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Original Paper

Sensitivity of Testis Tumour Cells to Chemotherapeutic Drugs: Role of Detoxifying Pathways

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In contrast to most other types of cancer, metastatic testicular germ cell tumours (TGCT) are cured in most patients using cisplatin-based combination chemotherapy. The biochemical mechanisms underlying this sensitivity have not been defined. Drug detoxification can modulate response to chemotherapy *in vivo* and *in vitro*, and therefore we measured levels of glutathione (GSH), glutathione-S-transferase (GST) and both constitutive and cisplatin- and dexamethasone-induced levels of metallothionein (MT) in five human testis tumour cell lines. The levels were compared with those in five human bladder cancer cell lines and two cell lines with cisplatin resistance acquired *in vitro*. GSH levels were relatively low in the testis tumour cell lines, as might be expected in drug-sensitive cells, and there was a 77-fold increase in GSH levels in the cisplatin-resistant testis tumour cell line. GST levels were similar in the two cell types, while metallothionein levels were relatively high in the testis tumour cell lines. These data indicate that GSH may contribute to the sensitivity of TGCT to chemotherapy, and that GSH expression may be involved in the acquisition of cisplatin resistance in these tumours.

Key words: testis tumour cells, drug sensitivity, GSH, GST, metallothionein
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INTRODUCTION

METASTATIC TESTICULAR germ cell tumours (TGCT) are cured in over 80% of patients using cisplatin-based combination chemotherapy [1, 2]. Similar combinations are used to treat advanced stages of ovarian, bladder and small cell lung cancers [3, 4]. In contrast to TGCT, long-term survival is rare in these other types of cancer. Failure of cisplatin-based chemotherapy is a consequence of drug resistance, either at the time of first treatment or acquired following an initial response.

Cell lines derived from human testicular germ cell tumours are also highly sensitive to cisplatin [5–7], certain other chemotherapeutic drugs [8] and gamma-irradiation [9]. In contrast, bladder cancer cell lines are relatively resistant to cisplatin and other anticancer agents [6, 8, 9], reflecting the difference in the response of testis and bladder cancers in the clinic.

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In experimental systems *in vitro* and *in vivo*, there is evidence that detoxification pathways can be associated with acquired resistance to cisplatin [10]. For example, elevation of glutathione (GSH) levels is associated with cisplatin resistance in human ovarian and small cell lung cancer cell lines [11–13], and depletion of GSH with agents, such as buthionine-S-sulphoxamine, can increase sensitivity to cisplatin [14]. Similarly, upregulation of glutathione-S-transferase (GST) [15–17] or metallothionein [18–20] can be associated with resistance to cisplatin. The aim of this study was to determine whether levels of GSH, GST and metallothionein are related to the inherent differences in sensitivity to DNA damaging agents of testis tumour cells.

MATERIALS AND METHODS

All the cell lines were grown under identical conditions in RPMI 1640 medium (Gibco) supplemented with glutamine (2 mM) and 5% fetal calf serum (Imperial Labs) in a humidi-

fied atmosphere of 5% CO_2 in air. Details of the cell lines are summarised in Table 1 [21–30].

Protein estimation

Pellets of cells (1×10^7–2×10^7 cells) were frozen at –80°C and then mixed with 500 μl of 10 mM Tris–HCl (pH 8.0) per 10 million cells. The samples were sonicated using three 5-s bursts, and then centrifuged at 13000 rpm on an MSE microfuge for 10 min. Protein was measured in the supernatant by the method of Bradford [31], using reagents supplied by Bio-Rad and according to the manufacturer's instructions. Bovine serum albumin was used as a standard.

Glutathione measurement

Cytoplasmic GSH levels were measured by the method of Colgreave and Moldeus [32], with minor modifications. Briefly, pellets of washed cells were resuspended in phosphate-buffered saline (PBS). Aliquots of 250 μl of suspended cells were mixed with an equal volume of 8 mM monobromobimane in 50 mM N-ethylmaleimide, pH 8.0. After incubation for 5 min in the dark at room temperature, 15 μl of 100% trichloroacetic acid were added and the sample stored at 4°C for 30 min. For the estimation of derivatised GSH [33], samples were centrifuged at 13000 rpm for 10 min using a microfuge and 25 μl aliquots of the supernatant applied to a 5 μm Spherisorb ODS column (4.6 × 150 mm, Jones Chromatography, Hengoed, Wales, U.K.), equilibrated with 10% acetonitrile, 0.25% acetic acid (solvent A). High-pressure liquid chromatography (HPLC) was performed using a Waters model 625 pump. The flow rate was 1 ml/min. After 5 min isocratic flow, elution of derivatised GSH was achieved using 75% acetonitrile, 0.25% acetic acid (solvent B) using a convex gradient (curve 6) over 8 min followed by a 4-min flow with solvent B. Fluorescence of eluted products was measured at 480 nm with excitation at 394 nm. GSH concentrations were estimated from a calibration curve generated using reduced glutathione mixed with 1.5 mM dithiothreitol. The lower limit of detection using this assay was found to be 0.5 μmol/l.

Glutathione-S-transferase

Western blotting. Western blotting was performed using antisera raised against human α-, μ- and π-class enzymes. The preparation of the antisera and the technique used for immunoblotting have been described in detail in a previous publication [34]. Immunocomplexes were detected using ^{125}I-labelled protein A (Amersham, Buckinghamshire, U.K.). Radioactivity was detected using a PhosphorImager (Molecular Dynamics). α-, μ- and π-GST purified from human liver (for α and μ) and spleen (for π) were used as positive controls. Immunoblotting of a serial dilution of π-class GST demonstrated a linear response from 160 ng to greater than 10 μg protein. For the determination of isoform expression in cells, lysates were prepared as described above and 10 μg total protein loaded per track. The amount of GST per mg total protein was estimated by comparison of the signal obtained following immunoblotting with that of an internal control of purified protein.

Metallothionein

To investigate the induction of metallothionein, 2×10^{-7} M dexamethasone (Sigma, Poole, U.K.) or 100 ng/ml cisplatin (Sigma) were added to fresh culture medium 20 h prior to harvesting. A 20 h time-point was selected to permit maximal levels of protein to be expressed. For both constitutive and induced levels, approximately 10^7 cells were trypsinised, washed in PBS and TBS (25 mM Tris–HCl, 140 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM Na_2HPO_4) and resuspended in 1 ml 10 mM Tris–HCl, pH 7.4. Metallothionein levels were estimated using the cadmium-haemoglobin affinity method of Eaton and Toal [35]. The lower limit of detection is approximately 0.5 μg/ml.

RESULTS

Constitutive levels of GSH are shown in Table 2. The mean levels of GSH were lower in the testis tumour cell lines. Taking the mean value for the five cell lines of each cell type, the bladder lines contained 2.2-fold more GSH. Sensitivities to cisplatin amongst the testis tumour cell lines were similar,

Table 1. Characteristics of cell lines

Cell line	Doubling time (h)	Origin of biopsy	Prior treatment	[Ref.]
RT112	22	TCC, bladder primary	None	[21]
MGH-U1	20	TCC, bladder recurrence	None	[22]
HT1197	61	TCC, bladder recurrence	None	[23]
HT1376	31	TCC, bladder primary	None	[23]
RT4	37	TCC, bladder recurrence	Radioactive gold	[24]
SuSa	28	Testis primary, embryonal carcinoma, teratoma	None	[25]
833K	22	Testis abdominal metastatis, embryonal carcinoma, teratoma	Methotrexate, actinomycin-D, cyclophosphamide 2 months before biopsy	[26]
GCT27	26	Testis primary, embryonal carcinoma	None	[27]
GH	25	Testis primary	None	[28]
1618K	32	Testis primary, embryonal carcinoma, choriocarcinoma, seminoma	Four cycles vinblastine, cisplatin, bleomycin, five cycles cisplatin, bleomycin, doxorubicin and etoposide	[29]
SuSa-CP	30	SuSa	11 months *in vitro* exposure to increasing concentrations of cisplatin	[30]
RT112-CP	22	RT112	14 months *in vitro* exposure to increasing concentrations of cisplatin	[30]

Table 2. Glutathione (GSH) levels expressed as nmol/mg protein, GST levels expressed as ng/mg protein and metallothionein levels expressed as μg/mg protein in five bladder and five testis cancer cell lines and two cisplatin-resistant derivatives

Cell lines	Cisplatin sensitivity IC_{50} (ng/ml)	GSH (nmol/mg protein)	GST (ng/mg protein)	MT (μg/mg protein)
Bladder cell lines				
RT112	120.8 ± 5.2*	79.3 ± 1.4	36 ± 2	0.22 ± 0.02
MGH-U1	72.3 ± 3.9*	40.0 ± 2.3	20 ± 1	4.47 ± 0.8
HT1197	112.7 ± 5.0*	91.8 ± 10.0	18 ± 4	0.85 ± 0.15
HT1376	85.1 ± 14.5*	45.4 ± 1.1	14 ± 2	2.9 ± 0.7
RT4	113.6 ± 10.1*	45.3 ± 0.2	13 ± 3	0.6 ± 0.06
Testicular cell lines				
SuSa	38.0 ± 2.9*	2.3 ± 0.2	21 ± 1	5.2 ± 1.5
833K	25.3 ± 6.4*	25.5 ± 0.5	17 ± 4	18.7 ± 4.7
GCT27	21.1 ± 5.5*	25.4 ± 0.9	14 ± 1	17.6 ± 3.7
GH	28.3 ± 4.2*	23.3 ± 1.5	12 ± 1	10.5 ± 1.9
1618K	32.6 ± 3.3*	61.6 ± 7.6	14 ± 5	19.5 ± 2.5
Cisplatin-resistant cell lines				
SuSa-CP	87.0 ± 12.0†	176.6 ± 3.6	21 ± 6	33.0 ± 2.0
RT112-CP	638 ± 144†	104.3 ± 5.1	17 ± 6	0.48 ± 0.08

*From Masters and associates [8]. †From Walker and associates [30].

but the one cell line (1618K) derived from a patient who had been treated with cisplatin had a much higher level of GSH, similar to that of the bladder cancer cells.

The amount of GST protein present was determined from the immunostaining for π-GST, as levels of μ- and α-GST were below the level of detection. Levels of GST, expressed as staining intensity on Western blots (see Figure 1), are shown in Table 2. There was complete overlap between the two cell types in levels of GST and no correlation with sensitivity to cisplatin. One anomalous finding was that the testis tumour cell line 1618K contained an intermediate level of GST by Western blotting.

Constitutive levels of metallothionein are shown in Table 2. Mean levels were 7.9-fold higher in the testis tumour cell lines. These findings were the reverse of expectation, as we had thought that MT might be present at higher levels in the bladder cancer cells, protecting them from cisplatin toxicity. However, levels of MT in the individual cell lines did not correlate with cisplatin sensitivity.

The influence of cisplatin and dexamethasone on the induction of metallothionein is shown in Table 3. In general, there was little or no upregulation of metallothionein, with the exception of SuSa, in which MT levels increased 2-fold following exposure to each of the inducing agents. In contrast, in the cisplatin-resistant subline of SuSa, both inducing agents caused a downregulation of MT expression.

The levels of GSH, GST and metallothionein in the two cell lines with cisplatin resistance acquired *in vitro* are shown in Tables 2 and 3. Both cell lines are approximately 4-fold more resistant to cisplatin than their parental cells. In the cisplatin-resistant subline of the testis line SuSa, there was a 77-fold increase in GSH and a 6.3-fold increase in metallothionein levels. In contrast, levels of GSH and metallothionein were similar in the cisplatin-resistant subline of RT112 to those of the sensitive parental cells.

DISCUSSION

TGCT provide a paradigm for the cure of metastatic disease, but the mechanisms underlying sensitivity to chemotherapy are unknown. Among the factors that have been studied are topoisomerase II levels [36], mutation frequencies [37], DNA repair capacity [38, 39], O^6-alkylguanine-DNA-alkyltransferase activity [40] and cisplatin-induced DNA damage recognition [41]. This study has demonstrated that glutathione levels are generally low in testis tumour cells, and may be a contributory factor to their sensitivity to DNA damaging agents.

Raised GSH levels can be associated with acquired resistance to cisplatin [13] and other anticancer agents, and depletion of GSH by pretreatment with buthionine sulphoximine (BSO) can sensitise cells to cisplatin [42]. BSO depletion of GSH might also enhance cisplatin sensitivity through inhibition of DNA repair [43] or possibly through an interaction with the multidrug resistance-associated protein [44]. However, the degree of protection afforded by GSH is variable [44]. In this study, the mean levels of GSH were 2.2-fold higher in the more resistant bladder cancer cells. With regard to acquired resistance, the cisplatin-resistant testis subline showed a 77-fold increase in GSH levels, while the cisplatin-resistant bladder cancer subline showed only a 1.3-fold increase. In other cisplatin-resistant sublines of human testicular tumours, no change in GSH levels [45] or a 50% increase [46] has been reported. Similarly, in a series of sublines of the testis tumour cell line, SuSa, made resistant to etoposide by *in vitro* exposure, which were also cross-resistant to cisplatin, no change in GSH levels has been reported [47].

The relationship of GST levels to drug resistance is variable [10], and in some cases higher levels of GST are observed in cisplatin-resistant cells. In 3T3 cells transfected with GST-π, resistance to doxorubicin has been observed, but cisplatin sensitivity was unaffected [48]. In our study, little difference in GST expression was found between the two cell types, and only π-class (acidic) GST was detectable by immunostaining. In all samples from a wide variety of normal and neoplastic human tissues, GST-π mRNA [49] and protein [50] have been shown to be uniformly present at similar concentrations, with this being the most abundant isozyme [50]. However, GST-α and -μ have also been found in most of the cell lines, although at a much lower concentration [50]. Immunostaining of human testis tumours has also shown that all stained for

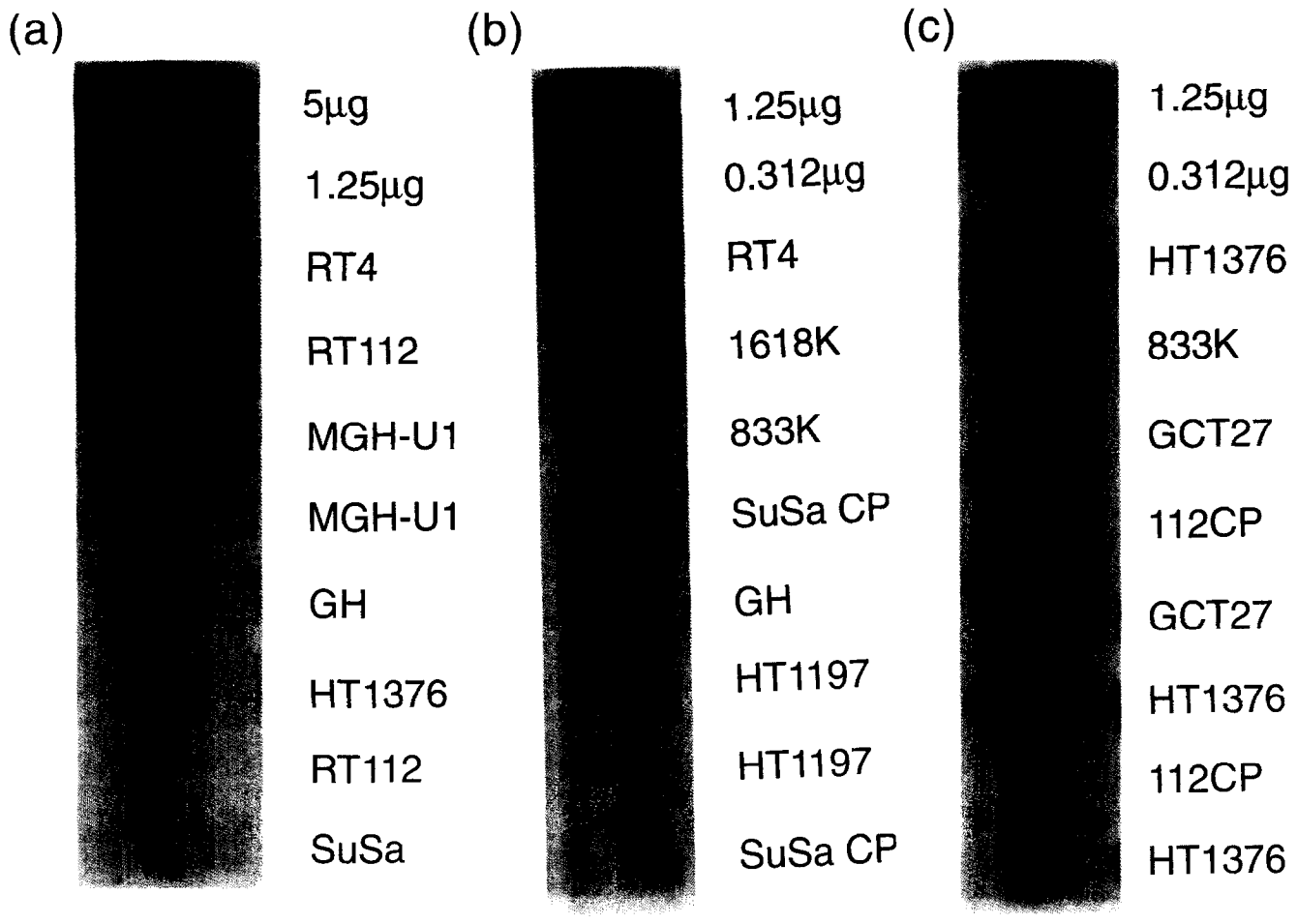

Figure 1. Three Western blots stained for GST-π. The first two lanes were loaded with purified GST-π protein.

Table 3. Constitutive and induced levels of metallothionein. The figures in parentheses show the fold induction

Cell line	Constitutive (μg/mg protein)	Cisplatin-induced (μg/mg protein)	Dexamethasone-induced (μg/mg protein)
RT112	0.22 ± 0.02	0.25 ± 0.06 (1.1)	0.25 ± 0.05 (1.1)
MGH-U1	4.47 ± 0.8	4.6 ± 0.6 (1.0)	4.2 ± 0.3 (0.9)
HT1197	0.85 ± 0.15	Not detectable	0.75 ± 0.35 (0.9)
HT1376	2.9 ± 0.7	2.5 ± 1.0 (0.9)	6.4 ± 2.4 (2.2)
RT4	0.6 ± 0.06	1.1 ± 0.18 (1.8)	0.55 ± 0.09 (0.9)
SuSa	5.2 ± 1.5	10.2 ± 0.9 (2.0)	9.5 ± 1.2 (1.8)
833K	18.7 ± 4.7	12.9 ± 1.2 (0.7)	17.9 ± 3.4 (1.0)
GCT27	17.6 ± 3.7	18.4 ± 1.9 (1.0)	21.3 ± 4.4 (1.2)
GH	10.5 ± 1.9	9.6 ± 0.6 (0.9)	9.1 ± 0.7 (0.9)
1618K	19.5 ± 2.5	14.3 ± 1.5 (0.7)	11.3 ± 2.7 (0.6)
SuSa-CP	33 ± 2.0	12.2 ± 0.76 (0.4)	13.5 ± 0.5 (0.4)
RT112-CP	0.48 ± 0.08	1.25 ± 0.15 (2.6)	1.15 ± 0.75 (2.4)

GST-π [51, 52]. Some of these also stained for GST-α, depending on the pattern of cell differentiation, and microsomal GST and GST-μ [52]. In keeping with the findings of this study, immunocytochemical staining for GST-π did not correlate with response to cisplatin-based chemotherapy in bladder cancers [53].

Resistance to cisplatin and alkylating agents *in vitro* and *in vivo* is sometimes associated with overexpression of metallothionein, and this is often also associated with cross-resistance to cadmium [18, 54–56]. Cadmium is toxic to the testis of the mouse and several other species at concentrations which cause little or no toxicity to other tissues [57, 58], so it was predicted that this might be associated with low levels of metallothioneins in testis cells. However, the testis lines, all of which are sensitive to cisplatin, contained higher constitutive levels of metallothioneins than the more cisplatin-resistant bladder cancer cell lines, in agreement with Sark and associates [11]. In keeping with these observations, immunocytochemical

staining of human testicular tumours indicated that non-seminomas stain heavily for metallothionein, with increasing amounts in more advanced tumours [59]. Subcellular distribution of metallothionein differs between cell types, and may be more important than metallothionein levels in cellular protection against cisplatin damage [60].

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