The Differential Expression of Cytokeratin 18 in Cisplatin-sensitive and -resistant Human Ovarian Adenocarcinoma Cells and Its Association with Drug Sensitivity

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Abstract

DNA is the primary target of cis-diaminedichloroplatinum (cisplatin), but the drug also interacts with the cellular cytoskeleton composed of microtubules and intermediate filaments. It was found that the cisplatin-resistant 2008/C13 cell line contained markedly lower levels (6-fold) of cytokeratin 18, when compared to the cisplatin-sensitive 2008 cell line. Northern blot analysis revealed a markedly decreased level of cytokeratin 18 mRNA in the resistant cell line. Southern blot analysis of the DNA extracted from the two cell lines and then digested with HpaII and its methylation-sensitive isoschizomer, MspI, revealed no detectable differences in the methylation status of the cytokeratin gene. Neither 5-azacytidine (5 μM) nor retinoic acid (1 μM) treatment enhanced the expression of cytokeratin 18 in the resistant cell line. However, transfection of full-length cytokeratin 18 cDNA into the cisplatin-resistant 2008/C13 cells resulted in clones with increased levels of cytokeratin 18, which was accompanied in the majority of clones by a marked increase in their sensitivity to cisplatin. These results demonstrate that modulating the expression of an intermediate filament protein results in sensitization of a drug-resistant human ovarian cell line to cisplatin.

Introduction

Cisplatin is an anticancer drug used widely for the treatment of various cancers. However, the development of resistance to its cytotoxic effect is a major problem in its use. Experiments using tumor cell lines sensitive and resistant to cisplatin indicate that resistance acquired to the drug is due to one or more factors acting alone or in conjunction with each other. These factors include decreased intracellular drug accumulation, increased drug metabolism or inactivation via conjugation to glutathione or metallothionein, increased DNA repair, and/or the activation of certain oncop genes (1–3).

Cisplatin binds to the N7 position of guanosine and forms intrastrand and interstrand cross-links. In addition to its direct binding to DNA, the other cellular effects of cisplatin that have been reported include disruption of the mitochondrial membrane potential, depolymerization of the microtubules, and collapse of the intermediate filament network (4–7). Recently, decreased expression of cytokeratin 14 was reported in a cisplatin-resistant variant of a human lung squamous cell carcinoma line (8). Since intermediate filament proteins bind to cisplatin-damaged DNA (9–11), the possibility that they may play a role in the cytotoxic action of cisplatin cannot be ignored.

Cytokeratins are intermediate filament proteins that are subdivided into type I (acidic) and type II (basic) keratins (12). One member of type I preferentially binds with one member of type II via a hydrophobic interaction to form an 8–10-nm filament (12). Intermediate filaments, along with microtubular proteins, play a key role in the maintenance of cell shape and in the spatial organization of the cellular organelles. They are also thought to be involved in the modulation of membrane transport (13). Thus, changes in intermediate filament content/structure could potentially alter the cellular cytoskeleton and affect a multitude of cellular events, including drug transport.

Christen et al. (14) have shown that the ultrastructure of the microtubules was altered in a cisplatin-resistant human ovarian adenocarcinoma cell line (2008/C13*) as compared to the parental cisplatin-sensitive 2008 cell line, and decreased expression of a membrane-associated, β-tubulin fraction was observed in the cisplatin-resistant cells. Interestingly, using a radiolabeled analogue of cisplatin, this resistant cell line (2008/C13*) was shown to have a defect in the initial uptake rate, but over a period of 24–48 h, the cisplatin accumulation was found to be similar in 2008 and 2008/C13* cells (2). Also, cisplatin accumulation studies performed in our laboratory using atomic absorption spectroscopy after treatment of 2008 and 2008/C13* cells with 100 μM cisplatin for 4 h revealed no significant differences between these two cell lines. Since intermediate filaments exist in close juxtaposition with the microtubules, an investigation as to the role of intermediate filaments in the development of cisplatin-resistance in human ovarian carcinoma cells (2008 and 2780) and their cisplatin-resistant variants (2008/C13* and C70, respectively) was undertaken.

Materials and Methods

Materials. Cisplatin was purchased from Aldrich (Milwaukee, WI). α-[32P]dCTP (3000 Ci/mol) was purchased from NEN-Dupont Research Products (Boston, MA). The multiprime DNA labeling system was obtained from the Amersham Corp. (Arlington Heights, IL). The Cam 5.2 and the CK4 mouse monoclonal antibodies were purchased from Becton Dickinson (San Jose, CA) and Oncogene Science (Manhasset, NY), respectively. A mouse monoclonal antibody to vimentin was purchased from Boehringer Mannheim (Indianapolis, IN). The alkaline phosphatase conjugated antimouse rabbit monoclonal antibody was obtained from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). The full-length cytokeratin 18 cDNA (15) was kindly provided by Dr. R. Oshima (La Jolla Cancer Center, La Jolla, CA). The eukaryotic expression vector pCDNA3 was purchased from Invitrogen (San Diego, CA).

Cell Culture Conditions. The parental (2008 and 2780) human ovarian carcinoma cells and their cisplatin-resistant variants (2008/C13* and C70, respectively) used in this study were maintained in RPMI medium as described previously (16).

Cytotoxicity Studies. The sensitivity of 2008 and 2008/C13* cells to the cytotoxic effect of cisplatin was assessed using the tetrazolium dye 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (16).

Protein Determinations. The protein content of the cytoskeletal fractions was determined by the Coomassie Brilliant Blue dye-binding assay (17) using the commercially available Bio-Rad Protein Assay reagent and BSA as the standard.

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2 The abbreviation used is: cisplatin, cis-diaminedichloroplatinum.
Intermediate Filament Protein Immunoblots. Cells in the exponential phase of growth were harvested and washed with a 10 mM phosphate buffer (pH 7.4) containing 120 mM NaCl and 2.7 mM KCl. The cell pellets were suspended in 20 mM Tris (pH 7.4), 0.6 mM KCl, 1% (v/v) Triton X-100, and 1 mM MgCl2 in the presence of 0.5 mM phenylmethylsulfonyl fluoride and 1000 units of DNase I for 5 min at room temperature. The lysate was then centrifuged at 13,000 × g, and the pellet (cytoskeletal fraction) was solubilized in 8 M urea and 0.5% (v/v) NP40. Fifty μg protein from the solubilized cytoskeletal fraction were mixed with SDS-PAGE buffer and heated at 100°C for 15 min. The protein samples were then separated on a 10% polyacrylamide gel and electrophoretically transferred onto polyvinylidene difluoride membranes using a Bio-Rad semidysem blotter. Immunostaining was performed as described earlier (16), using a 1:100 dilution of each of the intermediate filament antibodies. Densitometric analysis was performed using a LKB laser scanning densitometer to assess the difference in the color intensity of the immunostained bands.

RNA Extraction and Northern Blotting. Total cellular RNA was extracted from exponentially growing cells and fractionated through a 1% agarose gel containing 2.2 M formaldehyde (18). RNA was transferred to Nytran Plus nylon membranes (18), and Northern blot analysis was performed using a full-length cytokeratin 18 cDNA as the probe (18). Equal loading of RNA was ascertained by ethidium bromide staining of the agarose gels. The developed autoradiogram was subjected to densitometric analysis on a LKB laser scanning densitometer to assess the differences in the amount of the cytokeratin 18 mRNA between 2008 and 2008/C13* cells.

DNA Extraction, Restriction Endonuclease Digestion, and Southern Blotting. High molecular weight genomic DNA was isolated from exponentially growing 2008 and 2008/C13* cells using phenol-chloroform extraction and ethanol precipitation. The DNA (10 μg) was then digested overnight at 37°C using 30 units each of HpaII and its methylation-sensitive isoschizomer MspI. The digested DNA was subjected to electrophoresis in a 0.8% (w/v) agarose/1× Tris-Borate-EDTA gel at a constant voltage of 1 V/cm. After depurination and denaturation, the separated DNA was transferred onto Nytran membranes, and Southern blot analysis was performed as described (18) using the full-length cytokeratin 18 cDNA as a probe.

Plasmid Construction and Transfection. The full-length cytokeratin 18 cDNA was cloned into the EcoRI site of the eukaryotic expression vector, pCDNA3. Orientation was checked with restriction enzyme digestion. The plasmid carrying the full-length cytokeratin 18 in the right orientation termed pCDK18 was used for the transfection of the 2008/C13* cells. Subconfluently growing cells were transfected with pCDNA3 (10 μg/ml) or pCDK18 (10 μg/ml) using the calcium phosphate-DNA coprecipitation method. Cells were propagated in a medium containing geneticin (500 μg/ml; 0418 sulfate) for 3 weeks. Individual G418-resistant colonies were picked, grown, and screened for expression of the cytokeratin 18 protein using the Cam 5.2 mouse monoclonal antibody. For cisplatin chemoresistance studies, 18 clones from the 2008/C13* cells transfected with the pCDK18 vector and exhibiting cytokeratin 18 expression were analyzed. One clone of 2008/C13* cells transfected with the pCDNA3 vector alone (C13*/pCDNA3) was used as a control in these studies.

Statistical Analysis. The regression analysis and paired t test were performed using SigmaStat Statistical Analysis System, version 1.01.

Results and Discussion

The intracellular levels of the cytoskeletal proteins, vimentin and cytokeratin, were determined by Western blot analysis. High, but identical, vimentin levels were observed in the 2780 and C70 cells; however, only very low levels of vimentin were detected in the 2008 and 2008/C13* cells (data not presented). In contrast, expression of the simple epithelial cytokeratins (cytokeratins 8 and 18) was observed in the 2008 and 2008/C13* cells (Fig. 1). The level of cytokeratin 8 (M, 55,000), as judged by immunostaining with the Cam 5.2 antibody, were similar in the 2008 and 2008/C13* cells (Fig. 1A), but those of cytokeratin 18 (M, 45,000) were 6-fold lower in the cisplatin-resistant cells (2008/C13*) compared to the cisplatin-sensitive cells (2008; Fig. 1A). Similar results were observed with the cytokeratin 18-specific antibody, CK4 (Fig. 1B). Interestingly, the levels of cytokeratin 8 and cytokeratin 18 in the 2780 and C70 cells were low and similar (Fig. 1A). The mRNA level of cytokeratin 18 was assayed by Northern blot analysis using a full-length human cytokeratin 18 cDNA as a probe. A 5-fold lower cytokeratin 18 mRNA level was observed in the 2008/C13* cells as compared to the 2008 cells (Fig. 1C), results which correlate with the Western blot data.

Thus, a relationship apparently exists between decreased expression of cytokeratin 18 and resistance to cisplatin. A similar association between reduced expression of cytokeratin 14 and cisplatin resistance was reported recently in a human lung squamous cell carcinoma cell line (8). Interestingly, the cisplatin-resistant lung cells accumulated less intracellular cisplatin then the sensitive cells. Recent studies with the 2008/C13* cells report alterations in the ultrastructural morphology of the microtu-
The human ovarian carcinoma cells sensitive (2008) and resistant (2008/C13*) to cisplatin were treated with 5-azacytidine (5 μM) or retinoic acid (1 μM), either 24 h prior to exposure to cisplatin or simultaneously. Cell survival was determined by the tetrazolium blue dye assay. IC₅₀ values were calculated from a regression analysis of the survival curves and correspond to the cisplatin concentration that produces 50% inhibition of cell survival. IC₅₀ values are expressed ± SD and are the mean of three individual experiments performed in triplicate.

Table 1: Effect of treatment with 5-azacytidine and retinoic acid on cisplatin cytotoxicity

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<th>2008/C13*</th>
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<td>Cisplatin alone</td>
<td>1.8 ± 0.5</td>
<td>22 ± 3</td>
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<tr>
<td>5-Azacytidine (24-h pretreatment) + cisplatin</td>
<td>1.1 ± 0.7</td>
<td>16 ± 7</td>
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<td>5-Azacytidine + cisplatin (simultaneously)</td>
<td>1.9 ± 0.8</td>
<td>20 ± 6</td>
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<tr>
<td>Retinoic acid (24-h pretreatment) + cisplatin</td>
<td>1.2 ± 0.8</td>
<td>18 ± 3</td>
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<td>1.8 ± 0.5</td>
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Despite the presence of multiple HpaII/MspI sites in the 5' end of the murine cytokeratin 18 gene, no gross alterations in the methylation pattern were observed (data not presented). Using a RNA probe corresponding to 461 nucleotides from the 5' end of the murine cytokeratin 18 gene, Kulesh and Oshima (15) have demonstrated that it is possible to study the methylation status of the cytokeratin 18 gene (20). However, when DNA was isolated from the 2008 and 2008/C13* cells and subjected to restriction endonuclease digestion with HpaII and its methylation-sensitive isoschizomer MspI, followed by Southern blot analysis with a full-length cytokeratin 18 cDNA probe, no gross alterations in the methylation pattern were observed (data not presented). Using a RNA probe corresponding to 461 nucleotides from the 5' end of the murine cytokeratin 18 gene, Kulesh and Oshima have demonstrated that it is possible to study the methylation status of the cytokeratin 18 gene (20). However, in this study we have used a full-length cytokeratin 18 cDNA as a probe, and due to the presence of multiple HpaII/MspI sites in the 5' end of the gene (10 sites in the murine cytokeratin 18 gene), a full-length cDNA probe may not be specific enough to detect subtle differences in the methylation status of this region, especially since, of the 15–20 genes similar to cytokeratin 18 present in the human genome, only one may be active (15). 5-Azacytidine inhibits the DNA methyltransferase enzyme, and the resultant DNA hypomethylation induces gene expression (21). Treatment with 5-azacytidine has been shown to induce the expression of cytokeratin 8 in mesenchymal cells and Endo B (the mouse form of human cytokeratin 18) in myoblast cells (21, 22), although the addition of 5-azacytidine to an embryonic teratocarcinoma cell line did not result in the induction of Endo B (15). Retinoic acid treatment of epithelial cells also modulates the expression of various cytokeratins, including cytokeratins 8 and 18 (23, 24). However, treatment of the 2008 and 2008/C13* cells with 5-azacytidine (5 μM) and retinoic acid (1 μM) did not affect the expression of cytokeratin 18 (data not presented). Furthermore, a simultaneous exposure with cisplatin or a 24-h pretreatment of 2008 and 2008/C13* cells with 5-azacytidine (5 μM) and retinoic acid (1 μM) had no effect on cisplatin cytotoxicity (Table 1).

In an attempt to determine if increasing the expression of cytokeratin 18 in the 2008/C13* cells could lead to increased sensitivity to cisplatin, 2008/C13* cells were transfected with a full-length cytokeratin 18 cDNA cloned in the eukaryotic expression vector, pCDNA3. Twenty-six clones were picked to assess the relationship between expression of cytokeratin 18 and cisplatin sensitivity. Increased cytokeratin expression was observed in 18 clones (which represent 70% of the total number of clones). A Western blot of nine of these clones is presented in Fig. 2. Although the pCDNA3 vector leads to a high level of transcription, the modest increase observed in cytokeratin 18 expression is probably due to rapid turnover of this protein. Similar results have been observed in cytokeratin 18-transfected F9 cells and L-cell fibroblasts (15). Forced expression of one type of cytokeratin has also been shown to induce expression of the other type (25, 26), but the observed increase in cytokeratin 18 expression in these human ovarian carcinoma cells did not result in an increased expression of cytokeratin 8. The cisplatin sensitivity of the transfected clones (C13*/pCK1–C13*/pCK18) was assessed. A significant decrease in the IC₅₀ of cisplatin in 11 of the 18 transfected clones was observed (Table 2). Of particular importance was the observation that in 4 of the 18 clones, the IC₅₀ was decreased to 70–80% of the value observed for the 2008/C13* cells, and in 7 more clones, the IC₅₀ was reduced to 40–60% of that observed with the resistant cell line (Table 2). In the remaining seven transfected clones,

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Cytokeratins 8 and 18 are expressed in simple epithelia (12), and their expression is regulated by the differentiation state of the cell and possibly DNA methylation in the promoter region of the gene (15, 20). Kulesh and Oshima (15) have demonstrated that the 5'-flanking region of the human cytokeratin 18 gene is G/C rich; thus, it is possible that methylation of this region could be involved in its transcriptional regulation. However, when DNA was isolated from the 2008 and 2008/C13* cells and subjected to restriction endonuclease digestion with HpaII and its methylation-sensitive isoschizomer MspI, followed by Southern blot analysis with a full-length cytokeratin 18 cDNA probe, no gross alterations in the methylation pattern were observed (data not presented). Using a RNA probe corresponding to 461 nucleotides from the 5' end of the murine cytokeratin 18 gene, Kulesh and Oshima have demonstrated that it is possible to study the methylation status of the cytokeratin 18 gene (20). However, in this study we have used a full-length cytokeratin 18 cDNA as a probe, and due to the presence of multiple HpaII/MspI sites in the 5' end of the gene (10 sites in the murine cytokeratin 18 gene), a full-length cDNA probe may not be specific enough to detect subtle differences in the methylation status of this region, especially since, of the 15–20 genes similar to cytokeratin 18 present in the human genome, only one may be active (15). 5-Azacytidine inhibits the DNA methyltransferase enzyme, and the resultant DNA hypomethylation induces gene expression (21). Treatment with 5-azacytidine has been shown to induce the expression of cytokeratin 8 in mesenchymal cells and Endo B (the mouse form of human cytokeratin 18) in myoblast cells (21, 22), although the addition of 5-azacytidine to an embryonic teratocarcinoma cell line did not result in the induction of Endo B (15). Retinoic acid treatment of epithelial cells also modulates the expression of various cytokeratins, including cytokeratins 8 and 18 (23, 24). However, treatment of the 2008 and 2008/C13* cells with 5-azacytidine (5 μM) and retinoic acid (1 μM) did not affect the expression of cytokeratin 18 (data not presented). Furthermore, a simultaneous exposure with cisplatin or a 24-h pretreatment of 2008 and 2008/C13* cells with 5-azacytidine (5 μM) and retinoic acid (1 μM) had no effect on cisplatin cytotoxicity (Table 1).

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despite an increase in cytokeratin 18 expression, the IC50 was only 20–40% of that observed with the cisplatin-resistant 2008/C13* cells (data not shown). Different turnover rates of the newly synthesized cytokeratin 18 in the different clones and the possibility that the newly synthesized cytokeratin 18 forms intermediate filaments with differing degrees of efficiency in the individual clones may explain why not all of the clones exhibit a significantly increased sensitivity to cisplatin.

The major cellular lesion responsible for cisplatin toxicity is thought to be the generation of DNA intrastrand cross-links, although interstrand and protein-DNA cross-links have also been observed. Nonhistone proteins were found to be cross-linked to DNA in cells or isolated nuclei treated with cisplatin, and these proteins were later identified as cytokeratins (9–11). Several other studies also support the notion of an intimate association of intermediate filaments with the cell nucleus (13). Thus, it is plausible that the formation of cytokeratin-DNA cross-links is associated with cisplatin cytotoxicity and that the reduced expression of cytokeratin 18 in the 2008/C13* cells can lead to a reduced formation of protein-DNA cross-links, and thus decreased sensitivity to cisplatin.

The association of cytokeratins and anticancer drug toxicity is not limited to cisplatin. Dalton and colleagues (27, 28) have demonstrated a binding of mitoxantrone to cytokeratins. Furthermore, a mouse fibroblast cell line transfected with and expressing cytokeratins 8 and 18 was found to be multidrug resistant, although the transfected cells did not exhibit decreased intracellular drug accumulation (29). The authors suggested that the cytokeratin network may influence the intracellular drug distribution in such a manner that the nuclear targets are spared, resulting in a resistant phenotype. It has also been demonstrated that, compared to the cisplatin-sensitive 2008 cells, the cisplatin-resistant 2008/C13* cells (which express a 6-fold lower level of cytokeratin 18) are hypersensitive to the cytotoxic effects of taxol, vincristine, and vinblastine (16). This correlates, in an inverse manner, with the observation of Bauman et al. (29) who showed that mouse fibroblast cells expressing higher levels of cytokeratin 8 and 18 become relatively resistant to the mitotic spindle poisons, taxol, and the anthracyclines. The identification of cytokeratins as proteins that have the potential to modulate the effects of anticancer drugs opens a new area of investigation.

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References

20. Oshima, R. G., Trevor, K., Shevinsky, L. H., Ryder, O. A., and Cecena, G. Identification of the gene coding for the Endo B murine cytokeratin and its methylated, inactive, and protein-DNA cross-links have also been observed. Nonhistone proteins were found to be cross-linked to DNA in cells or isolated nuclei treated with cisplatin, and these proteins were later identified as cytokeratins (9–11). Several other studies also support the notion of an intimate association of intermediate filaments with the cell nucleus (13). Thus, it is plausible that the formation of cytokeratin-DNA cross-links is associated with cisplatin cytotoxicity and that the reduced expression of cytokeratin 18 in the 2008/C13* cells can lead to a reduced formation of protein-DNA cross-links, and thus decreased sensitivity to cisplatin.

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