Cisplatin-resistant bladder carcinoma cells: enhanced expression of metallothioneins

Received: 25 February 1998 / Accepted: 15 July 1998

Abstract Cisplatin is one of the most potent cytotoxic drugs and in chemotherapy has ameliorated numerous tumors. Nevertheless, resistance to cisplatin is a problem that is encountered in the chemotherapy of urologic tumors, especially transitional cell carcinomas. In order to improve definition of the mechanisms of cisplatin resistance we established a series of cisplatin-resistant sublines from the cell line RT 112 in increasing concentrations of cisplatin. The most resistant subline CP3 is approximately 10 times more resistant than the parental line and shows a 10-fold cross-resistance against methotrexate, whereas vinblastine and doxorubicin are equally effective in the parental and sublines. Combined treatment of CP3 cells with cisplatin and buthionine sulfoximine (BSO) does not result in enhanced cell kill, thereby ruling out glutathione as a resistance mechanism. However, in comparison with parental cells, CP3 cells are about 1.5 times more resistant against cadmium. On the protein level, the cisplatin-resistant cells reveal an enhanced expression of metallothionein II (MTII), but not MTI, suggesting that the cisplatin resistance we observed in these sublines is at least partly mediated by MTII. These sublines will in the future serve as valuable tools for the analysis of cisplatin resistance, especially in view of metallothionein-mediated resistance mechanisms.

Key words Transitional cell carcinoma · Cisplatin resistance · Cross-resistance · Methotrexate · Metallothioneins · Capillary electrophoresis

Introduction

Polychemotherapy with methotrexate, vinblastine, doxorubicine and cisplatin (M-VAC) has become established in the treatment of metastatic transitional cell carcinoma (tcc). A response rate of up to 60%, with complete response of approximately 40% has been reported for this type of treatment. Unfortunately, these responses are usually of limited duration and cures are rare. No response may be expected from recurrent tumors after M-VAC therapy as they have become chemoresistant. Several different resistance mechanisms are possibly involved: vinblastine and adriamycine (doxorubicin) are substrates of the membrane-bound efflux pumps P-glycoprotein and multidrug resistance-associated protein (MRP), which remove these agents from resistant cells before they develop toxicity. Doxorubicin exerts its toxic effect by stabilizing complexes between nicked DNA and topoisomerase (topo) II, thus preventing DNA religation. Reduced activity of topo II results in doxorubicin resistance. Amplification and overexpression of the dihydrofolate reductase gene may account for methotrexate resistance.

As cisplatin is probably the most active drug in M-VAC therapy, cisplatin resistance may play a vital role in tumor recurrence. Several properties of cisplatin resistance have been described for different cell compartments. Cisplatin-resistant cell lines have been defined that accumulate less cisplatin than their sensitive counterparts. This may be attributed to the inhibition of an active cisplatin uptake mechanism [6, 18]. Membrane-bound Na/K-ATPase has been shown to be less active in resistant cells, thereby reducing the active uptake. The amount of intracellular cisplatin in sensitive cells may be reduced by 60% [17] following Na/K-ATPase inhibition by ouabain. Reduced glutathione
(GSH), its related enzymes and metallothioneins (MTs) are important cytosolic defense mechanisms that act against various toxic substances. GSH is a radical scavenger that prevents oxidative cell damage. Electrophilic substances as cisplatin are conjugated to GSH by glutathione S-transferases, which, like GSH, were overexpressed in certain cisplatin-resistant cell lines [1, 24]. MTs are small cytosolic proteins of about 6000 to 7000 kDa and are located in the cytosol and nucleus. Their physiological function is probably the regulation of intracellular zinc homeostasis and detoxification of heavy metals. The numerous thiol groups of these proteins enable the binding and detoxification of oxidizing substances. Certain authors described enhanced metallothionein expression, especially of the MTII subtype in cisplatin-resistant cells [12, 14, 19, 27]. A possible role of MT in cisplatin resistance has been demonstrated by the transfection of sensitive cells with a MTII cDNA that confers resistance to the cytotoxic action of this drug [11]. In transitional cell carcinomas, MT expression correlates with the response to cisplatin-based chemotherapy [4, 14]. Recent immunofluorescence studies demonstrated that the resistance to cisplatin correlates with nuclear MT expression and probably prevents oxidative damage to DNA [13].

Cisplatin-resistant cells also possess better tolerance towards cisplatin-DNA adducts [22] or improved repair of cisplatin-induced DNA crosslinks [2, 7, 10, 16, 28]. No explanation exists so far for better tolerance, but several overexpressed DNA-repair genes have been found in cisplatin-resistant cells. ERCC (excision repair cross-complementing) genes belong to the latter category, particularly ERCC1.

In order to further characterize mechanisms of cisplatin resistance in urothelial cancers, we established several cisplatin-resistant sublines of the bladder carcinoma cell line RT112. We examined the cross-resistance profiles to different cytotoxic drugs and measured the expression of MTI and MTII with capillary electrophoresis.

**Material and methods**

Cells and cell culture

RT112 cells were generously donated by Dr. Loerke of the German Cancer Research Center, Heidelberg. The multidrug-resistant cell line RT112/D21 was established in our laboratory by culturing RT112 cells in increasing concentrations of doxorubicin. The cisplatin-resistant cell line CPF was donated by Dr. Schuldes, Department of Urology, University of Frankfurt. All lines were kept in Dulbecco’s MEM medium supplemented with 10% FCS, 1-glutamine and 1% penicillin/streptomycin (10 000 IU/ml, all from GibcoBRL). Medium was changed every third day and the cells were subcultured once a week.

Chemicals and drugs

Methotrexate, vinblastine and doxorubicin were purchased from Sigma.

Establishment of cisplatin-resistant sublines

RT112 cells were first incubated in 0.5 μg/ml cisplatin (Asta Chemicals) for 4 weeks. These cells were then periodically treated with 10 μg/ml cisplatin for 2 hours followed by incubation in 0.5 μg/ml until the cells were again almost 100% confluent. This cyclic treatment was repeated five times over a period of 18 weeks. The cells were then cultured in 1.5 μg/ml cisplatin, thus generating the cisplatin-resistant subline CP1.5. On stable growth, the cisplatin concentration was increased by 0.5 μg/ml every 6 to 10 weeks. Stable sublines were acquired from each concentration increase between 1.5 and 3 μg/ml CDDP and named CP2, CP2.5 and CP3.

Cloning of cisplatin-resistant cells

CP1.5 cells were diluted in medium at a concentration of 100 cells/10 ml. Of this suspension 100 μl were pipetted into each well of a 96-well microtiter plate. The wells that contained only a single cell were marked under the microscope. The clones acquired from these wells were pipetted into a culture flask without cisplatin. Following stable growth, cisplatin was added at a concentration of 1 μg/ml and was continuously increased. By this method, it was possible to establish two stable clones, K₁ and K₂.

MTT cytotoxicity assay

The yellow MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) was reduced in vital cells to a purple formazan precipitate. Cell number was counted on a hemocytometer and 2000 cells were pipetted into each well of a 96-well microtiter plate in 100 μl MEM medium. The cells were left to adhere overnight and cisplatin was then added at increasing concentrations in a volume of 100 μl medium. To achieve glutathione synthesis inhibition, BSO was added together with cisplatin at a concentration of 0.1 mM. After a 10-day incubation period, 20 μl of MTT stock solution (0.05 mg/100 ml) were added to each well. Four hours later, the liquid was removed, the formazan crystals released and then solubilized by the addition of 150 μl DMSO (Merck). The extinction of the purple color, which is directly proportional to the number of viable cells, was measured at a wavelength of 540 nm in an ELISA photometer (Titerk Multiscan Plus MKIII). The percentage of viable cells was calculated by the following formula:

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\% \text{ living cells} = \frac{\text{sample ext} - \text{blank ext}}{\text{control ext} - \text{blank ext}} \times 100
\]

Capillary electrophoresis

After centrifugation, pellets consisting of 5 million cells were lysed in 2.1 ml of 20% trichloric acid. The resulting cell suspension was incubated with 50 μl of sodium solution (0.2 mg/ml) and centrifuged at 400 g for 5 minutes. The supernatant was neutralized with 0.8 ml of 1 M TRIS-HCl pH 7.5 and 2.5 ml of this solution were loaded on PD-10 columns (Pharmacia Biotech) to remove salt and small proteins of less than 2000 Da. The resulting solution was frozen at −80°C and thawed under vacuum after 3 hours. The samples were solubilized in 400 μl demineralized water, filtered with a 30-kDa filter (Ultrafree MC, Millipore) and centrifuged for 16 minutes; 30 μl of the lower phase were used for capillary electrophoresis (PACE 2100, Beckman). The capillary had a total length of 57 cm with a detector window length of 50 cm and an internal diameter of 50 μm. The internal capillary wall was coated with hydroxy propylmethyl cellulose (Sigma) to prevent sticking of the proteins. The buffer was NaH₂PO₄-monohydrate (Merck). The samples were run for 50 minutes at 60 μA. Detection was performed with an UV-detector at a wavelength of 200 nm. The electrophoresis was calibrated with purified mouse MTI.