Expression of the Cisplatin Resistance Phenotype in a Human Ovarian Carcinoma Cell Line Segregates with Chromosomes 11 and 16

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Abstract

Many mechanisms have been proposed to explain cisplatin resistance, suggesting that this phenomenon is multifactorial. In an attempt to define the chromosome(s) responsible for cisplatin resistance in the human ovarian carcinoma 2008/C13* cell lines, somatic cell hybrids were obtained following fusion of the cisplatin-resistant 2008/C13* cells with an A9 rodent fibroblast cell line. The hybrids were then analyzed for segregation of the human chromosomes with the drug-resistant phenotype. Chromosomes 11 and 16 were present in all of the resistant somatic cell hybrids, with the highest concordance for chromosome 16. The role of both of these chromosomes was further established with microcell hybrids. Microcell hybrids of A9 cells with chromosome 16 from the 2008/C13* cells did not exhibit cisplatin resistance, but the presence of a normal chromosome 11 with chromosome 16 (derived from cisplatin-resistant 2008/C13* cells and not from the cisplatin-sensitive 2008 cells) resulted in increased resistance to cisplatin. In addition, loss of chromosome 11 from a resistant somatic cell hybrid resulted in the hybrid becoming sensitive to cisplatin, implicating this chromosome in maintaining the resistant phenotype. The results demonstrate that resistance to cisplatin is a dominant trait in the 2008/C13* human ovarian cells, and both chromosomes 11 and 16 are required for its expression.

Introduction

Ovarian carcinoma is a major cause of cancer deaths in women (1). Cisplatin [cis-diaminedichloroplatinum (II)] is an effective agent in the treatment of ovarian, testicular, head, neck, and bladder cancers (2). The cytotoxic effects of cisplatin are thought to result from the covalent binding of the cisplatin to DNA (reviewed in Refs. 3–5). The most common adduct is the intrastrand cross-link bridging adjacent guanines at the N(7) position (4, 6, 7). The use of cisplatin, however, is limited due to the emergence of resistance (1, 8). Several biochemical mechanisms of cisplatin resistance have been elucidated (9–11) and include reduced intracellular cisplatin accumulation (12–14), increased intracellular levels of glutathione (15–17), increased dTMP synthase activity (18), decreased DNA binding (19), enhanced drug inactivation by metallothionein (20–23), and increased cisplatin-DNA adduct removal (24, 25). However, the precise genes involved in the development of cisplatin resistance remain unknown, but the list of possible mechanisms suggests that the acquisition of the resistant phenotype is multifactorial.

To analyze the gene(s) responsible for cisplatin resistance, a cisplatin-resistant human ovarian cell line, 2008/C13*, which had been generated by the in vitro selection of the parental human ovarian carcinoma cell line 2008 (26), was used. Studies with the 2008/C13* resistant cell line have demonstrated unique characteristics, such as reduced accumulation of intracellular cisplatin (13), an enhanced replicative bypass of platinum-DNA adducts (27), and reduced cyto-keratin 18 (28) and membrane associated β-tubulin (29). In addition, the mitochondria appear morphologically aberrant, and these cells are hypersensitive to lipophilic cations (30). Variations in protein kinase activity (31, 32) and the cAMP signal transduction pathway (33) have also been reported in the 2008/C13* cell line. These studies suggest that there are multiple mechanisms by which 2008/C13* cells became drug resistant.

To identify the chromosomes involved in cisplatin resistance, a panel of somatic cell hybrids was established by the fusion of a cisplatin-sensitive rodent cell line, A9, with the drug-resistant 2008/C13* cell line. The individual human chromosomes in the somatic cell hybrids were then characterized and correlated to the resistant phenotype.

Materials and Methods

Materials. Cisplatin was obtained from Aldrich Chemical Co. (Milwaukee WI). Cell culture reagents and geneticin (G418 sulfate) were purchased from Life Technologies, Inc. (Grand Island, NY). Immobilon-P membranes were purchased from Millipore (Bedford, MA). Protease inhibitors were purchased from Dr. P. Andrews (Georgetown University, Washington, DC). The human DNA probe, a chromosome 16-specific α satellite probe, and other probes for the FISH analysis were purchased from Oncor (Gaithersburg, MD). PCR was performed with Taq polymerase from Perkin Elmer (Branchburg, NJ). The alkaline phosphatase detection kit was obtained from Bio-Rad (Hercules, CA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide and all other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Cell Lines and Culture Conditions. The human ovarian carcinoma cell lines sensitive and resistant to cisplatin, 2008 and 2008/C13*, respectively, were obtained from Dr. P. Andrews (Georgetown University, Washington, DC). 2008/C13* cells were grown in RPMI 1640 supplemented with 10% bovine calf serum and gentamicin at a final concentration of 10 μg/ml. 2008/C13* cells possess HPRT and APRT and, therefore, survive in HAT and AAT media, but they are sensitive to ouabain (10−3 M). A9 is a mouse fibroblast cell line that was originally isolated from wild-type L cells (34), these cells were grown as a monolayer in D10. A9 cells are HPRT and APRT deficient; therefore, HAT and AAT media are cytotoxic; however, these cells are resistant to the ouabain and grow in the presence of 10−3 M ouabain. The A9 cell line was obtained from Dr. R. Athwal (Fels Institute, Temple University). AAT media is D10 containing 4 × 10−4 M adenine, 4 × 10−3 M aminopterin, and 8 × 10−3 M thymidine. HAT media is D10 containing 20 μg/ml hypoxanthine, 4 × 10−6 M aminopterin, and 20 μg/ml thymidine (35). The MCH 556.5 cell line consists of A9 cells, which contain a normal human chromo-

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2 The abbreviations used are: GST, glutathione S-transferase; FISH, fluorescence in situ hybridization; AAT, adenine-azaserine-thymidine; APRT, adenosine phosphoribosyl transferase; D10, DMEM supplemented with 10% bovine calf serum; HAT, hypoxanthine-aminopterin-thymidine; HPRT, hypoxanthine phosphoribosyl transferase; IC50, 50% inhibitory concentration; TBS, Tris-buffered saline; LRF, lung resistance protein; MRP, multidrug resistance-associated protein; MT-II, metallothionein.
some 11, tagged with the G418-resistant gene. The MCH 556.5 cell line was obtained from Dr. B. Weissman (University of North Carolina, Chapel Hill, NC) and were grown in RPMI with 10% FCS and further supplemented with 600 μg/ml G418 to ensure that it selectively retained the human chromosome.

**Somatic Cell Hybrids.** The derivation and use of somatic cell hybrids for mapping of several human genes have been described previously (36). Primary somatic cell hybrid clones were derived by fusion of the HPRT-deficient A9 cells with 2008/C13* cells. The 2008/C13* and A9 cells were fused in the presence of 47% polyethylene glycol (M, 1300–1600) for 1 min, followed by extensive washing with DMEM, and incubated overnight in D10. Following overnight incubation, the recipient cells were plated into HAT media containing 10−4 M ouabain. Somatic cell hybrid clones were visible within 2–3 weeks, and individual colonies were isolated using glass cloning cylinders. The colonies were grown in monolayers in the presence of HAT and ouabain, and after several passages, the selection medium was replaced by D10. This procedure was repeated twice to ensure an identical cell population within each hybrid clone. Each individual colony now represented a somatic cell hybrid clone and was tested for either sensitivity or resistance to cisplatin using the cytotoxicity assay.

**Microcell Hybrids.** Microcell-mediated chromosome transfer was carried out as described previously (37). Briefly, the donor cell was incubated in the presence of colcemid (0.1 μg/ml) in the D10 medium for a period of 48 h at 37°C. The cells were then micronucleated in the presence of cytochalasin B (10 μM/ml) in DMEM. The micronucleated cells were centrifuged at 25,000 × g for 60 min at room temperature. The resulting preparation of microcell nuclei was suspended in 100 μg/ml phytohemagglutinin P and applied to the recipient cell monolayer. After allowing for agglutination at 37°C for 15 min, the cells and microcells were fused by 45% polyethylene glycol (M, 1300–1600) for 1 min, followed by extensive washing with serum-free medium. After an overnight incubation in a nonselective medium, the recipient cells were plated in selection media. Oubain/AAT media were used for the selection of 2008/C13* microcells fused with the recipient A9 cells. The microcell hybrid thus obtained was designated MCH16. Fusion was performed with the MCH16 microcells and the recipient MCH556.5 cells, resulting in MCH11/16 hybrids, and microcells derived from the cisplatin-sensitive 2008 cells with MCH556.5 cells, resulting in the MCH11/16 hybrids, respectively (hybrids were selected using G418/AAT media).

**Chromosome Preparation.** Exponentially growing cells were treated with colcemid (0.1 μg/ml) for 3 h. The cells were harvested, hypotonically treated (0.56% KCl for 30 min at room temperature), and subsequently fixed in freshly prepared acetic acid:methanol (1:3). The cell suspension was dropped onto chilled slides and dried at room temperature. The slides were aged overnight and processed for FISH.

**FISH.** The procedure for FISH was carried out according to conditions defined by the manufacturer (ONCOR). Briefly, the slides were denatured in 70% formamide and 2× SSC (0.3 M NaCl and 0.03 M sodium citrate (pH 7.2)) at 73°C for 2 min, immersed for 2 min in an ethanol series (70, 80, and 95%) at −20°C, and dehydrated. The slides were incubated with the denatured DNA probes in a humidified box at 37°C for 16–18 h. After washing, the slides were incubated with avidin-FITC (for the total human DNA probe) or with FITC-labeled antidigoxigenin (for the chromosome 11 cosatome and chromosome 16 α satellite probe) at 37°C for 10 min and then counterstained with propidium iodide and antifade (0.6 μg/ml). PCR Analysis. PCR reactions were carried out with 1 unit Taq enzyme, 1 μg genomic DNA, 150 μM of each deoxynucleotides, and 600 ng primer per reaction (total volume, 50 μl). The buffer and cycling conditions varied for all of the primers and were identical to those described by Abbot and Povey (38), Theune et al. (39), and Triputti et al. (40). The PCR products were electrophoretically separated on 1% agarose in 0.5× TBE buffer (0.09 M Tris-borate-EDTA buffer [pH 8.3]). The samples were then transferred to a 1× Southern杂交 wash solution containing 0.1 N HCl-isopropanol, and the absorbance of the colored product was measured at 570 nm in an automated microplate reader (model EL311s; BIO-TEK Instruments, Inc., Winooski, VT). Wells containing only the medium were used as blanks, and wells containing cells in the absence of the drug were used to determine the survival of the control untreated cells. A linear regression analysis of cell survival at the various drug concentrations was compared with the control, and the percentage of survival was calculated. The IC50 was determined and was defined as the concentration of the drug at which only 50% of the cells survived.

**Immunoblot Analysis.** For the detection of GST-π, the total cellular homogenate was prepared by lysing the cells in 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.5% Triton-X-100, and 0.1% PMSP. The proteins in the cell lysate (100 μg) were mixed with an equal volume of 2× SDS buffer [40% glycerol, 6% SDS, 0.25 M Tris-HCl (pH 6.8), 0.1% bromophenol blue, and 0.7 M mercaptoethanol] and held at 100°C for 3 min. The samples were then subjected to electrophoresis in a 15% separating gel according to previously published procedures (41). The separated proteins were electrophoretically transferred to Immobilon-P membranes. The membranes were preincubated with a blocking solution containing TBS [25 mM Tris-HCl (pH 7.5) and 150 mM NaCl] and 5% (w/v) nonfat dried milk for 60 min at room temperature with agitation. The membranes were then incubated with the primary antibody in TBS for 2 h, washed with TBS containing 0.05% (v/v) Tween-20 three times for 10 min, and then treated with the alkaline phosphatase-conjugated secondary antibody for 60 min. After thorough washing with TBS containing 0.05% (v/v) Tween-20, the membranes were stained with an alkaline phosphatase detection kit.

**Results**

Human ovarian carcinoma cells resistant to cisplatin, 2008/C13*, with an IC50 of 27.5 μM (Table 1), were used as the chromosome donor cell line and were fused with the recipient mouse A9 cells, which are cisplatin sensitive, with an IC50 of 5.2 μM. The mouse A9 × human 2008/C13* somatic cell hybrids thus generated were selected in the presence of HAT and ouabain, which ensured selection of only true somatic cell hybrids (mouse A9 cells are sensitive to HAT, and human 2008/C13* cells are sensitive to ouabain). The somatic cell hybrids thus obtained were analyzed for expression of the cisplatin resistance phenotype by the cytotoxicity assay. Twenty somatic cell hybrid clones were analyzed, and those somatic cell hybrid clones exhibiting values similar to the A9 parental cell line were classified as the sensitive clones; alternatively, somatic cell hybrid clones exhibiting IC50 values closer to the 2008/C13* cell line were classified as resistant clones. Initial screening of 20 somatic cell hybrid clones for the drug-resistant phenotype established the presence of 6 resistant and 14 sensitive somatic cell hybrid clones (Table 1). PCR was performed to determine the presence of the individual human chromosomes in these somatic cell hybrids (38–40). Table 1 shows the segregation of the human chromosomes with the drug-resistant phenotype. The presence of the drug-resistant phenotype and the presence of a human chromosome defined the concordant fraction; alternatively, the absence of the chromosome and the drug-resistant phenotype also defined a concordant fraction. On the other hand, the absence of either the phenotype or the chromosome in the presence of the other defined the discordant fraction. The higher the concordance for an individual chromosome, the higher the probability that it is responsible for the observed phenotype; alternatively, the lower the discordance of a chromosome, the greater the probability of its association with the observed phenotype (42). The ratio of the concordance:discordance fraction was calculated for each chromosome. It was evident that there was no single chromosome that correlated to resistance to cisplatin, but chromosomes 11 and 16 were present in all of the cisplatin-resistant somatic cell hybrid clones, with chromosome 16 possessing the highest concordance:discordance ratio.
FISH studies performed on the resistant somatic cell hybrids confirmed the PCR results. Fig. 1 shows the FISH analysis of the drug-resistant somatic cell hybrids for the presence of chromosome 11 (Fig. 1A) in the N2p1 resistant somatic hybrid clone and chromosome 16 (Fig. 1B) in the K2 resistant somatic hybrid clone. The coatasome probe for chromosome 16 could not be used, because this probe cross-reacted with the mouse genome; therefore, centromeric staining was performed with a chromosome 16-specific α satellite probe. Similar results were obtained for the other drug-resistant somatic cell hybrids (data not shown), and the results agreed with the PCR data (Table 1).

Because it was apparent that the either the presence of chromosome 16 (due to its high concordance:discordance ratio) or the presence of both chromosomes 11 and 16 (due to their universal presence in all the resistant somatic cell hybrid clones) was required for resistance, microcell hybrids containing either chromosome 16 of the 2008/C13* cell line or a combination of chromosomes 11 and 16 were observed in the MCH11/16 microcell hybrids (Fig. 1C), whereas both chromosomes 11 and 16 were observed in the MCH11/16 microcell hybrids (Fig. 1D).

The hybrid preparation and the mapping procedure are described in "Materials and Methods." The presence or absence of the human chromosomes was analyzed by PCR analysis. The human chromosomes present in these hybrids are indicated by x. The IC50 was calculated from linear regression analysis of the survival curves. The IC50 value presented is the mean of three different experiments (each performed in triplicate), and the SD was less than 10% in all cases. Concordancy is the simultaneous presence of the chromosome and the resistant phenotype or the absence of both. Discordancy is the absence of one trait in the presence of the other.

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Another approach taken to demonstrate the role of chromosomes 16 and 11 in cisplatin resistance was to attempt to correlate the loss of the resistant phenotype with the loss of a chromosome. Because the retention of human chromosomes in these somatic cell hybrids was not under any selective pressure, these hybrids had the tendency to lose human chromosomes over a period of time. Thus, the resistant somatic cell hybrid clones were maintained continuously in culture, and their resistance was determined periodically. N2p1, a resistant...
clone (Table 1), evolved into clone N2p46, which had become sensitive to cisplatin (Table 3). The IC$_{50}$ value for the N2p46 clone was 6.6 μM, compared with an IC$_{50}$ value of 25.0 μM for the original clone N2p1. The presence of absence of the human chromosomes in these two clones was determined by both PCR and FISH analysis. Table 3 shows the composite results of FISH, PCR, and the cytotoxicity analysis of the N2p1 and N2p46 clones. PCR and FISH analysis confirmed the presence of both chromosomes 11 and 16 in the N2p1 clone.
Cisplatin resistance and is located on chromosome 11. Western blot chromosome 11.

Thus, the drop in the IC50 value correlated with the loss of originally present in the N2pl clone were also present in the N2p46 clone. However, the N2p46 clone had lost chromosome 11, whereas the IC50 was calculated from at least three different experiments (each done in triplicate), and the error is expressed as ±SD.

The presence or absence of each chromosome was determined by using chromosome-specific PCR primers for both arms of each chromosome. FISH was performed to confirm the PCR results. The IC50 was calculated from at least three different experiments (each done in triplicate), and the error is expressed as ±SD.

Table 2 Cisplatin resistance of microcell hybrids

Chromosome 16 was transferred into A9 cells by microcell-mediated chromosome transfer, and the resultant hybrid MCH16 was assayed for resistance by the cytotoxicity assay. Chromosome 16 was transferred from MCH16 into MCH556.5 cells (an A9 cell line with the human chromosome 11) to establish the MCH11/16 microcell clone. Chromosome 16 from 2008 cells was transferred into the MCH556.5 cells to establish the MCH11/S16 microcell hybrid. The presence or absence of each chromosome was determined by using chromosome-specific PCR primers for both arms of each chromosome. FISH was performed to confirm the PCR results. The IC50 was calculated from at least three different experiments (each done in triplicate), and the error is expressed as ±SD.

Table 3 The loss of human chromosome 11 from a human X mouse somatic cell hybrid results in the loss of resistance

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Discussion

Resistance to cisplatin is undoubtedly a multifactorial characteristic. Hence, the observation that it is associated with more than one chromosome is not surprising. The results with the somatic cell hybrids indicated that chromosomes 11 and 16 are associated with drug resistance. The presence of chromosome X in many of the somatic cell hybrids was also not unexpected, because this chromosome carries the HPRT gene, and the somatic cell hybrid clones were selected for this trait following their fusion with the A9 cells (43). The level of concordance for chromosome 16 was at least 3–4-fold greater than that for any of the other chromosomes. Two conclusions emerge from these results: (a) chromosome 16 plays a major role in cisplatin resistance in 2008/C13* cell lines; and (b) chromosome 11 is essential for its expression. However, the presence of a cisplatin resistance gene cannot be assigned to chromosome 11, because the discordance ratio for this chromosome is not sufficiently high.

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The GST-raphic gene has been implicated in playing a major role in cisplatin resistance and is located on chromosome 11. Western blot analysis was performed to compare the expression of this protein in both the sensitive and resistant somatic cell hybrid clones. The expression of the GST-raphic enzyme was expressed in both the sensitive and resistant somatic hybrid clones (Fig. 3). The level of expression of the GST-raphic protein was greatest in the 2008/C13* resistant cells compared with the other somatic cell hybrids. However expression of this enzyme in sensitive somatic cell hybrid clones C3 and S2 (Table 1) was greater than that observed in the N2p1 resistant somatic cell hybrid clone. Thus, no correlation between this enzyme and cisplatin resistance in the somatic cell hybrids could be detected.

Fig. 2. PCR analysis of human chromosomes 11 and 16 was performed with the microcell hybrids with chromosome-specific primers. DNA (1 μg) from the indicated parental cells and the microcell hybrid clones was amplified with chromosome-specific specific primers, and the products were analyzed on a 2% agarose gel, as described in "Materials and Methods." PCR product sizes for the chromosome-specific primers are as indicated. The primers used were 11p13-catalase, 11q21-q22-collagenase I, 16p13.3-hemoglobin a, and 16q22.1-hepatoglobin.

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parental cell line. It is highly likely that expression of another gene present only on a chromosome 11 derived from the drug-resistant 2008/C13* cells would result in even higher levels of cisplatin resistance associated with the presence of chromosome 11 alone is not sufficient for drug resistance, and that the presence of both chromosomes 16 and 11 is necessary for the resistance phenotype.

Chromosomes 11 and 16 have both been implicated in different aspects of drug resistance. Genes for MRP, LRP, excision repair cross-complementing protein 4, and MT-IIA have been reported on chromosome 16. MRP is a membrane glycoprotein, which is found in cell lines exhibiting multiple drug resistance but failing to show the presence of P-glycoprotein (44). The cisplatin-sensitive parental cell line 2008 and the cisplatin-resistant derivative 2008/C13* have been analyzed by reverse transcription-PCR for mRNA expression of the MRP gene, but no discernible differences were observed (45). LRP is another protein associated with drug resistance that has mapped to the 16p chromosome (46). LRP is a vault protein, i.e., a cellular organelle mediating intracellular (nucleocytoplasmic) transport of a wide variety of substrates (47). It has been demonstrated that the level of LRP expression is directly correlated to the response of ovarian cancer cells to cisplatin and other alkylating agents in ovarian cancer cells (48). The role that this protein plays in 2008/C13* cisplatin resistance has yet to be investigated. Another gene product of interest occurring in the genes of the sensitive cells; and (b) the expression has been proposed as possible mechanisms of cisplatin detoxification (52). Recent studies on tumor cells transfected with the human MT-IIA gene showed resistance to cisplatin as well as other alkylating agents (20). However, the role of the MT-IIA gene in 2008/C13* cells remains to be determined.

Genes present on chromosome 11 that have been implicated in cisplatin resistance, include GST-σ and the structure-specific recognizing protein. The former has been widely implicated in cisplatin resistance, but its expression in the sensitive somatic cell hybrid clones C3 and S2 was found to be higher than the resistant somatic cell hybrid N2p1 suggesting that the GST-σ enzyme does not play a major role in resistance to cisplatin in the 2008/C13* cell line. In the resistant and sensitive somatic cell hybrid clones, glutathione levels were not significantly different (41).

Recently, a protein named structure-specific recognition protein has been shown to specifically recognize platinum-DNA adducts and to be present on chromosome 11 (53, 54). Its function in cisplatin resistance of the 2008/C13* cells is presently unknown.

The results of this study have shown that: (a) the phenotype for cisplatin resistance is dominant, and it is also a result of mutations occurring in the genes of the sensitive cells; and (b) the expression of one or more genes on both chromosomes 11 and 16 is required for the expression of the cisplatin resistance phenotype in 2008/C13* cells.

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References


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