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Overexpression of glutathione S-transferase A1-1 in ECV 304 cells protects against busulfan mediated G2-arrest and induces tissue factor expression

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1 The antineoplastic drug busulfan is frequently used in preconditioning regimens for bone marrow transplantation. Pharmacokinetics vary tremendously between patients due to extensive metabolism in the liver *via* conjugation to glutathione catalysed by glutathione S-transferase (GST) A1-1. Since elevated busulfan plasma levels have been reported to be a risk factor for developing veno-occlusive disease (VOD), metabolism of busulfan may play a pivotal role in the induction of VOD.

2 Therefore, we developed a cell model to investigate the influence of busulfan metabolism on its biological effects. GSTA1-1 cDNA was transfected into the cell line ECV 304 and protein expression was demonstrated by Western blotting. Enzymatic activity could be detected by formation of tetrahydrothiophene. Additionally, effects of busulfan treatment on cell cycle and expression of tissue factor have been investigated.

3 A busulfan-induced G2-arrest was reduced in GSTA1-1-transfected cells, which consequently displayed a significantly higher activity of cdc2 kinase (24.1 ± 1.5 AU mg^{-1} protein) after busulfan treatment compared to controls (14.7 ± 2.3 AU mg^{-1} protein; $P < 0.01$). Elevated basal expression of tissue factor in GSTA1-1-transfected ECV 304 cells could be 4 fold increased by busulfan treatment.

4 These data demonstrate that ECV 304 cells transfected with GSTA1-1 provide a valuable tool to assess busulfan metabolism *in vitro*. Furthermore, overexpression of GSTA1-1 leads to a partial protection against cell cycle effects of busulfan and affects tissue factor expression.
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Abbreviations: GST, glutathione S-transferase; THT, tetrahydrothiophene; VOD, veno-occlusive disease

Introduction

Bone marrow transplantation is a well-established therapeutic approach in the treatment of acute and chronic leukaemia. Preconditioning therapy is necessary to eliminate pathologic tissue and create space for the receiving bone marrow. The antineoplastic drug busulfan is frequently used in preconditioning treatment alone (Olavarria *et al*., 2000) or in combination with mitoxantrone, melphalan, thiotepa, etoposide or cyclophosphamide (Khalil *et al*., 1995; deMagalhaes Silverman *et al*., 1997; Schiffman *et al*., 1997; Zander *et al*., 1997).

Unlike total body irradiation, which suppresses overall growth in prepubertal children, conditioning regimens containing busulfan are particularly valuable in the treatment of paediatric patients (Giorgiani *et al*., 1995). However, the risk of developing severe adverse side effects must not be underestimated. One major side effect, which is correlated with high-dose oral busulfan therapy, is veno-occlusive disease (VOD) of the liver. Among patients treated with high-dose busulfan, approximately 20% develop VOD and half of the patients displaying a severe form of this disease do not survive (Grochow *et al*., 1989). Initial events in VOD include endothelial damage and the release of numerous factors resulting in activation of the coagulation cascade (Bearman, 1995). Among these, tissue factor may play a pivotal role since this protein is involved in both the intrinsic and the extrinsic pathway of the coagulation cascade (Kirchhofer & Nemerson, 1996).

Many studies have been undertaken to investigate possible risk factors for developing VOD. Injury of the liver, resulting mainly from earlier antineoplastic treatments, is a well established risk factor (Rozman *et al*., 1996). In addition, although the literature is controversial, increased busulfan plasma levels have been implicated as a risk factor. Grochow *et al*. (1989) identified busulfan plasma levels above which the incidence of developing VOD was significantly increased. Although subsequent studies have confirmed this observation, others have failed to show a correlation between plasma

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levels of busulfan and VOD (Dix *et al*., 1996; Vassal *et al*., 1996).

If busulfan plasma levels influence the development of VOD, it is important to note that pharmacokinetics of busulfan show a wide interindividual variability. For instance, given the identical oral dose of the substance, busulfan plasma levels vary by a factor of 10 (Grochow *et al*., 1989). Even if applied intravenously, an up to 4 fold variation in plasma levels occurs (Schuler *et al*., 1998). In fact, busulfan undergoes extensive metabolism in the liver by conjugation with glutathione (Ehrsson *et al*., 1983). This reaction is catalysed by cytosolic glutathione S-transferases (GST), which have been divided into alpha, mu, pi, theta, sigma, zeta and omega classes (Hayes & Pulford, 1995; Board *et al*., 1997; 2000). Among the alpha class the homodimeric A1-1 form has the highest affinity for busulfan (Czerwinski *et al*., 1996; Ritter *et al*., 1999). Conjugation with glutathione is an effective mechanism to metabolically detoxify a number of alkylating agents, such as chlorambucil, melphalan, phosphoramide mustard, or thiotepa (Dirven *et al*., 1995; Cnubben *et al*., 1998; Paumi *et al*., 2001). On the other hand, the same reaction bioactivates numerous carcinogens, such as dihalogenated alkanes (Inskeep & Guengerich, 1984), which show structural similarities to busulfan. Conjugation of busulfan with glutathione results in a positively charged sulfonium ion that is highly unstable under physiological conditions (Hassan & Ehrsson, 1987a). The sulfonium ion is cleaved to the lipophilic compound tetrahydrothiophene (THT), which undergoes a series of oxidation reactions. THT and its oxidation products do not have any toxic effects in a CHO-viability assay (Hassan & Ehrsson, 1987b). Thus, whether busulfan, the sulfonium ion or both are responsible for the treatment-related side effects, remains an open question.

In order to investigate whether the metabolism of busulfan contributes to its toxicity, we generated an endothelial cell model in which busulfan metabolism is simulated, resulting in the generation of the unstable sulfonium ion. Cytotoxicity of busulfan was determined using viability assays and cell cycle analysis. In addition, influence of busulfan on functional procoagulative parameters was investigated by measuring tissue factor expression using ELISA.

Methods

Materials

ECV 304 cells were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). Medium 199 and foetal calf serum were from Gibco (Karlsruhe, Germany). Busulfan, glutathione, MTT, 2-ethylthiophen, tetrahydrothiophene and propidium iodide were supplied by Sigma (Deisenhofen, Germany), Pefabloc® was from Roth (Karlsruhe, Germany) and RNaseA from Roche Diagnostics (Mannheim, Germany). The rabbit polyclonal antisera directed against human GST alpha, mu and pi were supplied by NOVO Castra Laboratories (Newcastle upon Tyne, U.K.) and the antiserum directed against human GFP was from ALEXIS Biochemicals (Grünberg, Germany). The transfection vector pTracer-SV40 containing green-fluorescent protein (GFP)- and Zeocin-resistance-genes was purchased from Invitrogen (Groningen, Netherlands) and human GSTA1-1 full-length cDNA was kindly provided by J.D. Hayes (Dundee, U.K.).

Cloning methods

GSTA1-1 full-length cDNA was cloned into pTracer-SV40 by standard cloning procedures (Sambrook *et al*., 1989). Recombinant *E. coli* clones (XL-1 blue, Stratagene, La Jolla, CA, U.S.A.) were identified by antibiotic selection with Zeocin™ (Invitrogen, Groningen, Netherlands) and sequenced by the Thermo Sequenase Cycle Sequencing Kit of Amersham Lifescience (Little Chalfont, U.K.).

Cell culture and transfection

ECV 304 cells were grown in medium 199 supplemented with 10% foetal calf serum. Cultivation was carried out at 37°C in an atmosphere containing 5% CO_2. For treatment with busulfan cells were seeded in six-well dishes at a density of 2.5×10^4 cm^{-2}. Medium was changed after 3 days of culture and compounds (dissolved in DMSO) were added at a final DMSO concentration of 0.1%. For detection of GST by Western blotting cells were scraped, resuspended in lysis buffer (20 mM Tris-HCl, pH 7.4, 0.2% Triton X-100, 1 mM Pefabloc®) and incubated on ice for 30 min with several intermediate-mixing steps. After centrifugation (5 min at 13,000 r.p.m. in a table top centrifuge) protein concentration of the supernatant was determined by the bicinchoninic acid method (Smith *et al*., 1985).

Subconfluent (approximately 80%) ECV 304 were transfected with 10 μg of the plasmid pTracer-SV40 containing the GST A1-1-gene in serumfree medium using positively charged liposomes (Lipofectin, Gibco, Karlsruhe, Germany) with a DNA : Lipofectin ratio of 1 : 9. Incubation time was 10 h and selection was started after 72 h.

Western blotting

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and Western blot analysis of ECV 304 cell lysates were carried out following standard protocols (Harlow & Lane, 1988). Briefly, 50 μg of total protein were subjected to 12.5% SDS gels, transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany) using a semidry blotter (Bio-Rad, München, Germany) and incubated with rabbit polyclonal antisera against GST alpha, mu, pi and GFP (diluted 1 : 5000) for 1 h at room temperature. As second antibodies, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins (DAKO, Hamburg, Germany) were used.

Determination of GST A1-1 activity

Busulfan was used as substrate and glutathione as cosubstrate for the conjugation reaction. The resulting sulfonium ion was hydrolytically cleaved into tetrahydrothiophene (THT) and quantified as described previously (Ritter *et al*., 1999). Briefly, cells in a density of 2.5×10^4 cells cm^{-2} were incubated with 600 μM busulfan and 1 mM glutathione up to 6 h. THT concentration in cell lysates was measured by gas-chromatography using a HP-5 MS column followed by mass selective detection. Calibration samples consisted of

heat-inactivated cell lysates and standard solutions of THT and 2-ethylthiophen as internal standard. Calibration curves were linear over a concentration range of 10 ng ml^{-1} to 1000 ng ml^{-1}.

Cell proliferation and cell cycle analysis

Cell proliferation was investigated by MTT-test. Briefly, cells were seeded into 96-well-plates and incubated with 10 μl MTT solution (10 mg ml^{-1}) for 2 h at 37°C. After adding 90 μl of lysis buffer, plates were rocked overnight at room temperature and in the absence of light. Absorbance was measured at 550 nm.

For cell cycle analysis cells were washed with phosphate buffered saline and fixed with cooled ethanol 70%. After centrifugation cells were resuspended in assay buffer containing phosphate buffered saline with 1% glucose, 2 mg ml^{-1} RNase A and 50 μg ml^{-1} propidium iodide to give a concentration of about 5×10^5 cells and incubated for 30 min at room temperature. The DNA content of the cells was determined on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.) using an argon laser with excitation wavelength at 488 nm. Data were accumulated by the CellQuest™ software from Becton Dickinson (San Jose, CA, U.S.A.) and histograms were analysed using ModFit LT (Verity Software House, Topsham, ME, U.S.A.). Percentage of cells in different phases of the cell cycle was calculated as area under the distribution curve and G2 arrest was characterized by the ratio of percentage G2 phase to percentage G1 phase.

To avoid glutathione depletion and hence busulfan effects that are not directly related to the generation of the sulfonium ion and its reactive ability, all incubations were performed in the presence of 1 mM glutathione.

Determination of cdc2 activity and tissue factor expression

Cdc2 activity was measured using the MESACUP® cdc2 Kinase Assay Kit (MBL, Nagoya, Japan) according to the manufacturer's instructions. Standardized cell lysates (30 μg protein) were incubated with 5 μl of a biotin-labelled oligopeptide and 5 μl of 1 mM ATP for 30 min at 30°C. After transfer to antibody-coated microwells samples were incubated for 60 min at 25°C followed by incubation with peroxidase-conjugated streptavidin for 30 min at 25°C. The substrate o-phenylenediamine was oxidized in the presence of hydrogen peroxide for 3 min and absorbance was measured at 490 nm.

Expression of tissue factor in cell lysates was determined using the IMUBIND® Tissue Factor ELISA Kit from American Diagnostica, Inc. (Greenwich, CT, U.S.A.). Cells were lysed as described above and ELISA was performed according to the manufacturer's instructions.

Statistical analysis

Data were analysed with a two-tailed Student's *t*-test, using the software GraphPad Prism 3.02 (GraphPad, San Diego, CA, U.S.A.). Results are presented as the means ± s.d. Differences were considered to be statistically significant when $P<0.01$.

Results

Stable transfection of ECV 304 cells with the GSTA1-1 cDNA

Initial determination of GST alpha, mu and pi by Western blot analysis showed expression of GST pi, but lack of GST alpha and mu in ECV 304 cells. Therefore, GSTA1-1 full-length cDNA was subcloned into the GFP gene-containing expression vector pTracer-SV40 and transfected into ECV 304 cells. Transfection of the empty vector was performed as mock control. Selection of the transfected cells was started after 72 h of cultivation in normal medium using the antibiotic agent Zeocin. Western blot analysis of GSTA1-1-transfected cells and pTracer-SV40-transfected control cells as well as untransfected ECV 304 cells is shown in Figure 1a. Whereas untransfected ECV 304 cells expressed only GST pi, mock-transfected cells exhibited additional expression of GFP. As expected, GSTA1-1-transfected cells showed expression of GST pi, GFP and marked amounts of GST alpha. Busulfan metabolizing activity of GSTA1-1 was determined using a CG-MS method. Formation of tetrahydrothiophene (THT) increased in a linear manner during incubation time and lysates from GSTA-1-transfected ECV 304 cells showed THT concentrations of 62.7 ± 9.7 ng ml^{-1} after 6 h compared to 13.0 ± 1.9 ng ml^{-1} and

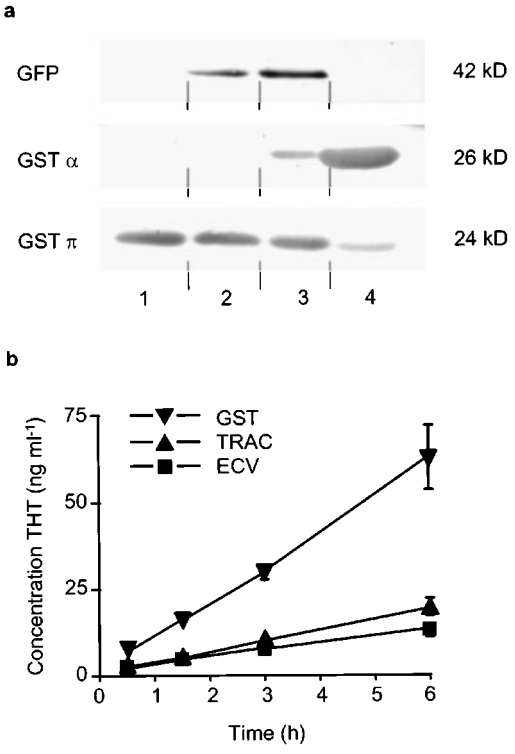

Figure 1 Determination of GSTA1-1 expression and function. (a) Western blot analysis of different lysates from ECV 304 cells. Blots were probed against the indicated antigens. Lane 1: untransfected, lane 2: vector-transfected, lane 3: GSTA1-1 transfected ECV 304 cells, lane 4: liver cytosol. (b) Untransfected (ECV), vector-transfected (TRAC) and GSTA1-1-transfected cells (GST) were incubated with 600 μM busulfan for 0.5, 1.5, 3 and 6 h. Quantification of THT formation in whole cell lysates (1 mg protein) was carried out using GC-MS (mean ± s.d., $n=3$).

19.8 ± 2.5 ng ml^{-1} for untransfected and mock-transfected ECV 304, respectively (Figure 1b). As activities of untransfected and mock-transfected ECV 304 cells were comparable, mock-transfected cells were used as controls in all further investigations.

Influence of busulfan incubation on proliferation and cell cycle

Cell proliferation was determined by MTT-test. Following incubation with 250 μM busulfan for 72 h the proliferation rate for GSTA1-1-transfected ECV 304 was $82.9 \pm 3.6\%$ of DMSO-treated cells. In mock-transfected ECV 304 cells, however, the proliferation rate was found to be $67.1 \pm 2.9\%$ of DMSO-treated cells ($P = 0.004$ for GSTA1-1-transfected *vs* mock-transfected cells, $n = 3$). In order to further investigate these effects, cell cycle analysis has been performed. Cell cycle histograms of busulfan treated mock-transfected and GSTA1-1-transfected ECV 304 cells are shown in Figure 2a. Cell cycle arrest in the G2 phase is markedly reduced in GSTA1-1-transfected compared to mock-transfected cells. After busulfan incubation for 24, 48 and 72 h ratios of percentage G2 phase to percentage G1 phase were found to be 0.10 ± 0.07, 2.26 ± 0.15 and 7.75 ± 2.62 in mock-transfected cells (0.49 ± 0.13 in DMSO-treated cells) and 0.52 ± 0.05, 1.69 ± 0.54 and 3.71 ± 0.14 in GSTA1-1-transfected ECV 304 cells (0.53 ± 0.02 in DMSO-treated cells), respectively. Incubation with 50 and 250 μM busulfan revealed G2 : G1 ratios of 7.52 ± 0.50 and 19.83 ± 4.63 in mock-transfected ECV 304 cells (0.66 ± 0.12 in DMSO-treated cells), respectively, whereas G2 : G1 ratios of 3.65 ± 1.89 and 4.77 ± 0.55 were found in GSTA1-1-transfected cells (0.41 ± 0.02 in DMSO-treated cells, Figure 2b).

An important regulator of the cell cycle, particularly at the entry from G2- into the M phase is cyclin dependent kinase cdc2, which complexes with cyclin B and is thereby activated to phosphorylate several cell cycle dependent substrates. Thus, activity of cdc2 kinase serves as a biochemical marker for cell cycle events in the G2/M phase. After incubation with 250 μM busulfan for 72 h, cdc2 kinase activity in mock-transfected cells was found to be 14.7 ± 2.3 AU mg^{-1} protein compared to 36.5 ± 5.5 AU mg^{-1} protein in untreated cells. In GSTA1-1-transfected cells cdc2 kinase activities of 24.1 ± 1.5 and 40.2 ± 8.7 AU mg^{-1} protein for busulfan treated and untreated cells, respectively, were found. Activity of cdc2 kinase after busulfan incubation was significantly increased ($P = 0.004$, $n = 3$) in GSTA1-1-transfected as compared to mock-transfected ECV 304 cells (Figure 3).

Expression of tissue factor in transfected ECV 304 cells

As induction of the coagulation system is a major aspect in the development of VOD, the influence of busulfan and its metabolism on procoagulative proteins was investigated. Determination of tissue factor protein expression in untreated cells revealed concentrations of 1.3 ± 0.1 ng mg^{-1} total protein and 8.9 ± 0.4 ng mg^{-1} total protein in mock-transfected and GSTA1-1-transfected cells, respectively. After a 72 h incubation with 250 μM busulfan 0.8 ± 0.2 ng mg^{-1} and 35.0 ± 0.8 ng mg^{-1} of tissue factor protein were found in mock-transfected and GSTA1-1-transfected ECV 304 cells, respectively (Figure 4). These data demonstrate an elevated basal expression of tissue factor in GSTA-1-transfected cells and a further 4 fold increase after busulfan treatment as compared to the DMSO treated cells. A significant increase of tissue factor expression after busulfan treatment could not be observed in mock-transfected cells.

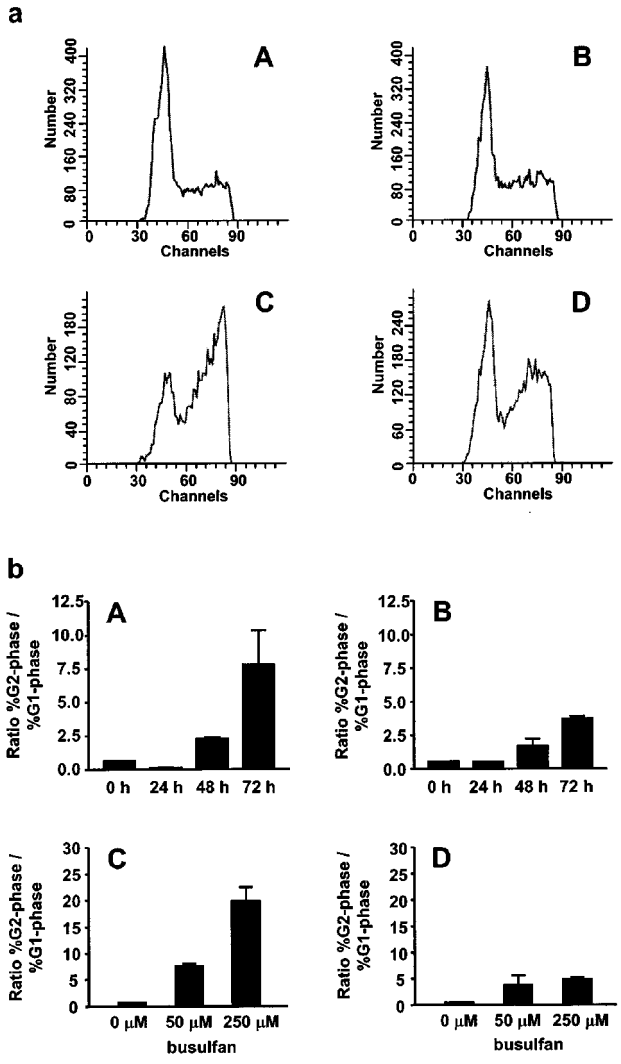

Figure 2 Determination of the effects of busulfan on cell cycle. Cells incubated with busulfan were stained with propidium iodide and cell cycle analysis was performed using flow cytometry. (a) Histograms of mock-transfected (A, C) and GSTA1-1-transfected (B, D) ECV 304 cells treated with DMSO (A, B) or 250 μM busulfan (C, D) for 72 h. (b) Time course (A, B) and concentration dependence (C, D) of the effect of busulfan in mock-transfected (A, C) and GSTA1-1-transfected (B, D) ECV 304 cells (mean $\pm$ min/max, $n = 2$).

Discussion

The antineoplastic compound busulfan displays a high myelosuppressive potential and is therefore used in preconditioning regimens for bone marrow transplantation. Pharmacokinetics vary tremendously between patients possibly due to extensive metabolism in the liver *via* conjugation to glutathione, which is catalysed by glutathione S-transferases. Since elevated busulfan plasma levels are

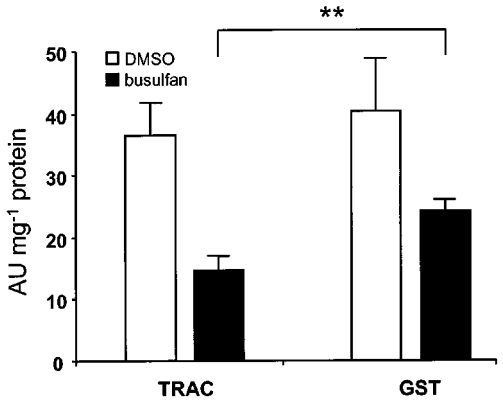

Figure 3 Determination of the effects of busulfan on cdc2 activity. GSTA1-1-transfected ECV 304 cells (GST) and control cells (TRAC) were treated for 72 h with either 250 μM busulfan or DMSO. Specific cdc2 activity was determined by ELISA. Mean ± s.d. of three independent experiments are given (**$P<0.01$).

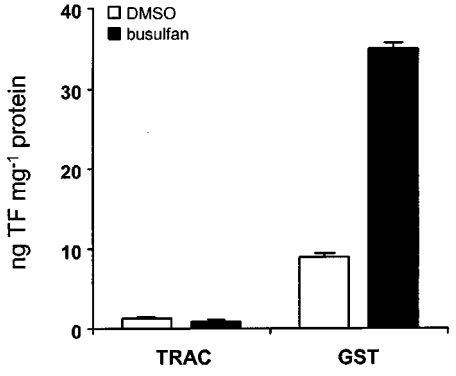

Figure 4 Determination of the effects of busulfan on tissue factor expression. GSTA1-1-transfected ECV 304 cells (GST) and control cells (TRAC) were treated for 72 h with either 250 μM busulfan or DMSO. Tissue factor expression in cell lysates was determined by ELISA against a standard curve (means ± s.d., $n=5$).

thought to be a risk factor for developing veno-occlusive disease (VOD), a serious adverse side effect in high-dose busulfan therapy, metabolism of busulfan may play a pivotal role in the induction of VOD. We therefore established a cell model to investigate the influence of busulfan metabolism on the biological effects of this antineoplastic agent.

Initial events in the development of VOD include endothelial damage and activation of the coagulation cascade. Therefore, the cell line ECV 304 was chosen as a cell system of endothelial origin. This cell line has been characterized by Takahashi *et al.* (1990), is derived from human umbilical vein endothelial cells, can bind UEA-1 lectin, expresses adhesion molecules and uPA (Takahashi & Sawasaki, 1991), can be stimulated by NO to release IL-8 (Villarete & Remick, 1995), can adhere to T-cells (Qu *et al.*, 1996), forms new blood vessels (Hughes, 1996) and expresses tissue factor (Lopez-Pedrera *et al.*, 1997). Moreover, recent publications describe cell polarity of endothelial cells (Haller *et al.*, 1998), regulation of adhesion molecules (Chen *et al.*, 1999) and modulation of the coagulation system (Hansen *et al.*, 2000) in ECV 304 cells, demonstrating the value of this cell line as an endothelial model.

Among the glutathione S-transferase (GST) enzyme family, GST alpha in the homodimeric A1-1 form has been reported to display the highest affinity for busulfan (Czerwinski *et al.*, 1996; Gibbs *et al.*, 1996). Western blot analysis of ECV 304 cells showed that GST alpha was not expressed in these cells, whereas GST pi was found. However, we recently demonstrated that the busulfan metabolizing activity of GST pi was only 13% of the activity of GST alpha (Ritter *et al.*, 1999). In order to simulate busulfan metabolism, to generate the unstable sulfonium ion and to investigate metabolic effects we transfected ECV 304 cells with the vector pTracer-SV40 containing GSTA1-1 cDNA. Formation of tetrahydrothiophene (THT) as an indicator for GSTA1-1 activity was substantial in GSTA-1-transfected cells. Remaining activity in mock-transfected cells probably resulted from activity of GST pi and from non-enzymatic formation of THT, which has been reported previously (Gibbs *et al.*, 1996; Ritter *et al.*, 1999). The ability of this cell model to metabolize busulfan and hence generate the glutathione conjugated metabolite is particularly valuable, since it is not possible to add the sulfonium ion directly to the medium. In addition, effects directly associated with busulfan metabolism can be determined simultaneously.

Busulfan is well known as an alkylating agent (Tong & Ludlum, 1980), which causes DNA-damage and induces cells to cell cycle arrest, providing time for genetic repair. In fact, it has been reported that incubation of a Chinese hamster ovary (CHO) cell line with busulfan results in an increased number of cells reversibly arrested in the G2 phase of the cell cycle (Millar *et al.*, 1986). Those results were confirmed in transfected ECV 304 cells, and found to be time and concentration dependent. However, the effects of busulfan treatment on mock-transfected cells were significantly more pronounced in terms of growth inhibition, cell cycle arrest in G2 phase and a decrease of cdc2 activity as compared to the GSTA-1-transfected cells. Busulfan concentrations used in these experiments were approximately 25 times higher as compared to plasma levels that are discussed to increase the risk of developing VOD (Grochow *et al.*, 1989). It is likely, however, that the liver concentrations of busulfan will exceed those in plasma after oral administration.

Taken together, these data show that transfection of ECV 304 cells with GSTA1-1 led to a partial rescue from busulfan induced cell cycle arrest and its growth inhibitory effects. Therefore, the conjugation of busulfan with glutathione in this model displays a mechanism of detoxification rather than bioactivation, which has been demonstrated for other alkylating agents such as melphalan, chlorambucil, phosphoramide mustard or thiotepa (Dirven *et al.*, 1995; Cnubben *et al.*, 1998; Paumi *et al.*, 2001). While most of those reports show an increased expression of the enzyme in cell lines that were selected for resistance against the drugs, this report shows the direct consequences of an increased busulfan metabolism towards cellular responses.

Since activation of the coagulation cascade is one of the pathological events in the development of VOD, we investigated the influence of busulfan and its metabolism on the expression of tissue factor. Increased levels of tissue factor in patients have been associated with thrombosis and atherosclerosis, both leading to arterial occlusion (Asada *et*

al., 1998). The data generated by measuring tissue factor expression and secretion in transfected ECV 304 cells upon busulfan treatment demonstrate that overexpression of GSTA1-1 in ECV 304 cells not only increased the constitutive level of tissue factor expression but also generated a cellular environment that allowed busulfan to further increase expression of tissue factor, which might be due to activation of busulfan-sensitive transcription factors. Investigations to address the underlying mechanisms will be performed. The clinical relevance of these findings is underlined by a recent report identifying vascular endothelial growth factor (VEGF) as causative and predictive in a small number of patients developing VOD (Iguchi *et al*., 2001). VEGF is well known to stimulate tissue factor expression in the endothelium (Armesilla *et al*., 1999) supporting a role for tissue factor in the development of VOD. In this context, elevated expression levels of GSTA1-1 in patients, who are treated with busulfan, could very likely accelerate expression of tissue factor and shift these patients into an increased procoagulative state. Moreover, expression of GST alpha in human liver has been reported to be highly variable (Hayes *et al*., 1989). Variations in protein expression are often related to genetic modifications. For the GSTA1 gene, seven single nuclear base substitutions in the promoter region have been described recently (Coles *et al*., 2001; Bredschneider *et al*., 2002). However, the outcome of those modifications on GST alpha expression and activity is controversial. Whereas Coles *et al*. (2001) showed clear correlations of GSTA1 and GSTA2 expression in two distinct genotypes, investigations by Bredschneider *et al*. (2002) failed to show any correlation between the possible haplotypes and GST alpha expression and function. Nonetheless, variability in the expression of GST alpha does exist, which could explain the unpredictable occurrence of VOD. This report highlights the importance of including GST alpha expression into the search for clinical parameters that define the risk of developing VOD.

In summary, we have shown that ECV 304 cells transfected with GSTA1-1 provide a valuable tool to catalyze conjugation of busulfan with glutathione and generate the unstable primary metabolite. Overexpression of GSTA1-1 in ECV 304 cells led to a protective stage against toxic effects of busulfan on the cell cycle and increased the basal expression of tissue factor. Moreover, under GSTA1-1-overexpressing conditions busulfan was found to further increase tissue factor expression in our cell model, suggesting that GSTA1-1 plays a pivotal role for developing coagulation disorders like VOD in patients treated with busulfan.

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