Reduced expression of small GTPases and hypermethylation of the folate binding protein gene in cisplatin-resistant cells

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Reduced accumulation of cisplatin is the most consistent feature seen in cisplatin-resistant (CP-r) cells that are cross-resistant to other cytotoxic compounds, such as methotrexate. In this report, defective uptake of a broad range of compounds, including [¹⁴C]-carboplatin, [³H]MTX, [¹²⁵]epidermal growth factor, [⁵⁷Fe], [³H]glucose, and [³H]proline, as well as [⁷³As]⁺, was detected in CP-r human hepatoma and epidermal carcinoma cells that we have previously shown are defective in fluid-phase endocytosis. Downregulation of several small GTPases, such as ras, rac, and rhoA, which regulate endocytosis, was found in CP-r cells. However, expression of an early endosomal protein and clathrin heavy chain was not changed, suggesting that the defective endocytic pathway is clathrin independent. Reduced expression of the cell surface protein, folate-binding protein (FBP), which is a carrier for the uptake of MTX, was also observed in the CP-r cells by confocal immunofluorescence microscopy and Real-Time PCR. Reactivation of the silenced FBP gene in the CP-r cells by a DNA demethylation agent, 2-deoxy-5-aza-cytidine (DAC) demonstrates that hypermethylation occurred in the CP-r cells. The uptake of [¹⁴C]carboplatin, [³H]FA, and [³H]MTX increased in an early stage CP-r cell line (KB-CP1) after treatment with DAC. Both a defective endocytic pathway and DNA hypermethylation resulting in the downregulation of small regulatory GTPases and cell surface receptors contribute to the reduced accumulation of a broad range of compounds in CP-r cells.

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Cisplatin (cis-diaminedichloroplatinum II) has become a major chemotherapeutic agent for treatment of a wide spectrum of solid tumours. Biochemical, cellular, and molecular approaches have been used to identify the molecular basis of resistance to cisplatin. Development of resistance to cisplatin in vivo and in cell lines is multifactorial, including changes in DNA repair proficiency (Fink et al, 1998; Branch et al, 2000; Fojo, 2001), proteins and enzymes involved in detoxifications of cisplatin, such as metallothionein and glutathione-related enzymes (Kelly et al, 1988; Godwin et al, 1992; Daubeuf et al, 2002), chaperones (Yamamoto et al, 2001), signal transduction pathways (Basu et al, 1996), and cell cycle regulators (Kondo et al, 2001). Alterations in the expression of proto-oncogenes, apoptosis-related genes, and cancer susceptibility genes have also been described in association with cisplatin resistance (Lowe et al, 1993; Husain et al, 1998; Slupianek et al, 2001). Recently, molecular approaches using cDNA microarrays and differential display have provided useful tools for further understanding of the molecular mechanisms that contribute to cisplatin resistance (Grottke et al, 2000; Niedner et al, 2001).

The reduced accumulation of cisplatin is the most consistent feature seen in cisplatin-resistant (CP-r) cells (Loh et al, 1992; Johnson et al, 1996; Shen et al, 2000). Mechanisms for drug resistance resulting from the reduced accumulation of drugs have been categorised as decreased influx (impaired uptake) and increased efflux (active export) (Gottesman et al, 2002). We have previously reported that a pleiotropic defect resulting in the reduced accumulation of [³H]MTX, [⁷³As]⁻, and [⁷³As]⁺ in the CP-r cells was found to be associated with reduced expression of folate-binding protein (FBP) and arsenic-binding proteins (Shen et al, 1998). The reduced accumulation of [¹⁴C]carboplatin in CP-r cells in association with crossresistance to this compound was also documented to be due to a defect in energy-dependent uptake and not due to active efflux (Shen et al, 2000). However, the cell biology and molecular bases of the reduced accumulation in CP-r cells need to be further elucidated.

A defect in fluid-phase endocytosis was found in higher level CP-r cell lines isolated in multiple steps (Chauhan et al, 2003). To investigate the cellular and molecular genetic events involved in cisplatin resistance, particularly for the reduced uptake of a variety of compounds, reduced expression of several small GTPases involved in the endocytic pathway and FBP was found in the CP-r cells as detected by confocal immunofluorescence microscopy,
immunoblotting, and realtime (RT)-PCR. Reactivation of the silenced FBP gene in the CP-r cells by a demethylation agent, 2-deoxy, 5-aza-cytidine (DAC), indicated that hypermethylation of the gene occurred during the development of cisplatin resistance, and that the uptake defect could be reversed to some extent by DAC. The data presented here provide evidence that CP-r cells have a global defect in the uptake of cisplatin and several other related and unrelated compounds at least related in part to reduced expression of small GTPases that regulate in endocytic pathways and DNA hypermethylation in the CP-r cells may play a role in this pleiotropic phenotype.

**MATERIALS AND METHODS**

**Cell lines and cell culture**

Two populations of CP-r cell lines and their parental cell lines were studied: the human epidermoid carcinoma cell line KB-3-1 and its CP-r derivative, KB-CP20, and the human liver carcinoma cell line BEL-7404 and its CP-r derivative, 7404-CP20.

Both human CP-r cell lines were selected in 20 μg cisplatin ml⁻¹ medium as described previously (Shen et al., 1998). The early-stage CP-r cell line KB-CP1 was isolated from the KB-3-1 cells in a two-step selection including 0.3 and 1 μg ml⁻¹ cisplatin (Liang et al., 2003). Figure 1 shows a flow diagram of the derivation of CP-r cell lines isolated from KB-3-1 and BEL-7404 cells that were used in this study. All cell lines were grown as monolayer cultures at 37°C in 5% CO₂. MTSNa buffer (10 mM Tris-HCl pH 7.45, 250 mM sucrose, 50 mM NaCl, and 1% bovine serum (BioWhittaker, Walkersville, MD, USA).

**Drugs and radiochemicals**

All chemicals were purchased from Sigma (St Louis, MO, USA) unless otherwise noted. Radioactive labelled compounds, such as [¹⁴C]carboplatin, [³H]MTX, [³⁵S]folic acid (FA), [¹²⁵I]epidermal growth factor (EGF), and [⁵⁹Fe], were purchased from Amersham Biosciences (Piscataway, NJ, USA). Arsenic-⁷³ at specific activity 200 Ci ml⁻¹ was prepared by the reduction of ⁷³As⁵⁺ with As(III) in 50 μl of reduction solution consisting of 0.1 mM NaAsO₂, 66 mM Na₂S₂O₅, 27 mM Na₂S₂O₃, and 82 mM H₂SO₄ was added to 50 μl of ⁵⁹Fe. The cell suspensions were transferred from the dishes into counting vials with cocktail Ecoscint A (National Diagnostics, Atlanta, GA, USA) and counted in a Beckman LS801 liquid scintillation counter. The radioactivity of the cisplatin-sensitive (CP-s) parental KB-3-1 cells incubated with various radioactive compounds was calculated as 100% accumulation, in comparison to the KB-CP20 cells. Duplicate dishes of cells for each determination were analysed.

**Preparation of enriched plasma membrane proteins**

Membrane proteins were purified according to the method of Cornwall et al. (1987). Briefly, 1 × 10⁶ cells from each cell line were disrupted on ice by nitrogen cavitation with constant stirring for 40 min. Two cycles of sucrose gradient ultracentrifugation were followed. The purified membrane pellets were resuspended in 3 ml TSNa buffer (10 mM Tris-HCl pH 7.45, 250 mM sucrose, 50 mM NaCl, and 1% aprotinin) and stored at –80°C until use.

**Measurement of the accumulation of radiochemicals**

Dishes containing a subconfluent monolayer of cells were used for the assay. For the uptake analysis of [³H]FA and [³H]MTX, cells were washed once with FA-deficient DMEM (Invitrogen), then incubated at 37°C with the same medium containing 0.5 μCi ml⁻¹ of radioactive materials. For all other radioactive compounds, cells were incubated with the regular medium containing 0.5 μCi ml⁻¹ of radioactive materials. After a 1- or 2-h incubation, uptake was stopped by washing the dishes three times with ice-cold phosphate-buffered saline, then harvesting cells by trypsinisation. The cell suspensions were transferred from the dishes into counting vials with cocktail Ecoscint A (National Diagnostics, Atlanta, GA, USA) and counted in a Beckman LS801 liquid scintillation counter. The radioactivity of the cisplatin-sensitive (CP-s) parental KB-3-1 cells incubated with various radioactive compounds was calculated as 100% accumulation, in comparison to the KB-CP20 cells. Duplicate dishes of cells for each determination were analysed.

**Confocal image analysis**

Cells were cultured in a Lab-Tek Chamber Slide (Nalgé Nunc International, Naperville, IL, USA) and fixed with 70% ethanol at –20°C for 15 min. The fixed cells were reacted with the primary antibodies and then followed by rhodamine-labelled anti-mouse IgG second antibody. FBP-specific monoclonal antibody MOv19 was a gift from MI Colnaghi (Mantovani et al., 1994). Immunofluorescent images of cells were monitored under a laser scanning confocal microscope (Bio-Rad) at × 600 magnification.

**RT-PCR**

DNA was extracted by Qiagen RNAeasy kits as described by the manufacturer (Qiagen, Valencia, CA, USA). RT-PCR on the LightCycler instrument (Roche Diagnostics, Indianapolis, IN, USA) was performed in a total volume of 20 μl in the presence of 2 μl of RNA (0.5 μg) or water as control. The reverse transcription solution (Roche, STBR Green I kit) was applied with 2 μl per reaction for a better resolution of melting curves and a significant increase in sensitivity as for GC-rich templates. Specific primers for desired genes were added to a final concentration of 10 pmol of each.
RESULTS

Reduced accumulation of radioactive compounds

In our previous reports, decreased accumulation of $^{14}$C-carboplatin, $[^3]$HMTX, $^{73}$As$^{3+}$, and $^{73}$As$^{5+}$ was found in the CP-r cells. In this report, we demonstrate that the KB-CP20 cells have a more global defect in the uptake of a variety of radioactively labelled compounds. As shown in Figure 2, the accumulation of $^{14}$C-carboplatin, $[^3]$HMTX, and $[^3]$HFA (FA) was decreased over 90% in the CP-r cells compared with the CP-s cells. The decreased accumulation of $[^3]$Hglucose and $[^3]$Hproline was detected in the CP-r cells. The uptake of $^{73}$As$^{3+}$, $^{73}$As$^{5+}$, $^{59}$Fe, and $^{125}$I]EGF was also decreased from 52% to over 80% in the CP-r cells compared to CP-s cells. The reduced accumulation of $[^3]$HFA is correlated with decreased expression of the epidermal growth factor receptor (EGF-R) in our CP-r cells as described previously (Chauhan et al., 2003).

Reduced expression of several small GTPases and cell surface proteins

We have found that KB-CP20 cells have a defect in nonreceptor-mediated endocytosis (Chauhan et al., 2003) and wondered if this endocytosis defect in CP-r cells might account for some of the reduced uptake of nutrients and cytotoxic compounds in CP-r cells. To explore the endocytosis pathway further, we checked whether small GTPases and other related proteins known to regulate endocytosis might also be affected. By immunoblotting analysis on small GTPases, the expression levels of rab5 and rac1 were noticeably decreased, while rhoA was almost undetectable in both KB-CP20 and 7404-CP20 cells in comparison to their parental CP-s cell lines (Figure 3). Quantitative RT-PCR analysis of mRNA for these small GTPases showed only modest decreases in mRNA levels (Table 1), indicating that post-transcriptional regulation might also be involved in the reduction in protein levels. The expression levels of EEA1, an early endosome marker, and a clathrin-heavy chain that is involved in receptor-mediated endocytosis, were similar in these two pairs of human CP-r and CP-s cell lines, indicating that the reduced accumulation of a variety of radioactive compounds is not associated with changes of these two intracellular components. Expression of caveolin was increased significantly in both KB-CP20 and 7404-CP20 (data not shown).

Reactivation of silenced FBP genes by a DNA methylation inhibitor

Since mRNAs encoding many different proteins were reduced in amount in CP-r cells, we sought to determine whether DNA hypermethylation might account for decreased transcription of certain genes in CP-r cells. In our previous paper, we reported that FBP and its mRNA were almost absent in the CP-r cells by Northern and immunoblot analysis (Shen et al., 1998). To study further the mechanism by which the FBP gene was downregulated, DAC, a DNA demethylation agent, was used to determine whether the silenced FBP gene could be reactivated as would be expected if the reduced expression of FBP in CP-r cells was due to hypermethylation of the gene. The expression levels of the FBP gene in CP-r cells were significantly elevated after exposure to DAC (Figure 4A, upper panel). There were no changes in CP-s cells after treatment with DAC (data not shown), indicating that DAC has a specific effect on the activation of silenced FBP gene in CP-r cells.

There are at least three genes that are known to be commonly involved in MTX resistance: FBP, reduced folate carrier (RFC), and dehydrofolate reductase (DHFR) (Hsueh and Dolnick, 1994; Kelland et al., 1995; Moscow et al., 1995). Figure 4A shows clearly that only the FBP gene was silenced in the KB-CP20 cells, whereas
Cells were treated with DAC, 1 μM for 4 days followed by DAC-free medium for 12 days. There was little effect of DAC on the parental KB-3-1 and KB-CP20 cell lines. The uptake of [3H]FA and (Figure 5A). There was little effect of DAC on the parental KB-3-1 cells only seen in DAC-treated two-step selected KB-CP1 cells compared to untreated cells (Figure 5B). For FA and methotrexate uptake, DAC also had less than a two-fold effect on the parental KB-3-1 cells. However, the KB-CP20 cells were nonresponsive to DAC treatment in the accumulation of [3H]FA. In parallel with the increased uptake of the above radioactively labelled compounds, the expression of the FBP gene was elevated as the concentrations of DAC increased. decreased accumulation of cisplatin and many other drug accumulation by blocking normal modes by which agents interact with the cell surface protein and decreased endocytosis. These changes have the net effect of decreased accumulation of cisplatin and many other compounds. Unlike other forms of multidrug resistance due to alterations in gene expression resulting in altered expression of cell surface protein and decreased endocytosis. This form of multidrug resistance causes decreased drug accumulation by blocking normal modes by which agents such as cisplatin enter cells. In this work, we show an association between the reduced uptake of a variety of cisplatin-related or - unrelated radioactively labelled compounds and a defective endocytic

**DISCUSSION**

In this work, we have examined the cellular and molecular basis of an extremely pleiotropic phenotype of CP-r cells that creates global alterations in gene expression resulting in altered expression of cell surface protein and decreased endocytosis. This form of multidrug resistance causes decreased drug accumulation by blocking normal modes by which agents such as cisplatin enter cells. In this work, we show an association between the reduced uptake of a variety of cisplatin-related or - unrelated radioactively labelled compounds and a defective endocytic
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...accumulation of [14C]carboplatin was due to impaired energy-dependent transport process (Shen et al... DNA hypermethylation has been reported in multidrug-resistant cell lines, including CP-r cells (Plumb et al, 2000; Effrth et al, 2001; Borst and Ellerink, 2002). A link between mismatch repair deficiency and cytotoxic drug resistance was ascribed to hypermethylation of the promoter regions of genes involved in DNA repair (Strathdee et al, 1999).

In our work, the FBP gene was chosen as a model because of its association with reduced uptake and crossresistance to MTX in CP-r cells. Preliminary results have shown differences in methylation patterns of the FBP gene in parental and CP-r cells (data not shown). To study the relation between the downregulation of gene expression and DNA hypermethylation, DAC, a demethylating agent, was applied to see if the silenced FBP gene and other genes could be reactivated. DAC has been shown to replace cytidine residues in replicating DNA and prevent methylation, thereby demethylating or inducing hypomethylation of DNA (Hsuieh and Dolnick, 1994). It is evident from this work that the silenced FBP gene in KB-CP20 cells could be reactivated by DAC (Figure 4). The duration of reexpression of the FBP gene was at least 12 days after initial exposure to this compound for 4 days.

FBP, as mentioned above, and RFC are both involved in the uptake of MTX, while DHFR is a cytotoxic target for MTX. The data reported here indicate that only FBP expression was remarkably reduced and that there was little change in the expression of RFC and DHFR genes in the CP-r cells (Figure 4A). No response of the RFC gene to DAC treatment was observed, whereas the expression of DHFR was elevated about 2–3-fold after DAC induction.

Uptake assays on [14C]carboplatin, [3H]FA, and [3H]MTX in DAC-treated or -untreated cells revealed that DAC treatment increased the accumulation of these compounds in KB-CP1 cells. The parental KB-3-1 cells had no response to DAC for uptake of [14C]carboplatin with some increase in the uptake of [3H]FA and [3H]MTX. In contrast, DAC showed little effect on uptake in the high-level Cp-r KB-CP20 cells. Why did expression of the FBP gene in the KB-CP20 cells after treatment with DAC not result in increased uptake of [3H]FA and [3H]MTX? Confocal microscopic images revealed that expression of the FBP gene was elevated in the KB-CP20 cells, but FBP was not localised at the cell surface. Instead, it was distributed in the cytoplasm. Therefore, it could not function as a cell surface carrier for MTX. In the KB-CP1 cells, FBP induced by DAC was largely localised at the cell surface, explaining the increased uptake of MTX and FA under these conditions.

Taken together, these data suggest the existence of a novel cellular defense system or systems that result in cisplatin resistance and crossresistance to other related and unrelated drugs. Defects in this system, which cause decreased uptake of many compounds, lead to reduced expression and mislocalisation of certain genes, such as transporters, that is, MRPI (Liang et al, 2003) and MRP2-4 (data not shown), carriers, that is, FBP, and genes involved in endocytic pathways and protein trafficking, that is, some small GTPases. In this work, we show that the FBP gene was turned down or off by DNA hypermethylation triggered by selection in cisplatin. Whether DNA methylation is a primary or secondary event is not clear, but demethylation by DAC results in at least partial reexpression and function of some of the gene products. These findings may provide valuable information for the design of...
regimens for cancer chemotherapy as well as improving the ability to detect drug resistance before and after chemotherapy with cisplatin by monitoring changes of DNA methylation status and expression levels of small GTPases and FBPs in clinical tumor specimens.

In an effort to see if cisplatin resistance is related to the overexpression of the copper transporter, we inserted the human copper transporter hCTR1 (Zhou and Gitschier, 1997) into the expression vector pcDNA3.1, then transfected this into CP-s cells. Figure 7 shows killing curves indicating little difference in cells transfected with the control mock vector and two individual hCTR1 expression vectors in levels of resistance to cisplatin.

Recent data from Howell’s lab demonstrated that two P-type of ATPases ATP7A and ATP7B are involved in cisplatin resistance in human ovarian carcinoma cells by modulating cellular pharmacology of cisplatin and other related mechanisms, suggesting that a sequestration and secretory system may exist (Katano et al., 2002, 2003). Therefore, it is possible that there might be at least two pathways for the reduced accumulation of cisplatin and other related chemicals in CP-r cells: impaired uptake and active secretion. These results will help us to understand the multifactorial mechanisms in acquisition and development of cisplatin resistance in human cancer cells.

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Figure 7 Cell killing curves show that there was little difference in cisplatin resistance between two individual stable transfected KB-3-1 cell lines with hCTR1/pcDNA3 and cells transfected with the empty vector in a 3-day assay as described in a previous study (Shen et al., 2000). KB-3-1 cells were described in Figure 1.
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