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Biochemical Pharmacology 67 (2004) 1587–1599
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Analysis of cell-cycle kinetics and sulfur amino acid metabolism in methionine-dependent tumor cell lines; the effect of homocysteine supplementation

Valérie Pavillard[a,b], Abedalnaser A.A. Drbal[a], David J. Swaine[b], Roger M. Phillips[b], John A. Double[b], Anna Nicolaou[a,*]

[a]*School of Pharmacy, University of Bradford, Bradford BD7 1DP, UK*
[b]*Cancer Research Unit, Tom Connors Cancer Research Centre, University of Bradford, Bradford BD7 1DP, UK*

Received 8 October 2003; accepted 12 January 2004

Abstract

Methionine dependence is a feature unique to cancer cells, exhibited as inability to grow in a methionine-depleted environment supplemented with homocysteine, the immediate metabolic precursor of methionine. This study explores the effect of methionine depletion and homocysteine supplementation on the viability, sulfur amino acid metabolism and cell-cycle kinetics of normal and cancer cells, as well as their ability to recover from the treatments. An array of cells including hepatomas (HTC, Phi-1), prostate adenocarcinomas (PC-3) and transformed (3T3) and normal (HS-27) fibroblasts, has been used aiming to evaluate the importance of tissue specificity. All cell lines proliferated well in methionine-complete media (M+H−), whilst only the normal fibroblasts HS-27 grew in methionine-depleted homocysteine-supplemented media (M−H+). None of the tested cell lines were able to grow in media without methionine or homocysteine (M−H−). HTC was the only cell line that did not recover from the M−H+ treatment whilst PC-3 did not recover from the M−H− treatment. Methionine and homocysteine depletion (M−H+ and M−H−) were found to induce arrest at different phases of the cell cycle, depending on the cell line: the methionine-dependent HTC, PC-3 and 3T3 arrested at the S and G2/M phase, whilst Phi-1 and the methionine-independent HS-27 accumulated in the G1 phase. The cell-cycle kinetics showed that the observed blockades were reversible. The information resulting from these studies is important for not only the behavior of cancer cells, but also for appreciating the potential of developing cancer therapies based on methionine-depletion strategies.

Keywords: Methionine dependence; Homocysteine; Sulfur amino acids; Cancer cells; Cell-cycle kinetics; Transsulfuration

1. Introduction

A broad range of human and animal cancer cell lines and tumors have been described as methionine dependent. Methionine dependence is termed as the inability of cancer cells to grow in a methionine-depleted environment supplemented with homocysteine, the immediate metabolic precursor of methionine. In contrast, normal cells are able to efficiently utilize homocysteine and sustain growth in the absence of externally provided methionine (methionine-independent cells) [1–4]. A number of cancer cells show methionine-independent phenotypes although they exhibit significantly lower growth rates when compared to methionine-containing control cultures [3,4]. Impaired 5,10-methylene tetrahydrofolate reductase and methionine synthase activities [4–7], impaired transamination (methionine salvage) pathway [8], as well as differential use of exogenous and de novo produced methionine for increased rate of transmethylations [9,10], are among the lines of investigation that have been explored in an attempt to identify the origins of methionine dependence. However, none of these paths has provided a hypothesis that can fully explain all the features of this metabolic defect, hence the biochemical basis of methionine dependence remains to be elucidated.

Abbreviations: AdoMet, *S*-adenosylmethionine; SBD-f, 7-fluorobenzofurane-4-sulfonic acid, ammonium salt; MEM, Modified Eagle's medium; DMEM, Dubelcco's modified Eagle medium; RPMI, Roswell Park Memorial Institute medium; Cys, cysteine; Hcy, homocysteine; Cys-Gly, cysteinyl-glycine; GSH, glutathione.

* Corresponding author. Tel.: +44-1274-234717; fax: +44-1274-235600.
E-mail address: a.nicolaou@bradford.ac.uk (A. Nicolaou).

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doi:10.1016/j.bcp.2004.01.006

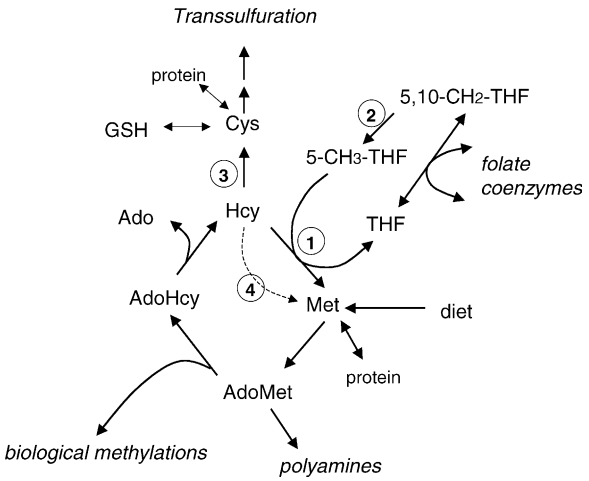

Fig. 1. Major biochemical pathways related to methionine and homocysteine metabolism. AdoMet: *S*-adenosylmethionine; AdoHcy: *S*-adenosylhomocysteine; Met: methionine; Hcy: homocysteine; GSH: glutathione; THF: tetrahydrofolate; 5-CH_3-THF: 5-methyltetrahydrofolate; 5,10-CH_2-THF: 5,10-methylenetetrahydrofolate; (1) methionine synthase; (2) methylene tetrahydrofolate reductase; (3) cystathionine β synthase; (4) betaine-synthase.

Methionine is an essential amino acid provided by the diet and the de novo recycling of homocysteine [11]. Two major intersecting pathways are involved in methionine metabolism, the methionine cycle and the sequential transsulfuration reactions (Fig. 1). Whilst the methionine cycle is ubiquitously expressed, the transsulfuration pathway is limited to liver, kidney, small intestine and pancreas. Apart of its role in protein synthesis, methionine is the substrate for the formation of *S*-adenosylmethionine (AdoMet), the broadly utilized methyl donor for DNA, protein and lipid methylating reactions. Homocysteine is involved in the salvage of the one-carbon pathway, recycling of intracellular folates, catabolism of choline and betaine in the liver, and the transsulfuration pathway as precursor of cystathionine, cysteine and, eventually, glutathione. Excessive concentrations of homocysteine and methionine are exported from the cells [12,13]. Cysteine is used in protein synthesis and the synthesis of glutathionine, a thiol-containing tripeptide with multiple function ranging from antioxidant defence to modulation of cell proliferation [14]. Cysteine is provided by the diet or tissues expressing the enzymes of the transsulfuration pathway and has also been shown to reduce the requirements for methionine in murine [15] and human [16] cells. However, due to constitutive expression of the homocysteine transmethylation pathway as opposed to the limited tissue distribution of transsulfuration [17], methionine sparing by cysteine appears to be limited to liver cells.

One of the hallmarks of cancer cells is the loss of cell-cycle checkpoints [18]. This leads to loss of genomic integrity, and dysregulation of oncogenes and tumor suppressor genes, all contributing to tumorigenesis. Therapeutic manipulation of cell-cycle checkpoints has been one of the major modern directions for development of cancer therapeutics [19,20]. Methionine depletion has been shown to reversibly arrest the cell cycle of methionine-dependent cells at the S and G2/M phase [21,22]. This feature has been considered as a target for selective targeting of methionine-dependent cancer cells using chemotherapeutic agents [10,23,24]. The majority of these studies have focused on the cell-cycle effects during methionine depletion but little is known about the biochemical and cell-cycle events that take place when the cell-cycle block is released by replacement with methionine-rich media. Since most studies on methionine depletion utilize homocysteine supplementation, it is important to investigate and appreciate the effect of homocysteine in the cell cycle and sulfur amino acid metabolism during treatment and recovery studies. It is conceivable that the cell cycle of methionine-dependent cells may be synchronized for a period of time during this recovery phase thereby providing opportunities for therapeutic intervention using cell-cycle phase specified anti-cancer drugs. The principle objective of this study is to characterize the cell-cycle effects and sulfur amino acid biochemistry of normal and cancer cells during methionine depletion, with and without homocysteine supplementation, as well as during recovery from these treatments. Since the two major pathways that regulate the methionine and homocysteine pathways are tissue dependent [11], an array of cells including hepatomas, epithelial cells and fibroblasts has been used aiming to evaluate the importance of tissue specificity.

2. Materials and methods

2.1. Materials

Modified Eagle's medium (MEM), Dubelcco's modified Eagle medium (DMEM), Roswell Park Memorial Institute medium (RPMI), trypsin-ethylenediaminotetraacetic acid solution (Trypsin-EDTA), MEM non-essential amino acid solution, L-glutamine, sodium pyruvate, penicillin and streptomycin solutions, insulin, D,L-homocysteine thiolactone, propidium iodide, RNase A, glutathione (reduced form), DL-homocysteine, cysteine, cysteinyl-glycine and *N*-acetyl cysteine were obtained from Sigma (Dorset, UK). MEM, DMEM and RPMI without methionine were from ICN. Hepes was purchased from Gibco BRL. One kilodalton cut-off dialyzed fetal calf serum was obtained from First Link. 7-Fluorobenzofurane-4-sulfonic acid, ammonium salt (SBD-f) was obtained from Fluka.

2.2. Cell lines

Five different cell lines were used: HTC and Phi-1 (rat hepatocarcinoma), PC-3 (human prostate adenocarcinoma), 3T3 (mouse transformed fibroblasts) and HS-27 (non-transformed human new-born foreskin fibroblasts).

All the cell lines were obtained from the European Collection of Cell Cultures.

2.3. Cell culture

Cells were maintained in vitro at 37 °C in a 5% carbon dioxide 95% air humidified incubator. HTC and Phi-1 cells were grown in MEM, PC-3 cells in RPMI, 3T3 and HS-27 cells in DMEM media. For the different treatments, the cells were grown in the following media: MEM or RPMI or DMEM containing 100 μM of L-methionine (abbreviated M+H−); methionine-free MEM or RPMI or DMEM media containing 100 μM of L-homocysteine thiolactone (abbreviated M−H+); methionine-free MEM or RPMI or DMEM media (abbreviated M−H−). All media were supplemented with 10% 1 kDa cut-off dialyzed fetal calf serum, 1 mM sodium pyruvate, 50 units/ml penicillin, 50 μg/ml streptomycin, non-essential amino-acid solution, 2 μM glutamine, 50 μg/ml insulin (used only for Phi-1 and HTC cells), and 24 μM Hepes (for PC-3 cells only).

2.4. Cell-treatments and cell number determination

Cells in exponential growth were trypsinised, counted and plated in 6-well plates at a cell density of 10^4–3×10^4 cells per well depending of the cell line, in M+H− media. The medium was changed after 24-h incubation to start the experiment: control cells were grown in M+H− medium, while test cells were treated with M−H+ or M−H− media for 5 days. At the end of the treatments, media were changed to M+H− (recovery). Cells were harvested at appropriate time points. Cells were trypsinised, stained with Trypan Blue to determine viability, and counted by using a haemocytometer, the final count being the average of two counts. Each experiment was performed in duplicate, and repeated three independent times.

2.5. Cell-cycle studies

Cells (between 10^5 and 5×10^5 cells depending of the cell line) were plated in M+H− medium in 50 cm^2 Petri dishes. The medium was changed 24 h later to start the experiment: control cells were maintained in M+H− medium while test cells were treated with either M−H+ or M−H− media. Following 5 days of M−H+ or M−H− treatment, media were replaced by M+H− and the cells were allowed to recover. The cells were harvested at appropriate time points using trypsin, centrifuged for 5 min at 200 × *g*, re-suspended in 200 μl of PBS and fixed by adding 2 ml of 70% ethanol. Fixed samples were stored in −20 °C awaiting analysis. Fixed samples were centrifuged for 5 min at 200 × *g*, washed in 2 ml of PBS and pelleted. Cell pellets were re-suspended in 400 μl of PBS; 50 μl of 1 mg/ml RNase A and 50 μl of 400 μg/ml propidium iodide were then added, and the samples were incubated for 30 min at 37 °C (DNA staining). The reaction was stopped on ice, and the samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, BDIS) equipped with a 15 mW argon-ion air-cooled laser with excitation wavelength 488 nm. Pulse height signals of forward light scatter (FSC), side light scatter (SSC) were collected, and propidium iodide emission was measured using fluorescence parameter FL-2 with a bandpass filter 585/42 nm. Data on scatter parameters were collected in linear mode as was FL2-Area and FL2-Width (double determination) used for DNA histogram analysis. Ten thousand events were acquired and stored in list mode using Cellquest software (BDIS). DNA histograms analysis was performed based on the algorithm first described by Watson et al. [25], and modified by Ormerod et al. [26] using the software WinMDI and Cylchred.

2.6. Metabolite analysis

Cell culture media was collected during the different treatments and stored at −80 °C awaiting analysis. Total homocysteine (free thiol, disulfide, homocysteine–cysteine mixed disulfide), cysteine, cysteinyl-glycine and glutathione (total reduced glutathione and glutathione disulfide) were measured by fluorimetric detection (derivatization with SBD-f) after separation by HPLC as described by Kenyon et al. [4]. *N*-Acetylcysteine was used as internal standard. Results are expressed as concentration (μM) of metabolite present in the medium.

2.7. Statistical analysis

All results are expressed as means ± S.E.M. Statistical analysis was performed with the SPSS 11.0 Software. Independent samples' Student's *t* test was used to analyze cell-cycle events.

3. Results

3.1. The effect of methionine and homocysteine supplementation on cell proliferation

Growth curves for all the cell lines (i.e. HTC, Phi-1, PC-3, 3T3 and HS-27) were performed for up to 20 days (depending of the cell line) in (a) methionine-complete media, containing 100 μM L-methionine (M+H−), (b) methionine-depleted media supplemented with 100 μM L-homocysteine thiolactone (M−H+) and (c) media without methionine or homocysteine (M−H−). In all cases dialyzed serum was used to avoid supplementing the cells with traces of amino acids potentially present in the serum.

All cell lines proliferated well in the M+H− media (Fig. 2). When methionine was replaced with homocysteine (M−H+), HTC, Phi-1, PC-3 and 3T3 cells ceased to proliferate, showing methionine dependence (Fig. 2A, C

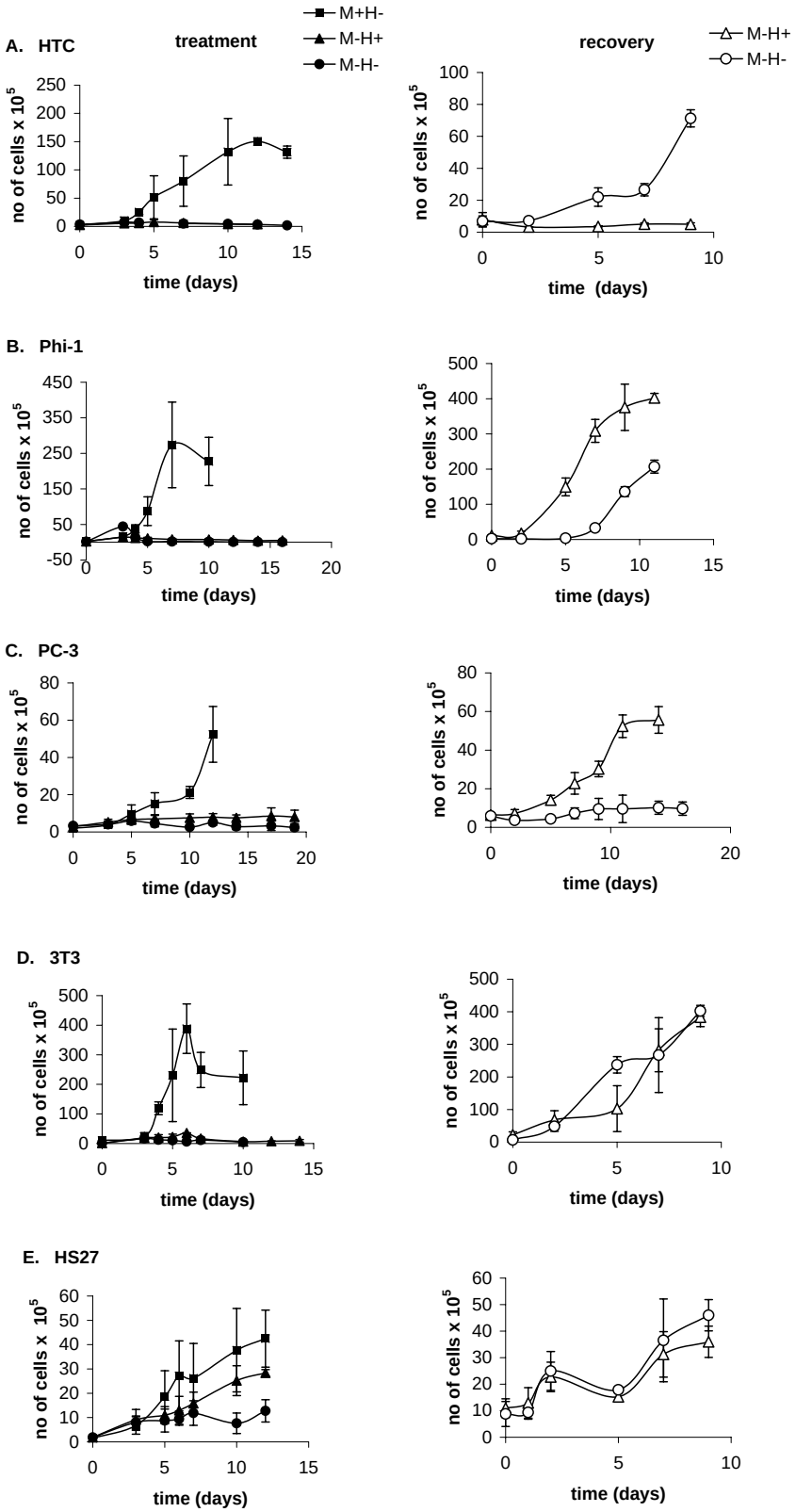

Fig. 2. Progress curves for (A) HTC, (B) Phi-1, (C) PC-3, (D) 3T3 and (E) HS-27 cells treated with media containing 100 μM methionine (M+H−), 100 μM homocysteine (M−H+) or none (M−H−) (treatment). Cells treated for 5 days with M−H+ or M−H− media were allowed to recover in methionine containing media (recovery). Values are means ± S.E.M. of at least three independent experiments.

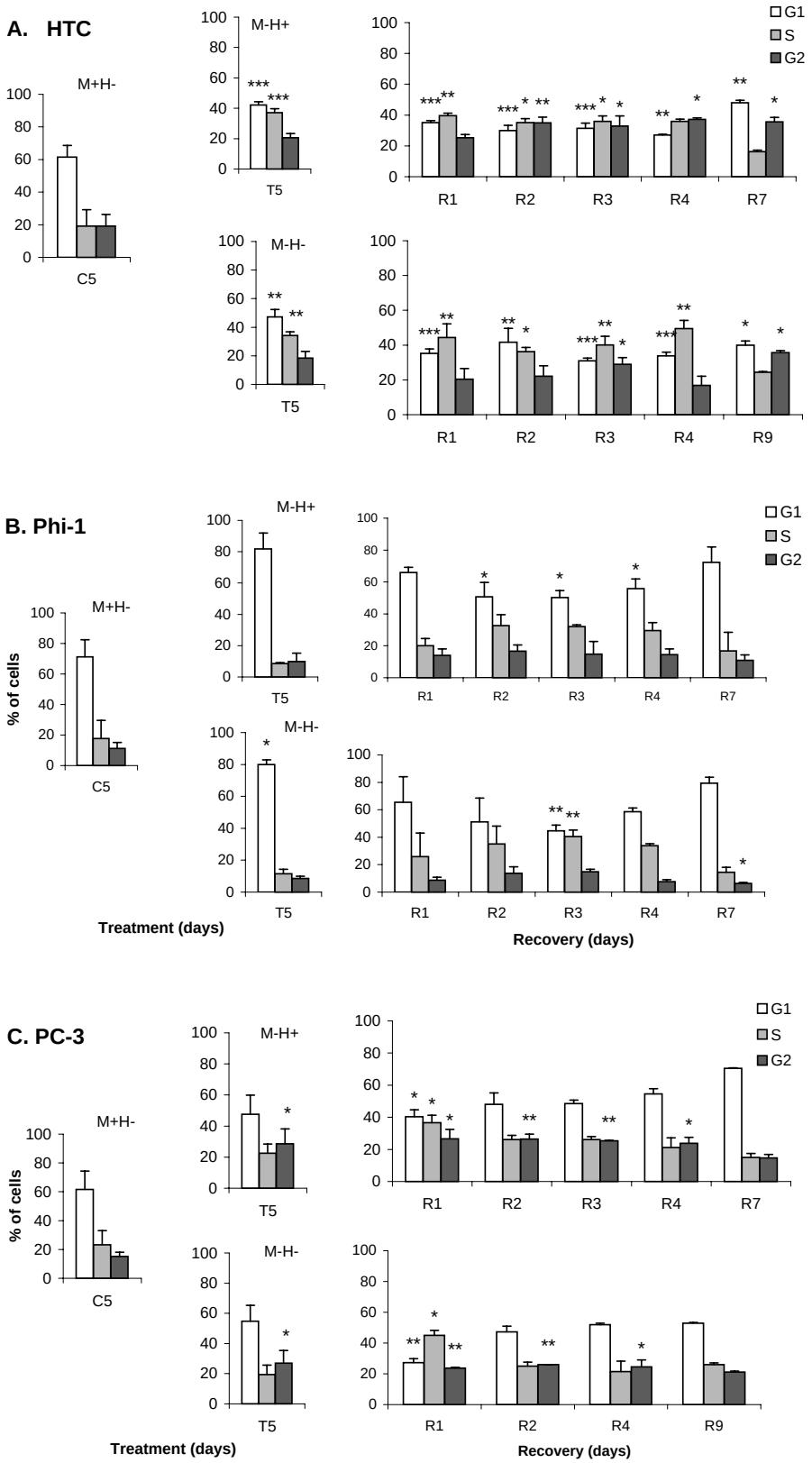

Fig. 3. Cell-cycle analysis of (A) HTC, (B) Phi-1, (C) PC-3, (D) 3T3 and (E) HS-27 cells treated with media containing 100 μM methionine (M+H−), 100 μM homocysteine (M−H+) or none (M−H−) (treatment). Cells treated for 5 days with M−H+ or M−H− media were allowed to recover in M+H− media (recovery). Results are expressed as percentage of cells in G1, S and G2 phases of the cell cycle. C5: cell-cycle profile obtained after 5 days of culture in M+H− (control); T5: cell-cycle profile obtained after 5 days of culture treatment in either M−H+ or M−H− (treatment); Rn: cell-cycle profile obtained during the *n* days of recovery after treatment in methionine-containing media (M+H−). Values are means ± S.D. of at least three independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). All comparisons were made using a two-tailed Student's *t* test.

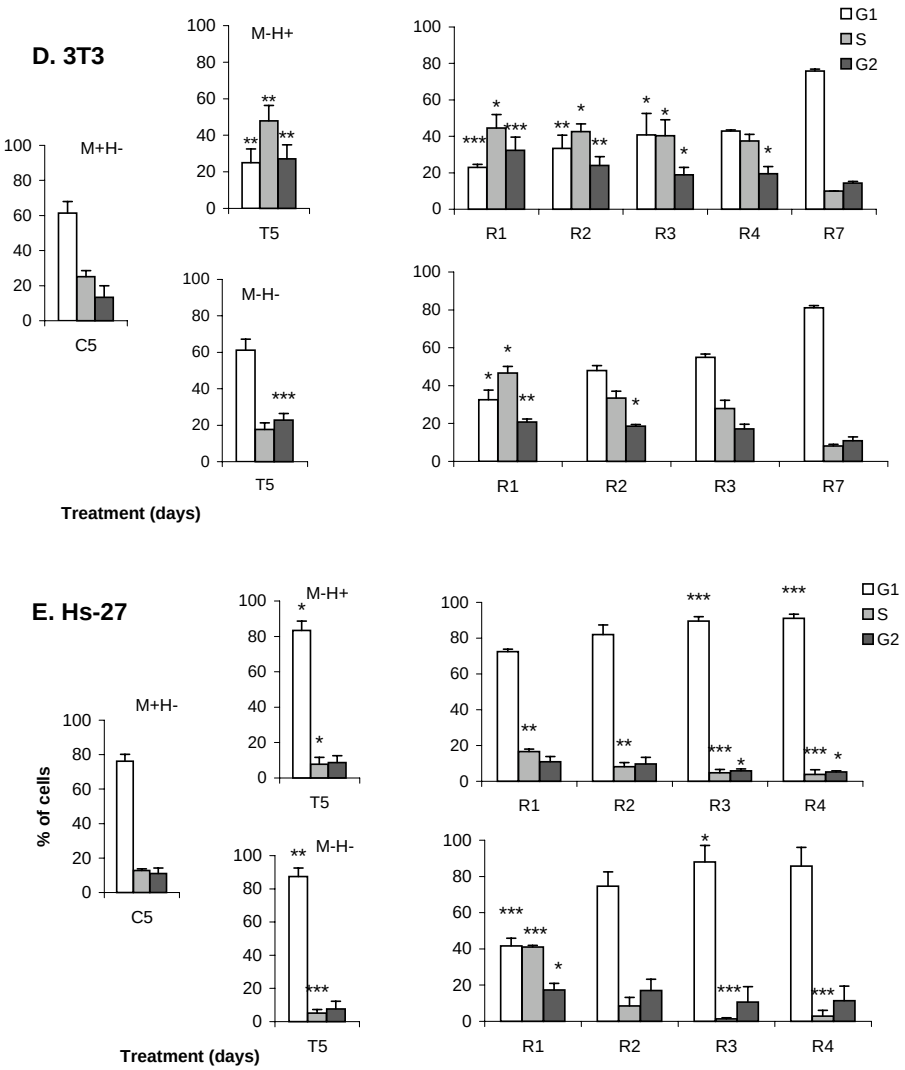

Fig. 3. (*Continued*).

and D, respectively). Normal fibroblasts HS-27 were able to grow in M−H+ media, showing, as expected, a methionine-independent phenotype (Fig. 2E). Our results are in agreement with previously reported studies on HTC and PC-3 being methionine dependent [4,22]. Phi-1 did not show any growth when treated with M−H+ media for up to 17 days (Fig. 2B), appearing thus to be methionine dependent. None of the tested cell lines were able to grow in the M−H− media. However, the cells remained viable for up to 15 days with the exception of Phi-1 cells that appear to be very sensitive to this treatment and cell numbers decreased almost to zero at the end of the treatment (day 17) (Fig. 2B).

The ability of the cell lines to survive the methionine-depletion treatments (M−H+ and M−H−) was tested by assessing cell viability and ability for growth after the depletion. Following 5-day treatment with M−H+ and M−H− media, the cells were transferred to complete (M+H−) media and allowed to proliferate. Growth curves were performed for up to 15 days. Although HTC cells were able to recover from the M−H− treatment, they did not grow after the 5-day homocysteine supplementation (M−H+) treatment. (Fig. 3A). The second hepatoma cell line, Phi-1, recovered from both treatments although a lag period of 5 days was observed following the M−H− treatment (Fig. 2B). Conversely, PC-3 cells recovered and proliferated after the M−H+ treatment (Fig. 3C) but were not able to proliferate after the M−H− treatment. 3T3 and the normal fibroblasts HS-27 recovered from both treatments (Fig. 2D and E) and were able to proliferate.

3.2. The effect of methionine and homocysteine supplementation on cell-cycle kinetics

In order to evaluate the effect of methionine and homocysteine on the cell cycle, the cell-cycle kinetics for all cell lines were followed under normal (M+H−), methionine depletion (M−H+) and starvation (M−H−) conditions. The progress of the cell cycle when the cells were recovering from 5-day treatments with M−H+ and M−H− media was also followed up. DNA from ethanol fixed cells was stained with propidium iodide and the samples analyzed by

flow cytometry. Fig. 3 shows the DNA histograms obtained for the five cell lines, results expressed as percentage of cells in the G1, S and G2/M phases of the cell cycle. The relative distribution of cells in the G1, S and G2/M phases was calculated for all cell lines after 5 days of control treatment (M+H−) (Fig. 3) and the results of all treatments and recovery experiments were compared to this.

Treatment of HTC cells with M−H+ resulted in decrease of cells in the G1 phase and concomitant accumulation in the S phase. The cells recovered from the M−H+ treatment through a burst of cells in the G2/M phase followed by increase in G1. The M−H− treatment of the HTC cells showed the same profile as for M−H+, i.e. decrease of cell population in the G1 and increase in G2/M phase, followed by a recovery through reduction of cell number in G1, increase in S and finally the G2/M phase. Overall, methionine depletion (M−H+) and starvation (M−H−) appeared to have the same effect on these liver cells (Fig. 3A).

Methionine depletion (M−H+) in Phi-1 cells did not result in any statistically significant changes whilst starvation (M−H−) caused an accumulation of cells in the G1 phase. Recovery in both cases was observed through an initial decrease of cell numbers in the G1 phase accompanied by an increase of cells in the S phase, and the cell cycle normalized 7 days after the reversal of the treatment (Fig. 3B).

PC-3 cells showed an accumulation of cells in the S and G2/M phase following both methionine depletion (M−H+) and starvation (M−H−) treatments. The cells appeared to recover from both treatments in the same manner, i.e. increase of cells in the G2/M phase. The cell cycle appeared to normalize within 7–9 days from the reversal of the treatment (Fig. 3C).

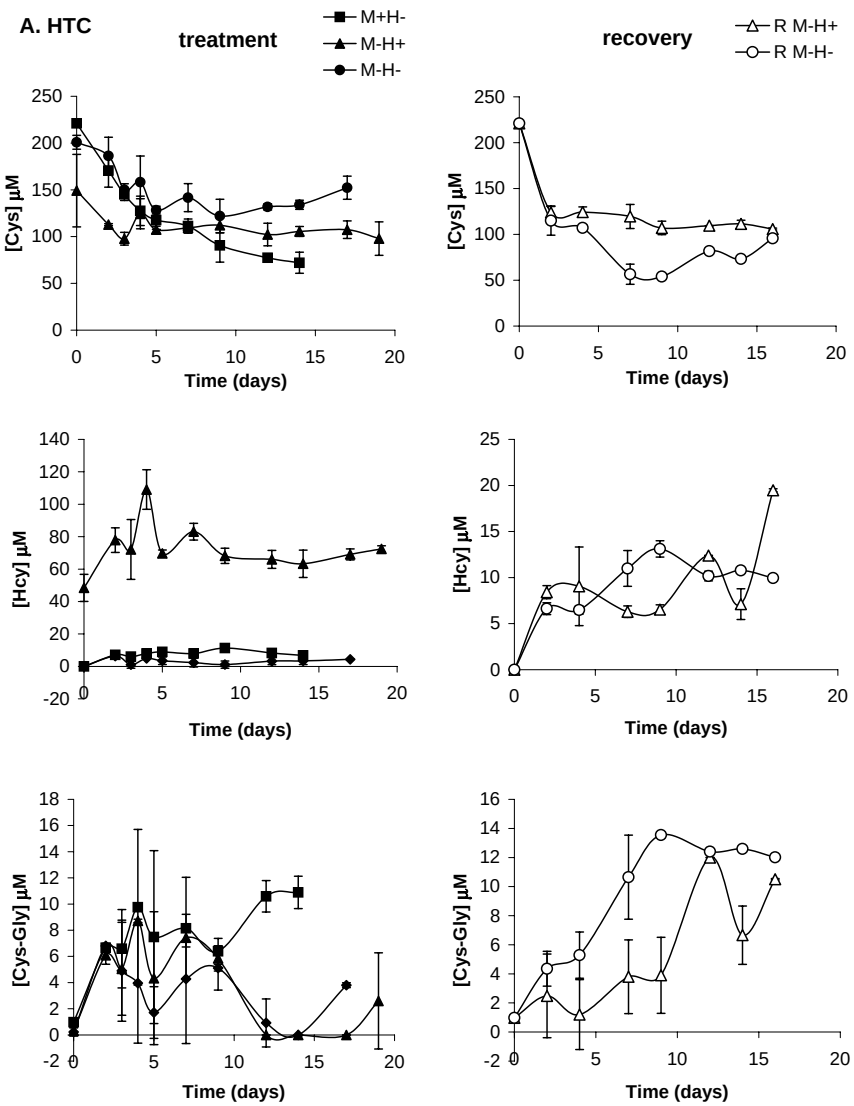

Fig. 4. Cysteine (Cys), homocysteine (Hcy), cysteinyl-glycine (Cys-Gly) and glutathione (GSH) in cell culture media of (A) HTC, (B) Phi-1, (C) PC-3, (D) 3T3 and (E) HS-27 cells treated with media containing 100 µM methionine (M+H−), 100 µM homocysteine (M−H+) or none (M−H−) (treatment). Cells treated for 5 days with M−H+ or M−H− media were allowed to recover in M+H− media (recovery). Values are means ± S.D. of at least three independent experiments.

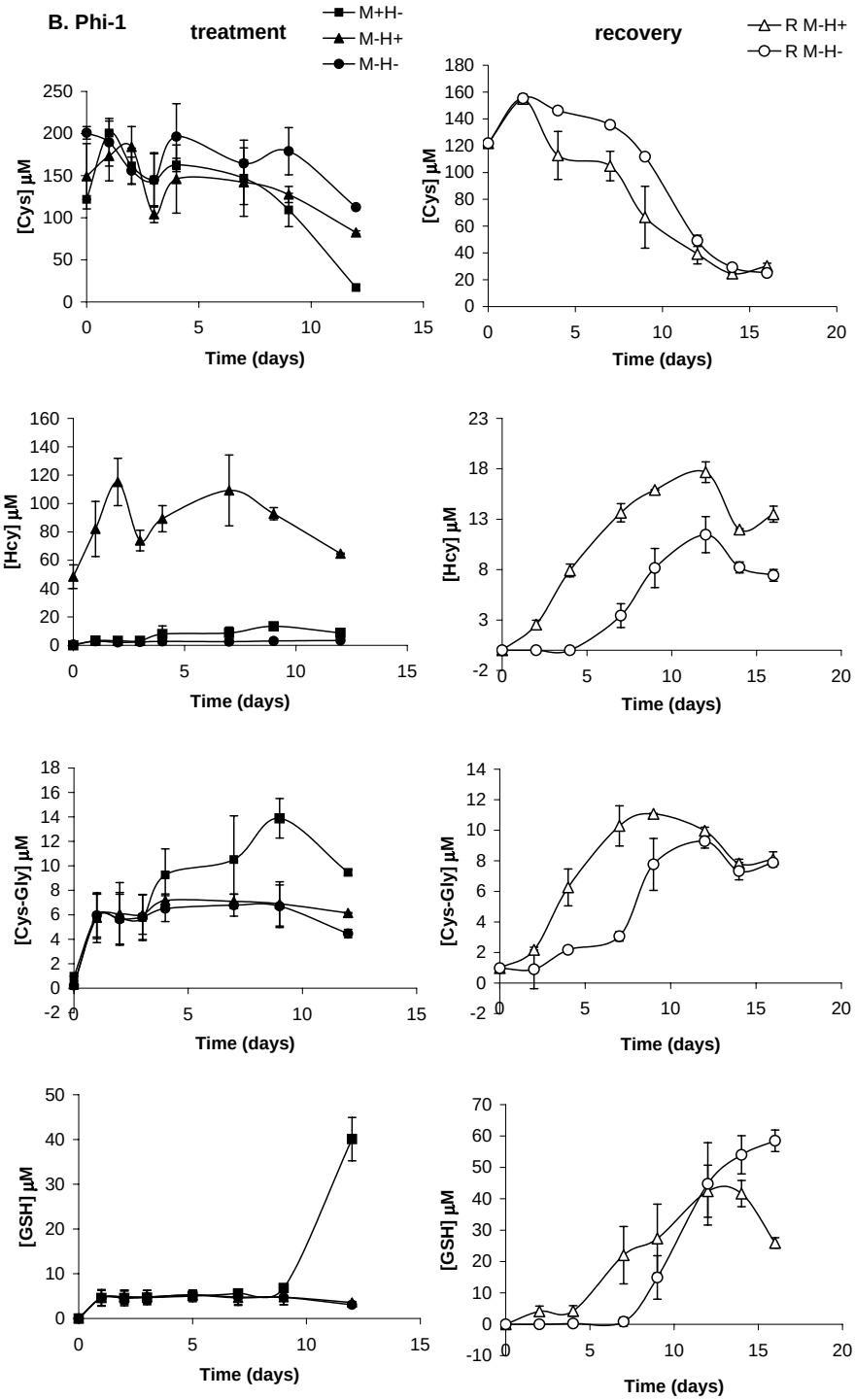

Fig. 4. (*Continued*)

3T3 cells appeared to accumulate on the S and G2/M phase of the cell cycle following a methionine-depletion treatment (M−H+) accompanied by reduced cell numbers in the G1 phase. However, the starvation (M−H−) treatment caused only an increase in the number of cells in the G2/M phase. 3T3 appeared to recover from both treatments in the same manner, with an initial increase of cells in the S phase followed by normalization of the cell cycle within 7 days from the reversal of the treatments (Fig. 3D).

The normal fibroblasts HS-27 responded to methionine-depletion (M−H+) and starvation (M−H−) with an increase in the number of cells in the G1 phase and reduction of cells in the S phase. Recovery from both treatments followed the same pattern with an initial increase of cell numbers in the S phase, followed by increase in the G1 and decrease in the S phases (Fig. 3E).

Overall, apoptotic cells have been observed in the DNA histogram analysis (data not shown) but this did not prevent

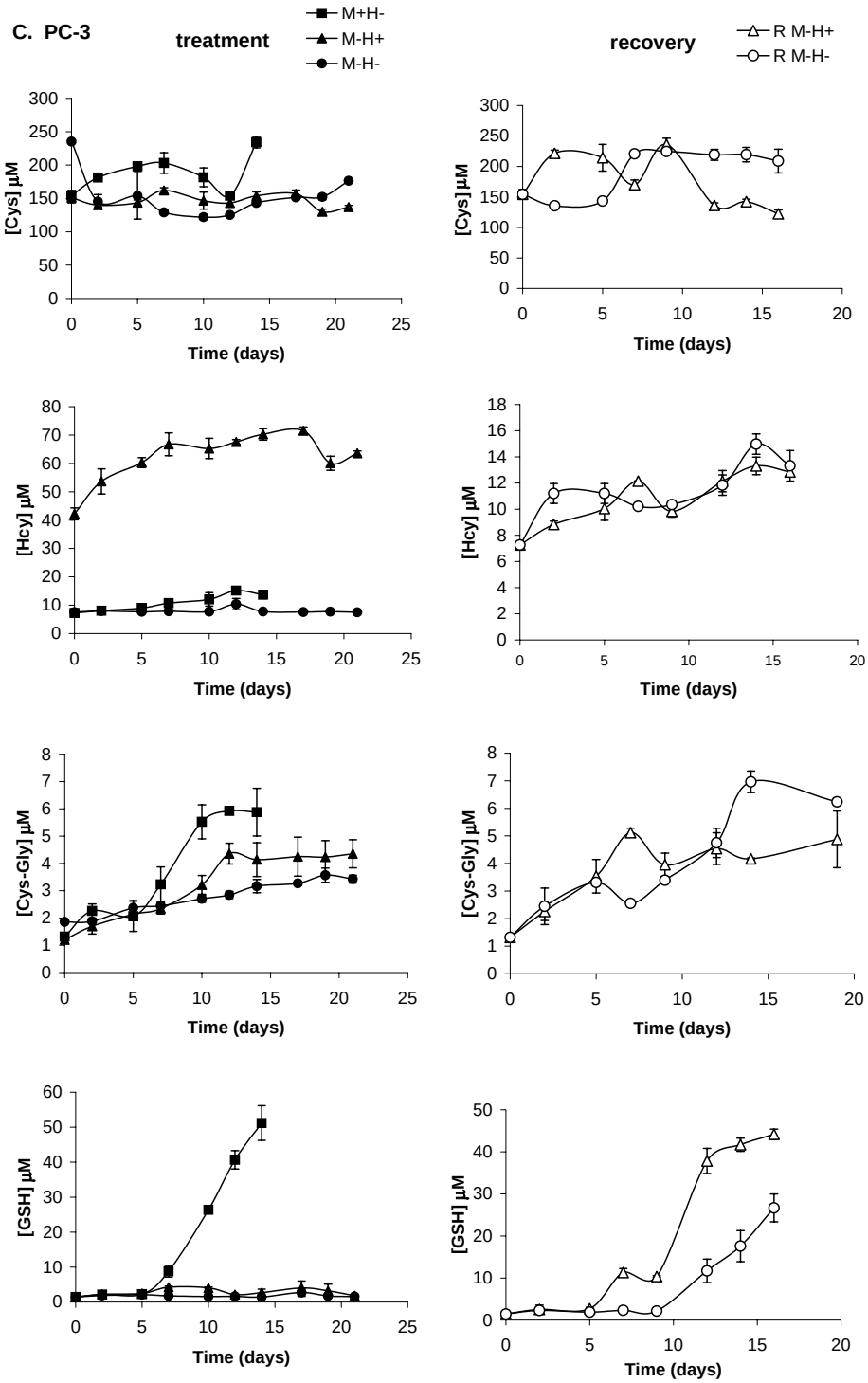

Fig. 4. (*Continued*)

the apparent recovery of the cells after M−H+ and M−H− treatments.

3.3. The effect of methionine and homocysteine supplementation on sulfur amino acid metabolism

The levels of cysteine (Cys), total homocysteine (Hcy), cysteinyl-glycine (Cys-Gly) and glutathione (GSH) were estimated in the media of all the treatments; results are presented in Fig. 4.

Under control conditions, (100 µM methionine), the hepatoma HTC cells utilized Cys, and exported low concentrations of Hcy and Cys-Gly (Fig. 4A). In the M−H+ and M−H− treatments, the cells showed similar patterns for Cys (uptake: although at lower rates) and Hcy (exported), whilst Cys-Gly was found to

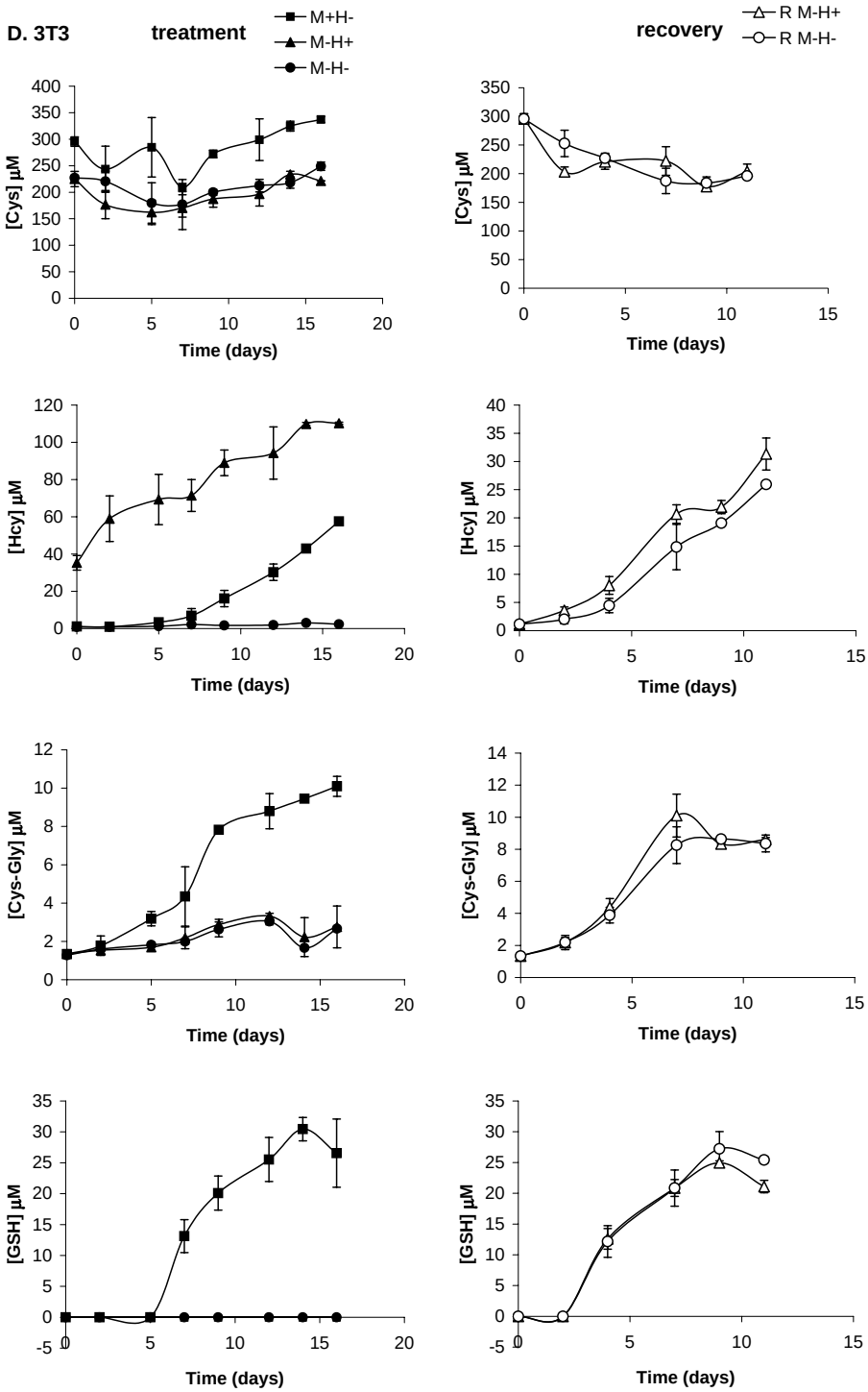

Fig. 4. (*Continued*)

decrease within 10 days of culture. The M−H+ and M−H− treatment did not affect the ability of HTC cells to uptake Cys from the media or export Hcy and Cys-Gly as shown from the results of the recovery studies (Fig. 4A). GSH was not detected in the media of HTC cells under any condition, in agreement with our previous report [4].

The hepatomas Phi-1 (Fig. 4D) utilized Cys and produced low concentrations of Hcy, Cys-Gly and GSH when treated with control (100 µM methionine) media. Methionine-depletion (M−H+ or M−H−) did not alter the ability of the cells to uptake Cys or produce Cys-Gly and GSH, although the levels of those two metabolites were lower (Fig. 4B). When the cells were allowed to recover from the M−H+ and M−H− treatments, they were found to follow the same trend, although the M−H+ treatment resulted in higher rate of Cys uptake and higher Hcy and Cys-Gly production.

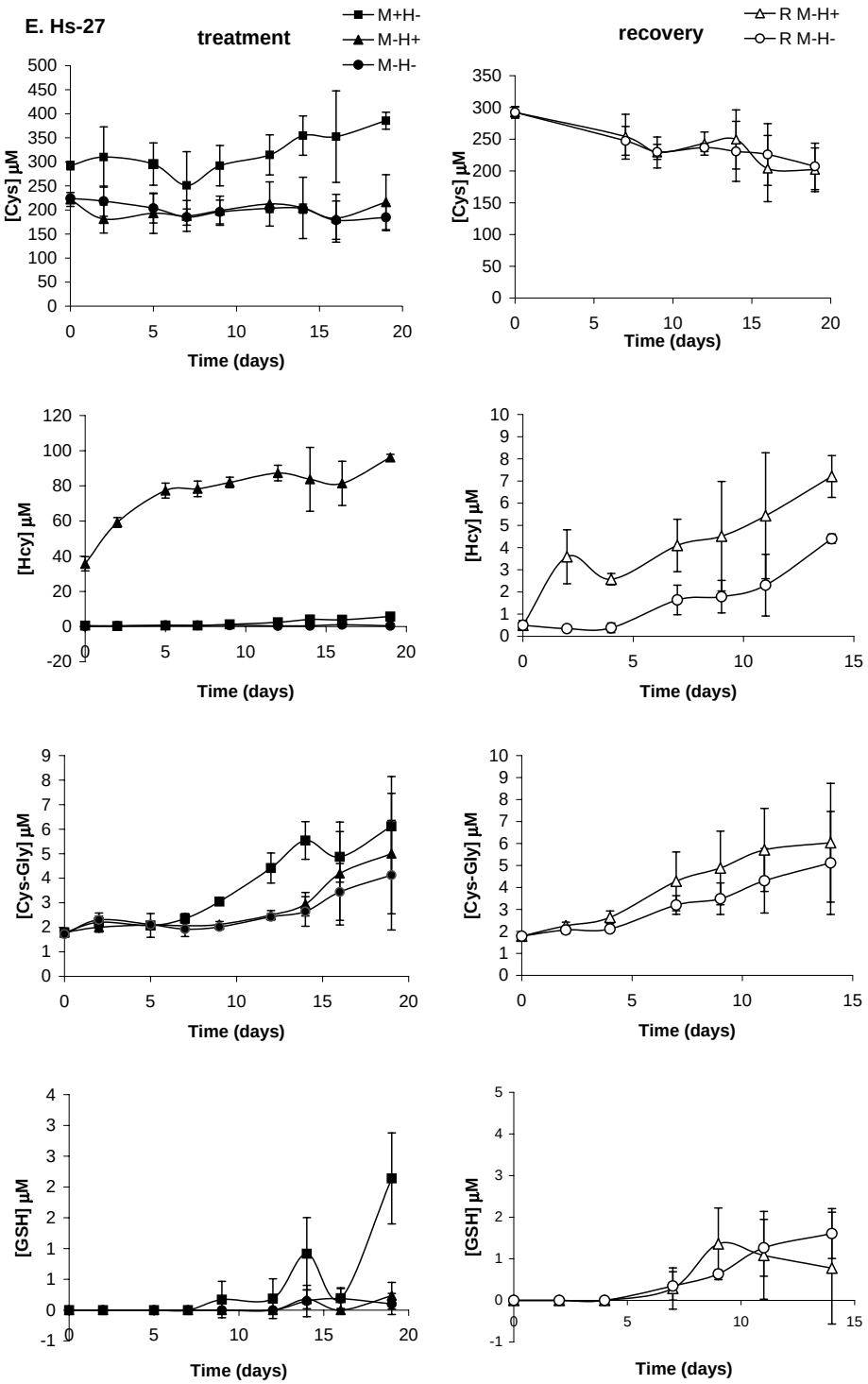

Fig. 4. (*Continued*).

Prostate adenocarcinoma PC-3 cells (Fig. 4C) utilized Cys and exported Hcy, Cys-Gly and GSH when grown in complete M+H− medium (Fig. 4C). The M−H+ and M−H− treatments did not affect the ability of the cells to uptake Cys, and produce Cys-Gly. However, Hcy production was very low in the M−H− medium and GSH production was very low in both M−H+ and M−H− conditions. Upon recovery, PC-3 were able to utilize Cys and produce Hcy, Cys-Gly and GSH showing patterns similar to the ones obtained under control (M−H+) conditions (Fig. 4C).

The transformed fibroblasts 3T3 were found to utilize Cys and export Hcy, Cys-Gly and GSH when grown in M+H− control media. When 3T3 cells were treated with M−H+ and M−H− media, they metabolized Cys in a similar to M+H− way (Fig. 4D). Although Hcy was

exported from the cells in M−H+ media, Hcy levels in M−H− media were very low. Cys-Gly was produced at very low levels in both treatments whilst GSH level were negligible. Upon recovery from a 5-day treatment with M−H− and M−H+, 3T3 were found to metabolize all thiols in the same manner, all were comparable to the corresponding control.

The normal fibroblasts HS-27 cells in control M+H− media (Fig. 4E), metabolized Cys, and exported, at low concentrations, Hcy, Cys-Gly and GSH. When grown in M−H+ media, the levels of Cys remained unchanged whilst Hcy and Cys-Gly were exported. Cys was not utilized when the cells were grown in M−H− media, and Gys-Gly was the only metabolite that was exported. 3T3 cells recovered from the 5-day M−H− and M−H+ treatments, and were able to utilize Cys, export Hcy, Cys-Gly and GSH.

4. Discussion

A broad range of cancers and transformed cells are characterized by their dependence on the essential amino acid methionine. This feature is unique to cancer cells and offers a means of selectively controlling the proliferation of methionine-dependent cells by depleting their environment of methionine. Although the role of many biological pathways and enzymes regulating methionine metabolism has been investigated, the molecular basis of this metabolic defect remains unclear. Methionine depletion either by nutritional manipulation [27] or use of the hydrolase methioninase [28] has been explored as therapeutic strategy against cancer.

All the cancer cell lines tested in this study were found to be methionine dependent (Fig. 2), i.e. they stopped proliferating in methionine-depleted media supplemented with homocysteine (M−H+). Cell growth was also arrested in methionine and homocysteine-depleted media (M−H−) (starvation). The only surprising result was the behavior of the hepatoma cells Phi-1 that we had previously reported to exhibit a partially methionine-independent phenotype [4] and are now exhibiting methionine dependence (Fig. 2B). However, in the present study, we have used a new batch of cells obtained from the European Cell Culture Bank. It is, therefore, possible that subculturing the cells has resulted in enhancing some characteristics of their methionine-dependent trait. Both hepatoma cell lines (HTC and Phi-1) were found to utilize cysteine during the methionine-depletion treatments whilst the fibroblasts 3T3 and HS-27, and epithelial cells PC-3 (Fig. 4) did not. These results agree with the presence of the transsulfuration sequence in the liver cells and their ability to use cysteine to spare methionine [11].

The methionine-depletion treatments were found to induce arrest of the cell-cycle events at two different phases, depending on the cell line: the methionine-dependent cells HTC, PC-3 and 3T3 exhibited arrest at the S and G2/M phase (Fig. 3A, C and D) whilst the methionine-independent HS-27 appeared to be blocked at the G1 phase (Fig. 3E). Surprisingly, Phi-1 did not show the profile exhibited by the other liver cell line HTC or the methionine-dependent cells PC-3 and 3T3, but arrested at the G1 phase of the cell cycle similarly to the methionine-independent normal fibroblasts HS-27. Lu et al. [22] have recently reported that upon methionine-depletion the methionine-dependent prostate cells PC-3 arrested predominantly in the G2/M phase whereas the partially dependent LNCaP accumulated exclusively in the G1 phase. This data is similar to our findings for Phi-1 and HS-27. These observations indicate that there is a wider diversity between the methionine-dependent cancer cells in terms of exhibiting different cell-cycle characteristics. Further studies using dependent and partially dependent cell lines are needed to prove this hypothesis. Should this be confirmed, the cell-cycle characteristics could be used to tailor cell cycle-dependent therapies to specifically target different cancers.

It is also important to note that both the homocysteine supplementation (M−H+) and starvation (M−H−) treatments had similar effects on the cell-cycle kinetics (Fig. 3), leading to the conclusion that the cellular events behind the arrest of the cell cycle are independent of the presence of homocysteine and can most probably be attributed to lack of methionine. Also, a differential analysis of the expression of cell cycle-dependent kinases controlling the individual check points may elucidate these events and offer new targets for intervention.

With the exception of HTC, all other cells recovered from the methionine-depletion homocysteine-supplementation treatment (M−H+). HTC were treated with homocysteine for 5 days prior to reversing the treatment to methionine-rich media. They were then cultured for a further 9 days in methionine-rich media (M+H−) without showing any growth. However, the cell-cycle kinetics (Fig. 3A) showed that the observed increase in S phase is induced by both the homocysteine (M−H+) and starvation (M−H−) treatments, and that both are reversible. Apparently, the cause that led to these cells being unable to recover from the M−H+ treatment did not have a major effect on the cell-cycle events. The lack of growth can be attributed to either a specific homocysteine-mediated effect or a complete depletion of the methionine and methyl-group pools (e.g. SAM or specific folate species) necessary for cell proliferation, an explanation that is in agreement with the hypothesis for higher methylation rates in methionine-dependent cancer cells [9]. The uptake of cysteine, formation of homocysteine and cysteinyl-glycine upon recovery were not found to be compromised in the HTC cells.

The prostate adenocarcinomas PC-3 appeared to be affected by the complete depletion of methyl group source (starvation) (M−H−) and did not recover when the treat-

ment was reversed (Fig. 2). This could again be explained if we consider the need of a minimum concentration of methionine and/or methyl groups for maintaining certain crucial for cell growth metabolic functions. However, the cell-cycle events following recovery did not differentiate between the two treatments (M−H+ and M−H−) and showed the same profile upon recovery (Fig. 3). PC-3 cells accumulated in the S and G2/M phase of the cell cycle, in accordance with the studies of Guo [21], Lu [22] and Poirson-Bichat [29].

Overall, methionine and homocysteine restriction induced arrest in cell growth. Although within the time frame of this study the arrest was not reversible for two of the tested cell lines (HTC and PC-3), it did not differentially influence the metabolic competence of these cells. The cell-cycle events also indicate an ability of these cells to recover and offer the potential of synchronizing them at specific point of the cell cycle. It is therefore possible to utilize methionine depletion to synchronize methionine-dependent cancer cells and devise a specific and selective therapeutic strategy.

Acknowledgments

The authors wish to thank Prof. R.M. Hoffman for useful discussions and advice. This project has been funded by Cancer Research UK (Program Grant No. C459/A 2579).

References

[1] Hoffman RM. Altered methionine metabolism, DNA methylation and oncogene expression in carcinogenesis. A review and synthesis. Biochim Biophys Acta 1984;738:49–87.
[2] Guo HY, Herrera H, Groce A, Hoffman RM. Expression of the biochemical defect of methionine dependence in fresh patient tumors in primary histoculture. Cancer Res 1993;53:2479–83.
[3] Mecham JO, Rowitch D, Wallace CD, Stern PH, Hoffman RM. The metabolic defect of methionine dependence occurs frequently in human tumor cell lines. Biochem Biophys Res Commun 1983;117: 429–34.
[4] Kenyon SH, Waterfield CJ, Timbrell JA, Nicolaou A. Methionine synthase activity and sulphur amino acid levels in the rat liver tumour cells HTC and Phi-1. Biochem Pharmacol 2002;63:381–91.
[5] Tisdale MJ. Methionine metabolism in Walker carcinosarcoma in vitro. Eur J Cancer 1980;16:407–14.
[6] Tang B, Li YN, Kruger WD. Defects in methylthioadenosine phosphorylase are associated with but not responsible for methionine-dependent tumor cell growth. Cancer Res 2000;60:5543–7.
[7] Fiskerstrand T, Riedel B, Ueland PM, Seetharam B, Pezacka EH, Gulati S, et al. Disruption of a regulatory system involving cobalamin distribution and function in a methionine-dependent human glioma cell line. J Biol Chem 1998;273:20180–4.
[8] Dumontet C, Roch AM, Quash G. Methionine dependence of tumor cells: programmed cell survival? Oncol Res 1996;8:469–71.
[9] Tisdale MJ. Effect of methionine deprivation on methylation and synthesis of macromolecules. Br J Cancer 1980;42:121–8.
[10] Stern PH, Hoffman RM. Enhanced in vitro selective toxicity of chemotherapeutic agents for human cancer cells based on a metabolic defect. J Natl Cancer Inst 1986;76:629–39.
[11] Finkelstein JD. Pathways and regulation of homocysteine metabolism in mammals. Semin Thromb Hemost 2000;26:219–25.
[12] Soriano-Garcia JF, Torras-Llort M, Ferrer R, Moreto M. Multiple pathways for L-methionine transport in brush-border membrane vesicles from chicken jejunum. J Physiol 1998;509:527–39.
[13] Finkelstein JD. The metabolism of homocysteine: pathways and regulation. Eur J Pediatr 1998;157:S40–4.
[14] Lu SC. Regulation of hepatic glutathione synthesis: current concepts and controversies. FASEB J 1999;13:1169–83.
[15] Djurhuus R, Svardal AM, Mansoor MA, Ueland PM. Modulation of glutathione content and the effect on methionine auxotrophy and cellular distribution of homocysteine and cysteine in mouse cell lines. Carcinogenesis 1991;12:241–7.
[16] Di Buono M, Wykes LJ, Ball RO, Pencharz PB. Dietary cysteine reduces the methionine requirement in men. Am J Clin Nutr 2001;74: 761–6.
[17] Finkelstein JD. Methionine-sparing effect of cystine in human subjects. Am J Clin Nutr 1998;68:224–5.
[18] Stewart ZA, Westfall MD, Pietenpol JA. Cell-cycle dysregulation and anticancer therapy. Trends Pharmacol Sci 2003;24:139–45.
[19] Gali-Muhtasib H, Bakkar N. Modulating cell cycle: current applications and prospects for future drug development. Curr Cancer Drug Targets 2002;2:309–36.
[20] Nabel EG. CDKs and CKIs: molecular targets for tissue remodelling. Nat Rev Drug Discov 2002;1:587–98.
[21] Guo H, Lishko VK, Herrera H, Groce A, Kubota T, Hoffman RM. Therapeutic tumor-specific cell cycle block induced by methionine starvation in vivo. Cancer Res 1993;53:5676–9.
[22] Lu S, Epner DE. Molecular mechanisms of cell cycle block by methionine restriction in human prostate cancer cells. Nutr Cancer 2000;38:123–30.
[23] Hoshiya Y, Kubota T, Matsuzaki SW, Kitajima M, Hoffman RM. Methionine starvation modulates the efficacy of cisplatin on human breast cancer in nude mice. Anticancer Res 1996;16:3515–7.
[24] Goseki N, Yamazaki S, Shimojyu K, Kando F, Maruyama M, Endo M, et al. Synergistic effect of methionine-depleting total parenteral nutrition with 5-fluorouracil on human gastric cancer: a randomized, prospective clinical trial. Jpn J Cancer Res 1995;86:484–9.
[25] Watson JV, Chambers SH, Smith PJ. A pragmatic approach to the analysis of DNA histograms with a definable G1 peak. Cytometry 1987;8:1–8.
[26] Ormerod MG, Payne AW, Watson JV. Improved program for the analysis of DNA histograms. Cytometry 1987;8:637–41.
[27] Epner DE, Morrow S, Wilcox M, Houghton JL. Nutrient intake and nutritional indexes in adults with metastatic cancer on a phase I clinical trial of dietary methionine restriction. Nutr Cancer 2002; 42:158–66.
[28] Tan Y, Sun X, Xu M, Tan X, Sasson A, Rashidi B, et al. Efficacy of recombinant methioninase in combination with cisplatin on human colon tumors in nude mice. Clin Cancer Res 1999;5:2157–63.
[29] Poirson-Bichat F, Gonfalone G, Bras-Goncalves RA, Dutrillaux B, Poupon MF. Growth of methionine-dependent human prostate cancer (PC-3) is inhibited by ethionine combined with methionine starvation. Br J Cancer 1997;75:1605–12.