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Transient cisplatin-resistant murine fibrosarcoma cell characterized by increased metallothionein content

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Abstract. Cisplatin-resistant mouse fibrosarcoma cells, SSK-R, were isolated after short and low-dose drug treatment of the sensitive SSK cells in vitro. These SSK-R sublines exhibit up to sevenfold cisplatin resistance and are characterized mainly by an increased metallothionein content. Loss of drug resistance after about 140–180 cell divisions in drug-free medium coincides with loss of metallothionein content. The glutathione level is the same in the sensitive and resistant sublines; inhibition of glutathione synthesis by buthionine sulphoximine enhances the sensitivity in both cells lines by a factor of 2.7. The resistant sublines are not cross-resistant to radiation; a radiation exposure followed immediately by cisplatin treatment results in an additive effect. The cellular cisplatin content is slightly reduced in SSK-R2 cells and it remains at this level also upon loss of drug sensitivity.

Key words: Cisplatin resistance in vitro – Metallothioneins – GSH – Irradiaton – Heat

Introduction

Cisplatin belongs to a group of cytostatic agents that is widely used in chemotherapy. However, its use is limited mainly by its toxicity to normal cells and the development of drug resistance. Several in vitro models have been developed to study the mechanisms leading to acquired drug resistance and to find methods to prevent or bypass drug resistance. Reported mechanisms include decreased drug accumulation (Richon et al. 1987; Mann et al. 1988; Waud 1987), reduced DNA cross-linking (Teicher et al. 1987; Hospers et al. 1988), an increased contant of glutathione or its related enzymes (Behrens et al. 1987; Sabouri et al. 1989; Meijer et al. 1990), or overexpression of metallothioneins (Kelley et al. 1988; Bakka et al. 1981). Most of the above studies have in common that the mechanisms leading to resistance are multifactorial for each cell system. Results of different cell systems, however, are rather controversial, especially those concerning cross-resistance against different agents of radiation and those describing detoxification of cisplatin by protein- or nonprotein thiols.

In our studies using mouse fibrosarcoma cells, metallothioneins play an important role in the development and reversion of cisplatin-resistant cells. After only a few low-dose drug treatment cycles, various drug-resistant clones have been isolated that exhibit a transient drug resistance upon subculturing in drug-free medium. In the following study, we investigated mainly the mechanisms mediating this cisplatin resistance and the efficacy of combined-modality treatment to bypass cisplatin resistance.

Abbreviations: BSO, buthionine sulphoximine; Cisplatin, *cis*-diamminedichloroplatinum (II); GSH, glutathione; R_F, resistance factor

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Materials and methods

Cell lines and tissue culture

Mouse fibrosarcoma cells (SKK) were induced by treating the skin of CH3 mice with methylcholanthrene; tumour cells have been isolated and established in our laboratory since 1976. The cells are adapted to growth in vivo and in vitro (Kummermehr and Trott 1976). They grow as monolayer culture in Eagle's minimal essential medium (MEA), supplemented with 10% calf serum, 0.01% neomycin, and 0.035% $NaHCO_3$ maintained in a humified CO_2 incubator at pH 7.4 and 37° C. The mean cell cycle time is 12 h.

Cisplatin resistance was generated in vitro either by permanent exposure of 5×10^4–10^5 SSK cells to low cisplatin concentrations (0.2–0.5 μg/ml) or by intermittent drug treatment. In the latter case cells were exposed to 10 μg/ml cisplatin for 1 h and then allowed to grow up to confluence, at which time the treatment was repeated. After only three to five treatment cycles, resistant clones were isolated. Most of these clones either showed transient resistance lasting about 10–15 cell cycles, underwent continuous degeneration with formation of giant cells, or exhibited only very moderate drug resistance with R_F resistance factor, defined as the ratio of the slopes of the survival curves of resistant over parent SSK cells after a 1-h exposure period).

Drug exposure

Exponentially growing cells were subcultured, appropriately diluted and allowed to attach to the glass surface overnight. Exposure to cisplatin (cisplatin solution, Behring, Marburg, BRD) was carried out in culture

medium for 1 h at different drug concentrations. The drug was diluted in Hank's solution and added to the culture medium. After the allotted exposure time the medium was decanted, the cells were rinsed twice with Hank's solution and fresh culture medium was added. When cellular drug efflux was tested, cells were exposed to the calcium antagonists verapamil or nifedipine (both Ratiopharm, Blaubeuren, BRD) simultaneously with cisplatin for 1 h. A 2-μg/ml dose of cisplatin was used for the sensitive SSK cells and isoeffective cisplatin doses for the resistant sublines together with increasing concentrations of calcium channel blockers. Data are corrected for the effect of cisplatin alone.

For inhibition of glutahione (GSH) synthesis, cells were pretreated with 50 μ*M* BSO for 18 h. Cisplatin was then added for 1 h buthionine-sulphoximine medium.

For cadmium chloride ($CdCl_2$) toxicity studies, cells were exposed for 1 h to various concentrations of $CdCl_2$.

Irradiation. For both radiation alone or the combined radiation plus cisplatin treatment the appropriate number of seeded cells were allowed to attach to the glass surface overnight as described for drug exposure. Cells were exposed to graded doses of γ-rays from a gamma-cell 40 caesium-137 source (AECL-Industria, Canada) at a dose rate of 1.2 Gy/min. Immediately after irradiaion, the flasks were returned at 37° C water bath, until the culture medium had regained normal temperature. At that time, i.e. about 10 min after irradiation, cells were treated with cisplatin for 1 h; then the medium was removed and all flasks were treated as described above.

Hyperthermic drug exposure. Immediately before drug treatment, the culture medium was replaced by 37° C MEM containing 50 m*M* HEPES buffer (Boehringer, Mannheim, BRD), to maintain the pH of the medium at pH 7.4 during drug exposure. Cisplatin was added, the culture bottles were stoppered and immediately immersed horizontally into a precision water bath (Julabo, Seelbach, BRD) with proportional temperature control, combined with disturbance-factor compensation and a temperature-control accuracy of ±0.02° C. The intended final exposure temperature of 42° C was achieved within 1–3 min. All other procedures were then continued as described above. The data are corrected for the effect of heat alone. Thermal enhancement ratios were calculated from the ratio of the slopes of the survival curves at 37° C over that at 42° C for each cell line.

Cell survival

Following any of the indicated treatments, cells were incubated for either 8 days (SSK cells) or 10 days (resistant sublines). Then the colonies were stained with methylene blue and those containing more than 50 cells were counted. The ratio of mean colony yield of treated to untreated cells, i. e. the surviving fraction was calculated. All experiments were carried out with four replicate bottles and repeated at least three times. Experimental data were accepted if the colony-forming efficiency of the untreated cells was higher than 35% and χ^2 of all replicates was within a probability of 95%.

Cellular cisplatin concentration

The cellular concentration of platinum was determined with proton-induced characteristic X-ray emission (PIXE) as described in detail by Eichholtz and Hietel (1990). Samples of 10^6 cells were exposed to cisplatin as described above. At the end of drug exposure the cells were rinsed twice with Hank's solution, scraped off the glass with a rubber spatula and centrifuged (1000 g, 10 min). Finally they were lysed by deep-freezing and thawing the pellet twice. The suspension was then transferred onto a filter-paper disc of 5 mm diameter, avoiding any contact with metal that would interfere with the platinum determination. The filter-paper disc was air-dried before measurement. The protein-induced characteristic X-rays emitted by the irradiated sample were determined and analysed by a semiconductor spectrometer. For calibration 20 μl cisplatin, diluted in Hank's solution to the appropriate concentration, was used. The detection limit was 3.5 ng/sample with a tolerance of about 8% at 20 ng/sample and 17% at 10 ng/sample.

GSH and protein determination

Cells in the logarithmic growth phase were used for GSH determination according to Tietze (1969). For protein determination the Lowry assay was used (Lowry et al. 1951).

Metallothionein test

Cells were tested for metallothionein content by binding of radioactive ^{109}Cd to the cytosol followed by fast protein liquid chromatography (FPLC). Samples of 5×10^6 exponentially growing SSK or SSK-R2 cells were harvested by trypsination and washed twice with phosphate-buffered saline (4° C). The cells were disrupted by sonification and then centrifuged (20 000 *g* for 20 min). The supernatant was incubated with trace amounts of ^{109}Cd for 30 min at 37° C. Thereafter the solution was centrifuged (12 000 *g*, 2 min) and filtered through a 0.45-μm membrane filter. Aliquots of the filtrate were subjected to FPLC gel filtration on a Superose-12 column (300×10 mm). Fractions of 0.5 ml wer collected and measured for radioactivity. Cadmium-binding metallothionein was identified by comparison with rabbit liver metallothionein (Sigma).

The conditions for chromatography were as follows: column, 300×10 mm Superose-12; eluent, 0.05 *M* sodium phosphate pH 7.0/0.15 *M* sodium chloride; flow rate, 0.5 ml/min; detector, UV, 280 nm.

Results

Characterization of cisplatin-resistant SSK sublines

Cisplatin sensitivity of SSK and all resistant sublines was determined after 1 h exposure to increasing drug concentrations at 37° C. For all strains tested so far cell survival is an exponential function of drug concentration (Fig. 1). Cisplatin resistance of SSK sublines is modest with RF =3.4 (SSK-R6), 4.7 (SSK-R1) and 7.1 (SSK-R2). Most of the resistant strains gradually lose their cisplatin resistance together with their $CdCl_2$ resistance (see below) and RF decreases to about 2 in SSK-R2 cells after 140–180 cell divisions. Clone R2,

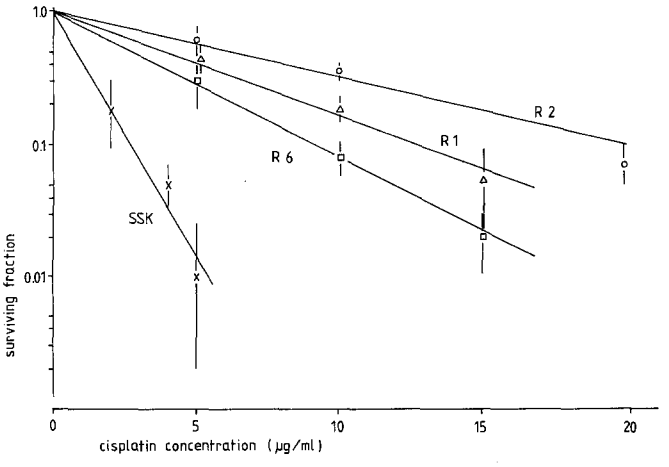

Fig. 1. Survival of parent SSK cells and three cisplatin-resistant sublines as a function of cisplatin concentration (1 h exposure at 37° C). ×, SSK; ○, SSK-R2; △, SSK-R1; □, SSK-R6. Each point represents the mean (±SD) of at least ten dishes from three or more experiments

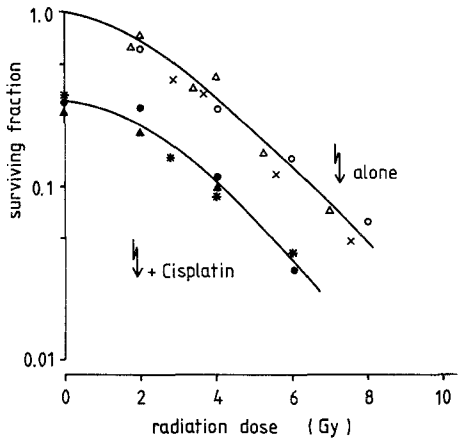

Fig. 2. Survival curves of SSK and resistant cells to radiation alone (○, △, ×) or to combined radiation and cisplatin treatment (●, ▲, *). Drug exposures used: 2 μg/ml, 1 h for SSK cells (×/*) and isoeffective drug concentrations for the resistant strains SSK-R2 (●) and SSK-R1 (▲)

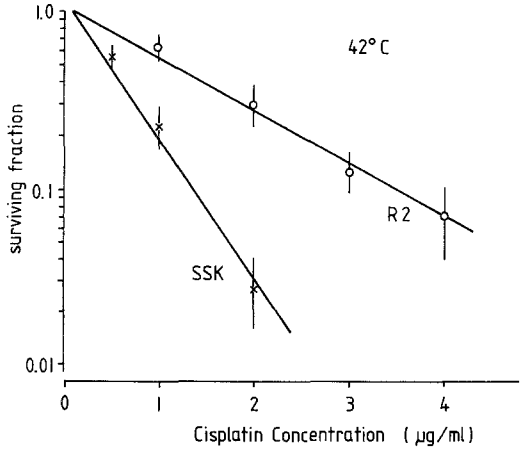

Fig. 3. Cell survival of SSK (×) and SSK-R2 (○) cells after cisplatin exposure under hyperthermia (1 h at 42° C). Results are corrected for the effect of heat alone

which was isolatd after intermittent drug treatment, is further characterized by the fact that it is relatively stable and can be distinguished from the parent strain morphologically – compared to other isolated clones. SSK-R2 cells are elongated, and colonies exhibit fascicular growth, while a typical 2-week-old SSK colony is densely piled up in the centre. Doubling times are longer in SSK-R2 cells (15–18 h compared to 12 h in SSK cells), whereas plating efficiency is in the range of 60%–90% in both strains. The protein content is not significantly different in SSK (161±8 μg/10^6 cells) and in SSK-R2 cells (153±9 μg^6 cells).

There is no cross-resistance to cytostatic agents such as Adriamycin, vinblastine and melphalan or to irradiation. Combined treatment of radiation followed immediately by a 1-h cisplatin exposure results in an additive effect of the two modalities in all cell lines tested (Fig. 2). Cell sensitivity to cisplatin may be enhanced by hyperthermic exposure conditions (42° C, 1 h). Heat alone has the same cytotoxic effect on both cell lines (data no shown); combined heat plus cisplatin treatment increases cell kill in both cell lines (Fig. 3). From the slopes of the survival curves at 37° C (Fig. 1) and 42° C (Fig. 3), thermal enhancement ratios are calculated, showing that the cell sensitivity is enhanced even more in SSK-R2 cells than in the parental SSK cells (the thermal enhancement ratio is 2.1 in SSK cells and 5.2 in SSK-R2 cells).

Possible mechanisms of cisplatin resistance

Drug uptake. Plasma membrane alteration is a common mechanism of cellular resistance, particularly after treatment with alkylating agents leading to reduced intracellular drug levels in the resistant sublines (Richon et al. 1987; Mann et al. 1988).

Cellular cisplatin concentrations were measured in SSK and SSK-R2 cells by PIXE. Regression lines of cisplatin uptake of 10^6 SSK and SSK-R2 cells after 1 h drug exposure are presented in Fig. 4 (correlation coefficient >0.99). Drug uptake is reduced by 37% in the resistant strains compared to

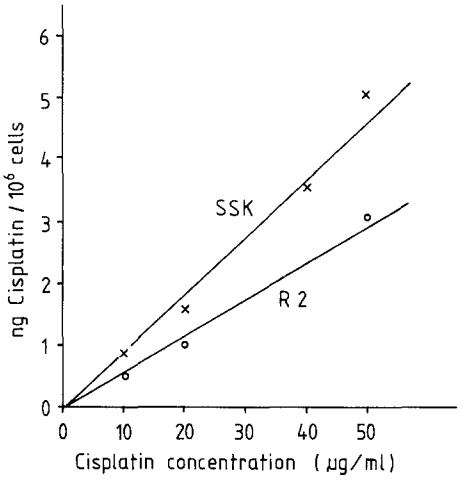

Fig. 4. Cellular cisplatin content of 10^6 SSK (×) and SSK-R2 (○) cells after 1 h exposure to various drug concentrations

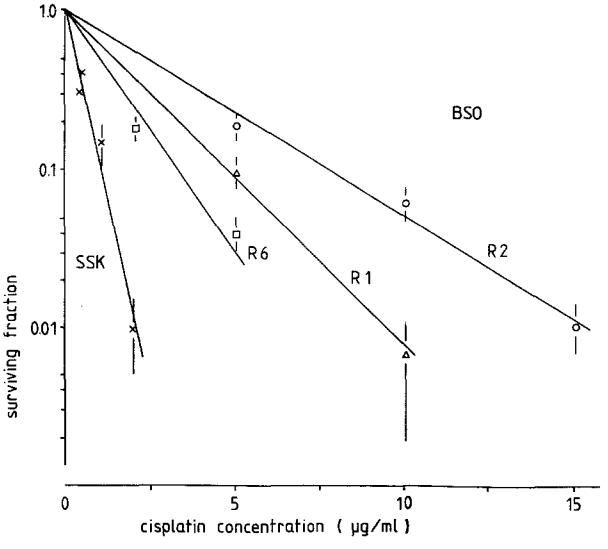

Fig. 5. Cell survival as a function of various cisplatin concentrations for 1 h following 18 h *BSO* treatment (50 μmol). *Symbols* are as in Fig. 1. Results are corrected for the effect of BSO alone; *points without error bars* give the mean of single experiments

SSK cells. When SSK-R2 cells lose their drug resistance after about 140–180 cell divisions, cellular drug concentrations remain unchanged, although RF is reduced from 7.1 to about 2.2–2.6 in the cell-survival assay.

Drug efflux. A major reason for drug resistance may be an increased drug efflux, as described mainly in pleiotropic drug-resistant cells. It is demonstrated for cisplatin only by Onoda et al. (1986) in B16 melanoma cells using calcium channel blockers. Exposure of SSK cells and various resistant sublines to the calcium antagonist nifedipine together with cisplatin tends to increase cell sensitivity slightly, but to almost the same degree in both cell lines and only at high concentra-

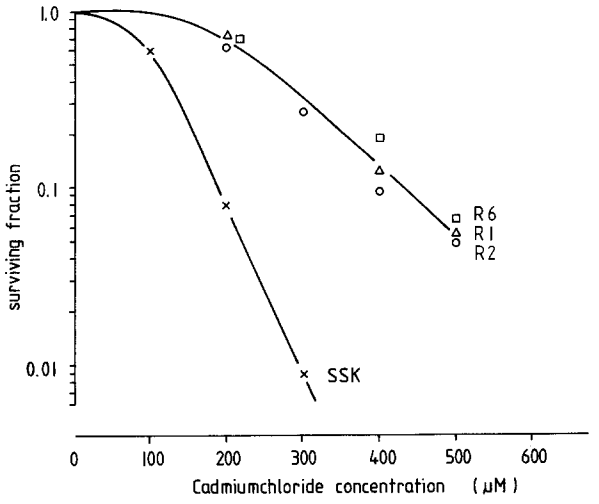

Fig. 6. Cell survival as a function of cadmium chloride concentration after a 1-h exposure. *Symbols* are as in Fig. 1.

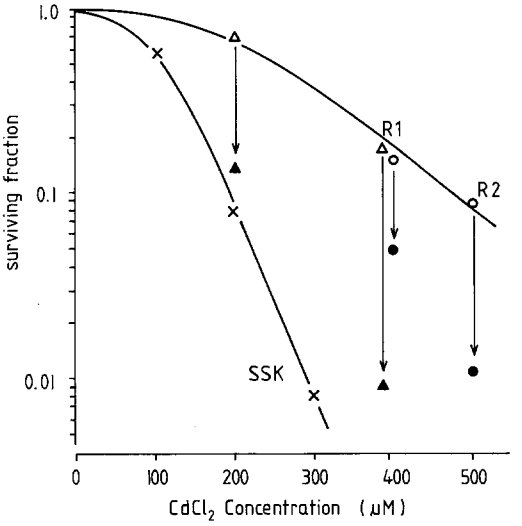

Fig. 7. Cell survival as a function of cadmium chloride concentration after 1-h exposure. Resistant sublines SSK-R1 (△) and SSK-R2 (○); ▲, ●, same sublines, when cisplatin resistance is diminished. ×, SSK cells

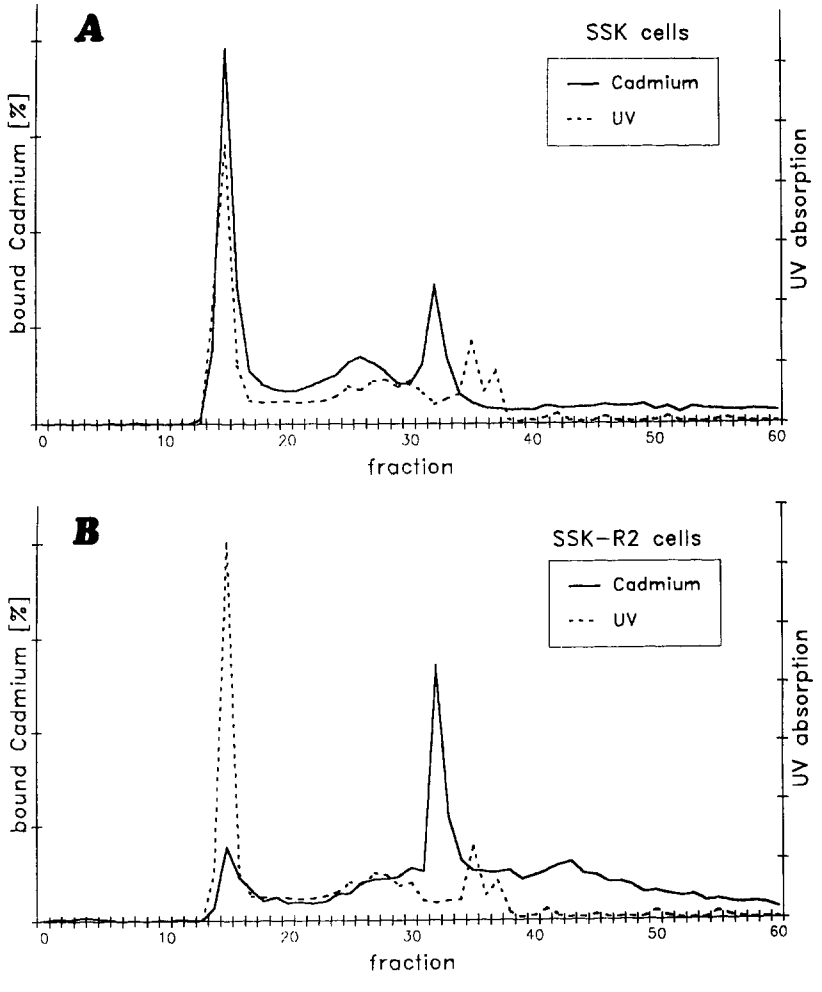

Fig. 8. FPLC chromatograms of ^{109}Cd-labelled cytosol fractions of SSK (**A**) and SSK-R2 cells (**B**) with ^{109}Cd binding (——) and UV absorption (. . .). Fractions 31–33 correspond to the metallothionein region

tions (10 μg/ml, data not shown). The calcium antagonist verapamil exhibits no enhancing effect.

Inhibition of GSH synthesis. Glutathione and its related enzymes alter drug cytotoxicity by a variety of mechanisms, especially detoxification and oxygen scavenging. Depletion of GSH by buthionine sulphoximine, a specific inhibitor of GSH synthesis, may therefore reduce drug resistance. The GSH content is similar in sensitive SSK and resistant SSK-R2 cells (14.5 and 16.5 nmol GSH/mg protein). If GSH synthesis is inhibited by BSO (50 μmol, 18 h), the GSH content is reduced by 85%–90%. If the cells are then exposed for 1 h to various cisplatin concentrations, cell sensitivity is enhanced (Fig. 5). However, the enhancement factors are virtually identical in sensitive and resistant cells, e. g. 2.7 for SSK and R1 cells, 2.6 for R2 and 2.8 for R6 cells.

Metallothioneins. Thiol proteins, such as metallothioneins, also play an important role in drug detoxification. They form complexes with the drug and thus inactivate the agent before it can produce toxic lesions. Metallothioneins can be demonstrated indirectly by measuring the toxicity to cadmium chloride. Decreased cellular toxicity to $CdCl_2$ is an indicator of elevated metallothinoein levels (Bakka et al. 1981; Teicher et al. 1987). Figure 6 shows the dependence of cell survival on $CdCl_2$ concentration for SSK cells and some resistant strains. In SSK-R strains, $CdCl_2$ toxicity is reduced by a factor of 2.3 compared to the parent SSK cells. When SSK-R cells lose their resistance to cisplatin, they concomitantly lose their resistance to $CdCl_2$ and the survival curves gradually approximate those of SSK cells (Fig. 7).

If the cells are pretreated with buthionine sulphoximine to inhibit GSH synthesiis, as described above, and then exposed to $CdCl_2$, sensitivity is increased to almost the same extent in both SSK and SSK-R2 cells (R_F=3.4 and 3.0, data not shown).

Increased metallothionein content of the resistant SSK-R2 cells is also demonstrated directly by specific binding of trace amounts of ^{109}Cd to the cytosolic fractions (Fig. 8). In this qualitative rather than quantitative assay, fractions 31–33 correspond to the metallothionein region (molecular mass=6–10 kDa). These fractions contain almost twice as much activity in the SSK-R2 cells compared to the sensitive SSK cells (19% and 11% respectively of the cumulative activity). In SSK cells, 30% of the ^{109}Cd activity is shifted to the high-molecular-mass region.

Discussion

Cisplatin resistance was induced in SSK cells after only three to five treatment cycles. These are very short and probably clinically relevant drug exposures in contrast to other protocols, where resistant clones were isolatd after treatment times of 14 months (Teicher et al. 1991) or several years (Richon et al. 1987). Resistance is modest, and in all clones that were tested the resistance is only transient.

So far the resistant SSK-R cells have not developed cross-resistance to other cytostatic agents or to ionizing radiation. Since many patients with drug-resistant tumors also receive radiotherapy, data are needed to associate drug resistance with radiation response. Reports on cisplatin resistance and cross-resistance to radiation are conflicting. They range from observed cross-resistance (Behrens et al. 1987; Louie et al. 1985; Schwartz et al. 1988), no effect (Wallner and Li 1987; Mitchell et al. 1988) to collateral sensitivity (Hill et al. 1988). Our studies demonstrate that drug resistance does not necessarily confer radiation resistance. In our cell system radiation may be used to bypass drug resistance, suggesting combined-modality therapy prior to development of drug resistance.

Hyperthermic drug treatment offers another possibility to bypass cisplatin resistance in SSK-R cells: heat does not only enhance drug sensitivity but it seems to increase drug action preferentially in resistant cells. This results in a thermal enhancement ratio that is higher by a factor of 2.5 in SSK-R2 cells compared to the enhancement ratio in the parental SSK cells. Our results are in accordance with those of Mansouri et al. (1989), who even describe a reversal of cisplatin resistance by hyperthermia, and of Teicher et al. (1991), who suggest that hyperthermia may be a membrane-active modulator that may increase the cytotoxic activity in the resistant cells.

Several mechanisms may be responsible for cisplatin resistance in SSK cells. Intracellular detoxification of cisplatin obviously plays an important role in SSK cells. Inhibition of GSH synthesis by BSO may be used to enhance cisplatin action in both sensitive and resistant cells. This will be especially useful if differential effects of GSH inhibition in normal versus tumor tissue are confirmed in various cell systems, as has been described by Russo et al. (1986) or Ozols et al. 1987. However, GSH does not cause cisplatin resistance in SSK-R2 cells, since the enhancement in cisplatin sensitivity after inhibition of GSH synthesis is the same for both sensitive and resistant SSK cells.

Besides this non-protein cellular thiol GSH, there are other cysteine-rich, low-molecular-mass proteins, that might interact with electrophilic metal compounds and thus prevent their binding to target DNA. These metallothioneins are shown directly and indirectly to be involved in the development and loss of cisplatin resistance in SSK cells. Intracellular detoxification of the drug correlates with cell survival: high cisplatin resistance corresponds to decreased $CdCl_2$ toxicity, an indirect measure of elevated metallothionein. A loss of cisplatin resistance coincides with increasing $CdCl_2$ toxicity. Elevated metallothionein levels are also demonstrated directly by increased of ^{109}Cd to the metallothionein region of the cytosol of SSK-R2 cells compared to the sensitive SSK cells. These data demonstrate the dominant role of metallothioneins in the development and loss of cisplatin resistance in SSK cells.

The survival data indicate that there is a second mechanism involved in cisplatin resistance of SSK cells, which operates independently: clones R2 and R6 differ in their cisplatin sensitivity by a factor of 2.1, however, they exhibit the same $CdCl_2$ toxicity (Fig. 1 and 6). For clone R2, the cellular drug content was determined and shown to be reduced by 37% compared to the parent sensitive strain. This clone retained its low cisplatin content also after loss of $CdCl_2$-correlated cisplatin resistance (R_F decreased from 7.1 to about 2). These data show that at least two independent mechanisms are leading to cisplatin resistance in SSK cells.

Several groups have indicated that an elevated methallothionein content is associated with protection

against ionizing radiation and with cross-resistance against alkylating agents. In our SSK-R cells, no change in radiation sensitivity was found in a number of clones with slightly different cisplatin sensitivity. Scavenging of radiation-induced hydroxyl radicals by metallothioneins would require a close proximity of thiols and DNA, as suggested by Kaina et al. (1990). These authors showed that metallothionein-overexpressing transfectants had the same radiosensitivity as the parent cells and concluded that free radicals could not be scavenged efficiently by metallothionein in vivo. The same cells, however, were resistant to some alkylating agents although there was no correlation between metallothionein level and the degree of protection. They assume that these levels are either saturating and/or additional other factors are involved.

Protein thiols are less frequently associated with cisplatin resistance than the more common non-protein thiols and they do not always correlate with the degree of resistance in all cell systems. Thus, Schilder et al. (1990), evaluating cisplatin resistance and expression of metallothionein mRNA in various human ovarian carcinoma cell lines, conclude that there is no causal relationship between metallothionein expression and cisplatin resistance. Their data are in contrast to our findings, which may result from the use of different methods. These authors have used far higher drug doses to induce cisplatin resistance, leading to very high degrees of resistance (a range of 13- to 68-fold resistance compared to maximally 7 in our cells) and also to stable resistance. In their human ovarian cancer cell lines, the degree of resistance is dependent upon the method of selection, which is different from what is observed in our SSK cells, where even higher drug doses or longer exposure times do not result in higher degrees of resistance than presented in Fig. 1. Moreover, these authors show that selection for cisplatin resistance is associated with increase in GSH. This is not true for SSK cells, where GSH depletion increases cisplatin toxicity in both sensitive and resistant cells to the same extent and does not explain differences in cadmium toxicity. Our results are supported by data of Teicher et al. (1987, 1991), who demonstrated an increased metallothionein content in histologically different cisplatin resistant human tumor cell lines. Since these authors tested the stability of resistance for 2 months only, it was not observed whether the metallothionein level was transiently or permanently elevated. Also, Kelley et al. 1988, suggest a relationship between metallothionein and cisplatin resistance and assume that metallothioneins are overexpressed in a number of resistant human cells. Overexpression might be due either to an enhanced rate of gene transcription or to increased messenger RNA stability.

In conclusion, our results show that the dominant factor leading to transient cisplatin resistance in SSK cells is the increase in metallothioneins. Cisplatin resistance may be bypassed by a combined treatment modality with radiation, by drug exposure under hyperthermic temperature or by inhibition of GSH synthesis.

Our sensitive and resistant murine fibrosarcoma cells, which grow in vitro and in vivo, may now be used to study mosaic tumours of defined portions of sensitive and resistant cells and develop a therapeutic model.

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