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Growth support and toxicity of homocysteine and its effects on methionine metabolism in non-transformed and chemically transformed C3H/10T1/2 cells

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The effects of homocysteine (Hcy) on one non-transformed (Cl 8) and two malignant clones (Cl 16 and Cl T422) of the C3H/10T1/2 mouse embryo fibroblasts, were examined with regard to toxicity, ability to support growth and effects on methionine (Met) metabolism and glutathione level. Homocysteine in its reduced form (Hcy-SH) was toxic to all cell lines, and the LD_{90} was estimated to be 1.0×10^{-4} M for Cl 8 and Cl 16 cells measured by plating efficiency, 0.8×10^{-4} M for Cl 8 and 0.3×10^{-4} M for Cl 16 when measured by total cell growth. At toxic concentrations, Hcy-SH showed a drastic effect on cell morphology both in the presence and absence of Met. The same effect was demonstrated with L-cysteine. No toxic effect was seen with homocystine (Hcy-SS-Hcy) or homocysteine thiolactone (Hcy-tl) at similar concentrations. Hcy-tl supported growth of both the non-transformed and malignant cells in Met-deficient medium but with decreasing efficiency in the order Cl 8, Cl 16 and Cl T422. The growth rate constant compared to that of Met-supplemented medium was 0.62 for Cl 8, 0.44 for Cl 16 and 0.38 for Cl T422 cells. The intracellular level of S-adenosylhomocysteine (AdoHcy) increased in all three cell lines in Hcy-tl-supplemented medium. The S-adenosylmethionine (AdoMet) content increased in Cl 8 cells, was constant in Cl 16 cells and decreased in Cl T422 cells under the same conditions. This resulted in a constant ratio of AdoMet/AdoHcy in the non-transformed cells (Cl 8) whereas this ratio decreased by 40% in Cl 16 and by 72% in Cl T422 cells when Hcy-tl replaced Met in the medium. The ability of Hcy-tl to support growth thus seemed to correlate well with alteration in Met metabolism in this cell culture system. The intracellular level of glutathione (GSH) was measured during exponential growth, but showed small variations between non-transformed cells and Cl 16 cells. However, Cl T422 cells showed a distinct lower level of GSH in Met-supplemented medium, and this increased 3- to 4-fold when Met was replaced with Hcy-tl.

Introduction

The ability of cells in culture to grow in medium where methionine (Met*) is replaced by homocysteine (Hcy) has been the subject of numerous investigations (1–3). Several of these experiments indicated a link between oncogenesis and altered metabolism of Met in that malignant cells grew poorly or not at all in the presence of Hcy while normal cells grew well under the same conditions. Halpern *et al.* (4) showed that of three normal cell lines and three malignant cell lines tested, only the normal cells survived and grew when Met was replaced by Hcy in the medium. Similar results have been demonstrated with normal and transformed human fibroblasts as well as malignant rat breast cells (5) and normal and transformed rat liver epithelial cells (6). Based on these findings it has been postulated that the inability of cells to grow on Hcy, i.e. 'Met dependence', is a general metabolic defect of cancer cells, and a number of normal and malignant cell lines have been tested and found to support this hypothesis (1,7,8). However, some experiments have shown that Met dependence is not a characteristic feature of all malignant cells, and even normal cells have been found to be Met dependent (9,10). Recently, Christa *et al.* (11) demonstrated that of two normal and one malignant cell lines tested, only the malignant cells grew well, the normal cells grew either poorly or not at all when Hcy was substituted for Met in the medium. These results indicate that even if Met dependence is a result of an oncogenic transformation process, it may not be an obligatory event for the neoplastic stage.

The biochemical basis for this metabolic defect is not well understood. The high Met requirement and the increased overall transmethylation rate found in most malignant cells (1,6,12) indicate alterations of the enzymatic apparatus involved in the synthesis of Met and the important methyl-group donor S-adenosylmethionine (AdoMet). However, the activity of methionine synthetase, the enzyme catalyzing the conversion of Hcy to Met, seems to be comparable in Met-dependent and Met-independent cells, and Met is incorporated into protein in normal amounts in Met-dependent cells (1). Also, it has been shown (1) that AdoMet synthetase, the enzyme catalyzing the formation of AdoMet, is not altered in several Met-dependent cells, in spite of the fact that the AdoMet level is lowered and the S-adenosylhomocysteine (AdoHcy) level is raised, leading to a lowered AdoMet/AdoHcy ratio when malignant cells are grown in medium where Met is replaced by Hcy (13). These results may in part be explained by the altered isozyme pattern of methionine-adenosyl transferase seen in some malignant cells (3). The isozymes predominant in malignant cells are more sensitive to feedback inhibition by AdoMet thus lowering the amount of AdoMet synthesized.

In order to look for alterations in metabolism of Met and Hcy in an oncogenic transformation process and try to elucidate further the biochemical basis for this alteration, we have used non-transformed C3H/10T1/2 Cl 8 mouse fibroblasts (14) and two malignant cell lines obtained by chemical transformation of the former. The C3H/10T1/2 cell is a well-established system for carrying out classical two-stage transformation experiments (15) and is particularly suited for comparison of metabolic differences between non-transformed and malignant cells due to the close genetic relationship between the cell types. This close relationship is due to the fact that malignant cells are derived directly from the non-transformed cells by treatment with chemical carcinogens. The present paper describes the different requirements for growth with respect to Met for the different cell lines and

*Abbreviations: Met, methionine; Hcy, homocysteine; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; Hcy-SH, sulfhydryl-containing homocysteine; Hcy-tl, homocysteine thiolactone; GSH, glutathione; MCA, 3-methylcholanthrene; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; BME, Basal Medium Eagle; EMEM, Eagle's Minimum Essential Medium; FCS, fetal calf serum; Hcy-SS-Hcy, homocystine (the disulfide of homocysteine); PE, plating efficiency.

the metabolic effects on the intracellular levels of AdoMet and AdoHcy under the various growth conditions. The importance of introducing a non-toxic chemical form of Hcy in performing such experiments is shown, since the unreduced sulfhydryl-containing form of Hcy (Hcy-SH) has severe toxic effects on the cells. When the cells are exposed to homocysteine thiolactone (Hcy-tl), however, no toxic effects on cells could be detected, but still there is a possibility that when the lactone ring of Hcy-tl is opened intracellularly this may produce a toxic effect which is reflected by the lower growth rate compared to Met. Therefore, the content of one of the cell's potent reducing agents, glutathione (GSH), was measured to determine whether or not an increased intracellular reducing potential would lead to alterations of the GSH level. GSH is involved in cellular detoxification processes (16), and an altered level of GSH may reflect cellular responses to toxic agents.

Since Hcy in its reduced form was rather toxic to the cells, all growth experiments with regard to Met dependency were performed with L-Hcy-tl.

Materials and methods

Chemicals

DL-Hcy-SH, L-Hcy-tl, DL-Hcy-SS-Hcy, L-cysteine, L-Met, reduced GSH, cyanocobalamin (B_{12}) and folic acid were obtained from Sigma Chemical Co., St Louis, MO. L-[^{35}S]Met was obtained from Amersham International plc, Amersham, UK. 3-Methylcholanthrene (MCA) was from Koch-Light Labs Ltd. and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was from P.L.Biochemicals Inc.

Synthesis and purification of L-[^{35}S]Hcy-tl

L-[^{35}S]Hcy-tl was prepared essentially as described by Stern *et al.* (17). L-[^{35}S]Met (2 mCi; 221.4 mCi/ml) was evaporated at 60°C under a stream of nitrogen. H_3PO_2 (20 μl; 50%) and HI (1.0 ml; 57%) were added. The reaction mixture was gently boiled with reflux for 18 h and then distilled at 60°C under a light stream of nitrogen. The residue was dissolved in 0.5 ml methanol and transferred to a column containing 1 ml dry alumina. The reaction vessel was washed six times with 0.5 ml methanol, each washing being added to the column. The eluate was collected in a vessel containing 10 μl 0.1 M HCl The methanol was distilled off at 60°C under a light stream of nitrogen and the residue was dissolved in 1.0 ml 0.01 M HCl and stored at −20°C.

The purity of [^{35}S]Hcy-tl was evaluated by TLC (17) and by determination of Met using a sensitive HPLC assay involving precolumn derivatization with *o*-phthaldialdehyde (18) and showed no detectable Met. When the preparation was analyzed by HPLC on a cation-exchange column coupled to an on-line scintillation detector (Ramona LS), ~80% of the radioactive material co-chromatographed with the Hcy-tl standard, and <0.1% with Met. The [^{35}S]Hcy-tl contained 1.12 mCi/ml as determined by liquid scintillation counting, and the total yield was 56%.

Cell lines and culture conditions

Stock cultures of C3H/10T1/2 mouse embryo fibroblasts were grown on tissue culture plastic flasks (Nunc, Denmark) in Basal Medium Eagle (BME) (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sera-Lab, Ltd., Sussex, UK). The non-transformed Cl 8 cells and the chemically transformed Cl 16 cells were originally obtained from the laboratory of Dr C.Heidelberger, University of Southern California, Los Angeles (14,19). The Cl T422 cells were obtained by chemical transformation following a two-stage transformation protocol (15): C3H/10T1/2 Cl 8 cells were treated with an initiating dose of MCA (0.037 μM) for 24 h followed by continuous treatment with the tumor promoter TPA (0.17 μM). After 6 weeks in culture, transformed foci were picked and cloned using the ring isolation technique (20). One of these, Cl 42, was recloned from single cell colonies three times and injected subcutaneously into immunosuppressed female C3H mice. Cells from one of the resulting tumors were isolated and designated Cl T422 (R.Male and J.R.Lillehaug, in preparation). In all experiments cells were grown at 37°C in an atmosphere of 5% CO_2 in air and a relative humidity of 95%.

Analysis of cell growth

Cells were seeded at a density of 26 000 cells per dish (3.5 cm; Nunc, Denmark) in Eagle's Minimum Essential Medium (EMEM) (methionine-free; Flow Laboratories, UK) supplemented with 1.5 μM vitamin B_{12} (cyanocobalamin), 11.3 μM folic acid, 10% dialyzed, heat-inactivated FCS (Sera-Lab Ltd), and either 0.1 mM L-Met, 0.1 mM L-Hcy-tl, 0.2 mM DL-Hcy-SH, 0.1 mM DL-homocystine, 0.2 mM L-cysteine or a combination of the amino acids as indicated in each experiment. Due to rapid oxidation of free thiol groups, solutions of DL-Hcy-SH and L-cysteine were prepared freshly for each experiment immediately before use. At the times indicated, two parallel dishes from each group were trypsinized and the cells counted in a Coulter Counter Model ZM (Coulter Electronics Ltd., Luton, UK).

Toxicity of DL-Hcy-SH

Toxicity of DL-Hcy-SH was estimated by plating efficiency or by measurement of total cell growth. For plating efficiency, 200 cells were seeded per dish (6 cm, Nunc, Denmark) in BME medium. One day later the medium was replaced by BME containing increasing concentrations of DL-Hcy-SH. Twenty-four hours later the medium was again removed and replaced by ordinary BME. Ten days after seeding, the cells were washed with 0.9% NaCl, fixed with methanol and stained with Giemsa. Colonies were scored and plating efficiency was expressed as number of colonies in test group as a percentage of control. Each group represents the average of five separate dishes.

For estimation of toxicity by total cell growth, 26 000 cells were seeded per dish (3.5 cm, Nunc, Denmark) in EMEM medium supplemented with vitamin B_{12}, folic acid and dialyzed FCS as described above and with increasing concentrations of DL-Hcy-SH. On day 5 and day 10 (Cl 8 and Cl 16 respectively), the cells were trypsinized and counted using a Coulter Counter Model ZM. Total cell growth is expressed as number of cells in test group as a percentage of control. Each group represents the average of three separate dishes.

Both the BME and the EMEM used for the toxicity studies were ordinary media containing Met.

Labelling of cellular AdoMet and AdoHcy with ^{35}S

Cells were seeded at a density of 60 000 cells per dish (6 cm; Nunc, Denmark) in EMEM supplemented with vitamin B_{12}, folic acid and dialyzed FCS, as described above, and with either 0.1 mM L-Met or 0.1 mM M-Hcy-tl. While the cells were growing exponentially, the medium was removed and replaced with 1.5 ml fresh medium containing either 15 μCi/ml L-[^{35}S]Met or 5 μCi/ml L-[^{35}S]Hcy-tl. The total concentration of Met/Hcy was adjusted to 0.1 mM by addition of unlabelled amino acids. After labelling for 24 h the medium was removed, the cells gently washed twice with ice-cold PBS and then frozen at −85°C until analyzed.

Two parallel dishes from each group were treated with unlabelled amino acids and used for determination of cell number at the end of the labelling period.

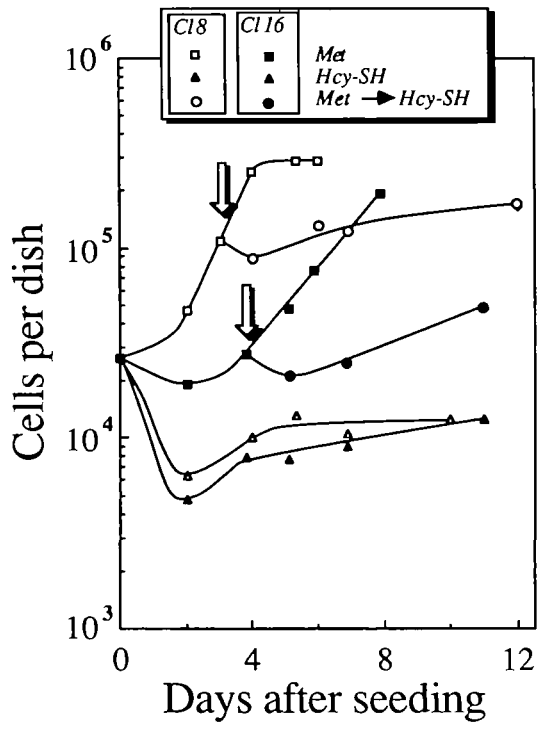

Fig. 1. Growth of C3H/10T1/2 cells in medium supplemented with either Met, Hcy-SH or both. 26 000 cells were seeded per dish (35 mm) at day 0 in Met-deficient medium supplemented with either 0.1 mM Met, 0.2 mM Hcy-SH or both. In one group of Cl 8 cells, Met was replaced by Hcy-SH at day 3, in one group of Cl 16 cells Met was replaced by Hcy-SH 3.8 days after plating. The growth curves shown are representative examples of four separate experiments.

Determination of AdoMet and AdoHcy

The cells were extracted with perchloric acid (300 μl per dish), and the extract was analyzed by HPLC on a cation-exchange column as described in detail elsewhere (21). The effluent corresponding to the elution of AdoHcy or AdoMet was collected using a programmable fraction collector, and the radioactivity determined by liquid scintillation counting.

The amount of AdoHcy in the cell was below the detection limit of the assay based on UV detection at 260 nm. Therefore, radioactivity co-chromatographing with AdoHcy is taken as a measure of cellular AdoHcy (21).

Determination of GSH

Cl 8, Cl 16 and Cl T422 were seeded at a density of 60 000 cells per dish (6 cm; Nunc, Denmark) in EMEM supplemented with vitamin B_{12}, folic acid, dialyzed FCS and with either 0.1 mM L-Met or 0.1 mM L-Hcy-tl. The cells were harvested during logarithmic growth (mid-log phase) by removing the medium and placing the dishes on ice. After gently washing twice with ice-cold PBS, the cells were frozen at −20°C followed by immediate extraction with ice-cold 5% sulfosalicylic acid. After removal of precipitated proteins by centrifugation, reduced GSH was determined as described (22) with a slight modification. Briefly, free sulfhydryl groups are derivatized in the presence of monobromobimane (Kosower's reagent) and the GSH-bimane derivative is then quantitated by chromatography on a 3-μm ODS Hypersil column, which is equilibrated and eluted with 14.2% methanol and 0.25% acetic acid adjusted to pH 3.9 with NaOH. The column is eluted by increasing the methanol concentration to 90%. The retention time of the GSH-bimane derivative was 3.2 min.

Parallel dishes from each group were used to determine the cell number at harvesting.

Determination of protein

Protein was determined according to Bradford (23) using the Bio-Rad Protein Assay Kit. Bovine γ-globulin was used as protein standard.

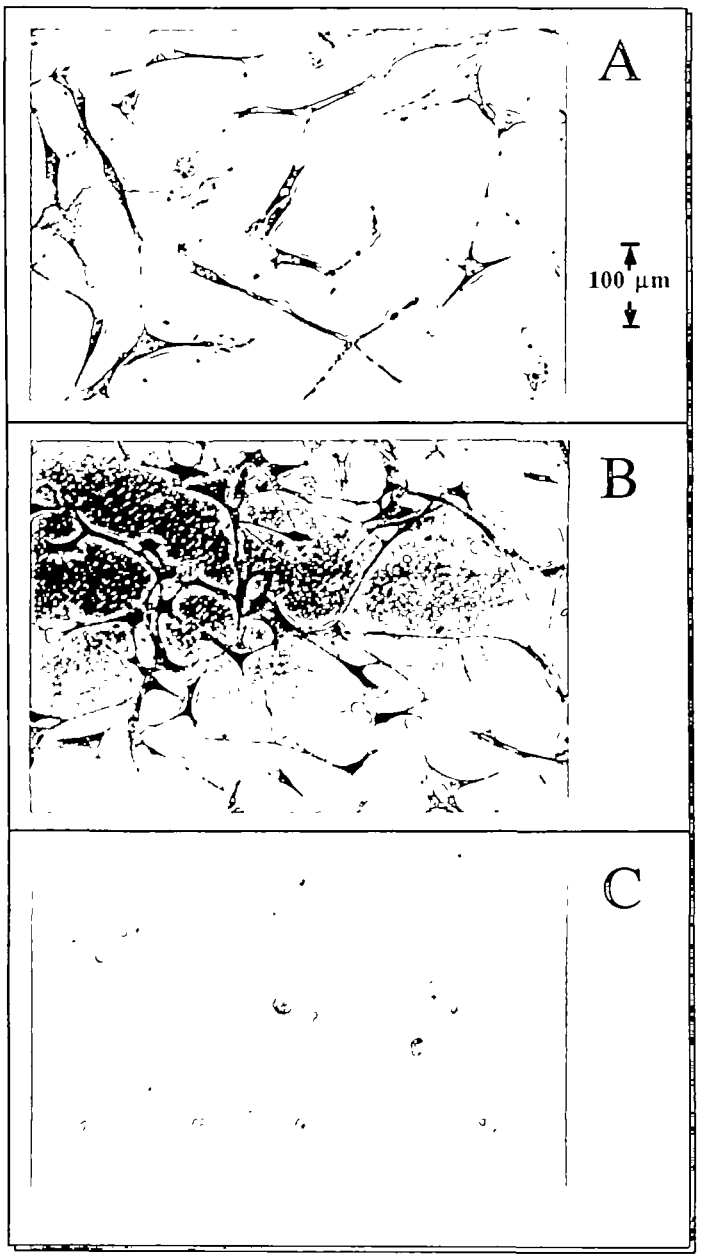

Fig. 2. Morphological effect of Hcy-SH on C3H/10T1/2 Cl 8 cells. 60 000 cells per dish (6 cm) were seeded in BME supplemented with either 0.1 mM Met (A), 0.1 mM Met + 0.1 mM Hcy-tl (B) or 0.1 mM Met + 0.2 mM Hcy-SH (C) and grown for 2 days. (Magnification = 108 ×; bar = 100 μm.)

Results

Toxicity of Hcy-SH

When non-transformed (Cl 8) and malignant (Cl 16) mouse fibroblasts were seeded in a medium lacking Met but containing DL-Hcy-SH none of the cells grew, while both cell types grew normally in Met-containing medium as shown in Figure 1. Co-addition of Met and Hcy-SH did not support growth more than Hcy-SH alone, demonstrating that the inability to grow under these conditions was not due to lack of Met (data not shown).

Substituting Met with Hcy-SH while the cells were growing exponentially caused an immediate arrest of cell growth (Figure 1). When L-Hcy-tl was used, the normal cells grew well but at a slower rate than with Met (Figure 4). Figure 2C shows the dramatic effect of cell morphology when Cl 8 cells were exposed to Hcy-SH for 2 days after plating. This experiment was performed in a medium containing both Met and Hcy-SH, again demonstrating that the observed effect was not due to lack of

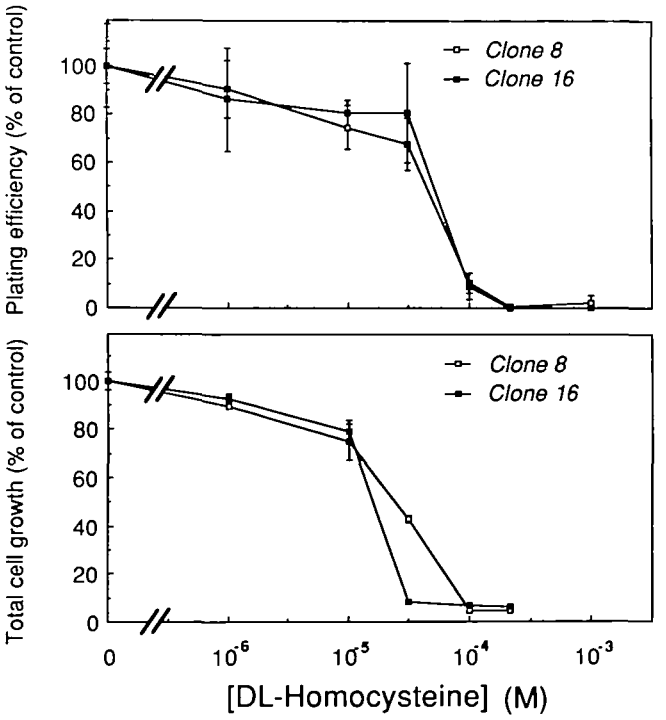

Fig. 3. Dose−response relationship of toxicity of Hcy-SH on C3H/10T1/2 cells. **Upper panel:** relative plating efficiency after treatment with Hcy-SH for 24 h. Results are expressed as the number of colonies as a percentage of control 10 days after seeding of 200 cells/dish. Each point represents the average of five determinations ± SD. **Lower panel:** total cell growth after continuous treatment with Hcy-SH. Results are expressed as total number of cells as a percentage of control 5 days (Cl 8) and 10 days (Cl 16) after seeding of 26 000 cells/dish. Each point represents the average of three determinations ± SD.

Met. Furthermore, cells exposed to Hcy-tl and Met did not show any morphological alterations (Figure 2A,B) and addition of Hcy-tl had no effect on growth in a Met-containing medium (data not shown). Co-addition of Met and L-cysteine (Cys-SH) to the medium caused exactly the same effects on growth rate and morphology of Cl 8 cells as did Hcy-SH (data not shown). However, when DL-Hcy-SS-Hcy replaced Met in the medium, Cl 8 cells grew at a rate similar to that for Hcy-tl (Figure 4). This shows that the toxicity of Hcy-SH was not due to the D-form of the amino acid, but strongly indicates that it was a result of exposure to free sulfhydryl (-SH) groups. The dose–response relationship of the toxicity of Hcy-SH was then estimated by plating efficiency (PE) and total cell growth (Figure 3). The results revealed small differences between the two cell types, the total cell growth measurements indicated a slightly higher sensitivity towards Hcy-SH of the malignant Cl 16 cells. The two types of experiments correlated rather well, but again the total cell growth appeared to be the most sensitive method. Both types of experiments showed a dramatic increase in toxicity between 10^{-5} and 10^{-4} M Hcy-SH. From these results the LD_{90} was found to be 1.0×10^{-4} M for both cell types as determined by PE, and 0.8×10^{-4} M for Cl 8 and 0.3×10^{-4} M Hcy-SH for Cl 16 as determined by total cell growth.

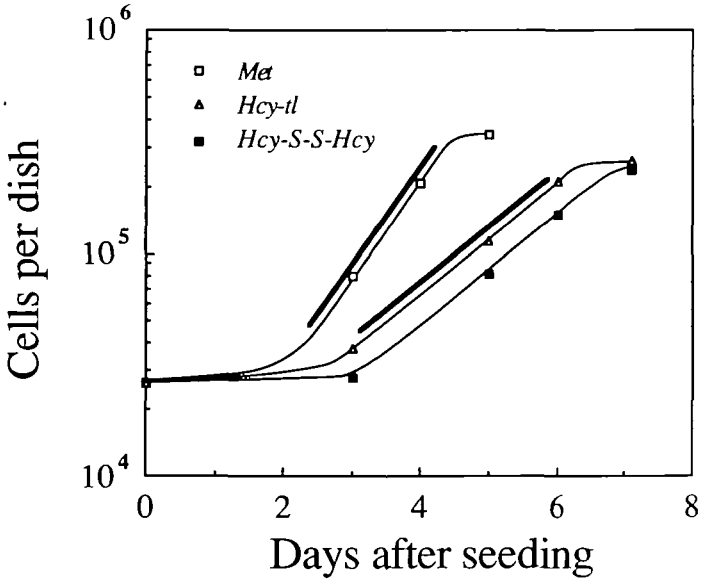

Fig. 4. Growth of C3H/10T1/2 Cl 8 cells in medium supplemented with either Met, Hcy-tl or Hcy-SS-Hcy. 26 000 cells were seeded per dish (3.5 cm) at day 0 in Met-deficient medium supplemented with either 0.1 mM Met, 0.1 mM Hcy-tl or 0.1 mM Hcy-SS-Hcy. The rule indicates the part of the growth curve which was used to calculate the growth rate constant k. The growth curves are representative examples of four separate experiments.

Growth support of homocysteine

As a consequence of the results demonstrating the toxicity of Hcy-SH, the ability of Hcy to support growth of C3H/10T1/2 cells was tested with L-Hcy-tl. Figure 4 demonstrates that the non-transformed cells (Cl 8) grew well, but at a slightly lower rate when Hcy-tl replaced Met in the medium. The malignant cells (Cl 16) also grew under these conditions (Figure 5) but at an essentially lower rate. Supplementing the medium with Hcy-SS-Hcy gave nearly identical growth curves to those obtained with Hcy-tl (Figures 4,5).

To investigate whether the behavior of Cl 16 was a general feature of malignant cells of this type, we tested another malignant cell line, Cl T422, which we had obtained by chemical transformation of Cl 8 cells with MCA and TPA, followed by inoculation into C3H mice and isolation of tumor cells. The growth curves shown in Figure 6 demonstrate a more pronounced 'Met dependence' for these cells since the growth rate obtained when Hcy-tl replaced Met was markedly lower than for the other two cell lines. In order to make a more direct comparison of the three cell lines with respect to the ability of Hcy to support their growth, we have calculated the growth rate constant k, defined by the formula

$$k = \frac{\log n_2 - \log n_1}{t_2 - t_1}$$

where n_1 and n_2 are the numbers of cells at times t_1 and t_2, respectively. The growth rate constants were calculated from the linear parts of the growth curves as indicated by the straight lines

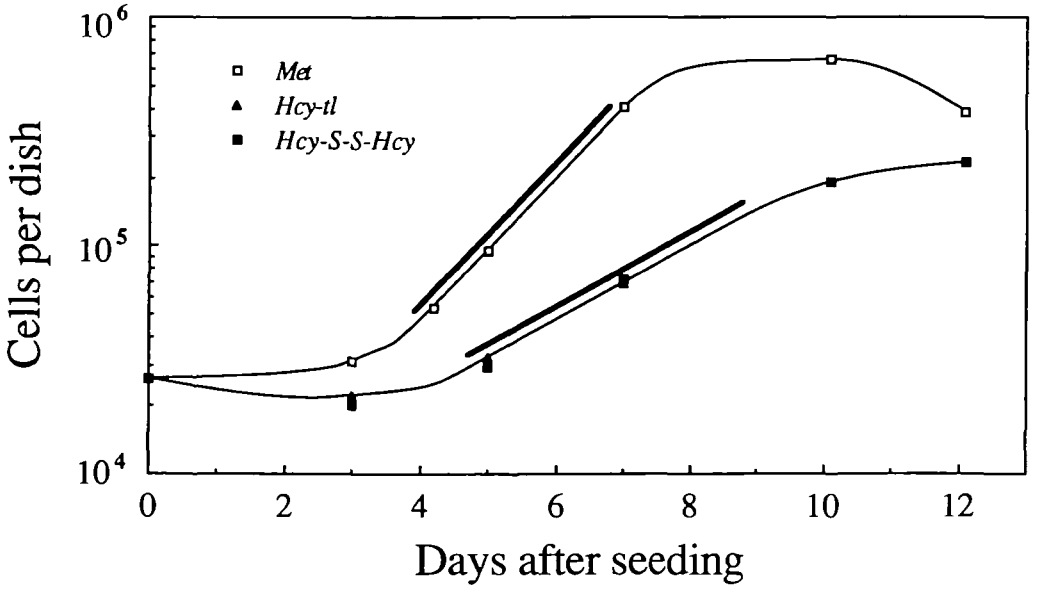

Fig. 5. Growth of C3H/10T1/2 Cl 16 cells in medium supplemented with either Met, Hcy-tl or Hcy-SS-Hcy. 26 000 cells were seeded per dish (3.5 cm) at day 0 in Met-deficient medium supplemented with either 0.1 mM Met, 0.1 mM Hcy-tl or 0.1 mM Hcy-SS-Hcy. The rule indicates the part of the growth curve which was used to calculate the growth rate constant k. The growth curves are representative examples of three separate experiments.

on Figures 4–6. The results are summarized in Table I which shows the growth rate constants for the different cells as well as the ratio between the growth rates for Hcy-tl- and Met-supplemented cells. The growth rate with Met in the medium was similar for the two malignant cell lines and slightly lower than for the non-transformed cells. The growth rate with Hcy-tl in the medium was lower than with Met in all three cell types and decreased in the order of Cl 8, Cl 16, Cl T422; this is reflected in the decreasing $k_{Hcy\text{-}tl}/k_{Met}$ ratio (Table I).

Table I. Growth rate constants of C3H/10T1/2 cells

Cells	k_{Met}	$k_{Hcy\text{-}tl}$	$k_{Hcy\text{-}tl}/k_{Met}$
Cl 8	0.37 ± 0.03	0.23 ± 0.03	0.62
Cl 16	0.30 ± 0.06	0.14 ± 0.02	0.44
Cl T422	0.31 ± 0.03	0.12 ± 0.01	0.38

Cells were grown in Met-deficient medium supplemented with either L-Met (k_{Met}) or L-Hcy-tl ($k_{Hcy\text{-}tl}$).
$k = (\log n_2 - \log n_1)/(t_2 - t_1)$ where n_1 and n_2 are the numbers of cells at the times t_1 and t_2 respectively. The values represent the average (± SD) of at least three separate experiments of which the growth curves shown in Figures 4–6 are typical examples.

Effects on Met metabolism and GSH level

To investigate whether the different ability of cells to utilize Hcy for growth was a result of alterations in the metabolism of methionine we measured the level of AdoMet and AdoHcy under the various growth conditions. The cellular content of AdoHcy was below the detection limit of the HPLC method based on absorption at 254 nm. Therefore, the cells were labelled with L-[^{35}S]Met and L-[^{35}S]Hcy-tl respectively for 24 h during exponential cell growth, and the amount of radioactivity in the HPLC peaks of AdoMet and AdoHcy was determined. The level of AdoMet was high enough to allow exact determination in molar amounts, and from these results it was possible to calculate the molar amounts of AdoHcy as well. The results are summarized in Table II and demonstrate significant differences between the three cell lines. The AdoMet content of the non-transformed cells (Cl 8) was raised when Met was replaced by Hcy-tl; in the malignant Cl 16 cells this level was nearly constant while in the Cl T422 cells the AdoMet content was significantly decreased. In contrast, AdoHcy increased in all three cell lines, but the extent of the increase was different. The increase was slightly higher in Cl 16 cells compared with Cl 8, but for Cl T422 the increase was far more pronounced. Accordingly, the AdoMet/AdoHcy

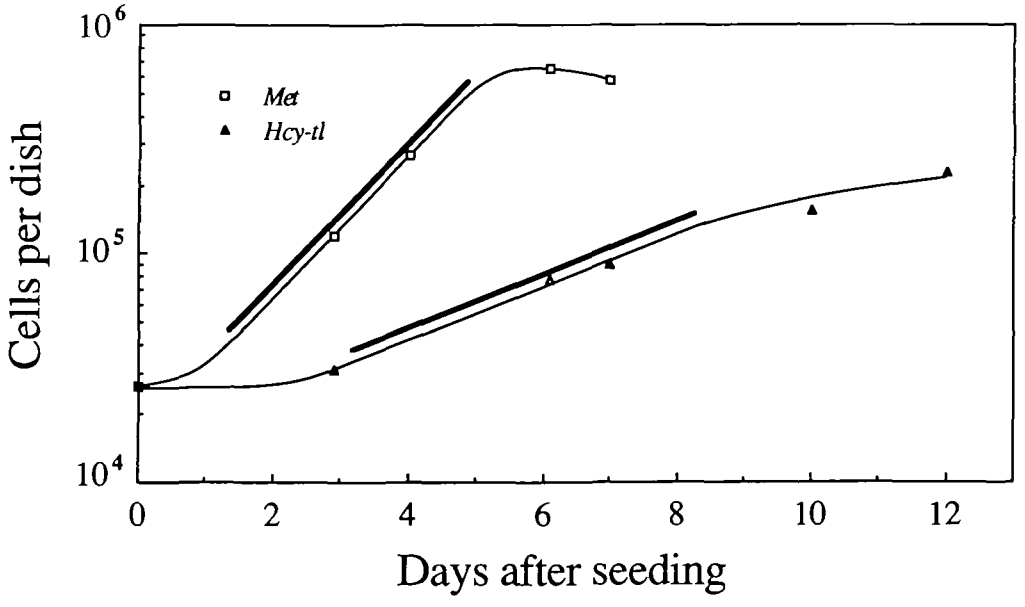

Fig. 6. Growth of C3H/10T1/2 Cl T422 cells in medium supplemented with either Met or Hcy-tl. 26 000 cells were seeded per dish (3.5 cm) at day 0 in Met-deficient medium supplemented with either 0.1 mM Met or 0.1 mM Hcy-tl. The rule indicates the part of the growth curve which was used to calculate the growth rate constant k. The growth curves are representative examples of three separate experiments.

Table II. Cellular content of S-adenosylmethionine (AdoMet), S-adenosylhomocysteine (AdoHcy) and glutathione (GSH) in mid-log phase C3H/10T1/2 cells

Cells	Medium suppl.	AdoMet/10^6 cells		AdoHcy/10^6 cells		AdoMet/AdoHcy ratio	GSH (nmol/10^6 cells)
		c.p.m.	pmol	c.p.m.	pmol		
Clone 8	Met	69408 ± 4410	249 ± 10	1010 ± 61	3.6 ± 0.1	68.7 ± 0.3	35.9 ± 5.0
	Hcy-tl	44775 ± 1350	400 ± 13[a]	661 ± 16	5.9 ± 0.5[a]	67.8 ± 3.5	28.3 ± 3.7
Clone 16	Met	55894 ± 551	270 ± 6	527 ± 9	2.5 ± 0.1	105.9 ± 0.5	37.0 ± 4.0
	Hcy-tl	31428 ± 978	281 ± 6	504 ± 43	4.5 ± 0.3[a]	62.6 ± 3.3[b]	44.1 ± 5.0
Clone T422	Met	96511 ± 3115	336 ± 5	1056 ± 29	3.7 ± 0.1	91.4 ± 3.7	9.6 ± 0.7
	Hcy-tl	37804 ± 4251	248 ± 8[a]	1441 ± 228	11 ± 3[c]	24.0 ± 5.2[a]	32.6 ± 0.5[b]

Cells were grown in Met-deficient medium supplemented with either 0.1 mM L-Met or 0.1 mM L-Hcy-tl. For determinations of AdoMet and AdoHcy cells were labelled for 24 h with [^{35}S]Met (22.5 μCi) or [^{35}S]Hcy-tl (7.5 μCi) in fresh medium. GSH was determined in cells harvested without medium change after plating. Results are expressed as the average of three separate dishes ± SD. Significance levels were calculated using Student's t-test.
[a] $P < 0.005$ compared to Met.
[b] $P < 0.001$ compared to Met.
[c] $P < 0.05$ compared to Met.

ratio remained constant in Cl 8 cells when Hcy-tl replaced Met, decreased ~40% in Cl 16 and as much as 72% in Cl T422 cells (Table II).

Cellular GSH content has been shown to change following exposure to toxic agents (16) and may be linked to AdoHcy metabolism (24). Since Hcy-tl may represent an intracellular reductive potential or produce other toxic metabolites and also may alter the amount of cysteine available for GSH synthesis, it was of interest to determine the level of GSH at the different growth conditions. Cells were grown in medium supplemented with either Met or Hcy-tl, harvested during mid-log-phase and assayed for the cellular content of GSH. The results are shown in Table II and demonstrate equal amounts of GSH in Cl 8 and Cl 16 cells when grown on Met. However, when Met was replaced by Hcy-tl, Cl 8 cells showed a slight decrease while Cl 16 cells showed a slight increase in their GSH content. In contrast, Cl T422 cells showed a level of GSH about one-third of that in Cl 8 and Cl 16 cells when grown on Met, and this level increased 3- to 4-fold when Hcy-tl replaced Met in the medium.

Discussion

Toxicity of Hcy-SH

The results presented in Figures 1–3 demonstrate the significance in providing a chemical form of Hcy which is non-toxic when cells in culture are tested for their ability to utilize Hcy instead of Met. This implies that results showing lack of cell growth in the presence of Hcy-SH should not be considered conclusive until a toxicity test has revealed the toxic effects on the actual cells.

The dose–response relationship of the Hcy-SH (Figure 3) showed a sharp increase in toxicity at concentrations required for Met to support growth. Hcy-SH in solution oxidizes rapidly to its disulfide, Hcy-SS-Hcy, which has a much lower solubility and thus may precipitate. This may in some experiments bring the concentration of Hcy-SH from a highly toxic level to a non-toxic level, depending on the time which elapses after preparation of the Hcy solution. The ability to utilize Hcy for growth may therefore be partially or even totally masked by a toxic effect of Hcy-SH and vary from one experiment to an other. To overcome these problems it is thus important that cells are grown in the presence of either Hcy-tl or Hcy-SS-Hcy when tested for Met dependence. Hcy-tl is preferable due to its greater solubility in water.

Cysteine has been shown to be highly toxic to several cell types in culture (25). Exposure of Cl 8 cells to cysteine in the present study mimicked the effect of Hcy-SH (data not shown), whereas no toxicity was observed in the presence of Hcy-tl or Hcy-SS-Hcy. These data suggest that the toxic effect is dependent on free sulfhydryl groups. The free thiol group may react with exposed sulfhydryl groups in proteins of the cell membrane and either alter the cell morphology directly or partly prevent the attachment of the cells to the growth surface. Another possibility is the generation of H_2O_2 which in turn will damage the cells. This was shown by Starkebaum and Harlan (26) who demonstrated a toxic effect on endothelial cells exposed to 0.1 mM Hcy-SH plus 2 μM $CuSO_4$. They observed a morphological response with a pronounced rounding of cells, similar to that shown in Figure 2. This effect, however, could be completely prevented by addition of catalase along with Hcy-SH and $CuSO_4$, indicating that the observed toxicity was mediated through H_2O_2 production.

Figure 3 indicates that measurement of total cell growth was slightly more sensitive than PE. The reason for this may be that the former method is sensitive to both cytostatic and cytotoxic effects, while the latter will reveal mainly cytotoxic effects, as the cells are exposed to the substance for only 24 h and then removed.

Growth support of homocysteine

The results obtained by growth in medium supplemented with either Met or Hcy-tl (Figures 4–6) showed significant differences between the three cell lines tested in their ability to utilize Hcy-tl for growth. Both the malignant cell lines grew at a slower rate than the normal cells when Hcy-tl replaced Met in the medium. Cl T422 was most affected and showed a nearly 3-fold reduction of growth rate (Figure 6, Table I). In addition, the observation that Hcy-tl and Hcy-SS-Hcy supported growth to the same extent (Figures 4 and 5) suggests that cleavage of the thiolactone ring or reduction of the disulfide bond do not cause growth reduction and are not rate-limiting processes during cellular utilization of the amino acid.

The absolute requirement for Met of several malignant cell lines demonstrated in earlier reports (4,5) could not be detected in our cells. Poirier and Wilson (27) postulated that the difference in growth rates of malignant cells in Met- and Hcy-supplemented media is due to a generally higher growth rate of malignant cells in Met-supplemented medium and not to a decrease in growth rate in Hcy-supplemented medium when compared to normal cells. This hypothesis is not supported by the results presented in this paper, as both the malignant cell lines grew at a slower rate than their parent cells in Met-supplemented medium (Table I).

Recently, Christa *et al.* (11) found that of two normal and one malignant cell line tested, one of the normal cell lines did not grow at all, one grew slowly and only the malignant Raji cells grew at a normal rate when Met was replaced by Hcy-tl. This is in contrast to a similar study where Hcy-SH did not support the growth of Raji cells (10). In the latter report a number of normal and malignant cell lines were tested and only one, a T-cell leukemia line, was able to utilize Hcy-SH; all other cell lines, including the normal ones, showed an absolute requirement for Met. However, the Hcy-SH used in that work is cytotoxic, as demonstrated in the present paper, and this could at least account for the lack of growth of the normal cell lines in Hcy-supplemented medium.

The present work supports previous observations made in nearly all the systems tested: non-transformed cell lines are fully capable of utilizing Hcy instead of Met, but the ability of malignant cell lines to utilize Hcy varies from 0 to 100% compared with non-transformed cells. This is supported by a recent paper where 20 human malignant cell lines were tested and 11 were found to have an absolute requirement of Met for growth, two were partly independent and seven were completely independent of Met for growth (13). The Met dependence thus seems to be a typical but not an obligatory event in carcinogenesis. These results also demonstrate the vital importance of using cells of the same genetic origin when testing for differences between normal and malignant cells with regard to methionine requirements.

Effects on Met metabolism

The intracellular level of AdoMet and AdoHcy (Table II) showed distinct differences between the non-transformed and the malignant cell lines. In Hcy-tl-supplemented medium both of the malignant cell lines (Cl 16 and Cl T422) had a lower AdoMet/AdoHcy ratio than in Met-supplemented medium. This is in contrast to the non-transformed cells (Cl 8) which showed a constant ratio of AdoMet/AdoHcy. These results correlate well with earlier reports which have demonstrated lower AdoMet/AdoHcy

ratio in Hcy-supplemented medium for a number of Met-dependent tumor cell lines compared with either Met-independent tumor cells or normal cells (13,30). However, there is a remarkable difference between our results and the data presented by others (13,28). The C3H/10T1/2 cell lines studied had a high AdoMet/AdoHcy ratio, between 68.8 and 106.2 in Met-supplemented medium, and the ratio was reduced to 26.3 in the Cl T422 cells when Hcy-tl replaced Met (Table II). In contrast, the AdoMet/AdoHcy ratio was reduced from 36 to 0.18 in SV40-transformed human fibroblasts (28) when Met was replaced by Hcy in the medium. Several other Met-dependent tumor cell lines had an AdoMet/AdoHcy ratio between 0.21 and 2.79 in Hcy-supplemented medium (13), whereas a number of Met-independent tumor cell lines had a ratio of ~8 (13). The reason for the maintenance of a high AdoMet/AdoHcy ratio in the malignant C3H/10T1/2 cells in Hcy-tl-supplemented medium is not known. However, the interaction of AdoMet and AdoHcy with numerous methyltransferases (29) indicates that a reduction of the ratio to 20 would not have a significant effect on the overall transmethylation rate. The data presented in this paper therefore suggest that alteration in the AdoMet/AdoHcy ratio, in the C3H/10T1/2 cells at least, may be linked to, but is not a mediator of, growth inhibition.

Although the ratio of AdoMet/AdoHcy was constant in non-transformed cells, it should be noted that the actual level of AdoMet increased in Hcy-tl-supplemented medium, and that this increase was balanced by a similar increase in AdoHcy to maintain the constant AdoMet/AdoHcy ratio. In Cl 16 cells, the AdoMet level was comparable to that in non-transformed cells in Met-supplemented medium and did not change when Met was replaced by Hcy-tl. However, the AdoHcy content was lower in Met-supplemented medium compared with the non-transformed cells and increased to a greater extent in Hcy-tl-supplemented medium. Again these results are in accordance with previous findings (13). Cl T422 showed the same pattern, but the effect on the metabolites was more pronounced. The AdoMet level was higher than in non-transformed cells in Met-supplemented medium and decreased in Hcy-tl-supplemented medium to the same level as Cl 8 and Cl 16 cells in Met-supplemented medium. The AdoHcy level increased 2- to 3-fold and resulted in a pronounced decrease in the AdoMet/AdoHcy ratio when Hcy-tl replaced Met. Some characteristic features of the results presented in Table II are the higher level of AdoHcy in all three cell lines when Hcy-tl replaced Met and the increase of this level from Cl 8 to Cl 16 to Cl T422. However, the change in AdoMet level is even more interesting as normal cells showed an increase in Hcy-tl-supplemented medium, in contrast to the malignant cells which showed either a constant level (Cl 16) or a decrease (Cl T422) in AdoMet when Hcy-tl replaced Met.

Comparing the effects on the Met metabolism presented here with the ability of the three cell lines to utilize Hcy-tl for growth (Table I), demonstrates a striking correlation between the results. The decrease in growth rate of the cell lines in Hcy-tl-supplemented medium was greater in the malignant cells than in non-transformed cells, and greater in Cl T422 than in Cl 16. The same pattern was observed in the change of the AdoMet/AdoHcy ratio in Hcy-tl-supplemented medium: constant in Cl 8 cells, ~40% decrease in Cl 16 and ~70% decrease in Cl T422.

The changes in AdoMet/AdoHcy ratio in Hcy-tl-supplemented medium could be explained by the fact that Hcy is a substrate for AdoHcy hydrolase in the synthetic direction (29), and accumulation of AdoHcy would result. AdoHcy is a potent inhibitor of a number of AdoMet-dependent methyl-transferases (31) and would reduce the AdoMet consumption. Isozymes of AdoMet synthetase (methionine adenosyl transferase) with different K_m values for Met and with different sensitivity to feedback inhibition by AdoMet, have been demonstrated in mammalian tissues. The isozyme pattern has been shown to be altered in malignant cells, in which the isozyme most sensitive to AdoMet is predominant (3). Elevation of the AdoMet level would then lead to an inhibition of AdoMet synthesis, and the overall result could be an increased AdoHcy level and a nearly constant or decreased AdoMet level.

However, this is not supported by Oden *et al.* (31) who found only a single AdoMet synthetase enzyme in several normal and transformed cell lines, and no alteration in the activity of the enzyme could be detected. They showed that the Met pool of Met-dependent cells was 20 times lower than that found in Met-independent cells. This may account for the lower AdoMet level in malignant cells in Hcy-supplemented medium.

Effects on GSH level

The variations of the GSH content in Cl 8 and Cl 16 cells under the various growth conditions were small and on a comparable level (Table II). The GSH content of Cl T422 cells, however, showed a 3- to 4-fold increase when Hcy-tl replaced Met in the medium. This difference seemed mainly to be the result of a decreased steady-state level of GSH in Cl T422 cells grown in Met-supplemented medium rather than an increase in Hcy-tl-supplemented medium. Since cellular GSH is increased in response to diverse cytotoxic agents (32) it is possible that the increase in GSH content observed in Cl T422 cells was a response of a toxic agent formed during cellular utilization of Hcy. Hcy-tl is hydrolyzed intracellularly to Hcy-SH which was toxic to C3H/10T1/2 cells, at least at high extracellular concentrations (Figures 1–3). Recent reports have shown that oxidation of Hcy-SH generates H_2O_2 and leads to injury of endothelial cells in culture (26), and that GSH may protect the cells against cellular injury by H_2O_2 (33).

In a recent work the intracellular GSH content was shown to be closely related to the growth state of 3T3 cells (34). GSH increased sharply during the initial period of exponential growth, reached a maximum about mid-log phase and then gradually decreased. In the present experiments the cells were harvested in the middle of the logarithmic growth period and therefore the GSH content should be at its maximum. Nevertheless, the small variations seen in Cl 8 and Cl 16 cells could reflect the difference in growth state between the experiments.

Increasing the AdoHcy content by inhibiting the AdoHcy hydrolase in cells in culture decreased the amount of GSH by 3- to 6-fold (24). In the present paper, however, we demonstrated an increased GSH content when AdoHcy was increased in Cl T422 cells due to growth in Hcy-supplemented medium. However, the cellular GSH content did not show any obvious correlation to the altered Met metabolism observed in the malignant cell lines used in this study.

Concluding remarks

The results presented in this study show that Hcy-SH was toxic to C3H/10T1/2 cells and is not suitable for evaluation of growth requirements in these cells. Hcy-tl was non-toxic and supported growth of both normal and malignant cells, but with decreasing efficiency in the order Cl 8, Cl 16 and Cl T422. The ability of Hcy-tl to support growth correlated with alterations in Met metabolism. The AdoMet/AdoHcy ratio was constant in normal cells, moderately reduced in Cl 16 cells and markedly reduced in Cl T422 cells when Hcy-tl replaced Met in the medium. The intra-

cellular GSH content showed small variations betwen the cell lines except for Cl T422 cells which showed a lower level under normal conditions and a distinct increase when Hcy-tl replaced Met. However, the lack of ability of malignant cells to utilize fully Hcy-tl for growth, and the possible formation of toxic agent(s) during growth in Met-deficient medium supplemented with Hcy-tl, should be further investigated.

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