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The relationship between nuclear glutathione levels and resistance to melphalan in human ovarian tumour cells

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Endogenous levels of thiols, such as glutathione (GSH), have been suggested as important determinants of the response of cultured human tumour cells to various chemotherapeutic agents, including alkylating agents [1, 2], platinating agents [3] and anthracyclines [4]. *In vitro* studies on cultured human and rodent cell lines have demonstrated that modulation of GSH levels by agents such as buthionine sulphoximine(BSO) [1] or oxothiazolidine(OTZ) [5], can significantly alter the chemosensitivity of human tumour cells. However, cellular GSH levels have not been shown to correlate closely with the observed degree of chemosensitivity in human tumour cells following BSO pretreatment [1, 2]. One report has shown GSH levels to be depleted to a lesser extent in the nucleus than in the cytoplasm following BSO pretreatment [6]. In this study, the relative importance of the nuclear and cytoplasmic thiol pools in determining the chemosensitivity of the human ovarian adenocarcinoma OAW42 cell line, and the melphalan-resistant (OAW42/MER) subline was examined.

Material and methods

Cell lines. The origins of the human ovarian adenocarcinoma parental cell line, OAW42, have been previously described [7]. Melphalan resistance was induced by the addition of melphalan to the growth media, initially every 10 days, and the surviving sub-clone was termed OAW42/MER. The frequency of drug administration was gradually increased to every 7 days, and the concentration of melphalan was subsequently raised in increments of 0.1 μg/mL to a final concentration of 1.0 μg/mL (3.2 μM). Resistance was maintained by the weekly administration of 1.0 μg/mL of melphalan to the growth media. The parental OAW42 line, and the OAW42/MER cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum, together with 10 units/mL insulin (the Sigma Chemical Co., Poole, U.K.). The cell cultures were sub-cultured every 3 days to ensure exponential growth.

Modulation and determination of glutathione levels. Total cellular glutathione was extracted as previously described [1], except that cell suspensions were sonicated until all cells were disrupted. Isolated nuclei were obtained by incubating 1×10^6 cells in a final concentration of 65 μM digitonin (Sigma) for 10 min (20° in the dark), followed by centrifugation for 5 min at 40 *g*. The centrifuged pellet was resuspended in PBS, and thereafter treated as the whole cell preparations. GSH levels were determined using the DTNB/GSH-reductase method [8], and expressed relative to cell number. Modulation of GSH levels was achieved by an 18 h incubation of the cell cultures in a final concentration of 50 μM BSO, conditions previously shown to be non-cytotoxic but capable of achieving significant reduction in GSH levels in human ovarian tumour cells [12]. The clonogenicity of our cell lines following BSO pretreatment were found to be 93.2 ± 6.6% (OAW42), and 96.2 ± 4.6% (OAW42/MER) of control cultures.

Cytotoxic drug exposure and clonogenic cell survival assay. Exponentially growing cell cultures, in appropriate cases following pretreatment with BSO or OTZ, were trypsinized in the presence of EDTA, washed twice in Dulbecco's PBS, resuspended in fresh culture media, and seeded at densities of between 10^2 and 10^4 cells in 60-mm petri dishes, and the cell cultures exposed to various concentrations of melphalan (Alkeran, Wellcome Foundation Ltd., Crewe, U.K.). After 2 hr at 37° in the dark, and in appropriate cases in the presence of 50 μM BSO, unincorporated drug was removed by washing the cells twice with PBS (previously equilibrated to 37°), following which 5 mL of Hams F12 media with 15% FCS was then added to each culture. After 12 days at 37° in humified 5% CO_2, the colonies were fixed in cold (4°) 70% ethanol, stained with 10% Giemsa, and colonies containing greater than 100 cells counted.

Flow cytometry. Cellular DNA content was determined as previously described [9], and cell cycle analysis was carried out according to the programme written by M. G. Ormerod and A. W. R. Payne (Institute of Cancer Research, Sutton, U.K.), based on an algorithm developed by J. V. Watson (MRC Centre, Cambridge, U.K.) [10].

Results and discussion

The responses of OAW42, and OAW42/MER cells to melphalan alone, or after BSO pretreatment are depicted in Fig. 1. The parental OAW42 line exhibited a biphasic response to melphalan (Fig. 1A). Linear regression analysis of the data points over the dose range 0 to 4 μg/mL yielded a D_{10} of 2.5 μg/mL, while similar analysis of dose points above 4 μg/mL resulted in a D_{10} of 8.04 μg/mL. The melphalan resistant line showed a single exponential response to melphalan (Fig. 1B) with a sensitivity (D_{10} = 9.08) similar to the resistant subpopulation of the parental line. It is therefore reasonable to conclude that repeated melphalan treatments selected out the resistant subpopulation of the OAW42 line. Comparison of the cellular DNA content profiles of the two lines show that the melphalan-resistant OAW42/MER line consists of a near-tetraploid (4.1C) population, whereas the parental OAW42 cell line is polyclonal with a near triploid (3.15C), and a near tetraploid

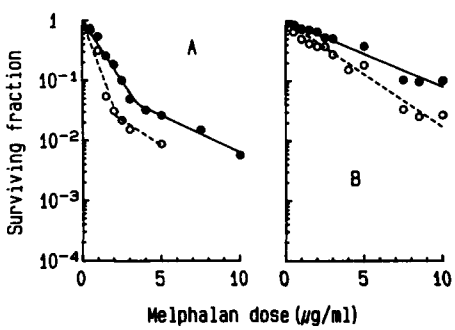

Fig. 1. Melphalan dose–response curve of OAW42(Panel A), and OAW42/MER(Panel B) cells, with (○) and without (●) BSO pretreatment. Each point represents the mean of at least six experiments.

Table 1. The effect of BSO on cellular and nuclear GSH levels

	Cellular GSH (pmol/10^6 cells)	Cytoplasmic GSH (pmol/10^6 cells)	Nuclear GSH (pmol/10^6 cells)
OAW42	4680.3 ± 299.0	4148.2	532.0 ± 52.9
OAW42 + BSO	532.0 ± 52.9	375.3	272.9 ± 3.0
OAW42/MER	8547.5 ± 579.5	7744.2	801.5 ± 113.2
OAW42/MER + BSO	720.1 ± 60.0	409.3	310.8 ± 42.9

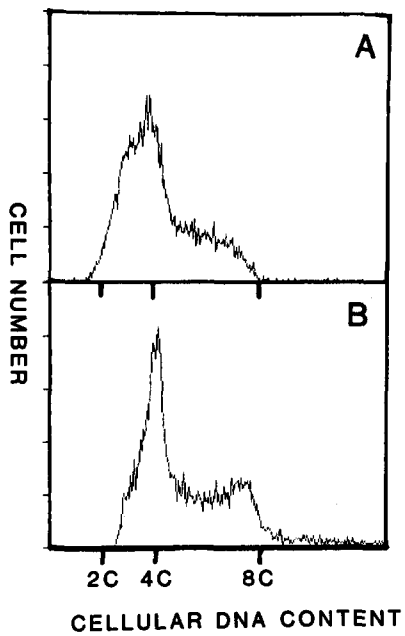

Fig. 2. Cellular DNA content profiles of (A) OAW42 cells and (B) OAW42/MER cells.

(4.1C) population (Fig. 2). Pretreatment with BSO appeared to sensitize both sub-populations of the parental OAW42 line ($D_{10}S = 1.4\ \mu g/mL$, $D_{10}R = 5.8\ \mu g/mL$), representing an enhancement factor of 1.7 and 1.4, respectively, whilst BSO pretreatment increased the sensitivity ($D_{10} = 5.64\ \mu g/mL$) of OAW42/MER cells to a similar level as the parental line.

The decreased sensitivity of the OAW42/MER cell line to melphalan was associated with a concomitant 1.8-fold increase in cellular GSH, and 1.5-fold increase in nuclear GSH content compared to the parental OAW42 line (Table 1), which is in accordance with previous data in other melphalan-resistant human tumour cell lines [1, 11]. However, following exposure to BSO, the altered cytotoxicity of melphalan did not correlate well with the changes in total cellular GSH levels, a discrepancy previously reported in murine cells [12, 13]. BSO exposure depleted cellular GSH levels to 14% (OAW42) and 8% (OAW42/MER) of control levels, the final GSH concentration being 1.1-fold greater in OAW42/MER cells than OAW42 cells.

In contrast, BSO pretreatment reduced nuclear GSH levels to 51% of initial levels in OAW42 and to 38% in OAW42/MER cells. Despite a 1.6-fold and 1.3-fold greater depletion of cellular and nucleic GSH levels by BSO in the OAW42/MER cell line compared to the parental OAW42 line, the final cellular and nuclear GSH levels in OAW42/MER were similar to those in OAW42 cells.

The present studies emphasize the importance of nuclear GSH levels in determining the cellular response to alkylating agents. GSH has been implicated in the repair of radiation induced [14] and *cis*-platinum induced DNA damage [15], and it may be that nuclear GSH levels may also be involved in the repair of drug-induced DNA damage. The closer association between changes in intranuclear GSH levels and the dose-modification factor could however be an epiphenomenon: melphalan is believed to be detoxified by glutathione-*S*-transferase catalysed conjugation reactions with GSH [16], yet these enzymes are primarily located within the cytoplasm. It is therefore difficult to suggest why nuclear GSH levels should be the more important determinant of alkylating resistance. Following BSO pretreatment, the proportion of total cellular GSH levels within the nucleus increased by 4-fold (10% to 42%), although the absolute levels fell by 2–2.5-fold. The apparent importance of nuclear GSH levels after BSO pretreatment may therefore reflect the greater relative contribution of this pool to the cellular GSH mediated detoxification mechanisms.

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Role of *N*-methyl-D-aspartate (NMDA) receptors in the response of extrapyramidal neurotensin and dynorphin A systems to cocaine and GBR 12909

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Changes in the activity of dopamine (DA) receptors substantially alter the cerebral concentration of neurotensin (NT) and dynorphin A (Dyn). The administration of a dopaminergic D-1 receptor agonist, SKF 38393, increases the concentration of neurotensin-like immunoreactivity (NTLI) in the striatum of the rat, whereas the D-2 receptor agonist, LY 171555, reduces the NTLI content [1]. SKF 38393 treatment also increases the concentration of nigral dynorphin-like immunoreactivity (DLI) [2]. Interestingly, because of their ability to enhance dopaminergic activity, the DA uptake blockers, cocaine and GBR 12909 [1-(2-[bis(4-fluorphenyl)-methoxy]-ethyl-4-(3-phenyl-propyl)piperazine], as well as the DA releaser, methamphetamine, cause substantial increases in striatal and nigral levels of NTLI and DLI [3–7].

Several studies suggest an interaction between the dopaminergic and glutamatergic systems. Glutamate can induce DA release [8, 9] while DA can alter glutamate reuptake [10] and release [11, 12]. Moreover, the release of both DA and glutamate is required for the development of postischemic damage in the neostriatum [13]. Consequently, the glutamatergic system could participate with the dopaminergic system in the regulation of peptidergic systems. This is supported by the ability of MK-801 ([+]-5-methyl-10,11-dihydro-5*H*-dibenzo [*a*,*d*]-cyclohepten-5,10-imine maleate; dizocilpine), a noncompetitive antagonist at the *N*-methyl-D-aspartate (NMDA)-type glutamatergic receptor, to prevent the methamphetamine-induced increase in striatal NTLI and DLI concentrations [14, 15]. Although the mechanisms vary, administration of DA uptake blockers causes increases in levels of extrapyramidal NTLI and DLI much like methamphetamine; thus, the objective of this study was to determine the role of NMDA receptors in the cocaine- and in the GBR 12909-induced changes in the extrapyramidal neuropeptide systems.

Materials and Methods

Male Sprague–Dawley rats (180–230 g) were housed six per cage in a temperature-controlled room with a 12-hr light/dark cycle and given access to food and water *ad lib*. Animals received five intraperitoneal injections of cocaine (30 mg/kg/dose; National Institute on Drug Abuse, Rockville, MD) dissolved in saline or of GBR 12909 (20 mg/kg/dose; NOVO Industri A/S, Copenhagen, Denmark) dissolved in propylene glycol at 6-hr intervals 15 min after an intraperitoneal administration of 0.9% NaCl or MK-801 (1.7 mg/kg/dose; Merck, Sharp & Dohme, Rahway, NJ) dissolved in saline. The drug doses are expressed as the free base. The animals were killed by decapitation 1 hr after the last dose, which is the time of maximum peptide response to cocaine [5]. The brains were removed rapidly and the striatum was dissected out on a cold plate. After freezing the brain on dry ice, the substantia nigras were excised with a microdissecting knife. The tissues were stored at −80°C until assayed.

The response of the NT and Dyn systems was assessed by measuring the tissue concentrations of NTLI and DLI according to previously reported techniques [3, 4] employing selective NT and Dyn antisera in a radioimmunoassay