Increased Expression of Dihydrodiol Dehydrogenase Induces Resistance to Cisplatin in Human Ovarian Carcinoma Cells

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We employed cDNA microarrays to identify the differentially expressed genes in a cisplatin-sensitive parental (2008) human ovarian carcinoma cell line and its cisplatin-resistant variant (2008/C13*). Differential expression of five genes was found in the 2008/C13* cells, a result confirmed by semi-quantitative reverse transcription-PCR. The five genes were identified as fibroblast muscle-type tropomyosin and skeletal muscle-type tropomysin, dihydrodiol dehydrogenase, apolipoprotein J and glucose-6-phosphate dehydrogenase variant-A. Treatment of the 2008 cells with cisplatin (at its IC50 concentration of 2 μM) induced expression of these genes, as determined by semi-quantitative reverse transcription-PCR analysis using gene-specific primers. In contrast, treatment of the drug-resistant 2008/C13* cells with cisplatin (at its IC50 concentration of 20 μM) did not lead to the induction of any of the aforementioned genes. Most importantly, constitutive overexpression of dihydrodiol dehydrogenase (but not the other genes) in the 2008 cells led to induction of cisplatin resistance, clearly indicating its role in the development of the resistance phenotype in the 2008/C13* cells. The development of cisplatin resistance in the transfected cells was associated with an increase in the dihydrodiol dehydrogenase enzyme activity. Although at present it is not clear how dihydrodiol dehydrogenase is involved in cisplatin resistance, the identification of this gene as a causal factor suggests the existence of a hitherto undefined pathway resulting in cisplatin resistance.

Cisplatin is one of the most widely used anticancer drugs. It is effective in the treatment of ovarian, testicular, bladder, head and neck, and small cell lung cancers (1). The basis of its therapeutic effect is generally considered to be the covalent binding of it to nucleophilic sites on cellular macromolecules, including DNA. Cisplatin reacts at the N7-position of guanosine and forms intrastrand and interstrand cross-links (2). Unfortunately, its efficacy is restricted due to the existence of intrinsic tumor cell resistance or by the acquisition of tumor cell resistance subsequent to drug treatment (3). An improved understanding of the cellular and molecular mechanisms by which cisplatin resistance develops is necessary for this drug to be used most effectively.

A large body of data from experimental and clinical studies suggests that the mechanisms underlying cisplatin resistance are multifactorial. They include decreased drug accumulation (4, 5), enhanced cellular detoxification due to increased levels of GSH (6) and intracellular metallothioneins (7, 8), increased DNA repair (9, 10), and/or loss of mismatch repair (11). In addition, alterations in oncogene expression and signal transduction pathways have also been proposed to be associated with cisplatin resistance (12). Furthermore, the altered expression of several other genes has been observed, but how these lead to cisplatin resistance is still poorly defined. Most of these genes were found to confer cisplatin resistance after they were artificially overexpressed in cells using gene transfection. They include c-fos, Ha-ras, c-myc, Her-2/neu, and Bcl-2, Bcl-XL (13–15). However, their disparate biochemical functions have made it difficult to define whether these genes play a primary or secondary role in the development of cisplatin resistance.

To identify the multiple changes occurring during the development of cisplatin resistance, a sensitive method was required that would measure genome-wide alterations in gene expression in a single well defined step. Indeed, utility of techniques such as suppressive subtraction hybridization in a cisplatin-resistant head and neck cell line (16) and genome wide screening in yeast (17) have resulted recently in the identification of changes in the expression of such unlikely drug resistance candidate genes as cytochrome oxidase I, ribosomal protein 28 S, elongation factor 1α, α-enolase, hsp70, PDE2, and ZDS2. However, the differential expression of these genes was not confirmed by quantitative PCR and/or Northern blot in either study. In addition, the effect of drug exposure on the expression of these “genes associated with cisplatin-resistance” was not investigated, and transfecants were not tested for drug resistance.

In the present study, we employed the cDNA microarray from PerkinElmer Life Sciences (2400 genes) to reveal the differentially expressed genes in cisplatin-resistant cells as compared with the sensitive, parental cells. The recent development of DNA microarray technology has enabled the measurement of temporal gene expression levels. We used the parental human ovarian carcinoma cell line 2008 and its cisplatin-resistant variant 2008/C13* cell line in our study. The 2008/C13* cells have been generated by in vitro selection of parental human ovarian carcinoma cells (2008) in the presence of increasing concentrations of cisplatin (18). The 2008/C13* cells exhibit between a 9- and 12-fold resistance to cisplatin (Ref. 19 and this article), and their resistant phenotype is stable (i.e. it
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**DNA sequence of the forward and reverse primers, annealing temperature used, and number of cycles performed for the RT-PCR-mediated confirmation of the differential expression of the cisplatin-resistance associated genes identified by cDNA microarray**

| Gene identity | Primers | Annealing temperature | No. of cycles |
|---------------|---------|-----------------------|---------------|
| Fibroblast muscle-type tropomyosin | Forward: 5'-ggagaagttgctctcgg-3' | 55 °C | 35 |
| Skeletal β-tropomyosin | Forward: 5'-ggagaaactctgctgcc-3' | 55 °C | 35 |
| Apolipoprotein J | Forward: 5'-ttggcccaaaacctctc-3' | 55 °C | 28 |
| Glucose-6-phosphate dehydrogenase var. A | Forward: 5'-catctatctggctggg-3' | 55 °C | 28 |
| Dihydrodiol dehydrogenase (U05598) | Forward: 5'-gggctggaagtgagg-3' | 55 °C | 28 |
| Dihydrodiol dehydrogenase (M86609) | Forward: 5'-ggagagggaggtgagg-3' | 55 °C | 22 |
| Glyceraldehyde-6-phosphate dehydrogenase | Reverse: 5'-ggagagggaggtgagg-3' | 55 °C | 22 |

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**EXPERIMENTAL PROCEDURES**

**Materials**—Cisplatin, camphorquinone, and (S)(+)-1-indanol were purchased from Aldrich. 1-Acetonephosphorl, dicumarol, NADP+, and NADPH were obtained from Sigma. Cell culture reagents and genta-mycin were obtained from Cellgro (Herndon, Virginia). RNAzol B was purchased from Tel-test Inc (Friendswood, TX). MessageClean kit was purchased from PerkinElmer Life Sciences. A polyclonal antibody against avian myeloblastosis virus RT.

**Cell Culture Conditions and Cisplatin Treatment**—The human ovarian carcinoma cells A2780 and SKOV-3 as well as the cisplatin-sensitive 2008/C13* cells were obtained from the American Type Culture Collection (ATCC). The cells were maintained at 37 °C and 5% CO₂ in a humidified incubator.

**DNA Extraction and Purification**—Total DNA was isolated from cells using the DNeasy kit (Qiagen). The DNA was quantified by fluorometry and was used as a standard marker.

**Cisplatin Sensitivity of Parental Cells Constitutively Overexpressing the Identified Cisplatin Resistance-associated Genes**—To correlate the function of each of the four candidate genes with the cisplatin resistance phenotype, it was necessary to evaluate the effects of forced overexpression of a recombinant protein of each of the candidate genes on cisplatin cytotoxicity. Thus, primer pairs from the cDNA sequence of each of the candidate genes (using the full-length sequence data from the sequence deposited in the GenBank™ data base; see Table II) were designed to enable us to generate a full-length cDNA. These were then cloned into the eukaryotic expression vector (pCR3.1, Invitrogen). Orientation of the full-length cDNA as well as its sequence were determined by restriction enzyme digestion and automated DNA sequencing, respectively. The expression vector with the insert in the right orientation was then transfected (20) into sub-confluent parental human ovarian carcinoma cells (2008, A2780, or SKOV-3) using the LipofectAMINE reagent (Invitrogen). The transfected cells were propagated in a medium containing 500 μg/ml Geneticin (G418 sulfate) for 3 weeks. Individual G418-resistant colonies were picked (20 colonies for each transfection experiment), grown, and screened for the expression of the recombinant message using the RT-PCR procedure described above.
The clones that expressed a high level of the recombinant message were then subjected to growth inhibition assays in the presence of different concentrations of cisplatin. The cytotoxic effects of cisplatin were assessed using a tetrazolium dye as described previously (20).

**Dot-blot Analysis of Dihydrodiol Dehydrogenase Expression**—RNA (0.1–2 ug) isolated from the cisplatin-sensitive (2008) and -resistant (2008/C13*) cells were spotted onto Nylon membrane. After air-drying the membrane, it was placed in a UV-cross-linker (Stratagene) set at optimal cross-link. Prehybridization was carried out for 4 h as described previously (20). Hybridization was performed with radiolabeled full-length dihydrodiol dehydrogenase cDNA overnight. This was followed by 1× low stringency wash and 2× high stringency washes at 55 °C as described previously (20). The nylon membrane was then exposed to Eastman Kodak XAR-5 film at −80 °C for 36 h and then developed on the X-Omat. Thereafter, the blot was stripped and hybridized with radiolabeled ubiquitin cDNA to ensure equal RNA concentrations. After high stringency washes at 65 °C (2×), the blot was exposed as before for 24 h and developed on the X-Omat.

**Dihydrodiol Dehydrogenase Enzyme Activity**—The dihydrodiol dehydrogenase enzyme activity was measured in the cytosol fraction of the 2008, 2008/C13*, and the dihydrodiol dehydrogenase-transfected 2008 cells as described previously (21). The rate of formation (and in some case, the rate of disappearance) of NADPH at 340 nm (extinction coefficient, 6500 m−1 cm−1) was assessed. For isolation of the cytosolic fraction, cells were plated at a density of 2×10⁶ cell/dish and allowed to adhere overnight. The cells were then washed (3×) with chilled phosphate-buffered saline and collected by scraping in a buffer containing 10 mM sodium phosphate, pH 7.4, 150 mM KCl, and 0.5 mM EDTA (Buffer A). After a brief centrifugation, the cells were resuspended in Buffer A containing a protease inhibitor mixture and homogenized with a glass Dounce homogenizer with a tight-fitting pestle. The lysed homogenate was centrifuged at 14,000 × g for 20 min. The supernatant fraction thus obtained was further centrifuged at 100,000 × g for 60 min to separate the cytosol fraction (supernatant) from the microsomal fraction (pellet). Aliquots of the cytosolic fraction were immediately stored at −70 °C. The protein concentration of the cytosol fraction was determined by the Coomassie Blue dye binding assay using commercial available Bio-Rad protein assay reagent and bovine serum albumin as standard.

The cytosolic fractions were then assayed for dihydrodiol dehydrogenase activity essentially as described previously (21). Briefly, the assay mixture consisted of 4 mM NADP+ (or 0.16 mM NADPH), 100 mM potassium phosphate, indicated concentrations of the substrates, and the cytosolic fraction. The reaction was started by addition of substrate, and the disappearance of NADPH at 25 °C was monitored with the aid of a Beckman DU-70 recording spectrophotometer. An assay mixture containing all of the components except the substrate served as the blank. Initial rates of NADPH disappearance were determined in duplicate.

**Immunocytochemical Analysis of Dihydrodiol Dehydrogenase Expression**—Polyclonal antibody that was reactive to the four subtypes of dihydrodiol dehydrogenase was used. For immunocytochemistry, freshly trypanoyzed cells (~1000 cells) were spotted onto a sterilized coverslip in a 6-well plate. After incubating at 37 °C for 4 h, most of the cells adhered to the coverslip, and an additional 1 ml of medium was added to fully cover the cells. After a 48-h incubation, the cells were harvested by a brief wash with warm phosphate-buffered saline twice, air-dried, and then fixed with cold acetone/methanol. Immunocytochemistry was performed as described previously by the immunoperoxidase method (22). The endogenous peroxidase was inactivated with 0.2% sodium azide in 3% H2O2. The slides were incubated with the antibodies to dihydrodiol dehydrogenase and then with biotin-conjugated goat anti-mouse immunoglobulin (Dako, Carpenteria, CA). The chromogenic reaction was visualized by peroxidase-conjugated streptavidin and aminomethylcarbazole (Sigma), and the crimson precipitate was identified as positive staining. The samples were counterstained with hematoxylin, and the slides were mounted with glycerol gelatin. Each batch had a positive and a negative control to ensure the staining quality.

**RESULTS**

**cDNA Microarray Analysis**—The parental human ovarian carcinoma cell line 2008 and its cisplatin-resistant variant 2008/C13* were used in cDNA microarray experiments as the source of the two different gene pools. Furthermore, to ascertain the reproducibility of the technique, the microarray assay was repeated employing DNA-free total RNA prepared from different cultures of each cell line at two different time points. To avoid possible differences caused by different labeling patterns, the labeling pattern was inverted when the experiment was repeated. Only those genes whose fluorescence ratio was at least 5-fold over background levels (in both cell lines) were considered in the final selection of differentially expression. Furthermore, a gene was considered as being differentially expressed only if its comparative signal (i.e. cyanine 3 to cyanine 5 ratio) demonstrated a greater than 5-fold increase/decrease. In addition to these quality control features, an experiment was considered valid only if the control genes spotted on the microarray (three plant genus-specific genes) and ~100 housekeeping genes) gave a composite cyanine-3 to cyanine-5 ratio of 1, i.e. the expression levels of these genes (as indicated by the fluorescence intensity) had to be similar in both cell lines, thus indicating that the amount of input total RNA from the two cell lines used for the differential expression analysis was similar. Thus, in the first experiment, we found 32 genes (increased at least 5-fold) when cDNA from 2008 cells was compared with that from the 2008/C13* cells. After the second experiment, a total of nine genes were identified that were differentially expressed in both experiments (Table II). These genes were all expressed at higher levels in the cisplatin-resistant cell line 2008/C13* as compared with their expression in the cisplatin-sensitive 2008 cells. Four of these nine genes belong to the family of proteins involved in maintaining the cytoskeletal architecture of a cell, viz. two isoforms of tropomyosin (fibroblast muscle-type tropomyosin and skeletal β-tropomyosin), profilin II, and the myosin regulatory light chain. The drug detoxifying enzyme dihydrodiol dehydrogenase spotted at two different locations/slide was also consistently identified as being overexpressed (>30-fold) in the 2008/C13* cells. In addition, up-regulation of apolipoprotein J (>6-fold), an inhibitor of programmed cell death, was also observed in the 2008/C13* cells. A >5-fold up-regulation of a variant form of glucose-6-phosphate dehydrogenase (variant A) and neurotrypsin, a ser-
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**Fig. 1.** Confirmation of the differential expression of the genes listed in Table II by semi-quantitative RT-PCR. RNA was prepared from the 2008 (lanes 1 and 2) and 2008/C13* (lanes 3 and 4) cells using the RNeasy B reagent (Qiagen). cDNA was synthesized using the Qiagen Omniscript RT with 1 μg of RNA as template. Thereafter, cDNA equivalent to 40 and 100 ng of RNA from each cell line was used for the PCR reaction using gene-specific primers and 1.5 mM MgCl₂. Each of the RT-PCR reactions was performed with three different batches of RNA to assess differential gene expression.

Semi-quantitative RT-PCR Analysis—To confirm the differential expression of the nine genes (Table II), semi-quantitative RT-PCR analysis was employed. Total cellular RNA from the 2008 and the 2008/C13* cell lines was used for the RT-PCR analysis. Nine gene-specific primers were designed to amplify the corresponding cDNA fragment of ~200 base pair. The PCR cycle numbers were optimized for each primer to ensure that the comparison of the level of expression of each gene was within the linear phase of amplification. Furthermore, to ensure that an equal amount of RNA was used for each RT-PCR reaction, primers for glyceraldehyde-6-phosphate dehydrogenase were employed as an internal control. The observed PCR product intensity for each of the experimental primer pairs was normalized against the PCR product intensity observed with the glyceraldehyde 6-phosphate dehydrogenase. The abundance of the PCR product was semi-quantified by densitometric scanning of the ethidium bromide-stained agarose gels, and the cDNA fragment corresponding to each amplified gene was compared between the 2008 and 2008/C13* cells. Six of the nine genes were confirmed to be highly overexpressed (3–50-fold) in the 2008/C13* cells as compared with the 2008 cells (Fig. 1). These six genes code for five different proteins. They are two isoforms of tropomyosin, fibroblast muscle-type tropomyosin and skeletal β-tropomyosin, dihydrodiol dehydrogenase, apolipoprotein J, and glucose-6-phosphate dehydrogenase variant A. The mRNA expression of the two isoforms of tropomyosin was negligible in the 2008 cells but significantly up-regulated in the 2008/C13* cells. The other four genes were expressed at basal levels in the 2008 cells, and their expression was found to be up-regulated in 2008/C13* cells. In contrast, the expression of the neurotrypsin gene, profilin II gene, and the myosin regulatory light chain gene was found to be similar in the 2008 and the 2008/C13* cells. The reasons for the discrepancies between the differences in gene expression observed by cDNA microarray analysis and the similar levels of expression observed with semi-quantitative RT-PCR analysis (for neurotrypsin, profilin II and myosin regulatory light chain) is not known at present. However, it clearly demonstrates the importance of confirming differential expression observed in a cDNA microarray analysis with RT-PCR or Northern blotting techniques.

**Fig. 2.** Dot-blot analysis of differential expression of dihydrodiol dehydrogenase in the 2008 and 2008/C13* cells. RNA (0.1–2 μg) was blotted onto Nylon membranes and hybridized with radiolabeled dihydrodiol dehydrogenase (DDH1) cDNA (A). The blot was exposed to x-ray films for 36 h and then developed. Then, the blot was stripped and hybridized to ubiquitin (Ubq) cDNA (B) to assess equal loading of RNA.

**Induction of Cisplatin Resistance-related Genes in the 2008 Cells**—When combined, the microarray and RT-PCR techniques demonstrated that six genes were overexpressed in cisplatin-resistant 2008/C13* cells. It was of interest to know whether this overexpression was directly associated with drug treatment and whether such treatment would alter their expression in parental cells treated with cisplatin. Thus, 2008 cells were treated with 2, 5, 10, and 25 μM cisplatin for 6 and 24 h, and the expression of each of the six candidate genes was assessed by the semi-quantitative RT-PCR analysis using gene-specific primers. The expression of the fibroblast muscle-type tropomyosin and skeletal β-tropomyosin was strongly induced by cisplatin treatment (Fig. 3). The expression of these two genes was increased after 6 h of cisplatin treatment and was observed to be cisplatin dose-dependent, i.e. the level of induction (10–50-fold) was directly correlated with the dose of cisplatin. In contrast, the level of expression of the other genes was found to be elevated only after 24 h of cisplatin treatment (dihydrodiol dehydrogenase, 3–5-fold; apolipoprotein J, 2–10-fold; and glucose-6-phosphate dehydrogenase, 2–5-fold). No significant change in the expressions of these genes was observed 6 h after exposure to cisplatin.

In contrast to the effects of cisplatin treatment observed in the 2008 cells, treatment of 2008/C13* cells did not result in significant changes in the mRNA levels of any of the aforementioned genes, except for skeletal β-tropomyosin, wherein a 3-fold increase in the mRNA levels was observed at 4 and 6 h (Fig. 4, lanes 5–8). However, at the end of 24 h of treatment with cisplatin, the levels of skeletal β-tropomyosin were found to be similar to those found in the untreated cells (Fig. 4, lanes 9–10).

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1.5 mM MgCl2. Each RT-PCR was performed with two different batches of RNA to assess the effects of cisplatin treatment. A representative photograph is displayed that shows the effect of cisplatin treatment on the mRNA levels of the candidate genes.

Fig. 3. Effects of cisplatin treatment on the mRNA levels of the differentially expressed genes in the 2008 cells. The 2008 cells were plated at a density of 2 x 10^6 cells/100-mm dish and treated with the indicated concentration of cisplatin for 2, 4, 6, and 24 h. Thereafter, total RNA was extracted from the cisplatin-treated cells using the RNAzol B reagent (TelTest). cDNA was synthesized using the Qiagen Omniscript RT with 1 μg of RNA as template. Thereafter, cDNA equivalent to 40 and 100 ng of RNA from each cell line was used for the PCR reaction using gene-specific primers and 1.5 mM MgCl2. Each RT-PCR was performed with two different batches of RNA to assess the effects of cisplatin treatment. A representative photograph is displayed that shows the effect of 6 h (for fibroblast muscle-type tropomyosin and mRNA for skeletal β-tropomyosin) and 24 h (for dihydrodiol dehydrogenase, apolipoprotein J, and glucose-6-phosphate dehydrogenase) of cisplatin treatment on the mRNA levels of the candidate genes.

Table III

| Cells               | Cisplatin IC_{50} (μM) | Degree of resistance |
|---------------------|------------------------|----------------------|
| 2008                | 2 ± 0.4                | 1                    |
| 2008/C13*           | 19 ± 2                 | 9                    |
| 2008 clones transfected with full-length tropomyosin cDNA     | 3 ± 1                  | 1.5                  |
| 2008/TM5            | 4 ± 1                  | 2                    |
| 2008 clones transfected with full-length apolipoprotein J cDNA | 2 ± 0.3                | 1                    |
| 2008/A5             | 2 ± 0.1                | 1                    |
| 2008 clones transfected with full-length glucose-6-phosphate dehydrogenase cDNA | 3 ± 1                  | 1.5                  |
| 2008/G3             | 3 ± 1                  | 1.5                  |
| 2008/G4             | 3 ± 1                  | 1.5                  |

overexpression of the recombinant genes were further subjected to growth inhibition assays as described under “Experimental Procedures.” As shown in Table III, overexpression of the both tropomyosin isoforms, apolipoprotein J, and glucose-6-phosphate dehydrogenase variant A did not induce cisplatin resistance in the 2008 cells.

In contrast, overexpression of dihydrodiol dehydrogenase (Fig. 5) induced between 6- and 9-fold cisplatin resistance in 2 of the clones analyzed (Table IV). Thus, the cisplatin IC_{50} value against 2008/D2 and 2008/D12 was 8- and 7-fold higher, respectively, than that observed with the parental 2008 cells (IC_{50} = 2 ± 0.4 μM). Also of note is our observation that overexpression of dihydrodiol dehydrogenase also induces resistance to the cisplatin analogue carboplatin, commonly used at present in the treatment of advanced ovarian carcinomas (Table IV). The 2008/C13* cells were found to be 16-fold resistant to the cytotoxic effects of carboplatin compared with the 2008 cells (IC_{50} = 36 ± 11 μM). In addition, the 2008/D2 and the 2008/D12 dihydrodiol dehydrogenase-transfected clones.

**Cells**—To establish a causal link between the development of cisplatin resistance and the observed overexpression of the candidate genes in the 2008/C13* cells, we decided to transfected the parental (2008) cells with the full-length cDNA of tropomyosin, apolipoprotein J, and glucose-6-phosphate dehydrogenase each. The transfected clones utilized were those that displayed an increased expression (between 3- and 10-fold; data not shown) of their respective mRNA compared to the 2008 cells as assessed by RT-PCR. The IC_{50} values were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described under “Experimental Procedures.” The values presented are as the mean ± S.D. and are from at least three independent experiments, each performed in triplicate. The degree of resistance was calculated as the ratio of cisplatin IC_{50} value of the individual cell line to the cisplatin IC_{50} value of the 2008 cells.

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| Cells               | Cisplatin IC_{50} (μM) | Degree of resistance |
|---------------------|------------------------|----------------------|
| 2008                | 2 ± 0.4                | 1                    |
| 2008/C13*           | 19 ± 2                 | 9                    |
| 2008 clones transfected with full-length tropomyosin cDNA     | 3 ± 1                  | 1.5                  |
| 2008/TM5            | 4 ± 1                  | 2                    |
| 2008 clones transfected with full-length apolipoprotein J cDNA | 2 ± 0.3                | 1                    |
| 2008/A5             | 2 ± 0.1                | 1                    |
| 2008 clones transfected with full-length glucose-6-phosphate dehydrogenase cDNA | 3 ± 1                  | 1.5                  |
| 2008/G3             | 3 ± 1                  | 1.5                  |
| 2008/G4             | 3 ± 1                  | 1.5                  |
were found to be 3-fold resistant to carboplatin compared with the 2008 cells (Table IV). These results correlated well with the higher expression of the protein observed in these dihydrodiol dehydrogenase-transfected cells (Fig. 5).

We performed immunocytochemical analysis using a polyclonal antibody against dihydrodiol dehydrogenase (Fig. 5). The expression of dihydrodiol dehydrogenase was significantly higher (as indicated by the crimson coloration localized in the cytosol) of the 2008/C13* cells (Fig. 5b) as well as the 2008/D2 (Fig. 5c) and 2008/D12 (Fig. 5d) clones as compared with the expression of dihydrodiol dehydrogenase observed in the cisplatin-sensitive, parental 2008 cells (Fig. 5a).

We then investigated the activity of dihydrodiol dehydrogenase in the 2008, 2008/C13* and the transfected 2008/D2 and 2008/D12 cells. The parental cells demonstrated an activity of 6.7 nmol/min/mg of protein when 1-aceanaphthenol was used as a substrate in the presence of NADP+ (4 mM). The dihydrodiol dehydrogenase activity in the 2008/C13*, 2008/D2, and the 2008/D12 cells was found to be 5-, 4-, and 4-fold higher, respectively (Table V). Similarly, using other substrates in the presence and/or absence of dicumarol (an inhibitor of NADPH quinone oxidoreductase), the dihydrodiol dehydrogenase activity was consistently found to be between 2- and 5-fold higher in the 2008/C13* cells and the transfected cells compared with the parental 2008 cells (Table V). Thus, the induction of cisplatin resistance in the transfected clones (as well as the 2008/C13* cells) was functionally associated with an increase in the dihydrodiol dehydrogenase enzyme activity (Table V), suggesting a direct correlation between the two phenotypes. To our knowledge this is the first report that indicates a causal relationship between overexpression of dihydrodiol dehydrogenase and the cisplatin resistance phenotype.

Effect of Constitutive Overexpression of the Dihydrodiol Dehydrogenase on the Cisplatin Sensitivity of the Parental A2780 and SKOV-3 Cells—Unequivocal evidence has been presented thus far that demonstrates a causal link between up-regulation of dihydrodiol dehydrogenase (expression as well as enzyme activity) and development of cisplatin and carboplatin resistance in the 2008 cells. In an attempt to understand whether such a phenomenon was cell line-specific or not, we transfected two additional human ovarian tumor cell lines (A2780 and SKOV-3). The cisplatin and carboplatin sensitivity of the parental and the dihydrodiol dehydrogenase-transfected clones is presented in Table VI. The cisplatin IC50 against the parental A2780 and the SKOV-3 cells was 0.3 ± 0.1 and 5 ± 1 μM, respectively. Forced, constitutive overexpression of dihydrodiol dehydrogenase (via transfection of full-length cDNA) resulted in a 4–5-fold increase in the resistance of the A2780-transfected clones. Similarly, the SKOV-3 transfected clones displayed a 3-fold resistance to cisplatin compared with the parental cells. Furthermore, the recombinant clones from A2780 and SKOV-3 cells (transfected with dihydrodiol dehydrogenase full-length cDNA) were found to be 4- and 3–4-fold resistant to the cytotoxic effects of carboplatin, respectively.

TABLE V

| Substrates | Dihydrodiol dehydrogenase activity | 2008 | 2008/C13* | 2008/D2 | 2008/D12 |
|------------|-----------------------------------|------|----------|--------|---------|
|            | nmol/min/mg of protein            |      |          |        |         |
| 1-Acenaphthenol | 7 ± 2 | 33 ± 8° | 26 ± 6° | 25 ± 8° |  |
| 1-Indanol | 0.5 ± 0.1 | 6 ± 2° | 2 ± 0.1° | 3 ± 0.3° |  |
| Reductase activity of dihydrodiol dehydrogenase using NADPH as cofactor | | | | | |
| Camphorquinone | 35 ± 1 | 65 ± 9° | 71 ± 4° | 41 ± 3° |  |
| Camphorquinone + dicumarol | 25 ± 1 | 45 ± 6° | 44 ± 4° | 35 ± 5° |  |

* P < 0.01 as compared to the enzyme activity observed in the cytosolic extracts of the 2008 cells.
The transfectected clones utilized were those that displayed an increased expression (greater than 5-fold; data not shown) of dihydrodiol dehydrogenase mRNA compared to the parental and the mock-transfected cells (as assessed by RT-PCR). The IC_{50} values were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described (20). The values presented are the mean ± S.D. and are from at least three independent experiments, each performed in triplicate. The degree of resistance was calculated as the ratio of IC_{50} value of individual transfected clone to the IC_{50} value of the parental cells.

| Cell line | IC_{50} cisplatin | Fold resistance | IC_{50} carboplatin | Fold resistance |
|-----------|-------------------|----------------|---------------------|----------------|
| A2780     | 0.3 ± 0.1         | 1              | 4 ± 1               | 1              |
| A2780/DDH5| 1.2 ± 0.1^a       | 4              | 18 ± 4^a            | 4.5            |
| A2780/DDH7| 1.6 ± 0.7^a       | 5              | 16 ± 5^a            | 4              |
| SKOV-3    | 5 ± 1             | 1              | 74 ± 22             | 1              |
| SKOV/DDH6 | 17 ± 0.4^a        | 3              | 219 ± 68^a          | 3              |
| SKOV/DDH13| 16 ± 5^a          | 3              | 286 ± 155           | 4              |

^a P < 0.005 compared to parental cells.

ovarian carcinoma cell line (2008) as compared with its cisplatin-resistant variant (2008/C13^*). In vitro studies have demonstrated that the 2008/C13^* cells exhibit a 9-fold resistance to cisplatin. Furthermore, the 2008/C13^* cells do not require the continuous presence of cisplatin to maintain their resistance, suggesting that the resistance phenotype is stable. Several biochemical alterations thought to be associated with cisplatin resistance have been identified in 2008/C13^* cells compared with the parental 2008 cells. Thus, previous work in our laboratory and that from other laboratories demonstrates that the 2008/C13^* cells exhibit a decreased intracellular accumulation of cisplatin (4), increased replicative bypass of cisplatin-DNA adducts (27), reduced expression of membrane-associated β-tubulin (28), and decreased expression of the intermediate filament, cytokeratin 18 (20). Moreover, the mitochondria in the 2008/C13^* appear morphologically aberrant, and these cells are hypersensitive to lipophilic cations as compared with the parental cells (18). Variations in the activation of protein kinase C activity (29) and in the cAMP signal transduction pathway (30) have also been observed in 2008/C13^* cells. An increased level of expression of the oncogene c-fos in the 2008/C13^* cells has recently been reported, and a partial reversal of the cisplatin resistance phenotype was achieved by treatment of the 2008/C13^* cells with an antisense oligonucleotide directed against c-fos (31). Examination of the basal levels of the drug-detoxifying enzyme glutathione S-transferase and the drug transport pump (multiple drug resistance-associated protein (MRP) involved in the transport of drugs conjugated to glucuronides) revealed no significant difference between the parental 2008 and the cisplatin-resistant 2008/C13^* cells (Ref. 32). All these data indicate that there are multiple mechanisms interconnected in a very complex way responsible for cisplatin resistance in the 2008/C13^* cell line, some of which may have clinical relevance.

Several techniques are currently available for identifying differentially expressed genes including high-throughput technology using cDNA microarray that allows simultaneous analysis of the expression of thousands of genes. Indeed, application of this powerful technique has led to the identification of differential gene expression patterns in doxorubicin-sensitive and -resistant cells (33). This technique allowed us to identify if any change in the expression of 2400 genes was observed when the 2008/C13^* was compared with the 2008 cells. Nine genes were identified that were overexpressed in the 2008/C13^* cells. The differential expression of six of these genes was further confirmed by semi-quantitative RT-PCR analysis. Although the cDNA microarray analysis identified several genes that were down-regulated in the 2008/C13^* cells, these alterations were not observed when the analysis was duplicated and, thus, were disregarded.

The six genes overexpressed in the 2008/C13^* cells included the two isoforms of tropomyosin, fibroblast muscle-type tropomyosin and skeletal β-tropomyosin, dihydrodiol dehydrogenase (two spots on the cDNA microarray slide with distinct GenBan™ accession numbers that code for the same protein), apolipoprotein J (also known as clusterin, sulfated glycoprotein-2, testosterone-repressed prostate message-2), and glucose-6-phosphate dehydrogenase-variant A (Table II).

Tropomyosins are a family of actin-binding proteins expressed ubiquitously in eukaryotic cells that stabilize actin in the microfilaments (34). Although the function of tropomyosin is well understood in muscle cells, their function is ill-defined in non-muscle cells. Several studies have found that overexpression of tropomyosin can suppress the transformation of malignant cells (35, 36). However, an association of tropomyosin with tumor cell sensitivity to anticancer drugs has never been reported. Our present result showed a 5–15-fold up-regulation of tropomyosin mRNA in 2008/C13^*, and upon treatment of the drug-sensitive 2008 cells with cisplatin, a rapid (4 h) and dramatic (10–50-fold) increase in tropomyosin expression was observed. However, transfection experiments showed that forced expression of tropomyosin in the parental cisplatin-sensitive cells did not result in the development of the resistance phenotype.

Apolipoprotein J was also found to be overexpressed in the 2008/C13^* cells. Also known as clusterin, testosterone-repressed prostate message-2, sulfated glycoprotein 2, SP 40–40, complement lysis inhibitor, gp80, glycoprotein III and T64, apolipoprotein J is a heterodimeric glycoprotein and has been proposed to have various biological functions, including sperm maturation, lipid transportation, regulation of the complement cascade, membrane recycling, cell-adhesion, and inhibition of apoptotic cell death (37). Overexpression of apolipoprotein J in hormone-refractory prostate cancer cells has been reported to contribute to the paclitaxel resistance through inhibition of apoptosis (38). However, transfection of the full-length cDNA in the 2008 cells did not support the role of apolipoprotein J in the development of cisplatin resistance.

The activity of the enzyme glucose-6-phosphate dehydrogenase is dependent on the presence of NADP^+, which is converted to NADPH during the oxidation of glucose 6-phosphate to 6-phosphogluconolactone in the pentose phosphate shunt. It is thought that glucose-6-phosphate dehydrogenase maintains the redox state of the cell by aiding in the detoxification of reactive oxygen species (39). A decreased expression of glucose-6-phosphate dehydrogenase has been shown to render cells more susceptible to oxidative stress and, thus, apoptotic death (40). In contrast, overexpression of glucose-6-phosphate dehydrogenase has been demonstrated to protect NIH 3T3 cells from the oxidative damage induced by tert-butyl hydroperoxide by elevating the intracellular levels of NADPH and GSH (41). In addition, increased expression of glucose-6-phosphate dehydrogenase has also been observed in a cyclophosphamide-resistant human leukemia cells (42). The cyclophosphamide-resistant K562 cells were also found to be 3-fold resistant to cisplatin. However, overexpression of glucose-6-phosphate dehydrogenase did not confer cisplatin resistance to the drug-sensitive 2008 cells.

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2 H. B. Deng, H. K. Parekh, K.-C. Chow, and H. Simpkins, unpublished observations.
Of particular importance was the observation that forced overexpression of dihydrodiol dehydrogenase did induce high levels of cisplatin and carboplatin resistance in the parental human ovarian carcinoma (2008, A2780, and SKOV-3) cells (Tables IV and VI). The induction of drug resistance was associated with increased enzyme activity in the transfected clones as well as in the 2008/C13* cells (Table V). Dihydrodiol dehydrogenase belongs to a superfamily of monomeric, cytosolic NADPH-dependent oxidoreductases that catalyze the interconversion of aldehydes and ketones to alcohol (43). The involvement of the oxidoreductase family of enzymes in drug resistance has been previously documented. Thus, up-regulation of a carbonyl reductase has been reported to induce the development of doxorubicin resistance in tumor cells (44). Increased expression of dihydrodiol dehydrogenase has also been observed in an ethacrynic acid-resistant human colon carcinoma cell line (21) and was thought to contribute to the drug-resistant phenotype of these cells, although increased expression of glutathione S-transferase was later demonstrated to be the causative factor. The overexpression of dihydrodiol dehydrogenase has been thought to be associated with an increased binding of trans-activating factors (transcription factors) to an antioxidant response element 5′-to the dihydrodiol dehydrogenase gene transcription start site (45). Ciaccio et al. (45) demonstrate indirectly the existence of an antioxidant response element-like element in the 5′-flanking region of the dihydrodiol dehydrogenase gene that is required for transcriptional activation by ethacrynic acid. To date, three proteins have been identified in an antioxidant response element binding complex, viz. Jun-D, c-Fos, and Jun-B. These proteins in different combinations oxidant response element used binding complex, acid. To date, three proteins have been identified in an anti-

addition, increased expression of peroxiredoxin II, a cytosolic enzyme with peroxidase activity, has been shown to confer cisplatin resistance in a gastric carcinoma cell line (26). Thus, it is plausible that an increase in the activity of dihydrodiol dehydrogenase in the 2008/C13* cells would be sufficient to repair the biochemical lesions induced by cisplatin (due to generation of free radicals), thus leading to development of drug resistance.

In conclusion, using the cDNA microarray technique, we have demonstrated that overexpression of dihydrodiol dehydrogenase in the 2008/C13* cell is responsible for the observed cisplatin-resistance phenotype. An understanding as to the role of this gene, in particular characterizing the regulatory proteins that bind to the transactivation site and control the expression of the dihydrodiol dehydrogenase gene, as well as the analysis of the cellular pathways associated with the detoxification activity of the expressed enzyme is currently under way.

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