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Status of reduced glutathione in the human hepatoma cell line, HEP G2

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There have been a number of reports recently suggesting that human hepatoma cell lines which retain differentiated parenchymal functions may provide an *in vitro* system for studying drug metabolism and cytotoxicity directly in man. Certain human hepatoma cell lines can carry out both cytochrome P-450-dependent mixed-function oxidase (MFO) reactions and conjugations with glucuronic acid and sulphate [1–4]. One cell line, Hep G2, has been used to activate chemicals for studies on mutagenesis and sister chromatid exchange [5, 6] and, furthermore, it has been shown to form similar adducts with benzo(*a*)pyrene as are formed in explants from normal human tissue [2]. In addition, the MFO activities and the glucuronic acid conjugation in these cells respond to *in vitro* exposure to the inducing agents phenobarbitone (PB)†, 1,2-benzanthracene (BA) and 3-methylcholanthrene [3, 7].

Intracellular reduced glutathione (GSH) plays an important role in the detoxification of a variety of chemicals by conjugating with electrophilic drug and carcinogen metabolites or by acting as a reductant in the metabolism of hydroperoxides and free radicals. Thus, the intracellular GSH content of cells controls the extent of carcinogenicity and cytotoxicity of many chemicals [8–10] and it is important therefore to investigate the status of GSH in the Hep G2 cells. This manuscript reports on the GSH levels in cultured Hep G2 cells, the ability of the cells to synthesise GSH, the effect of GSH depletion of cell viability and growth and compares the activity of glutathione-*S*-transferase (GST) in Hep G2 cells with that in freshly-isolated human adult hepatocytes. Two methods for depletion of GSH were used in this study. For measurement of GSH synthesis, the intracellular GSH was depleted by treatment with diethylmaleate (DEM), which acts by conjugating with the GSH [11]. The effect of GSH depletion on cell growth in culture was investigated using DL-buthionine-*SR*-sulfoximine (BSO), a potent irreversible inhibitor of γ-glutamylcysteine synthetase [12].

† Abbreviations used: GSH, reduced glutathione; DEM, diethylmaleate; BSO. DL-buthionine-*SR*-sulfoximine; CDNB, 1-chloro-2,4-dinitrobenzene; LDH, lactate dehydrogenase; PB, phenobarbitone; BA, 1,2-benzanthracene; GST, glutathione-*S*-transferase.

Materials

Reduced glutathione, diethylmaleate, 1-chloro-2,4-dinitrobenzene, Trypan Blue, 1,2-benzanthracene, and DL-buthionine-*SR*-sulfoximine were obtained from Sigma. Flow Laboratories supplied the Dulbecco's medium and foetal calf serum was purchased from Gibco. Hep G2 cells were obtained by Dr W. T. Melvin, Department of Biochemistry, Aberdeen University from Professor C. N. Hales, Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge.

Methods

1. *Cell culture.* Hep G2 cells were routinely grown in monolayer or multilayer culture in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) foetal calf serum. They were grown in a humidified atmosphere of 5% CO_2 in air and subcultured every 7 days at a split ratio of 1 to 3. For experiments on enzyme induction, confluent cells (day 7 after subculture) were treated with 2 mM PB or 25 μM BA for 3 days before GSH content and GST activity was measured on day 10. The medium was changed every day during these experiments.

2. *Isolation and incubation of Hep G2 cells.* Cell suspensions were prepared by treating confluent cultures with a 1:5 solution of 0.25% (w/v) trypsin: 0.02% (w/v) versene in phosphate buffered saline, pH 7.4. The viability of the suspensions was assessed by Trypan Blue exclusion (typically >90%). Cells were incubated in 50 ml round bottomed flasks in Krebs–Henseleit buffer, pH 7.4, containing 10 mM HEPES at 37° under an atmopshere of 95%O_2:5%CO_2.

3. *GSH resynthesis.* To deplete intracellular GSH, 10^6 cells/ml were incubated for 60 min at 37° with 0.02% (v/v) diethylmaleate (DEM). After this period the cells were sedimented by centrifugation at 600 *g* for 4 min and viability determined by Trypan Blue exclusion. For measurement of GSH resynthesis the cells were resuspended (10^6 viable cells/ml) in Krebs–Henseleit buffer supplemented with Dulbecco's amino acid solution deficient in sulphur containing amino acids.This amino acid solution was supplemented with 0.5 mM L-methionine, and/or 0.5 mM L-cysteine and in certain experiments 5 mM BSO was present as an inhibitor of GSH synthesis. Samples (1 ml) were

removed at timed intervals up to 2 hr for the determination of GSH by the method of Saville [13].

4. *The effect of GSH depletion on cell growth.* Cell viability, GSH content and growth rate (estimated by protein content and by cell numbers) were determined in the Hep G2 cells daily, for up to 6 days after subculture, in the presence and absence of 0.5 mM BSO in the medium. During these experiments the culture medium was changed daily in both control and BSO-treated flasks. The viability of the cultures was assessed by the lactate dehydrogenase (LDH) activity of the medium, and expressed as a percentage of the total LDH activity per flask after addition of 0.1% (v/v) Triton X-100 [14]. In addition, the time course of GSH depletion by 0.5 mM BSO was followed in cultured cells for up to 24 hr.

5. *Measurement of GST activity.* GST activity was measured in Hep G2 cell suspensions (10^6 cells/ml) in Krebs–Henseleit buffer, pH 7.4, by direct spectrophotometry as described by Habig and Jakoby [15] using 50 μM 1-chloro-2,4-dinitrobenzene (CDNB). The rate of CDNB conjugation in the Hep G2 cells was compared with values for freshly-isolated human adult hepatocytes which we have previously published [16].

For enzyme induction studies GST activity was measured in Hep G2 cell homogenates (0.1 ml homogenate) in the presence of excess GSH (1 mM). Cell homogenates were prepared as described previously for cultured rat hepatocytes [17]. Briefly, cultures were washed twice with phosphate buffered saline, pH 7.6, scraped off the flasks into 0.1 M sodium phosphate buffer, pH 7.6, using a rubber policeman, and homogenised using a Teflon–glass Potter-Elvehjem-type homogeniser. Homogenates were stored at −80° until analysis.

Results and discussion

Synthesis of GSH was investigated in the Hep G2 cells following depletion of the intracellular stores by DEM. Approximately 70% of the intracellular GSH was depleted after 60 min treatment with 0.02% (v/v) DEM (control cells contained 8.70 ± 0.56 nmol GSH/10^6 cells (N = 16) compared with 2.70 ± 0.22 nmol GSH/10^6 (N = 12) cells after 60 min treatment with DEM) without affecting cell viability. There is considerable variation in the rate and extent of DEM-mediated GSH depletion in different cell types [18] and this is presumably related to the activity of GST towards DEM. For example, in rat hepatocytes 90% of the GSH is depleted within 30 min by 0.02% (v/v) DEM [19] compared with 50% in Hep G2 cells in the same period.

The ability to resynthesise GSH after treatment with DEM for 60 min was measured in the presence of different amino acids. Figure 1 shows that no GSH resynthesis occurred without the addition of sulphur containing amino acids indicating that the cells did not contain a significant intracellular store of L-cysteine available for GSH synthesis. GSH synthesis occurred in the presence of L-cysteine but not of L-methionine and the L-cysteine-stimulated synthesis was prevented by inclusion of 5 mM BSO in the medium. Hepatocytes are one of only a few cell types that can synthesise GSH equally well from L-methionine or L-cysteine [20]. However, the resynthesis experiments indicate that Hep G2 cells have lost this differentiated hepatic function and do not contain a functional cystathionine pathway to utilise the sulfur atom of L-methionine for GSH synthesis.

Figure 2 shows the growth rate (in terms of cell numbers and protein content per flask) and GSH content of Hep G2 cells during the first 6 days after subculture. Between days 4 and 6 the cell growth rate slowed and at this time the cell GSH content declined from 12.2 ± 0.3 nmol/10^6 cells on day 4 to 8.8 ± 0.4 nmol/10^6 cells on day 5. This decline in GSH content may alter the cellular response to toxic chemicals. Thus particular attention should be paid to the standardisation of the growth condition and timing of

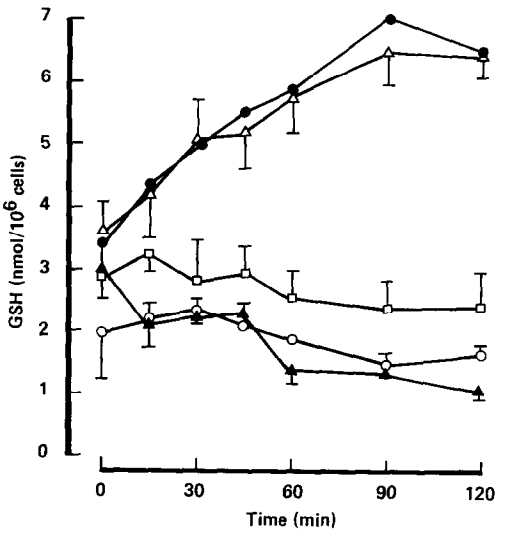

Fig. 1. GSH synthesis in Hep G2 cells following 60 min incubation with 0.02% (v/v) diethylmaleate. Synthesis was measured in cell suspensions (10^6/ml) in Krebs–Henseleit buffer, pH 7.4, containing 10 mM HEPES and supplemented with Dulbecco's amino acid solution deficient in sulphur containing amino acids (○—○, N = 3). This amino acid solution was further supplemented with 0.5 mM L-methionine (□—□, N = 4); 0.5 mM L-cysteine (△—△, N = 4); 0.5 mM L-methionine plus 0.5 mM L-cysteine (●—●, N = 2) or 0.5 mM L-cysteine plus 5 mM DL-buthionine-SR-sulfoximine (▲—▲, N = 5). Error bars represent the SEM.

experiments within the growth cycle when using Hep G2 cells to assess the cytotoxicity or genotoxicity of chemicals. At confluence, Hep G2 cells contain about 50% of the intracellular GSH content of freshly isolated human hepatocytes (19.4 ± 1.4 nmol/10^6 cels (N = 3); [16]) and this may render them more susceptible to xenobiotic-induced cytotoxicities than hepatocytes.

The effect of GSH depletion on cell growth was investigated by adding 0.5 mM BSO to the medium to inhibit GSH synthesis. Figure 2C shows that within 24 hr of adding BSO to the culture medium the GSH content had decreased to 3.8 ± 0.5 nmol/10^6 cells compared with 12.6 ± 1.0 nmol/10^6 cells in control flasks. Incubation of cells for 24 hr with higher concentrations of BSO (1 mM and 2 mM) did not cause greater depletion of GSH (results not shown). The rate of protein synthesis was not altered in BSO-containing flasks, but the BSO-treated cells divided more slowly (not significantly different at the 5% level, by non-paired Student's *t*-test) than control flasks between days 3 and 6. The effect of GSH depletion on the growth of cultured cells appears to depend on the particular cell line studied. BSO has been reported to depress the growth of cultured human breast cancer cells [21] whereas it has no effect on that of primary cultures of rat heart cells [22] or mouse lymphoma cells [23]. BSO is not cytotoxic to Hep G2 cells at the concentration required to deplete GSH (data not shown), so this method of GSH depletion is suitable for investigating mechanisms of xenobiotic induced cytotoxicities.

The time course of GSH depletion by BSO was investigated in cells 24 hr and 7 days after subculture. Figure 3 shows that the GSH levels declined to 40% of those in control untreated cells within 10 hr of BSO addition, and that the rate of this decline was similar after 24 hr and 7 days subculture. Thus, the decline in GSH levels with time after subculture does not appear to be accompanied by a change in the half-life of GSH.

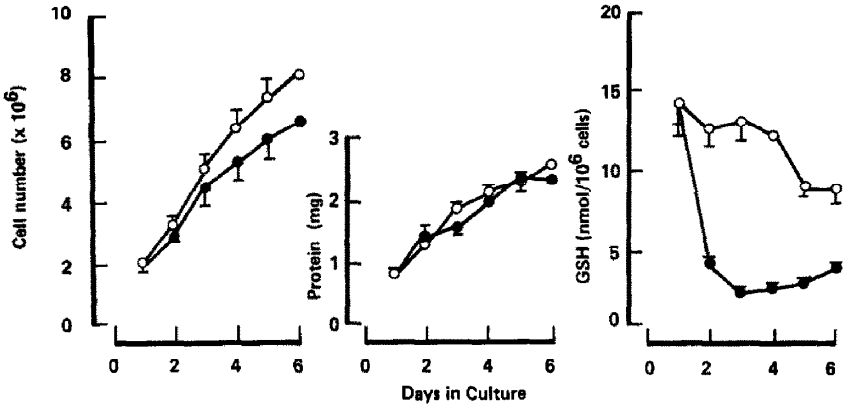

Fig. 2. Growth rate (Fig. A cell numbers per flask and Fig. B protein content per flask) and GSH content (Fig. C) of Hep G2 cells in the absence (○—○) and presence (●—●) of DL-buthionine-SR-sulfoximine. 0.5 mM DL-buthionine-SR-sulfoximine was added to treated flasks on day 1 (after 24 hr of culture). Results are the mean of between 5 and 7 experiments. Error bars represent the SEM.

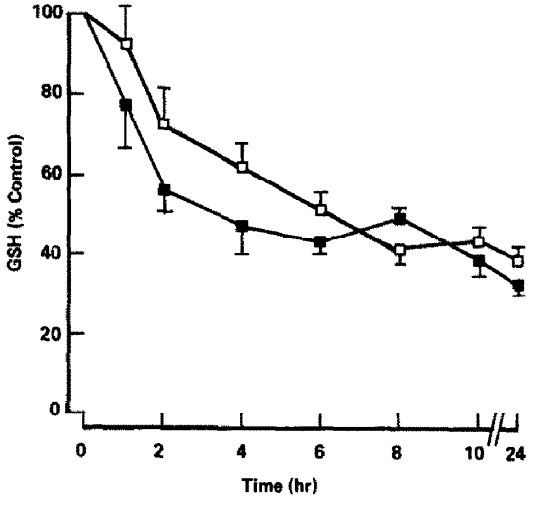

Fig. 3. Time course of GSH depletion by 0.5 mM DL-buthionine-SR-sulfoximine (BSO). The BSO was added at time zero to cells 24 hr (■—■) and 7 days (□—□) after subculture. GSH levels are expressed as a percentage of those in control untreated incubations. Results are the means of 4 experiments. Errors bars represent the SEM.

GSH conjugation with CDNB in Hep G2 cell suspensions compared well with that measured previously in this laboratory [20] in freshly-isolated human adult hepatocytes using the same method (1.22 ± 0.17 nmol/min/10^6 cells (N = 11) in Hep G2 cells and 1.69 ± 0.43 nmol/min/10^6 cells (N = 4) in hepatocytes). *In vitro* exposure to the inducers BA and PB increased GSH levels in Hep G2 cells but did not affect GST activity. BA increased GSH to 210.6 ± 8.9% (N = 5) and PB to 149.2 ± 5.9% (N = 5) of control cell values. Hepatic GST activities and GSH levels are known to be induced in the rat *in vivo* by treatment with barbiturate and polycyclic aromatic hydrocarbon type inducers [24, 25]. The concentrations of inducing agents used here, 25 μM for BA and 2 mM for PB, have been shown to induce MFO and UDP-glucuronyltransferase activities in Hep G2 cells [7]. However, GSTs in cultured hamster kidney cells are also resistant to *in vitro* BA induction [26] although the MFO activities are readily induced.

In conclusion, Hep G2 cells have not retained the cystathionine pathway which permits normal hepatocytes to utilise L-methionine for glutathione synthesis. Intracellular GSH levels in Hep G2 cells alter during growth of the cells in culture and, as this may affect their susceptibility to toxic chemicals, experiments investigating xenobiotic-induced toxicity require standardisation of the growth condition of the cells. The GSH content of Hep G2 cells can be manipulated using depletors such as DEM and BSO and by the inducing agents PB and BA.

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