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Methionine synthase activity and sulphur amino acid levels in the rat liver tumour cells HTC and Phi-1

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Abstract

Methionine dependence has been reported in tumour cells and suggested as a possible target for chemotherapeutic drugs. The underlying defect has not been extensively researched, nor have levels of sulphur amino acids been examined in these cells. This study compared two rat liver tumour cell lines. One was found to be methionine dependent (HTC) and the other found to be methionine independent (Phi-1). The methionine-dependent cell line (HTC) was discovered to contain markedly less methionine synthase activity, the enzyme activity being less responsive to methionine concentration than in the methionine-independent cells (Phi-1). HTC cells had lower cysteine requirements and contained larger concentrations of reduced glutathione (GSH) and taurine than the Phi-1 cells. Also, in contrast to Phi-1 cells, no glutathione was found in the media of the HTC cells, although large quantities of cysteinylglycine were detected. These results suggested that differences in methionine synthase activity might be partly responsible for methionine dependence and that methionine-dependent cells may have different metabolic requirements for other sulphur amino acids.

Keywords: Methionine synthase; Homocysteine; Glutathione; Taurine; Cysteine; Tumour cell; Methionine dependence; Hepatocytes

1. Introduction

The sulphur amino acids methionine, cysteine, taurine and homocysteine and the sulpho-peptide glutathione have many important functions. Methionine is required for initiation of protein synthesis by MET-tRNA formation [1] as well as for methylation reactions and polyamine production, via the production of *S*-adenosylmethionine [2]. Cysteine is required for protein synthesis; glutathione is responsible for detoxification of free radicals, conjugation with electrophiles and distribution of cysteine [3]. Taurine is an end product of sulphur amino acid metabolism and its levels can give an indication of their rate of metabolism through the transsulphuration pathway. It is also a putative antioxidant and is important for maintaining osmotic balance [4]. Homocysteine can be considered a toxic by-product of sulphur amino acid metabolism [5], but is also a precursor of cysteine, which is further utilised preferentially for protein or glutathione synthesis, the excess being catabolised to taurine and sulphate [4]. Elevated levels of plasma homocysteine have been recognised as an independent risk factor for cardiovascular disease [6]. The enzyme responsible for re-methylating homocysteine to methionine, is methionine synthase (EC 2.1.1.13). This enzyme requires methylcobalamin for activity and also produces tetrahydrofolate from 5-methyltetrahydrofolate (Fig. 1) [7]. Methionine synthase is the only enzyme capable of recycling folates at this point in the one carbon cycle. Homocysteine transmethylation is also catalysed by betaine homocysteine methyltransferase (EC 2.1.1.5), an intermediate step in the catabolism of choline that exhibits restricted tissue distribution and is believed to function to conserve methionine [5].

Whilst many important and expensive treatments for cancer are being pursued, cheaper strategies based upon the metabolic abnormalities of cancer cells should not be ignored. For this reason, reports in the literature that many tumour cells are methionine dependent [8] should be investigated. Methionine dependence is exhibited as the inability of the cancer cells to grow in a methionine-deprived environment, supplemented with homocysteine.

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Abbreviations: ATP, adenosine triphosphate; GSH, reduced glutathione.

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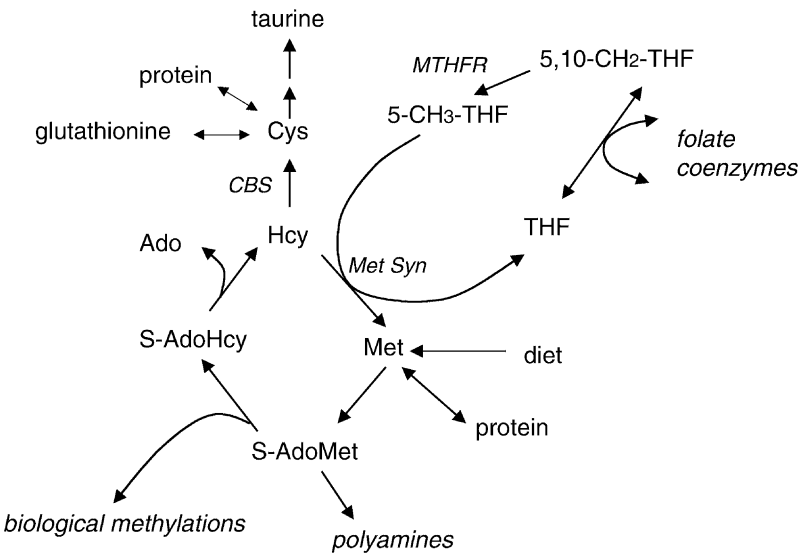

Fig. 1. Pathway showing how methionine is linked to the other sulphur amino acids. MetSyn: methionine synthase; CBS: cystathionine β-synthase; MTHFR: methylene tetrahydrofolate reductase; AdoMet: *S*-adenosyl-L-methionine; Ado: adenosine; Hcy: homocysteine; Met: methionine, THF: tetrahydrofolate; 5-CH_3-THF: 5-methyltetrahydrofolate; 5,10-CH_2-THF: 5,10-methylenetetrahydrofolate.

The biochemical basis of methionine dependence has not been fully elucidated [9].

Many of the enzymes and pathways summarised in Fig. 1 have been investigated in search of an explanation of this metabolic defect. Although methylenetetrahydrofolate reductase (MTHFR) deficiency has been considered as the main reason for methionine dependence, this has been ruled out as unlikely, since provision of high levels of the reaction product 5-methyltetrahydrofolate (5-MTHF) to methionine-dependent cells growing in methionine-deficient medium did not overcome growth inhibition [10,11]. It has also been suggested that transformed cells have a higher transmethylation rate and exhibit inability to compensate for a relative deficiency of methionine [11]. Defects in the methionine transamination (methionine salvage) pathway [13] and decreased methionine synthase activity have also been considered, although there are reports of both reduced and normal methionine synthase activity in methionine-dependent cells [12]. Altered cobalamin metabolism resulting in reduced methionine synthase activity [5,14] has also been considered as an explanation of this metabolic defect, whilst methionine-dependent cells were found to have their growth rate stimulated by growth limiting quantities of methionine and additional homocysteine, suggesting that methionine can still be formed from homocysteine [11]. To date, none of these hypotheses has successfully explained all the features of methionine dependence in cancer cells.

Removal of dietary methionine in cancer patients is reported to increase the effectiveness of chemotherapeutic agents [15,16]. In a similar study the effectiveness of methionine depletion has been attributed to a decrease in glutathione and ATP levels [17]. These studies show that although methionine dependence may not be fully understood, it has a great potential to be exploited clinically.

In the present study, two rat liver tumour cell lines were tested for methionine-dependence. HTC have previously been reported to be methionine dependent [18], but to our knowledge, Phi-1 cells have not previously been investigated. Primary rat hepatocytes were also investigated as they were assumed to give a 'normal' reference. We have also examined the effect that differences in methionine synthase and methionine concentration have upon the levels of sulphur amino acids in these cells.

2. Materials and methods

2.1. Materials

[^{14}C]5-methyltetrahydrofolate (55 mCi mmol^{-1}) was obtained from Amersham. AG1-X8 resin (chloride form mesh size 200–400) from Biorad. Dulbecco's modified Eagles medium (DMEM), modified Eagle's medium (MEM), modified Eagle's medium without methionine, non-essential amino acids (NEAA), glutamine, gentamicin and foetal calf serum were from Gibco. Hydroxycobalamin and 7-fluorobenzofurane-4-sulphonic acid, ammonium salt (SPD-f) were obtained from Fluka, D,L-homocysteine, methionine, dithiothreitol, phenylmethylsulphonylfluoride (PMSF), cyanocobalamin, glutathione (reduced form; GSH), cysteine, *O*-pthaldehyde, adenosine triphosphate (ATP), firefly lantern extract, *N*-acetylcysteine, Dowex 50 and all other chemicals were from Sigma.

2.2. Cells

HTC and Phi-1 cells were obtained from the European cell culture collection [19,20].

2.3. Cell culture

HTC and Phi-1 cells were routinely cultured in DMEM containing 10% foetal calf serum (FCS) undialysed, 1% NEAA and 0.12 mg mL^{-1} gentamicin. Treatment media consisted of: DMEM (as before) abbreviated to M200 since it contained 200 μM methionine. MEM containing 10% FCS, 1% NEAA, 0.12 mg mL^{-1} gentamicin, 1.5 μM cyanocobalamin and 10 μM folate, abbreviated to M100 as it contained 100 μM methionine. MEM containing 10% FCS, 1% NEAA, 0.12 mg mL^{-1} gentamicin, 1.5 μM cyanocobalamin, 10 μM folate and 100 μM D,L-homocysteine, abbreviated to MH100 as it contained 100 μM of homocysteine and 100 μM of methionine. MEM (containing all the components of MEM except methionine and glutamine) containing 10% FCS, 1% NEAA, 60 mg mL^{-1} gentamicin, 2 mM L-glutamine, 1.5 μM cyanocobalamin, 10 μM folate and 100 μM D,L-homocysteine abbreviated to H100 as it contained 100 μM homocysteine.

In experiments utilising confluent cells, cells were grown in 75 cm^2 flasks in DMEM until they were 70–80% confluent, as judged under the light microscope. Cells were washed with phosphate buffered saline (PBS) before treating with one of the media as described above.

In experiments using growing cells, cells previously grown in DMEM were seeded at a density of 3 million cells per flask in treatment media, except for cells treated with H100 media which were seeded at the higher density of 6 million cells per flasks, due to their slower growth rate. Each experiment was performed at least three times using duplicate flasks each time.

2.4. Treatment of cells at the experimental end point

At each time point, aliquots of media were removed from the flasks and stored at −80° for analysis of homocysteine, cysteine, glutathione, cysteinylglycine and taurine. The cells were then trypsinized and re-suspended in 5 mL media. Aliquots (0.5 mL) of the cell suspensions were removed, centrifuged in a microcentrifuge at 300 *g* for 5 s. The supernatant was removed and the cells resuspended in the same volume of 6.5% TCA and stored at −80° for use in GSH and ATP assays. Aliquots of the cells were also taken for cell number determination. The rest of the trypsinised cell suspension was centrifuged, the supernatant removed and the cell pellet stored at −80° for methionine synthase determination.

2.5. Isolation and handling of primary rat hepatocytes

Hepatocyte suspensions were prepared from rats using the two-stage collagenase perfusion, performed initially under anaesthesia (hypnorm:hypnovel:water 1:1:2) (3 mL kg^{-1}) by the method described by Moldeus *et al.* [21] as previously described [22]. Cells were used if viability was greater than 90% (assessed by Trypan Blue exclusion). The cells were resuspended in sterile Williams' medium E with Glutamax (0.75 million cells per millimeter), containing insulin (10 μM) 10% FCS, hydrocortisone (50 μg mL^{-1}) and gentamicin (0.12 mg mL^{-1}). Aliquots of the cell suspension (20 mL) were placed into 75 cm^2 flasks and incubated for 3 hr. After this time the cells were washed in PBS and the medium replaced. The different media used were: Williams' medium E containing insulin (10 μM), hydrocortisone (50 μg mL^{-1}), cyanocobalamin (1.5 μM), folate (10 μM) (again called M100). The previous media with an extra 100 μM methionine (M200), or 100 μM homocysteine (MH100), or Williams' medium E purchased without methionine to which 100 μM homocysteine and the previous ingredients were added (H100). The cells were cultured for 48 hr at which point they were harvested as described for the HTC and Phi-1 cells.

2.6. Cell number determination

Aliquots of cells trypsinized from the flasks were stained with Trypan Blue, for contrast and to determine whether the cells had been damaged. The cells were then counted using an haemocytometer, the final result being the mean of four counts.

2.7. Methionine synthase activity

Cell pellets (stored at −80°) were resuspended in 1 mL of homogenising buffer (50 mM phosphate, containing 0.1 M NaCl and 50 mg L^{-1} PMSF pH 7.2) and homogenised by sonicating for 3 × 5 s on ice using a probe sonicator. The homogenate was centrifuged at 300 *g* for 10 min and the supernatant assayed immediately for methionine synthase activity using a modified version of Weissbach *et al.* [23] described in Kenyon *et al.* [22]. Results were expressed as nmol of methionine produced per milligram of protein.

2.8. HPLC analysis of total homocysteine, cysteine, glutathione and cysteinylglycine in cell culture media

Levels of total homocysteine (free thiol, disulphide, homocysteine–cysteine mixed disulphide), glutathione, cysteinylglycine and cysteine were measured in media samples (stored at −80° until analysis) by fluorimetric detection (derivatisation with SPD-f) after separation by HPLC using a modified version of Fortin and Genest [24] described in Kenyon *et al.* [22]. *N*-acetylcysteine was used as an internal standard. Cysteine eluted after 2.5 min, homocysteine after 3.5 min, cysteinylglycine after 3.8 min, glutathione after 5.1 min and *N*-acetylcysteine after 6 min. Glutathione was measured as total reduced glutathione and glutathione disulphide. Results were expressed as total nmol of metabolite per milligram of total cellular protein.

2.9. Reduced glutathione measurement in cell cytosol

Reduced glutathione was determined as the total non-protein sulphydryls in the acid supernatant extract by a modification of Hissin and Hilf [25] adapted for use on the cytofluor series 400 fluorescent plate-reader (PerSeptive Biosystems). Aliquots of the acid extracts (7.5 μL) were placed onto clear 96-well plates. Phosphate buffer (1 M phosphate, 6 mM EDTA with NaOH pH 8.0) (277 μL), and 10 μL of OPA (1 mg mL^{-1} in methanol) was added to each well. Plates were incubated at room temperature for 25 min in the dark and subsequently read in the cytofluor plate reader (excitation wavelength: 350 nm; emission wavelength: 420 nm). Results were expressed as nmol of methionine produced per milligram of protein.

2.10. HPLC analysis of taurine

Taurine was measured in the media (stored at −80°) and cell homogenate (taken from homogenates prepared for methionine synthase activity and stored at −80° until analysis) by a modification of the method of Waterfield [26] as described previously [22]. Results were expressed as: internal taurine concentration (nmol per milligram protein) and external taurine concentration (nmol per milligram total cellular protein).

2.11. ATP measurement

ATP was determined in acid cell extracts according to the method of Stanley and Williams [27], but modified for use on a 96-well plate luminometer (Dynex Technologies). Briefly, 3 μL of supernatant remaining after centrifugation of TCA extracts (300 *g*, 5 min) or standard, was placed in a black 96-well plate. Buffer (80 mM $MgSO_4 \cdot 7H_2O$; 10 mM KH_2PO_4; 100 mM $Na_2HAsO_4 \cdot 7H_2O$ 1:1:1), 170 μL was added and the plate then placed in the luminometer. Luciferase (firefly lantern extract + 5 mL water + 15 mL buffer), was added (40 μL) and the luminescence measured on a the luminometer. Results were expressed as nmol per milligram protein.

2.12. Protein content analysis

After analysis of ATP and GSH the protein pellets from the TCA extracts were redissolved in NaOH; aliquots of homogenate (produced for methionine synthase analysis) were taken and both of these were stored at −20° until analysis. Protein content was assessed using Coomassie Blue Plus reagent for both types of sample. Briefly, equal volumes of diluted sample (or BSA standard) and reagent, were placed in clear 96-well plates and the absorbance read at 595 nm on the Spectromax 190 plate reader (Molecular Devices). Results were expressed as mg mL^{-1}.

2.13. Statistical analysis

All results are expressed as means ± SEM of the specified number of experiments. Statistical evaluation of data was carried out by analysis of variance and Duncan's *t*-test which compares all test groups with each other [28].

3. Results

3.1. Effect of different media methionine and homocysteine composition upon cell number

HTC and Phi-1 cells were treated with different media over 4 days. The end point of 4 days was chosen for the time span of the experiment as growing cells were confluent at day 4 due to the high seeding density. Both confluent and growing cells were counted every day.

Fig. 2A and B show the percentage increase in cell number from the seeding density of HTC and Phi-1 cells, respectively, over a period of 4 days. As can be seen, both cell types grew well when treated with the M200 media. Overall, HTC cells grew faster than Phi-1 cells, with the exception of those cells treated with H100 media where no growth was observed. Replacement of methionine with homocysteine (H100) also significantly decreased Phi-1 cell growth but cells were still able to grow; the percentage increase in cell number at day 4 was significantly greater

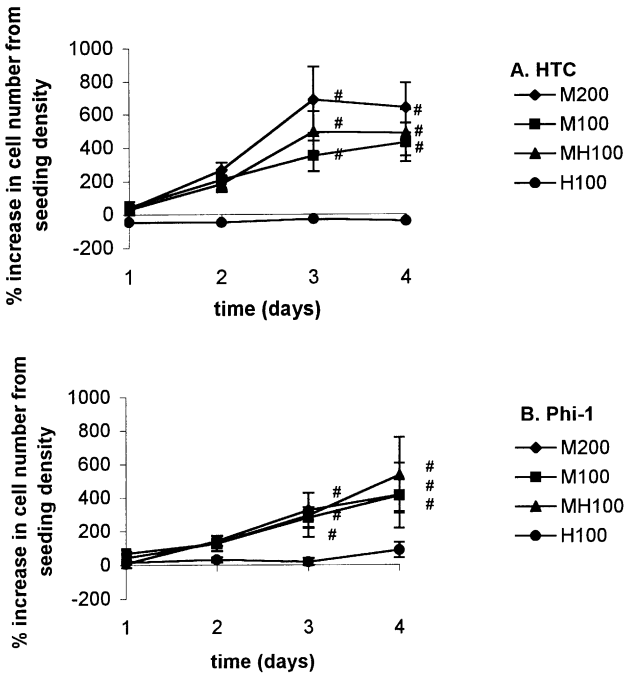

Fig. 2. The increase in cell number of (A) growing HTC and (B) Phi-1 cells treated with media containing different concentrations of methionine and/or homocysteine over 4 days. Data expressed as percentage difference from seeding density. Values are means ± SEM of three to four different experiments. (#) $P > 0.001$ comparing days 2, 3 and 4 to day 1 for each one of the treatments. All comparisons were made using Duncan's *t*-test.

than the seeding density ($P > 0.05$ comparing day 4 to day 1).

Confluent cells were also used in these experiments, to allow comparison of the behaviour of the tumour cells with primary rat hepatocytes, which do not grow in culture. The cell count for confluent HTC treated with M100, MH100 or H100 was between 20 and 30 $\times$ 10^6 cells and no significant change in cell number was observed over time (data not shown). When HTC cells were treated with M200, they continued to increase in number. Conversely, confluent Phi-1 cells did not show any significant change in cell number, under any condition of growth (cell count 27–37 $\times$ 10^6 cells) (data not shown).

3.2. Effect of different media methionine and homocysteine composition upon methionine synthase activity

Methionine synthase activity was measured in growing and confluent cells (Fig. 3). In general, methionine synthase activity in growing HTC cells (Fig. 3A) was found to be much lower than the corresponding value in

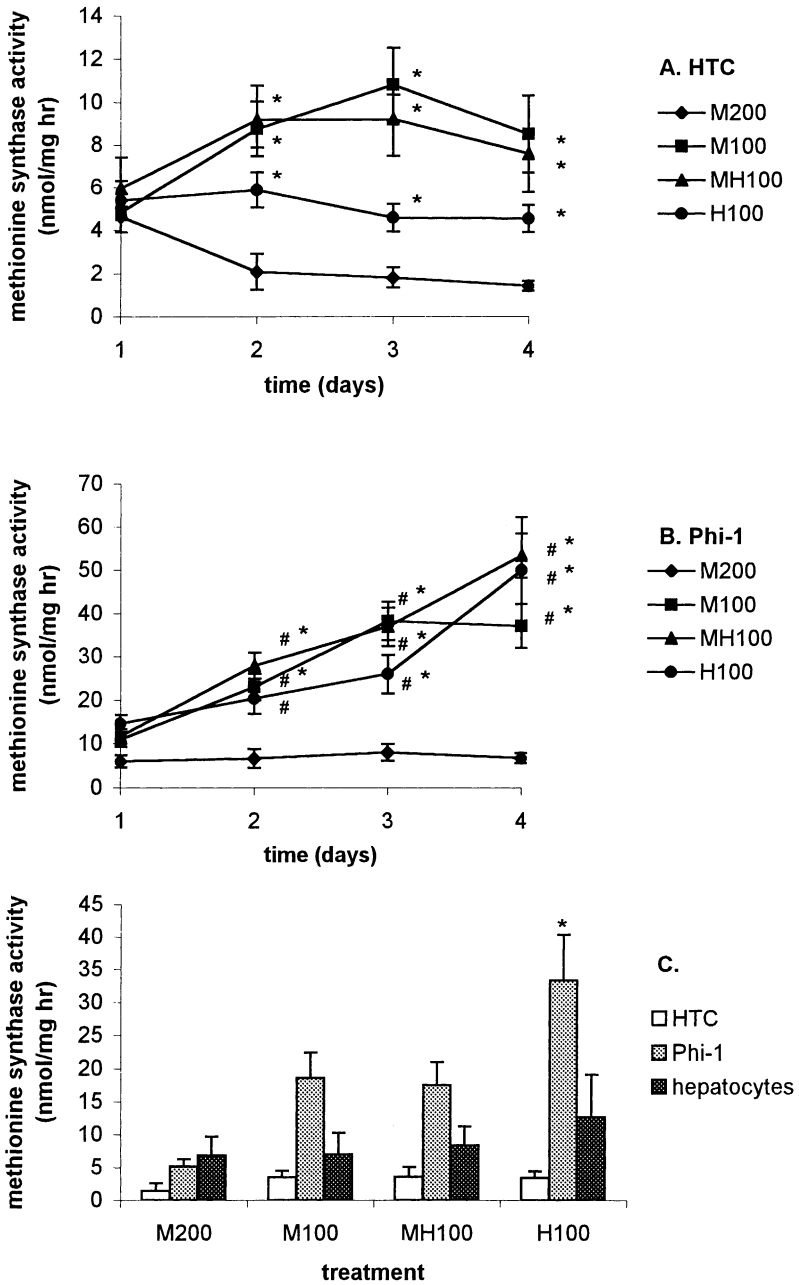

Fig. 3. Methionine synthase activity in (A) growing HTC; (B) growing Phi-1 and (C) confluent HTC, confluent Phi-1 and primary hepatocytes. Data shown over 4 days of treatment for growing cells, and after 2 days of treatment for confluent cells. Values are means ± SEM of three to four different experiments. (#) $P > 0.001$ comparing days 2, 3 and 4 to day 1 for each one of the treatments; (*) $P > 0.001$ comparing all treatments to M200. All comparisons were made using Duncan's *t*-test.

growing Phi-1 cells (Fig. 3B), with all treatments. When both cell lines were treated with high methionine media (M200), methionine synthase activity was found to be significantly lower than in all other cases (M100, MH100, and H100). For Phi-1 cells, methionine synthase activity in the M200 media did not change significantly over the period of 4 days (Fig. 3B) whilst in the HTC cells (Fig. 3A) a reduction in activity over time was observed.

Treatment of the growing HTC cells with different concentrations of methionine and homocysteine (M100, MH100 and H200) resulted in a small increase of methionine synthase activity, a result not exceeding 2-fold in any case (Fig. 3A).

Interestingly, when growing Phi-1 cells were treated with M100, MH100 and H100 methionine synthase activity increased significantly over time; the increase being more profound with the H100 and MH100 treatments, where a 5-fold increase in methionine synthase activity was observed after 4 days (Fig. 3B).

Primary rat hepatocytes, prepared using the collagenase perfusion method, were plated and kept in culture for 48 hr. These cells were used as a control system to give an insight on the effect of M100, MH100, H100 and M200 treatments on normal liver cells. In order to be able to directly compare the effect of these treatments, plated primary hepatocytes, and confluent HTC and Phi-1 cells grown with complete media, were treated with M100, MH100, H100 and M200 for 48 hr (Fig. 3C).

Methionine synthase activity in primary hepatocytes treated with M200 for 48 hr (4–10 nmol mg^{-1} hr^{-1}) was greater than that observed in confluent HTC cells (2 nmol mg^{-1} hr^{-1}), and comparable to that in confluent Phi-1 cells (5 nmol mg^{-1} hr^{-1}) treated in the same way. Although, methionine synthase activity in primary hepatocytes was not found to change upon reduction of the concentration of methionine (M100, MH100) an approximate 2-fold increase in enzyme activity was noted when methionine was removed from the media and replaced with homocysteine (H100) (Fig. 3C).

Compared with the M200 treatment, neither lowering the concentration of methionine (M100, MH100) nor replacing it with homocysteine (H100), resulted in a significant change in methionine synthase activity, in confluent HTC cells.

In confluent Phi-1 cells, methionine synthase activity increased inversely with the methionine content of the media. Treatment of Phi-1 cells with M100 or MH100 media resulted in increased methionine synthase activity, compared with M200 treated cells. When confluent Phi-1 cells were treated with H100 media the enzyme activity

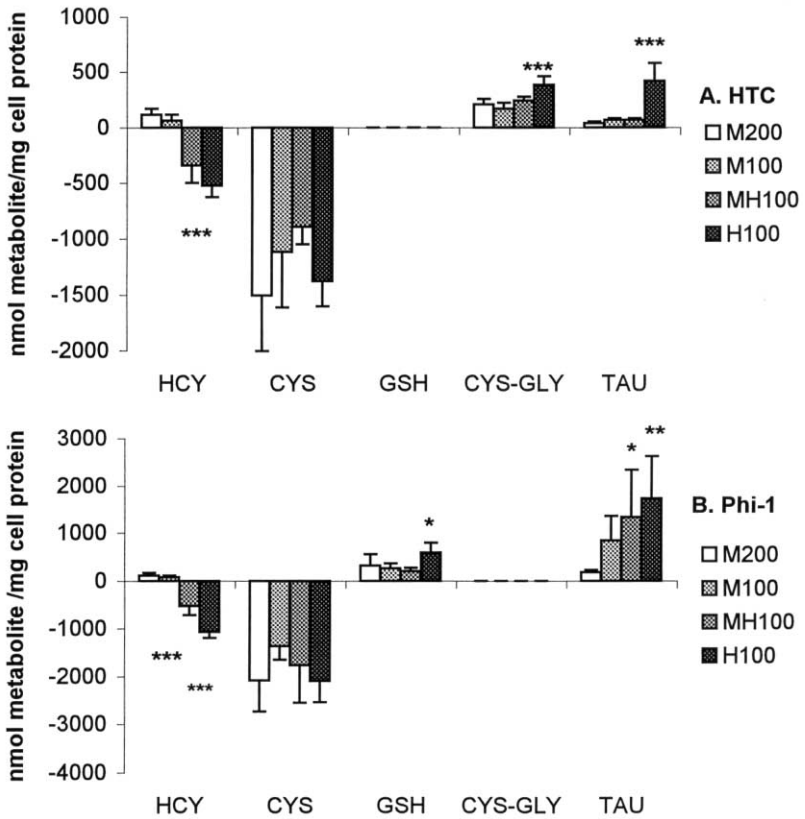

Fig. 4. Homocysteine, cysteine, glutathione (measured as total reduced glutathione and glutathione dissulphide), cysteinylglycine and taurine concentration in the media of growing HTC (A) and Phi-1 cells (B). Data for homocysteine and cysteine represent the uptake (negative values) or export (positive values) of those metabolites. Data shown after 2 days of treatment for all cell types. Values are means ± SEM of three to four different experiments, (∗) $P > 0.05$; (∗∗) $P > 0.01$; (∗∗∗) $P > 0.001$ comparing all treatments to M200. All comparisons were made using Duncan's *t*-test.

was found to increase 7-fold (Fig. 3C) compared to the M200 treatment and almost 2-fold compared to the M100 and MH100 treatments.

3.3. Effect of different media treatments upon homocysteine and cysteine concentrations

Homocysteine was removed by the cells from media supplemented with this amino acid (MH100 and H100) or exported into the unsupplemented media (M200 and M100) (Figs. 4 and 5). All cell types, when confluent (Fig. 5), removed more homocysteine from the H100 than from the MH100 media, with Phi-1 cells using the greatest quantity of homocysteine from the H100 media. When treated with MH100 media, confluent Phi-1 cells were found to export homocysteine in contrast to the HTC cells and primary hepatocytes. HTC cells used quantities of homocysteine that were similar to the amount used by the primary hepatocytes. Confluent Phi-1 cells exported more homocysteine than the HTC or primary hepatocytes when treated with M200 or M100. Both HTC and Phi-1 cells removed more homocysteine from MH100 or H100 media when they were growing (Fig. 4) compared to confluent (Fig. 5). Similarly both cell types exported less homocysteine when they were growing in M200 or M100 than when they were confluent (Figs. 4 and 5).

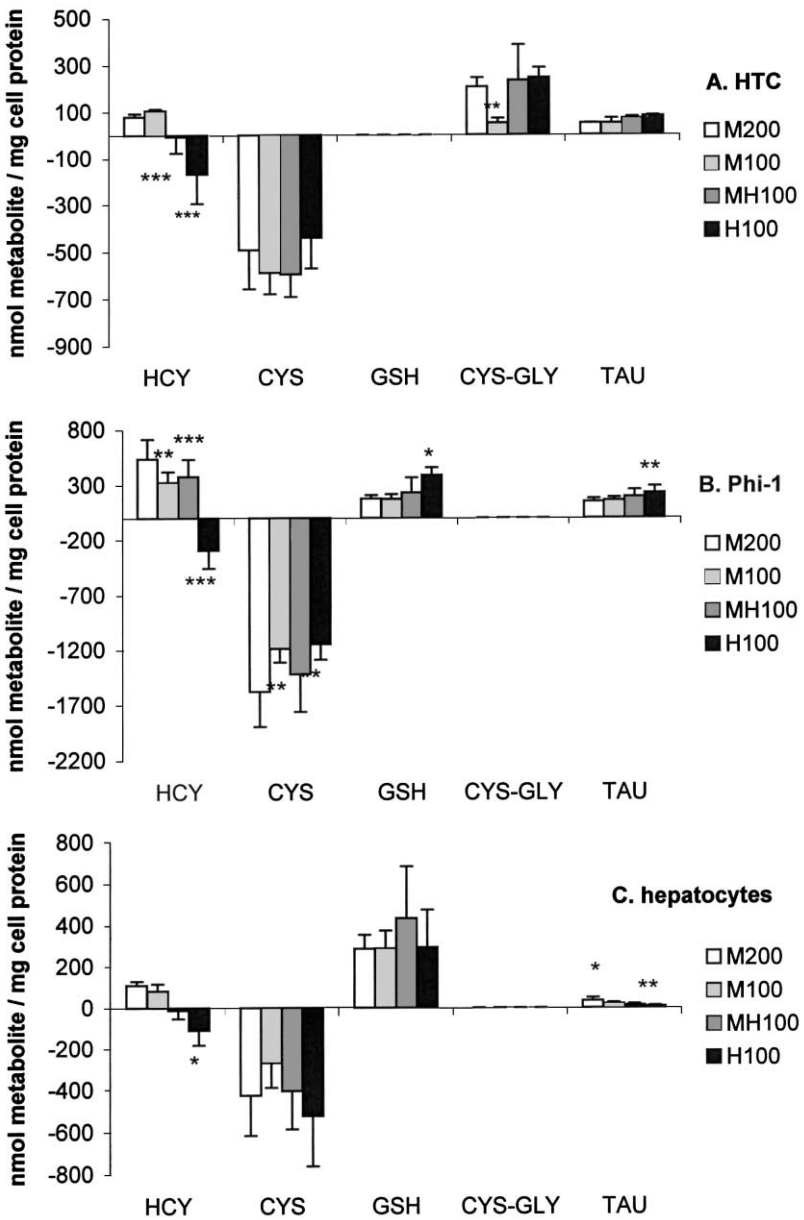

Fig. 5. Homocysteine, cysteine, glutathione (measured as total reduced glutathione and glutathione dissulphide), cysteinylglycine and taurine concentration in the media of confluent HTC (A); confluent Phi-1 (B) and primary hepatocytes (C). Data for homocysteine and cysteine represent the uptake (negative values) or export (positive values) of those metabolites. Data shown after 2 days of treatment for all cell types. Values are means ± SEM of three to four different experiments. (*) $P > 0.05$; (**) $P > 0.01$; (***) $P > 0.001$ comparing all treatments to M200. All comparisons were made using Duncan's *t*-test.

Fig. 5 shows the uptake of cysteine from the media by confluent Phi-1, HTC and primary hepatocytes. Phi-1 cells however removed larger quantities of cysteine from the medium than HTC or primary hepatocytes. As expected, growing cells required greater quantities of cysteine than confluent cells (Figs. 4 and 5). As the quantity of cysteine removed from the media per microgram of protein did not change over time (data not shown), the external cysteine concentration appeared to be in equilibrium.

3.4. Effect of different media upon glutathione concentration in the two cell types

Reduced glutathione concentrations measured in acidified cell lysate (*internal*) (Fig. 6) and total glutathione (total reduced glutathione and glutathione dissulphide) measured in the culture media (*external*) (Figs. 4 and 5) were assessed after the various treatments (M200, M100, MH100 and H100).

Internal: Reduced glutathione concentrations were higher in confluent HTC cells than confluent Phi-1 cells or primary hepatocytes, for all the treatments except MH100 (Fig. 6). Growing HTC cells also contained higher GSH concentrations than Phi-1 cells and Phi-1 contained more GSH than primary hepatocytes.

External: Similar quantities of total glutathione were detected in the media of confluent Phi-1 and primary hepatocytes (Fig. 5). No glutathione was detectable in the media of HTC cells. Cys-gly, the product of external glutathione breakdown, was detectable in the media of HTC cells (Fig. 5). Very low concentrations of cys-gly were detected in their media where Phi-1 cells were grown (around 0.6 nmol mg^{-1} protein). In growing Phi-1 cells (Fig. 4) similar quantities of glutathione were detected to those found in the media of confluent Phi-1 cells and primary hepatocytes (Fig. 5). Those cells treated with H100 media exported most glutathione. In the media of growing HTC cells most cys-gly was found with the MH100 and H100 treatments (Fig. 4).

3.5. Effect of different media upon taurine production

Taurine concentrations were measured in the cytosol (*internal*) (Fig. 6) as well as at the culture media (*external*) (Figs. 4 and 5) after the various treatments (M200, M100, MH100 and H100).

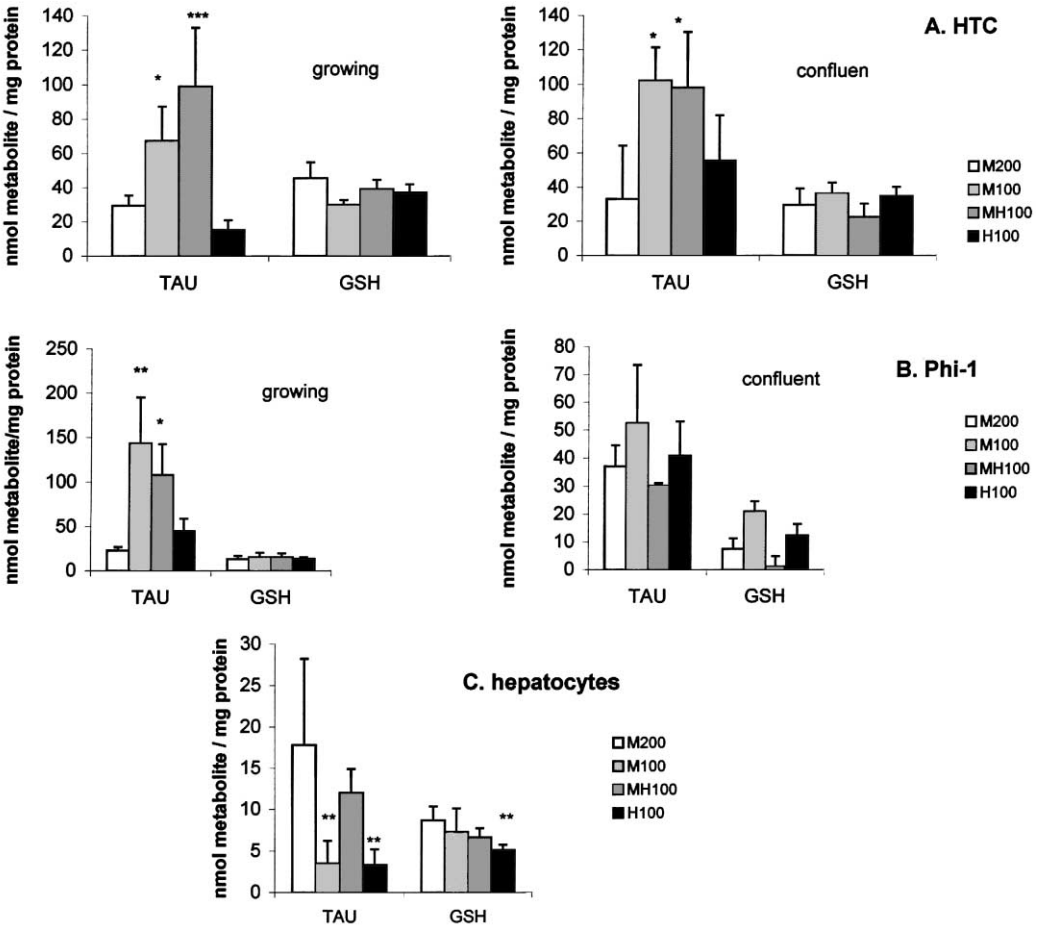

Fig. 6. Glutathione and taurine in the cytosol of growing and confluent HTC (A), growing and confluent Phi-1 (B) and primary hepatocytes (C). Data shown after 2 days of treatment for all cell types. Values are means ± SEM of three to four different experiments. (*) $P > 0.05$; (**) $P > 0.01$; (***) $P > 0.001$ comparing all treatments to M200. All comparisons were made using Duncan's *t*-test.

Internal: Confluent HTC and Phi-1 cells had higher internal taurine levels than primary hepatocytes (Fig. 6). There was no difference in taurine concentration between treatments in confluent Phi-1 cells. However, in confluent HTC, growing HTC and growing Phi-1 cells, M100 and MH100 treatments resulted in larger cytosolic amounts of taurine than in the M200 or H100 treatments. Confluent Phi-1 cells were found to contain less taurine than growing Phi-1 and, growing and confluent HTC cells.

External: Confluent (Fig. 5) and growing (Fig. 4) Phi-1 cells had higher external taurine levels than HTC cells or primary hepatocytes. The external concentration of taurine was higher in growing Phi-1 cells compared with confluent cells (Fig. 5). In HTC cells, the taurine concentration was much higher in the H100 treated cells than for the other treatments.

3.6. Effect of different media upon ATP levels

There was no difference in ATP levels between HTC or Phi-1 cells, whatever the treatment, whether the cells were confluent or growing (data not shown). Mean ATP concentration was: 13.6 ± 5.9 nmol mg^{-1} protein.

4. Discussion

Two rat liver cell lines HTC and Phi-1 were treated with media containing differing concentrations of methionine and homocysteine and compared to primary hepatocytes. Firstly, the survival of confluent cells treated with the different media was tested. Although homocysteine is considered to be toxic in high concentrations, treatment with H100 (or MH100) did not reduce cell number, or decrease the ATP concentration of the cells (a marker of reduced metabolic competence). The addition of homocysteine to a methionine containing medium (MH100) did not slow the growth of the cells, which would have been expected if homocysteine was toxic or cytostatic. Again ATP levels in growing cells were not affected by the MH100 treatment. This is in contrast to the findings of Poirson-Bichat *et al.* [29] who reported decreases in ATP concentration in PC-3 cells, grown in media containing homocysteine but no methionine.

When growing HTC cells were treated with the different media, the cells grew best in the M200 media (Fig. 2A), whereas the Phi-1 cells grew at the same rate in M200, M100 and MH100; this may indicate that the HTC cells have a greater requirement for methionine. Growing Phi-1 cells treated with the H100 media survived, but an increase in cell number was only obvious at day 4, indicating that a period of adjustment was required. The HTC cells did not grow in the H100 medium during the time course of this experiment, indeed many of the cells died. HTC cells were thus dependent upon methionine for growth confirming a previous report and our unpublished data [18]. Phi-1 cells were shown to be methionine independent. Methionine dependence was only noted when cells were grown in methionine-deficient media, since confluent cells survived in H100 media for up to 48 hr, in this study.

Methionine synthase activity has previously been proposed to be defective in methionine-dependent cells [8], therefore the activity of this enzyme in the two cell lines was assessed and compared to primary hepatocytes.

Methionine synthase activity has been measured in other tumour cell lines. HepG2 cells (human liver tumour) had enzyme activity of 15 ± 2 pmol mg^{-1} min^{-1} (equivalent to 0.9 nmol mg^{-1} hr^{-1}) [30]. A glioma cell line GaMg, P60 (methionine dependent) had methionine synthase activity of 6.9 nmol mg^{-1} hr^{-1} a methionine-independent revertant P60H had enzyme activity of 13.4 nmol mg^{-1} hr^{-1} [31]. In this study, confluent HTC cells (methionine dependent) were found to contain lower methionine synthase activity than either primary hepatocytes or Phi-1 cells (methionine independent) under all treatments (Fig. 3), in agreement with the results obtained from the methionine-dependent and -independent glioma cells [30]. Overall, methionine synthase activity in methionine- and methionine-independent cells was found to be altered in response to the concentration of methionine in the media, an effect also noted by Kamely *et al.* [32]. The exception to this trend was observed in confluent HTC cells, where the lack of change of methionine synthase activity could be attributed to a feature of those methionine-dependent cells. Other methionine-dependent cell types need to be tested to confirm this finding. Although the observed changes in the activity of methionine synthase suggest that methionine exercises a regulatory effect on the enzyme, we cannot ignore the possibility of homocysteine taking part in this mechanism. This hypothesis is further strengthened by the 7-fold increase of methionine syntase activity in H100 treated HTC as compared to M200 treated cells. Further studies are needed to elucidate this.

Methionine synthase activity was higher in growing HTC cells than in confluent cells; and in growing Phi-1 than in confluent Phi-1 cells, when given the same treatment. This suggests that the activity of methionine synthase also changes in relation to the growth status of the cells. This may be an important implication for potential cancer treatment as tumour cells generally grow faster than normal cells. In growing HTC cells, the H100 treatment resulted in lower enzyme activity than the M100 or MH100 treatments, in contrast to the effect observed in growing Phi-1 and confluent HTC and Phi-1 cells. Lack of methionine synthase activity may have been due to shortage of methionine available for protein synthesis. Interestingly in growing Phi-1 treated with H100 there was a delay before methionine synthase activity increased; this increase in specific activity corresponded with the observed increase in cell number of the Phi-1 cells.

Previous reports have indicated that methionine dependence in the GaMg glioma cell line could be attributed to

decreased production of methylcobalamin (the cofactor for methionine synthase) [14]. It has also been reported that the addition of cyanocobalamin to tumour cell culture media increases methionine synthase activity [30]. In the present study, cyanocobalamin concentrations in the media have not been altered and therefore none of the observed changed can be attributed to it. The possibility that the defect in methionine synthase activity in HTC may be caused by lack of available methylcobalamin cannot be ruled out, as this was not investigated.

Despite higher methionine synthase activity, confluent Phi-1 cells treated with M200 or M100 exported greater amounts of homocysteine than HTC and primary hepatocytes (Fig. 5). The greater uptake of homocysteine in growing cells may reflect the increase in methionine synthase activity in growing cells compared with confluent cells. As expected, more cysteine was removed from the media by growing cells than confluent cells as growing cells have a greater protein production. The quantity of homocysteine or methionine in the media did not appear to affect the cysteine requirements of growing or confluent cells.

Confluent and growing HTC cells had much higher cytosolic concentrations of GSH than Phi-1 or primary hepatocytes (Figs. 4 and 5), despite their lower cysteine uptake. Perhaps methionine dependence is advantageous to tumour cells by providing greater concentrations of glutathione. Decreasing the methionine concentration in the media did not decrease GSH concentration as previously observed [17]. The higher GSH concentration in HTC cells may also have resulted from differences in glutathione export; as Phi-1 cells were found to export glutathione (Figs. 4 and 5). No glutathione could be detected in the media of confluent or growing HTC cells, although cysteinylglycine was. External glutathione is taken up by the cells and broken down to cysteinylglycine by γ-glutamyltranspeptidase [3]. It appears that HTC cells metabolised exported glutathione at a much faster rate than Phi-1 cells as the concentration of cysteinylglycine in HTC media was similar to that of glutathione in Phi-1 media. Cysteinylglycine can be taken up into cells and used as a source of cysteine, which may explain the reduced cysteine requirements and reduced glutathione levels of the HTC cells.

Confluent HTC and Phi-1 cells appeared to have higher internal taurine levels than primary hepatocytes, but this was due to taurine contained in the serum used in the media used to grow the tumour cells. Phi-1 cells were found to remove less taurine from the media than HTC cells, even though internal concentrations of taurine in those two cell types were found to be similar. This finding may indicate that synthesis of taurine in the Phi-1 cells was more efficient than in HTC cells. Interestingly in the growing HTC cells treated with H100 the concentration of taurine in the media was far higher than with any other treatment, indicating that lack of methionine was affecting the uptake of taurine into HTC cells. It is also possible that homocysteine inhibits the uptake of taurine. Further studies are needed to explore this hypothesis.

This study has shown that methionine synthase activity and levels of sulphur amino acids are different in methionine dependent compared with methionine-independent tumour cells. The relationship between methionine synthase and glutathione needs to be examined more closely to determine whether differences between tumour cells and ordinary cells can be exploited to lower tumour cell glutathione and increase the effectiveness of chemotherapeutic drugs, without causing increased toxicity. The differences in sulphur amino acid concentrations and the different utilisation of them between the two cell types suggests that there may be other enzymes in the sulphur amino acid pathway that could prove to be useful drug targets.

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