RESEARCH COMMUNICATION

Studies on the differential inhibition of glutathione conjugate formation of (+)-*anti*-benzo[*a*]pyrene 7,8-dihydrodiol 9,10-epoxide and 1-chloro-2,4-dinitrobenzene in V79 Chinese hamster cells

Kathrin SUNDBERG*, Bengt JERNSTRÖM* and Stellan SWEDMARK†[1]

*National Institute of Environmental Medicine, Karolinska Institutet, S-171 77 Stockholm, Sweden, and †Department of DMPK Discovery, R&D Södertälje, AstraZeneca, S-151 85 Södertälje, Sweden

V79 Chinese hamster cells have previously been shown to lack the capacity to detoxify the mutagenic and carcinogenic compound (+)-*anti*-benzo[*a*]pyrene 7,8-dihydrodiol 9,10-epoxide [(+)-*anti*-BPDE] by Pi class glutathione transferase (GSTPi)-catalysed conjugation with GSH, although these cells contain such an enzyme [Romert, Dock, Jenssen and Jernström (1989) Carcinogenesis **10**, 1701–1707; Swedmark, Romert, Morgenstern and Jenssen (1992) Carcinogenesis **13**, 1719–1723; Swedmark and Jenssen (1994) Gene **139**, 251–256]. Previous findings also indicate that these results do not depend on an inactive GSTPi enzyme, since V79 cells conjugate 1-chloro-2,4-dinitrobenzene (CDNB) with GSH, but more likely on (a) factor(s) that inhibit(s) V79 GSTPi selectively [Swedmark, Jernström and Jenssen (1996) Biochem. J. **318**, 533–538]. The present study demonstrates that both human and V79 recombinant GSTPi enzymes are inhibited with respect to conjugating (+)-*anti*-BPDE, but not CDNB, after pre-incubation with V79-cell extract, but not with MCF-7-cell extract. In addition, it was found that the inhibition is dependent on the amount of cell extract present and that the factor(s) is heat-resistant and has a molecular mass of less than 10 kDa, suggesting that the factor(s) is (are) non-proteinaceous in nature.

Key words: glutathione conjugation, inhibitory factors, Pi class glutathione transferase, V79 cells.

INTRODUCTION

The soluble glutathione transferases (GSTs) constitute a family of multifunctional enzymes which catalyse the reaction of a large number of electrophilic compounds with GSH to yield more polar and excretable conjugates [1–3]. A function for GSTs as intracellular carrier proteins for various lipophilic compounds has also been proposed [4,5]. The GSTs are subdivided into several classes; in humans, for instance, the classes are Alpha, Mu, Pi, Theta and Zeta [6,7].

Isoenzymes belonging to the Alpha, Mu and Pi classes are all active towards ultimate mutagenic and carcinogenic diol epoxide intermediates of polycyclic aromatic hydrocarbons [8,9]. The GSTs belonging to class Pi are unique in this context, since the enzyme demonstrates an exclusive preference for the most potent diol epoxide enantiomers, including (+)-*anti*-benzo[*a*]pyrene 7,8-dihydrodiol 9,10-epoxide [(+)-*anti*-BPDE] [9–11].

The V79 cell line derived from Chinese hamster contains significant amounts of Pi class GST enzyme (GSTPi) [12], but apparently lacks the capacity to catalyse the conjugation of (+)-*anti*-BPDE with GSH [13]. The coding mRNA sequence of the V79 GSTPi has been determined and the similarity of the amino acid (aa) sequence to known GSTPi sequences was at least 84% [14]. None of the differences in aa sequence could explain the lack of activity of V79 cells towards (+)-*anti*-BPDE.

In a recent study, four different hamster-cell lines were compared with respect to their conjugating activities towards 1-chloro-2,4-dinitrobenzene (CDNB) and (+)-*anti*-BPDE [15]. The cytosolic fractions obtained from two of these cell lines, AHL-1 (derived from Armenian hamster lung cells) and BHK-C13 (derived from Syrian hamster kidney cells), demonstrated high activities towards both substrates. In contrast, the cytosolic fractions obtained from V79 and CHO-AA8 (both derived from Chinese hamster) were highly active with CDNB, but demonstrated no activity towards (+)-*anti*-BPDE. The aa sequences of GSTPi enzymes from AHL and BHK cells differed, by six and nine aa respectively, from the sequence of V79 GSTPi, but the aa sequence of GSTPi in V79 and Chinese hamster ovary (CHO) cells was identical. None of the differences in aa sequence involved positions constituting the active site of GSTPi. In contrast with the results observed with the cytosolic fractions, the purified GSTPi from each cell line were in all cases highly active towards (+)-*anti*-BPDE [15]. In fact the catalytic efficiencies were similar to what has been recently observed with human GSTP1-1 (the allelic variant with isoleucine at position 105) [9]. Taken together, the results indicate that Chinese hamster cells contain (a) factor(s) which inhibit(s) GSTPi-catalysed conjugation of (+)-*anti*-BPDE, but not of CDNB.

Here we have studied this phenomenon further by monitoring the effect of nuclei-free cell extracts on (+)-*anti*-BPDE-GSH conjugate catalysed by recombinant V79 GSTPi or recombinant human GSTP1-1 isoenzymes.

EXPERIMENTAL

Preparation of recombinant GSTPi enzymes

V79 cells were grown as described by Swedmark et al. [12]. The mRNA was prepared and the coding sequence for the V79 GSTPi enzyme amplified from the corresponding cDNA by PCR using primers covering the start and stop codons [14]. The

Abbreviations used: aa, amino acid; (+)-*anti*-BPDE, (+)-*anti*-benzo[*a*]pyrene 7,8-dihydrodiol 9,10-epoxide; CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione transferase; GSTPi, Pi class GST; LB, Luria broth; CHO, Chinese hamster ovary.

[1] To whom correspondence should be addressed (e-mail Stellan.Swedmark@astrazeneca.com).

© 2000 Biochemical Society

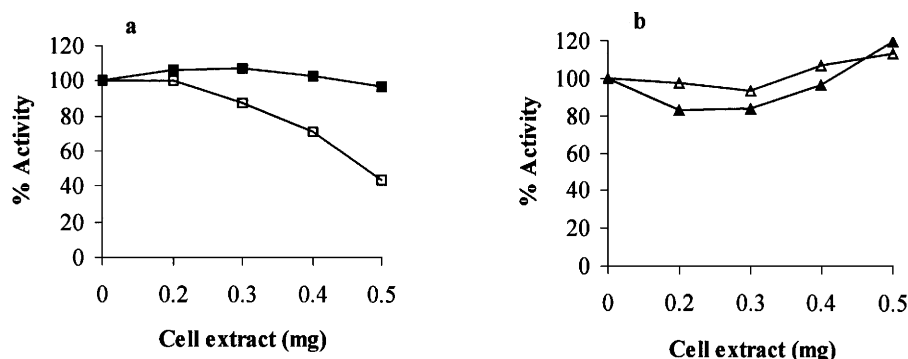

Figure 1 Effect of GSH-conjugate formation towards (+)-*anti*-BPDE and CDNB in the presence of nuclei-free cell extract

Recombinant V79 GSTPi corresponding to 100 μg of active protein/ml was pre-incubated for 24 h, at 4 °C with 0.2, 0.3, 0.4 or 0.5 mg of nuclei-free cell extract derived from V79 or MCF-7 cells. Prior to the incubation with (+)-*anti*-BPDE or CDNB, the protein mixtures were freed of microsomes by ultracentrifugation. The conjugation formation is expressed as percentage activity (the activity itself being expressed as nmol/min per mg of GSTPi). (**a**) BPDE-activity with V79 cell extract (□) and MCF-7 cell extract (■). Results are the means ($\pm$10%) for two independent experiments (□) or a single experiment (■). (**b**) CDNB-activity before (▲) and after (△) ultracentrifugation.

primers used were 5′-TTGAATTCATGCCGCCATACACCA-TTGTCTAC for the 5′ end and 5′-AAAAGCTTTTACTGCT-TGCCATTGCCATTAAT for the 3′ end. The primers were obtained from SGS, Köping, Sweden. The PCR products were purified using Wizard PCR Preps (Promega). The 5′- and 3′-end primers contain an *Eco*RI and a *Hin*dIII restriction-enzyme site respectively, and the PCR product was subsequently cleaved with these enzymes and purified with the Wizard PCR Preps. The pKK223-3 vector (Pharmacia) was cleaved with *Eco*RI and *Hin*dIII and purified with Magic DNA Clean-Up (Promega). The PCR products coding the V79 GSTPi enzyme was then ligated into the pKK223-3 vector using a ligation kit (Amersham) and subsequently transfected into competent JM105 *Escherichia coli* bacterial cells as described by Maniatis [16]. The bacterial cells were spread on to agar plates containing 100 μg of ampicillin/ml and left overnight at 37 °C. Antibiotic-resistant colonies were grown in the presence of 1 mM isopropyl thio-β-D-galactoside and 100 μg of ampicillin/ml in Luria broth (LB). Plasmid DNA from CDNB-positive colonies was prepared as described by Maniatis [16] and sequenced to confirm the presence of the sequence coding for V79 GSTPi GSTP1-1 protein. Two pKK223-3-specific primers, 5′-CGGCTCGTATAATGTGTG-GAATT and 5′-TGAAAATCTTCTCTCATCC-GCC (SGS) were used and the sequencing performed with the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Perkin–Elmer) and an IBI sequencing machine.

Bacterial cytosolic fractions were prepared as described by Kolm et al. [17] and assayed for CDNB activity by the method of Habig and Jacoby [18].

Recombinant human GSTP1-1 (the allelic variant with Ile at position 105) was obtained by expression in *E. coli* [19] and further purified by a modified affinity-chromatography method [20].

Preparation of cell extracts

Cell extracts were prepared from V79 and MCF-7 cells as follows; the cells were harvested in trypsin/EDTA and suspended in cold PBS, pH 7.4. Following sedimentation by low-speed centrifugation at 4 °C, the cells were resuspended in 2–3 vol. of ice-cold 20 mM Tris/HCl/0.25 M sucrose/0.5 mM EDTA, pH 7.4. Cells were lysed by pulsed sonication for 20 s at 35% energy using a Fisher model 300 sonicator with a micro-tip. Following centrifugation of the lysate at 12000 g, the supernatant was transferred to tubes and maintained at -80 °C until use. The protein concentration was determined by the method of Peterson [21].

GST-catalysed conjugation of CDNB or (+)-*anti*-BPDE

The activities of bacterial cytosolic fractions and recombinant human enzymes towards CDNB were measured as described by Habig and Jacoby [18]. The GST-conjugating activities of V79 cell extracts, bacterial cytosolic fractions and human GSTPi enzymes towards (+)-*anti*-BPDE were measured as described previously [15]. Following precipitation of the proteins by perchloric acid (final concentration 5%), the products formed were analysed by HPLC as described in [13]. The (+)-*anti*-BPDE was obtained through the Cancer Research Program of the NCI, Division of Cancer Cause and Prevention, Bethesda, MD, U.S.A. CDNB was obtained from Sigma.

Effects of cell fractions on GSTPi-catalysed conjugation of (+)-*anti*-BPDE and CDNB

Bacterial cytosolic fraction expressing V79 GSTPi or recombinant human GSTP1-1 corresponding to 100 μg of active protein/ml was pre-incubated for 24 h, at 4 °C with 200, 300, 400 or 500 μg of cell extract derived from V79 or MCF-7 cells. Prior to the incubation with (+)-*anti*-BPDE, the protein mixtures were centrifuged at 105000 g for 60 min in order to remove the microsomes. The activity towards CDNB was determined before and after centrifugation. In other experiments 500 μg of V79 cell extract was heat-inactivated (5 min at 100 °C) or dialysed using spin columns with a cut-off range of 10 kDa (Millipore) prior to incubation with human GSTP1-1.

RESULTS

Effect of cell extracts on GST-catalysed conjugation of (+)-*anti*-BPDE and CDNB

Increasing amounts of cell extract isolated from V79 or MCF-7 cells were incubated at 4 °C for 24 h with recombinant V79 GSTPi expressed in bacterial cytosol. Following incubation, the mixtures were freed of microsomes by centrifugation and briefly incubated with (+)-*anti*-BPDE in the presence of GSH. The

© 2000 Biochemical Society

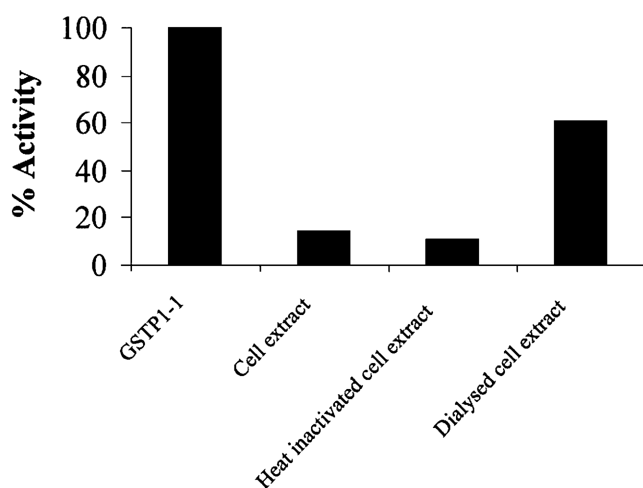

Figure 2 Activity of human GSTP1-1 towards (+)-*anti*-BPDE in the presence of 500 μg of nuclei-free V79 cell extract, heat-inactivated cell extract or dialysed cell extract

Results are means (±10%) for two independent experiments for all experiments, except for a single experiment with dialysed cell extract.

effect on GSH-conjugate formation is shown in Figure 1(a). The extract derived from V79 cells inhibited the GSTPi-catalysed diol epoxide conjugation in a concentration-dependent manner. In contrast, the MCF-7 extract had apparently no such effect. Ultracentrifugation of the V79 cell extract had no effect on the CDNB-conjugating activity of the recombinant V79 GSTPi enzyme in the extract (Figure 1b).

In order to substantiate the results obtained with recombinant GSTPi, the effect of V79 cell extract was studied with purified recombinant human GSTP1-1. The enzyme was incubated with V79 cell extract and centrifuged as described above prior to incubation with (+)-*anti*-BPDE and GSH. The results in Figure 2 show that the presence of extract inhibits conjugate formation by 85%. Repeating the experiments with preheated cell extract show that the inhibitory effect persists. Dialysis of the cell extract (cut-off range of 10 kDa) reveals that about 60% of the activity is recovered. Taken together, these observations seem to exclude a protein as the inhibitor of GSTP1-1-catalysed conjugation of (+)-*anti*-BPDE.

DISCUSSION

V79 cells are commonly used as targets in toxicity and genotoxicity studies, both alone or in co-cultivation experiments. Since these cells lack cytochrome P450 expression [22–24], they are particularly suitable in co-cultivation with other cell types or systems able to carry out metabolic activation. Less is known about the expression of deactivating enzymes in V79 cells, such as GSTs. However, we have recently shown that the cells contain GSTPi and are highly active towards CDNB, but inactive towards (+)-*anti*-BPDE [15]. This observation explained previous results obtained with V79 cells and (+)-*anti*-BPDE. An unexpected high number of mutations in the hypoxanthine:guanine phosphoribosyltransferase locus were observed [13], indicating that the V79 cells lack a functional GSTPi enzyme, since this enzyme from both rodents and man efficiently deactivates (+)-*anti*-BPDE by conjugation with GSH [10,11].

Further experiments revealed that the GSTPi enzyme, present in the cytosolic fraction obtained from V79 or CHO cells, has no capacity to conjugate (+)-*anti*-BPDE. Surprisingly, after isolation and purification of the GSTPi proteins by affinity chromatography, both enzymes were highly active towards (+)-*anti*-BPDE. In fact, the catalytic efficiency was similar to the corresponding one observed for human GSTP1-1 [9]. Interestingly, the GSTPi enzymes' capacity to catalyse conjugation of CDNB is not affected by their presence in cytosols [15]. Thus, on the basis of these results, it seems that both V79 and CHO cells contain (a) factor(s) that selectively inhibit(s) GSTPi-catalysed conjugation of the bulky (+)-*anti*-BPDE, but not of less complex CDNB.

In an attempt to elucidate the phenomenon of inhibition, the activity of recombinant V79 GSTPi or human GSTP1-1 towards (+)-*anti*-BPDE and CDNB in the presence of nuclei-free cell extracts was studied. From the results it can be concluded that V79 cell extract is inhibitory and that the effect clearly is dependent on the amount of extract used. In contrast, cell extract from MCF-7 cells demonstrates no such inhibitory effect.

One can argue that the inhibitory effect of V79 fraction relative to the MCF-7 fraction is due to difference in availability of (+)-*anti*-BPDE for conjugation. However, this was not the case, since the $t_{\frac{1}{2}}$ values of (+)-*anti*-BPDE were very similar in both experimental systems (8 min in V79 and 6 min in MCF-7). Another plausible explanation for the inhibitory effect of the V79 extract is reduced activity of GSTPi due to the removal of microsomes by centrifugation. Figure 1(b) clearly shows that this is not the case. The activity towards CDNB was constant in all mixtures.

However, it was necessary to prolong the pre-incubation of the GSTPi enzymes with the V79 cell extract to 24 h at 4 °C, since a pre-incubation of 30 min at 37 °C was insufficient to give rise to any inhibitory effect on (+)-*anti*-BPDE conjugation (results not shown). The reason for this difference remains obscure, but it might be explained by the association of GSTPi enzyme with the inhibitory factor(s) being time-dependent.

Available data indicate that the inhibitory factor(s) is not proteinaceous, since the inhibitory effect is insensitive to heat and, at least in part, dialysable (however, small peptides cannot be excluded). Furthermore, the inhibitory effect is not due to covalent modification of the enzyme, since purification of constitutive GSTPi from V79/CHO cells by affinity chromatography yields enzymes highly efficient in (+)-*anti*-BPDE conjugation [15].

Mitra et al. [25] observed that human placental and foetal liver GSTs were inhibited by fatty acids and fatty acid esters. Those authors found a reversible inhibitory effect, a finding that is in agreement with the present one, of stearic and palmitic acids as well as ascorbyl stearate and ascorbyl palmitate. However, in contrast with the study by Mitra et al. [25], the inhibition found in the present study does not affect CDNB, but only (+)-*anti*-BPDE.

In conclusion, V79 hamster cells, but not human MCF7 cells, contain (a) factor(s) that specifically inhibit(s) conjugation of bulky and complex substrates such as (+)-*anti*-BPDE by recombinant V79 GSTPi and human recombinant GSTP1-1, but not conjugation of the less complex CDNB. The identity and intracellular localization of the inhibitor is not known and requires further investigation.

This study was supported by grants from Swedish Match Sverige AB and the National Board for Laboratory Animals. We thank Dr. Dag Jenssen for supplying the V79 and MCF-7 cells.

REFERENCES

1 Mannervik, B. and Danielson, U. H. (1988) CRC Crit. Rev. Biochem. **23**, 283–337
2 Ketterer, B. and Christodoulides, L. G. (1994) Adv. Pharmacol. **27**, 37–69

© 2000 Biochemical Society

3 Hayes, J. D. and Pulford, D. J. (1995) Crit. Rev. Biochem. Mol. Biol. **30**, 445–600
4 Tipping E. and Ketterer B. (1978) in Transport by Proteins (Blauer G. and Sund, H., eds.), pp. 369–385, Walter de Gruyter and Co., Berlin and New York
5 Listowsky, I. (1993) in Structure and Function of Glutathione Transferases (Tew, K. D, Pickett, T. J., Mantle, T. J., Mannervik, B. and Hayes, J. D, eds.), pp. 199–209, CRC Press, Boca Raton, FL
6 Mannervik B. and Widersten, M. (1995) in Advances in Drug Metabolism in Man (Pacifici, G. M. and Francchia, G. M., eds.), pp. 408–459, European Commission, Brussels
7 Board, P. G., Webb, G. C. and Coggan, M. (1989) Ann. Hum. Genet. **53**, 205–213
8 Jernström, B., Funk, M., Frank, H., Mannervik, B. and Seidel, A. (1996) Carcinogenesis **17**, 1491–1498
9 Sundberg, K., Widersten, M., Seidel, A., Mannervik, B. and Jernström, B. (1997) Chem. Res. Toxicol. **10**, 1221–1227
10 Robertson, I. G., Guthenberg, C., Mannervik, B. and Jernström, B. (1986) Cancer Res. **46**, 2220–2224
11 Robertson, I. G., Jensson, H., Mannervik, B. and Jernström, B. (1986) Carcinogenesis **7**, 295–299
12 Swedmark, S., Romert, L., Morgenstern, R. and Jenssen, D. (1992) Carcinogenesis **13**, 1719–1723
13 Romert, L., Dock, L., Jenssen, D. and Jernström, B. (1989) Carcinogenesis **10**, 1701–1707
14 Swedmark, S. and Jenssen, D. (1994) Gene **139**, 251–256
15 Swedmark, S., Jernström, B. and Jenssen, D. (1996) Biochem. J. **318**, 533–538
16 Maniatis T, Fritsch, E. F. and Sambrook J. (1989) Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
17 Kolm, R. H., Stenberg, G., Widersten, M. and Mannervik, B. (1995) Protein Expression Purif. **6**, 265–271
18 Habig, W. H. and Jakoby, W. B. (1981) Methods Enzymol. **77**, 398–405
19 Stenberg, G., Björnestedt, R. and Mannervik, B. (1992) Protein Expression Purif. **3**, 80–84
20 Cameron, A. D., Sinning, I., L'Hermite, G., Olin, B., Board, P. G., Mannervik, B. and Jones, T. A. (1995) Structure **3**, 717–727
21 Peterson, G. L. (1977) Anal. Biochem. **83**, 346–356
22 Frantz, C. N. and Malling, H. V. (1976) J. Toxicol. Environ. Health **2**, 179–187
23 Krahn, D. F. and Heidelberger, C. (1977) Mutat. Res. **46**, 27–44
24 Kuroki, T., Drevon, C. and Montesano, R. (1977) Cancer Res. **37**, 1044–1050
25 Mitra, A., Govindwar, S., Joseph, P. and Kulkarni, A. (1992) Toxicol Lett. **60**, 281–288

Received 15 May 2000; accepted 26 May 2000

© 2000 Biochemical Society