of this drug (Shen et al., 1998). The uptake of both [^{14}C]carboplatin and [^{3}H]methotrexate was decreased in the KB and BEL7404 cells after pretreatment with cisplatin. The uptake of these drugs was reduced in proportion to the extent of cisplatin treatment from 1 to 5 μg/ml, as was the cleavage of γ-catenin (Fig. 5).

Localization of γ-Catenin Is Altered in CP-r KB Cells. We wanted to determine whether the altered localization of γ-catenin in parental KB-3-1 cells seen after treatment with cisplatin also occurs in untreated CP-r KB cells. The KB-3-1 and KB-CP-r cells of increasing resistance were analyzed by

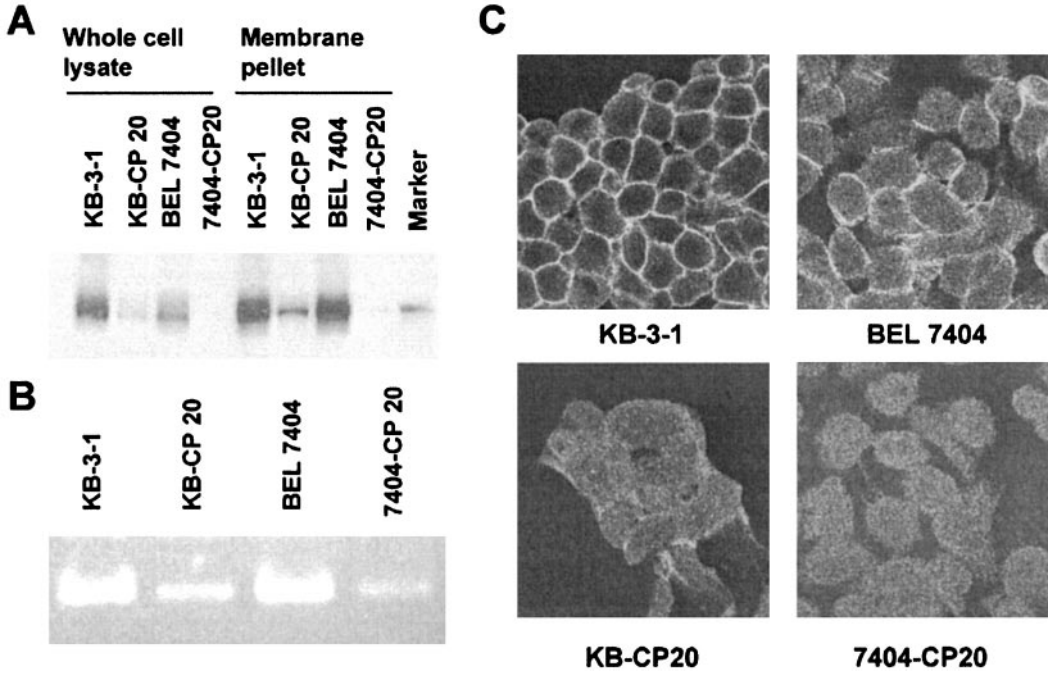

Fig. 2. Immunoblotting, RT-PCR, and confocal microscopic analysis of γ-catenin expression in CP-sensitive and -resistant cells. A, expression of γ-catenin in the CP-sensitive and -resistant cells in crude membrane and whole-cell lysates by a specific anti-γ-catenin monoclonal antibody. The membrane and whole-cell lysates were prepared as described under *Materials and Methods*. The results are shown as one of triplicate experiments. B, expression of γ-catenin mRNA determined by RT-PCR in the CP-sensitive and -resistant cells. Total cellular RNA was extracted and purified by the TRIzol reagent method. A specific primer for human γ-catenin was designed as shown under *Materials and Methods*. C, expression of γ-catenin in the CP-sensitive and -resistant cells observed by laser confocal microscopy. The protocol was the same as described under *Materials and Methods*.

confocal microscopy using a specific anti-γ-catenin antibody and a rhodamine-labeled second antibody. The expression of γ-catenin was reduced on the plasma membrane in parallel with increased cisplatin resistance in KB-CP-r cells. Also, γ-catenin increased in the cytosol of the KB-CP-r cells (Fig. 6). Thus, resistant cells grown in the absence of cisplatin display changes similar to sensitive cells undergoing short-term treatment with cisplatin.

Transfection of γ-Catenin Sensitizes Single-Step KB-CP.5 Cells to Cisplatin. To determine whether γ-catenin levels were directly related to cisplatin resistance, we transfected the γ-catenin gene into our CP-r cells to determine whether an increase in gene expression would reverse cisplatin resistance in our KB-CP cells. The γ-catenin gene was cloned from KB-3-1 epidermoid carcinoma cells by RT-PCR. The cloned γ-catenin gene was inserted into the pcDNA3.1/CT-GFP-TOPO vector using a CT-GFP fusion TOPO TA expression kit (Invitrogen). The vector with γ-catenin was transfected into single-step selected KB-CP.5 cells by liposome transfection with LipofectAMINE Plus Reagent from Invitrogen. A vector with no cloned insert was also transfected as a control. After G418 selection for 3 weeks, six individual clones of different sizes were selected from transfected KB-CP.5 cells for cell proliferation measurement and a colony-forming assay. Increased γ-catenin protein expression was confirmed in the stable transfected clones by Western blots (Fig. 7B). There was a 4.5-fold difference in cisplatin resistance between the KB-CP.5 cells transfected with the γ-catenin gene and control clones transfected with the control (empty) vector as determined by using an MTT cell proliferation measurement (Fig. 7A). Using a colony-forming assay, some decrease in resistance owing to γ-catenin expression is also seen. One series of stained dishes is shown as a representative of the triplicate experiments (Fig. 8). Interestingly, for the colony-forming assay, the size of the individual clones formed in the dishes is much larger for the KB-CP.5 cells transfected with the γ-catenin gene compared with the size of the cells transfected with the control vector, which corresponds to a shortening in doubling time of the γ-catenin transfectants from 18.2 h versus 20.8 h in the KB-CP.5 cells compared with 14 h in the KB-3-1 parental cells (Table 1).

Discussion

De novo or acquired resistance to chemotherapeutic drugs continues to be one of the most important obstacles hindering the successful treatment of cancer patients. Cisplatin is one of the most effective anticancer drugs and is widely used for clinical treatment. Resistance to cisplatin has been associated with several different mechanisms during the past several years, but no single mechanism has been shown to be uniformly present in various CP-r cell lines. In our laboratory, we have isolated two independent CP-r cell populations derived from human KB epidermoid adenocarcinoma cells (KB-CP-r) and human BEL7404 hepatoma cells (BEL7404-CP-r) by long-term exposure to escalating doses of cisplatin. It is clear that both CP-r cell lines showed reduced accumulation of cisplatin, carboplatin, methotrexate, and other an-

TABLE 1
Doubling times of parental and transfectant cells

Cell Lines	Doubling Time
	h
KB-3-1	14
KB-CP.5	20.8
KB-CP.5 with γ-catenin	18.2
KB-CP.5 with vector	20.4

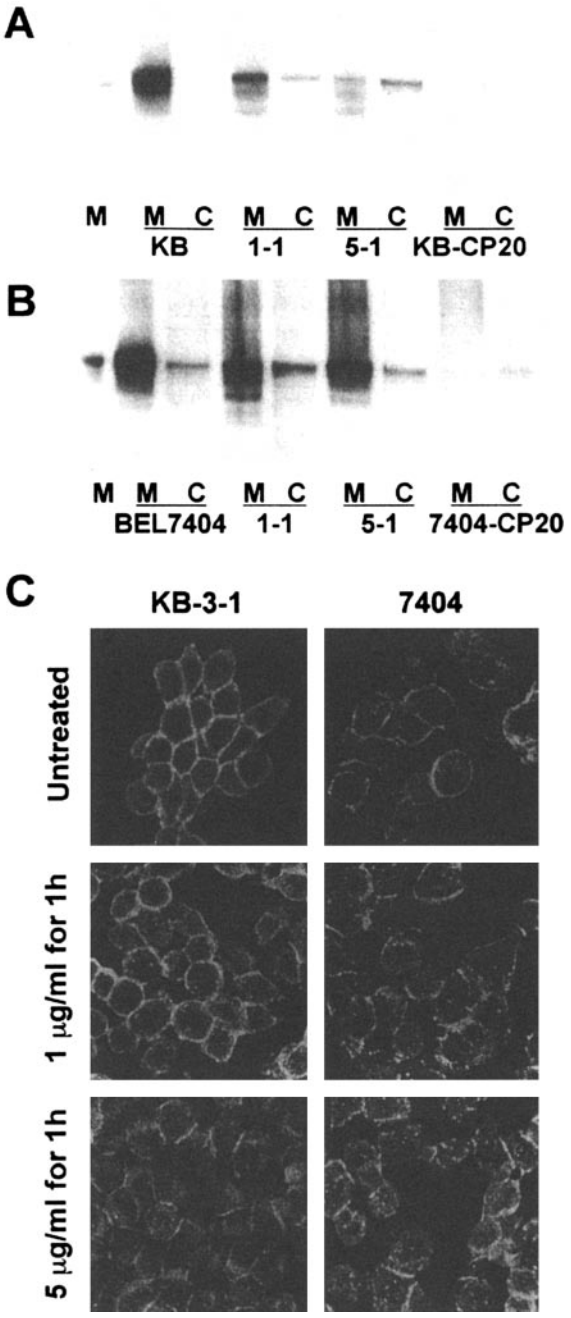

Fig. 3. Cleavage of γ-catenin on the plasma membrane of the CP-sensitive cells after treatment with cisplatin detected by Western blotting and immunofluorescence confocal microscopy. A, degradation of γ-catenin on the plasma membrane in the KB cells after treatment with 1 to 5 μg/ml cisplatin for 1 h. Plasma membranes were harvested by the process described under *Materials and Methods.* Each well was loaded with an equal amount of protein (10 μg). 1-1 indicates the cells treated with 1 μg/ml cisplatin for 1h; 5-1 indicates the cells treated with 5 μg/ml cisplatin for 1h. M and C represent the plasma membrane fraction and cytosol, respectively. B, degradation of γ-catenin on the plasma membrane in the BEL7404 cells after treatment with 1 to 5 μg/ml cisplatin for 1 h. The protocol was the same as described in A. C, degradation of γ-catenin on the plasma membrane in the KB and BEL7404 cells after treatment with 1 to 5 μg/ml cisplatin for 1 h evaluated by laser confocal microscopy.

ticancer drugs, especially those that depend on cell surface molecules to enter cells. CP-r cells have reduced expression of cell surface proteins on the plasma membrane (Shen et al., 1998). Recently, we have demonstrated reduced fluid-phase and receptor-mediated endocytosis in highly CP-r cells (Chauhan et al., 2003) and mislocalization of cell surface proteins into a low-density cytoplasmic compartment (Liang et al., 2003). In this study, we demonstrate reduced expression of the adherens junction protein γ-catenin in CP-r cells and show that cisplatin treatment itself induced cleavage and altered localization of γ-catenin, which is associated with decreased uptake of cisplatin and methotrexate.

How might the adherens junction protein γ-catenin be involved in the sensitivity of cells to cisplatin? Sutherland (1988) demonstrated that cell adhesion may significantly affect resistance of tumor cells to anticancer agents. More and more evidence showing a relationship between cell adhesion and drug resistance has accumulated. γ-Catenin interactions with cadherins are necessary for the maintenance of intercellular adhesion and proper functioning of cells (Karnovsky et al., 1995). The cellular role of γ-catenin is not well defined, but it may function within the desmosomal plaque, which attaches intermediate and actin filaments to the plasma membrane, linking cells together (Cowin et al., 1986). In addition to anchoring intermediate filaments to junctional complexes, γ-catenin has also been implicated in connecting cytoskeletal elements (Leung et al., 2002). Composed of actin filaments, microtubules, and intermediate filaments, the cytoskeleton is vital for many cellular processes, including mechanical strength, movement, adhesion, and intracellular trafficking (Klymkowsky, 1999). It has been shown that γ-catenin binds to more than one filament network, and it seems to be crucial for the integrity and survival of many eukaryotic cells and tissues (Troyanovsky et al., 1993).

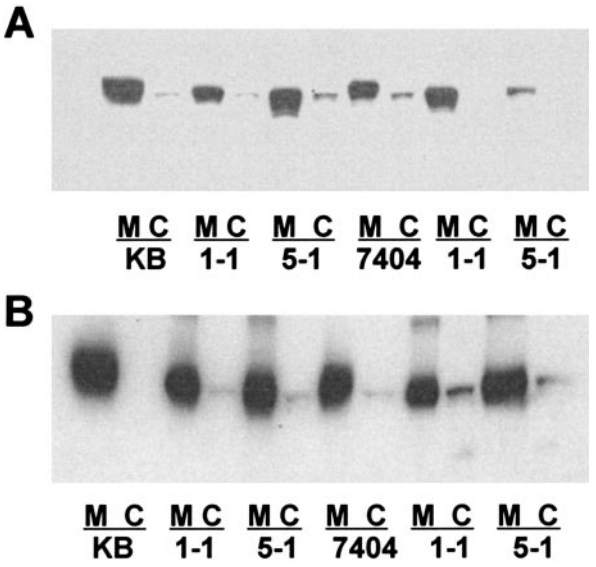

Fig. 4. Expression of γ-catenin in the CP-sensitive KB and BEL7404 cells treated with cytosine arabinoside and doxorubicin. A, KB and BEL7404 cells treated with various cytosine arabinoside concentrations and both plasma membrane and cytosol fractions isolated from KB and BEL7404 cells as described under *Materials and Methods.* 1-1 indicates the cells treated with 1 μg/ml cytosine arabinoside for 1 h; 5-1 indicates the cells treated with 5 μg/ml cytosine arabinoside for 1 h. M and C represent plasma membrane and cytosol, respectively. B, treatment with various doxorubicin concentrations of KB and BEL7404 cells was the same as described in A.

Schmeiser et al. (1998) demonstrated that γ-catenin can be specifically cleaved by caspases during cisplatin-induced apoptosis in Adz EIA + N-ras HER 313A cells and that caspase inhibitors appreciably reduced the proteolytic breakdown of γ-catenin. We compared the mRNA levels of caspase 1 through 10a genes using hAPO-1 C, a multiprobe RiboQuant RNase protection assay system (BD Biosciences PharMingen). We found that these mRNA levels did not change sig-

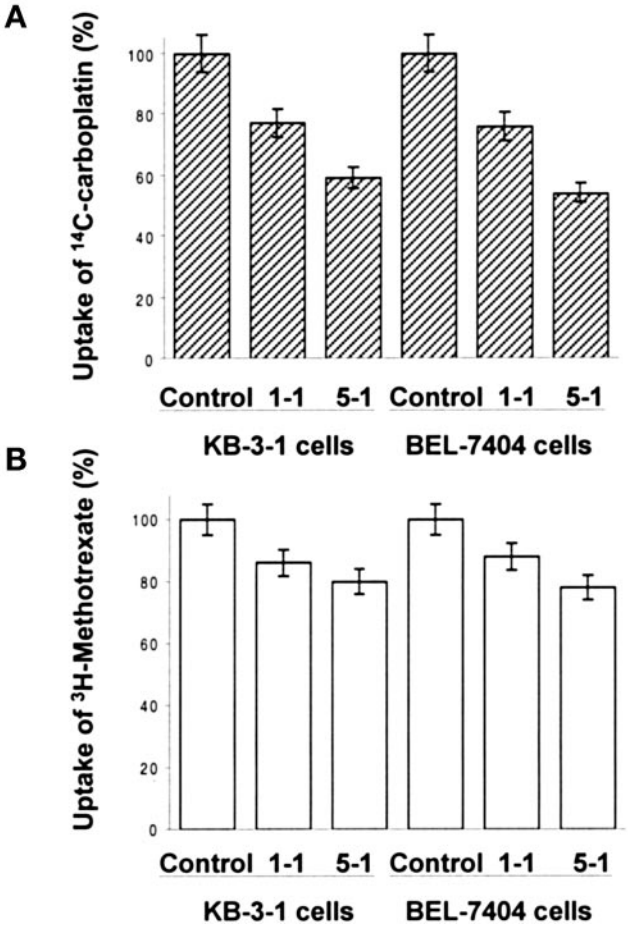

Fig. 5. Uptake of [^{14}C]carboplatin and [^{3}H]methotrexate of KB and BEL7404 cells after pretreatment with cisplatin. A, KB-3-1 and BEL7404 cells treated with 1 μg/ml and 5 μg/ml for 1 h and uptake of [^{14}C]carboplatin measured in KB-3-1 and BEL7404 cells, respectively, as described under *Materials and Methods.* The value is the average of triplicate experiments. B, uptake of [^{3}H]methotrexate measured in KB-3-1 and BEL7404 cells as described above.

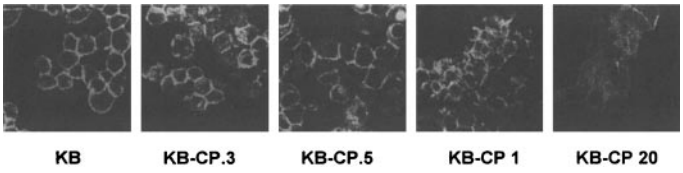

Fig. 6. Expression and localization of γ-catenin in the KB-CP-resistant series cells by confocal microscopy. Cells were cultured on 18-mm glass coverslips, fixed, and labeled with an anti-γ-catenin monoclonal antibody and rhodamine-labeled second antibody as described under *Materials and Methods*. Nonimmune IgG was used as a negative control. The cells were photographed by immunofluorescence under a laser scanning confocal microscope (Bio-Rad) at 600× magnification. Background fluorescence (not shown) was determined by applying the second antibody, a rhodamine-conjugated AffiniPure goat anti-mouse IgG, as described under *Materials and Methods.*

nificantly in untreated KB-3-1 cells compared with KB-3-1 cells treated with 1 μg/ml cisplatin for 1 h (data not shown). To prevent apoptosis from cisplatin treatment, we used *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, a specific synthetic peptide that irreversibly inhibits interleukin-1β-converting enzyme family protease/caspase activity and blocks apoptosis (Constantinou et al., 2003). Cleaved γ-catenin could still be found in the supernatant fraction of CP-treated KB-3-1 cells as detected by Western blotting (data not shown). Therefore, the exact mechanism of γ-catenin cleavage needs further study.

Cisplatin treatment may influence the shape and functional integrity of cells by causing the specific cleavage of γ-catenin, resulting in the dissociation between cytoskeleton and plasma membrane, which thereby results in a failure of entry mechanisms needed to bring cisplatin into the cell. Our finding of reduced endocytosis in CP-r cells (Chauhan et al., 2003) is consistent with this hypothesis, as is the altered localization of cell membrane proteins (Liang et al., 2003). By this hypothesis, cisplatin might be inducing changes in the cell that decrease uptake of cisplatin, a cellular defense mechanism that is constitutively activated in the CP-r mutants. The altered cytoskeleton in cisplatin-treated cells is consistent with our observation that parental KB and BEL7404 cells become circular after treatment with cisplatin when observed by phase contrast microscopy (data not shown).

As a major protein in regulating cadherin-mediated cell shape and tissue integrity, γ-catenin is normally localized to adherens junctions on the plasma membrane. It has also been reported that there are free γ-catenin molecules in the cytoplasm and occasionally in the nuclei of embryonic cells (Cowin et al., 1986; Karnovsky et al., 1995). Abnormal distribution of γ-catenin has been reported in pemphigus vulgaris (Muzio et al., 2001). γ-Catenin was increased in the cytosol of the parental sensitive KB and BEL7404 cells after treatment with cisplatin. In the KB-CP-r cell series (Fig. 6), the expression of γ-catenin on the plasma membranes was reduced with increasing levels of cisplatin resistance. At the same time, increased amounts of γ-catenin were found in the cytosol of the KB-CP-r cells. A role for γ-catenin in regulating cell proliferation is also possible, as has been demonstrated for β-catenin (Schweizer and Varmus, 2003). The KB and KB-CP-r cell series has been injected into nude mice. Interestingly, after 2 weeks, the number and size of tumors formed in the mice decreased in proportion to the level of cisplatin resistance for a series of KB-CP-r cells (data not shown). Because γ-catenin levels decrease as cells become more resistant to cisplatin, this result is consistent with γ-catenin also being involved with growth regulation (Gagescu, 2001).

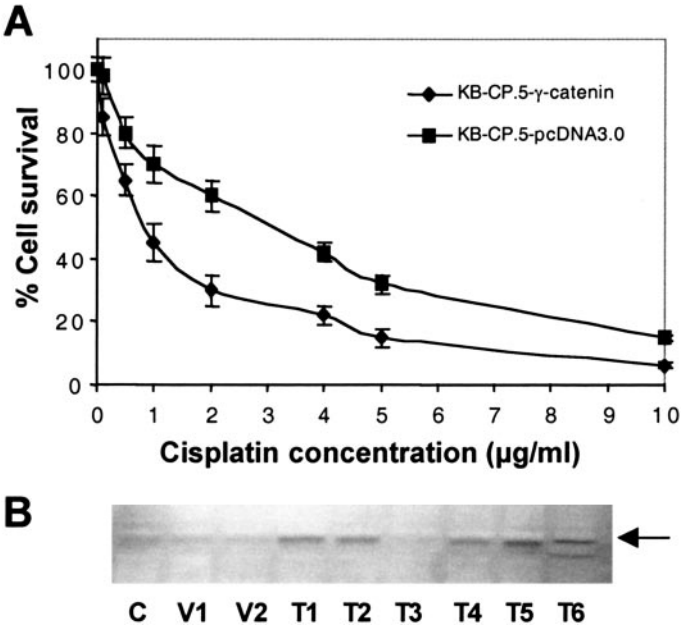

Fig. 7. Cisplatin killing curve assay for KB-CP.5 cells transfected with the γ-catenin gene. A, 10^3 cells seeded in each well of a 24-well tissue culture dish for 24 h and cisplatin added at various concentrations from 1 to 200 μg/ml for 2 h. After the addition of fresh medium, cells were continuously incubated for 3 days. Data were collected by a colorimeter after MTT staining. IC_{50} values were calculated based on the drug concentration that reduces the number of colonies to 50% of those in the control, drug-free medium. The experiments were repeated at least three times. The curve of KB-CP.5 cells transfected with γ-catenin shows the average of 6 individual clones. B, in the panel of Western blots of γ-catenin, C indicates KB-CP.5 cells as controls. V1 and V2 indicate different KB-CP.5 clones transfected with the pcDNA3.0 vector without the γ-catenin gene. T1, T2, T3, T4, T5, and T6 indicate different KB-CP.5 clones transfected with the γ-catenin gene. The arrow shows the position of γ-catenin.

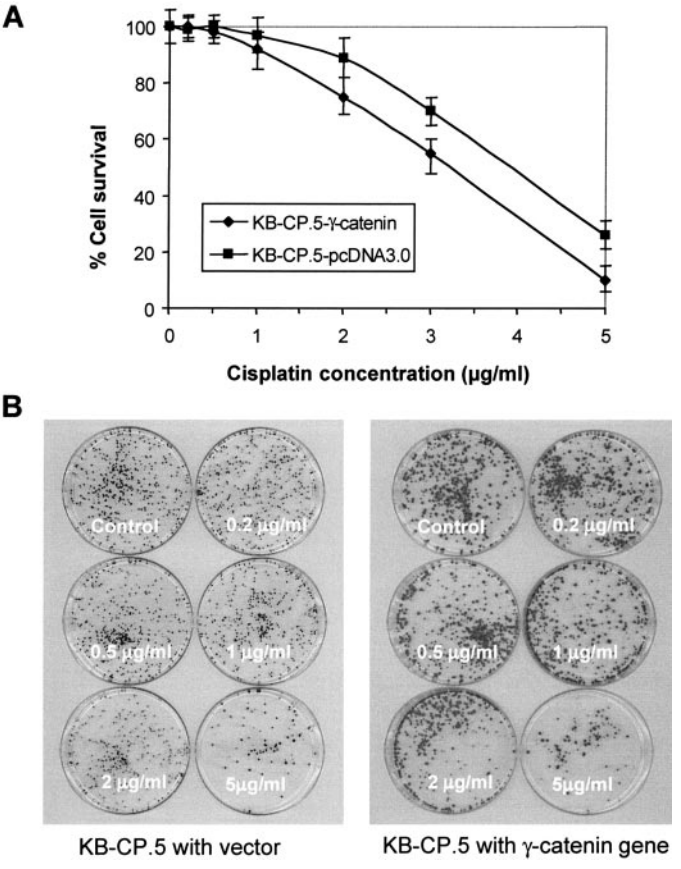

Fig. 8. Proliferating measurement for KB-CP.5 cells transfected with the γ-catenin gene by colony-forming assay. A, individual cell lines transfected with the γ-catenin gene and the control vector seeded at 300 cells per 60-mm dish. At the same time, different concentrations of cisplatin were added to the dishes for 2 h. The individual colonies were formed after incubation for about 15 days, stained with 0.5% methylene blue in 50% methanol, and counted. As before, the IC_{50} for each cell line was calculated based on the drug concentration that reduces the number of colonies to 50% of those in the control, drug-free medium. B, individual cells transfected with the γ-catenin gene and the control vector seeded for the colony-forming assay as described under *Materials and Methods*. Each series of stained dishes is represented by one of an experiment performed in triplicate.

In contrast to our results, Simcha and colleagues (1996) transfected γ-catenin into a human renal carcinoma cell line, resulting in the suppression of tumor formation in nude mice. In these cells, γ-catenin did not exhibit junctional localization but was diffusely distributed in the cytoplasm and nucleus. These results suggest that the antitumorigenic activity of γ-catenin in these carcinoma cells could be associated with a signaling activity rather than with its function in cell adhesion. γ-Catenin may be an important element in a largely uncharacterized signal transduction pathway that may modulate cell proliferation and architecture. The growth regulatory signal induced by γ-catenin seems to function in conjunction with other critical factors, such as BCL-2 protein (Hakimelahi et al., 2000). In addition, γ-catenin seems to be a component of a Wnt-dependent signaling pathway in which it (or its close relative β-catenin) forms a complex with transcription factors of the TCF/LEF-1 family. Furthermore, the amount of γ-catenin available for signaling and adhesion is thought to be regulated by a special mechanism(s) involving the product of the tumor suppressor gene *APC*. Experimental evidence has shown that signal pathways initiated by cell adhesion were operative in tumor cells and, furthermore, cause resistance to mechanistically distinct cytotoxic drugs (Simcha et al., 1996; Runswick et al., 2001). Numerous intercellular adhesion mechanisms may ultimately impinge on the same signal transduction pathways regulating cell cycle and drug resistance. Alternatively, it may be more feasible, at least in the case of solid tumors, to therapeutically target the downstream signal transduction pathways that are regulated by adhesive interactions at the cell surface. Further studies investigating the mechanisms of γ-catenin-mediated drug sensitivity may reveal novel targets directed at the reversal of de novo drug resistance.

In summary, our results suggest an important role for γ-catenin in the resistance of tumor cells to cisplatin and other chemotherapeutic drugs. Our data indicate that degradation of γ-catenin is an early marker for response of cells to cisplatin. Loss of γ-catenin from the cell surface, which occurs in CP-r cells and after cisplatin treatment, coincides with reduced uptake of cisplatin and may indicate that γ-catenin is an important factor in mediating or modulating resistance to cisplatin in cancer cells. Therefore, the development of therapeutic strategies designed to overcome initial cisplatin resistance should take into account the proteolysis of γ-catenin, which may be critical to the success of cisplatin-based chemotherapy.

Acknowledgments

We thank the National Cancer Institute, Center for Cancer Research Fellows' Editorial Board for their assistance in editing the manuscript. We are also grateful to George Leiman and Joyce Sharrar for manuscript preparation.

References

Akiyama S, Fojo A, Hanover JA, Pastan I, and Gottesman MM (1985) Isolation and genetic characterization of human KB cell lines resistant to multiple drugs. *Somatic Cell Mol Genet* **11:**117–126.

Boulikas T and Vougiouka M (2003) Cisplatin and platinum drugs at the molecular level. *Oncol Rep* **10:**1663–1682.

Chauhan SS, Liang XJ, Su AW, Pai-Panandiker A, Shen DW, Hanover JA, and Gottesman MM (2003) Reduced endocytosis and altered lysosome function in cisplatin resistant cell lines. *Br J Cancer* **88:**1327–1334.

Chitaev NA, Leube RE, Troyanovsky RB, Eshkind GG, Franke WW, and Troyanovsky SM (1996) The binding of plakoglobin to desmosomal cadherins: patterns of binding sites and topogenic potential. *J Cell Biol* **133:**359–369.

Constantinou C, Bushell M, Jeffrey IW, Tilleray V, West M, Frost V, Hensold J, and Clemens MJ (2003) p53-induced inhibition of protein synthesis is independent of apoptosis. *Eur J Biochem* **270:**3122–2132.

Cowin P, Kapprell HP, Franke WW, Tamkun J, and Hynes RO (1986) Plakoglobin: a protein common to different kinds of intercellular adhering junctions. *Cell* **46:**1063–1073.

Croix BS, Florenes VA, Rak JW, and Flanagan M (1996a) Impact of the cyclin dependent kinase inhibitor p27Kip1 on adhesion-dependent resistance of tumor cells to anticancer agents. *Nature (Lond)* **2:**1204–1210.

Croix BS, Rak JW, Kapitain S, Sheehan C, Graham CH, and Kerbel RS (1996b) Reversal by hyaluronidase of adhesion-dependent multicellular drug resistance in mammary carcinoma cells. *J Natl Can Inst* **88:**1285–1296.

Gagescu R (2001) Adhesion isn't everything. *Nat Rev Mol Cell Bio* **2:**229.

Gallicano GI, Kouklis P, Bauer C, Yin M, Vasioukhin V, Degenstein L, and Fuchs E (1998) Desmoplakin is required early in development for assembly of desmosomes and cytoskeletal linkage. *J Cell Biol* **28:**2009–2022.

Gottesman MM (2002) Mechanisms of cancer drug resistance. *Annu Rev Med* **53:**615–627.

Gottesman MM, Fojo T, and Bates SE (2002) Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* **2:**48–58.

Gumbiner BM (1996) Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84:**345–357.

Hakimelahi S, Parker HR, Gilchrist AJ, and Barry M (2000) Plakoglobin regulates the expression of the anti-apoptotic protein BCL-2. *J Biol Chem* **275:**10905–10911.

Hazlehurst LA, Damiano JS, Buyuksal I, Pledger WJ, and Dalton WS (2000) Adhesion to fibronectin via beta1 integrins regulates p27kip1 levels and contributes to cell adhesion mediated drug resistance (CAM-DR). *Oncogene* **19:**4319–4327.

Leung CL, Green KJ, and Liem RKH (2002) Plakins: a family of versatile cytolinker proteins. *Trends Cell Biol* **12:**37–45.

Liang XJ, Shen DW, Garfield S, and Gottesman MM (2003) Mislocalization of membrane proteins associated with multidrug resistance in cisplatin-resistant cancer cell lines. *Cancer Res* **63:**5909–5916.

Luna EJ and Hitt AL (1992) Cytoskeleton–plasma membrane interactions. *Science (Wash DC)* **258:**955–964.

Karnovsky A and Klymkowsky MW (1995) Anterior axis duplication in Xenopus induced by the over-expression of the cadherin-binding protein plakoglobin. *Proc Natl Acad Sci USA* **92:**4522–4526.

Klymkowsky MW (1999) Weaving a tangled web: the interconnected cytoskeleton. *Nat Cell Biol* **1:**E121–E123.

Kowalczyk AP, Bornslaeger EA, Borgwardt JE, Palka HL, Dhaliwal AS, Gorcoran CM, Denning MF, and Green KJ (1997) The NH2-terminal domain of desmoplakin binds to plakoglobin and clusters desmosomal cadherin-plakoglobin complexes. *J Cell Biol* **139:**773–784.

Lo Muzio L, Pannone G, Staibano S, Mignogna MD, Rubini C, Ruocco E, De Rosa G, and Sciubba JJ (2001) A possible role of catenin dyslocalization in pemphigus vulgaris pathogenesis. *J Cutan Pathol* **28:**460–469.

Machwe S, Arora S, Gupta R, and Sapra GR (2001) Cisplatin induces modifications in the development of cell surface patterns of ciliates. *Cell Biol Int* **25:**1131–1138.

Runswick S, O'Hare MJ, Jones L, Streuli CH, and Garrod DR (2001) Desmosomal adhesion regulates epithelial morphogenesis and cell positioning. *Nat Cell Biol* **3:**823–829.

Schmeiser K, Hammond EM, Roberts S, and Grand RJ (1998) Specific cleavage of gamma catenin by caspase during apoptosis. *FEBS Lett* **433:**51–57.

Schweizer L and Varmus H (2003) Wnt/Wingless signaling through beta-catenin requires the function of both LRP/Arrow and frizzled classes of receptors. *BMC Cell Biol* **2:**4.

Shain KH and Dalton WS (2001) Cell adhesion is a key determinant in de novo multidrug resistance (MDR): new targets for the prevention of acquired MDR. *Mol Cancer Ther* **1:**69–78.

Shen DW, Akiyama S, Schoenlein P, Pastan I, and Gottesman MM (1995) Characterization of high-level cisplatin-resistant cell lines established from a human hepatoma cell line and human KB adenocarcinoma cells: cross-resistance and protein changes. *Br J Cancer* **71:**676–683.

Shen DW, Cardarelli C, Hwang J, Cornwell M, Richert N, Ishii S, Pastan I, and Gottesman MM (1986) Multiple drug-resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, adriamycin, or vinblastine show changes in expression of specific proteins. *J Biol Chem* **261:**7762–7770.

Shen DW, Goldenberg S, Pastan I, and Gottesman MM (2000) Decreased accumulation of [^{14}C]carboplatin in human cisplatin-resistant cells results from reduced energy-dependent uptake. *J Cell Physiol* **183:**108–116.

Shen DW, Pastan I, and Gottesman MM (1998) Cross-resistance to methotrexate and metals in human cisplatin-resistant cell lines results from a pleiotropic defect in accumulation of these compounds associated with reduced plasma membrane binding proteins. *Cancer Res* **58:**268–275.

Siddik ZH (2003) Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* **22:**7265–7279.

Simcha I, Geiger B, Yehuda-Levenberg S, Salomon D, and Ben-Ze'ev A (1996) Suppression of tumorigenicity by plakoglobin: an augmenting effect of N-cadherin. *J Cell Biol* **133:**199–209.

Sutherland RM (1988) Cell and environment interactions in tumor microregions: the multicell spheroid model. *Science (Wash DC)* **240:**177–184.

Troyanovsky SM, Eshkind LG, Troyanovsky RB, Leube RE, and Franke WW (1993) Contributions of cytoplasmic domains of desmosomal cadherins to desmosome assembly and intermediate filament anchorage. *Cell* **72:**561–574.

Address correspondence to: Dr. Michael M. Gottesman, Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, 37 Convent Drive, Room 1A09, Bethesda, MD 20892-4254. E-mail: mgottesman@nih.gov