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GROWTH STATE DEPENDENT INCREASE OF GLUTATHIONE BY HOMOCYSTEINE AND OTHER THIOLS, AND HOMOCYSTEINE FORMATION IN GLUTATHIONE DEPLETED MOUSE CELL LINES

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Abstract—Homocysteine has been shown to increase glutathione levels in C3H/10T1/2 Cl 8 cells. The present paper confirms that this increase was specific for non-dividing cells. Several other thiols and disulfides, including cysteamine, mercaptoethanol and dithioerythritol, also increased glutathione, but the specificity for quiescent non-dividing cells was confined to homocysteine only. Cysteamine was most efficient, increasing glutathione 5-fold in confluent, non-dividing cells, and 3.2-fold in exponentially growing Cl 8 cells. The results indicate that the increase in glutathione was not specific for homocysteine or other cysteine generating agents, but rather related to the presence of potential thiol, either in free form, as thiolactone or in its oxidized, disulfide form. The effect of the glutathione synthesis inhibitor BSO was investigated in detail in both C3H/10T1/2 Cl 8 cells and in R1.1. mouse lymphoma cells. Twenty-four hours after addition of 20 μM BSO to exponential growing Cl 8 cells the glutathione content was reduced to 5.5%, with minimal toxic effect. To achieve the same GSH depleting effect on exponential growing R1.1. cells, the BSO concentration had to be increased to 50 μM, which had a slight, but distinct growth inhibitory effect on the lymphoma cells. Based on these data, the possibility that glutathione mediated homocysteine production was investigated in part by depleting the cells of glutathione and determining the homocysteine export rate as a measure of the intracellular production of the metabolite. The results showed that glutathione depletion by BSO had no effect on the homocysteine export rate in Cl 8 cells, while in R1.1. cells a moderate decrease in homocysteine export rate accompanied by a slight, but distinct decrease in growth rate, was observed when the cells were depleted of glutathione. In addition, these data indicate that BSO did not interfere with the overall transmethylation rate, and this observation supports the view of BSO as a specific inhibitor of GSH synthesis. A general difference between the homocysteine export rate in Cl 8 and R1.1. cells was observed. The former demonstrated a decreasing export rate during exponential growth, while the latter showed an initial decrease and then a slight increase in homocysteine export rate.

Homocysteine is a sulfur-containing amino acid not found in the diet and is a metabolic precursor for both methionine and cystathionine synthesis [1]. The only known source of homocysteine in vertebrates is the catabolism of *S*-adenosylhomocysteine (AdoHcy†), which has been the target for cytostatic action of numerous purine analogues [2, 3] including c^{3}Ari and c^{3}Ado [4–6]. The intracellular level of homocysteine seems to be strictly regulated by export mechanisms. Cells excrete considerable amounts of homocysteine under normal conditions, but perturbation of metabolism leading to accumulation of homocysteine leads to a similar increase in export of the amino acid [7]. Furthermore, inhibition of the catabolism of AdoHcy is followed by a drastic decrease in homocysteine export [4, 5], while an increased endogenous supply of methionine leads to a similar increase in homocysteine export [8]. The intracellular formation of homocysteine thus seems to be reflected in the export of the compound.

Recently, we showed that homocysteine induced a significant increase in glutathione in confluent monolayers of mouse embryo fibroblasts [6]. Since homocysteine is a precursor of cystathionine and subsequently cysteine synthesis, it is part of the metabolic pathway of the first step in glutathione systhesis [9, 10]. The increase in glutathione might therefore be a result of increase in a metabolic precursor of GSH synthesis. According to several reports [11, 12], cysteine and intracellular cysteine generating agents increase GSH levels. Since cysteine is closer to the metabolic endpoint, it might be expected to be even more effective than homocysteine in supporting GSH synthesis. Furthermore, thiols which are not part of the cystathionine/glutathione pathway have been shown to increase GSH levels [13], apparently by promoting cysteine uptake.

To reveal different mechanisms of GSH modulation we tested several related thiols with both free,

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† Abbreviations: BSO, L-buthionine-(*S,R*)-sulfoximine; c^{3}Ari, 3-deazaaristeromycin; c^{3}Ado, 3-deazaadenosine; Met, L-methionine; Hcy, homocysteine (not specified form); Hcy-SH, D,L-homocysteine; Hcy-tl, L-homocysteine thiolactone; Hcy-SS-Hcy, D,L-homocystine; DTE, dithioerythritol; MSH, 2-mercaptoethanol; GSH, reduced glutathione; GSSG, oxidized glutathione; GSSR, soluble glutathione mixed disulfides; AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine.

reduced sulfhydryl groups and some of the corresponding disulfides, for their ability to increase cellular GSH content with respect to the growth state of the cells.

The possible role of homocysteine and other thiols in mediating the GSH level, raises the question if GSH in some way is regulating the level of homocysteine. This question is addressed by depleting cellular content of GSH by buthionine sulfoximine (BSO), a well known, irreversible inhibitor of the first step in GSH-synthesis [14, 15]. BSO can sensitize cells towards the action of several cytotoxic agents and may become a useful drug in cancer chemotherapy [12, 16]. Studies on homocysteine export to BSO are important since homocysteine itself in the extracellular medium may represent a pathogenic factor provoking arterial lesions [1, 17].

The present paper describes the toxicity of BSO and the dose-dependent effect on GSH content in two different mouse cell lines. A suitable GSH-depleting concentration of BSO is picked to demonstrate the effect on homocysteine export when GSH is lowered to less than 5% of the control level in the two cell lines.

MATERIALS AND METHODS

Chemicals

L-Homocysteine thiolactone (Hcy-tl), D,L-homocysteine (Hcy-SH), D,L-homocystine (Hcy-SS-Hcy), L-cysteine, D, L-cystine, *N*-acetyl-L-cysteine, L(+)-penicillamine, cysteamine, dithioerythritol, L-buthionine-(*S*,*R*)-sulfoximine (BSO) and reduced (GSH) and oxidized (GSSG) glutathione were obtained from Sigma Chemical Co. (St Louis, MO). 2-Mercaptoethanol (MSH) was from Merck (Darmstadt, F.R.G.), sodium borohydride was from Fluka Chemie AG. (Buchs, Switzerland) and monobromobimane was from Calbiochem, Behring Diagnostics (La Jolla, CA).

The disulfide cystamine was obtained by bubbling air for 2 hr at room temperature through a solution of 5 mM cysteamine. After oxidation the content of free SH-groups was less than 2% of the original cysteamine solution (data not shown) as determined by the method of Ellman [18].

Cell lines and culture conditions

Non-transformed C3H/10T1/2 Cl 8 cells [19] were grown in Basal Medium Eagle (BME, Gibco, Paisley, U.K.) supplemented with 10% heat inactivated foetal calf serum (Sera-Lab, Ltd, Sussex, U.K.) as described previously [17].

The mouse T-lymphoma cell line R1.1 [20] was obtained from Dr Dennis A. Carson at Scripps Clinic and Research Foundation, La Jolla, CA. Stock cultures of R1.1 were maintained in suspension culture in RPMI 1640 medium supplemented with 10% heat inactivated foetal calf serum. The cells were grown in tissue culture plastic flasks (25 cm^2; Nunc, Denmark) and routinely diluted in fresh medium three times a week.

Both cell lines were maintained at 37° in an atmosphere of 5% CO_2 in air and a relative humidity of 95%.

Effect of thiols on glutathione content in C3H/10T1/2 Cl 8 cells.

Ten thousand cells were seeded per dish (6 cm, Nunc) in BME. During mid-exponential growth (22% confluence) and 3 days after reaching confluence, the cells were exposed to the different thiols by replacing the medium with fresh medium containing either no additions or thiol at a concentration of 100 μM unless otherwise indicated. Twenty-four hours after initiation of treatment, the cells were harvested for determination of glutathione by removal of the culture medium, gently washed twice with ice-cold PBS and immediately frozen at −85°.

For determination of cell number two parallel dishes from each group were harvested, the cells trypsinized and counted using a Coulter Counter Model ZM (Coulter Electronics Ltd, Luton, U.K.).

Effect of BSO on growth and glutathione content

Cl 8. Five thousand cells were seeded per dish (6 cm, Nunc) in BME. Five days later the medium was replaced by fresh medium containing increasing concentrations of BSO. At this point and 24 and 48 hr later cells were harvested for determination of glutathione as above. Two parallel dishes from each group were used to determine the cell number at harvesting.

*R*1.1. Exponentially growing R1.1. cells (4×10^4 cells/mL) were seeded in plastic vessels (25 cm^2, Nunc) in RPMI 1640 containing increasing concentrations of BSO. At the time of seeding and 24 and 48 hr later samples of the cell suspension were harvested for determination of glutathione by centrifugation at room temperature for 7 min at 500 *g*. The medium was removed and the cells were resuspended in 5 mL ice-cold PBS and centrifuged at 4° for 7 min at 500 *g*. The washing procedure was repeated once, the PBS removed and the cells frozen at −85°. Two parallel samples of the cell suspension were removed for quantitation of cell numbers.

Homocysteine export from glutathione depleted cells

Cl 8. Fifteen thousand cells were seeded per dish (6 cm, Nunc) in BME. Five days later, when cells were growing exponentially the medium was replaced by fresh medium containing either no additions or 20 μM BSO. At appropriate times the cells were harvested for determination of intracellular glutathione as described above. Samples of the medium were frozen at −20° for determination of extracellular homocysteine. Two parallel dishes from each group were used to determine the cell number at harvesting.

*R*1.1. Exponentially growing R1.1. cells (1×10^5 cells/mL) were seeded in plastic vessels (25 cm^2, Nunc) in RPMI 1640 containing either no additions or 50 μM BSO. At appropriate times the cells were harvested for determination of intracellular glutathione as described above. Samples of the medium were removed after centrifugation and frozen at −20° for determination of extracellular homocysteine. Two parallel samples of the cell suspension were removed for quantitation of cell numbers.

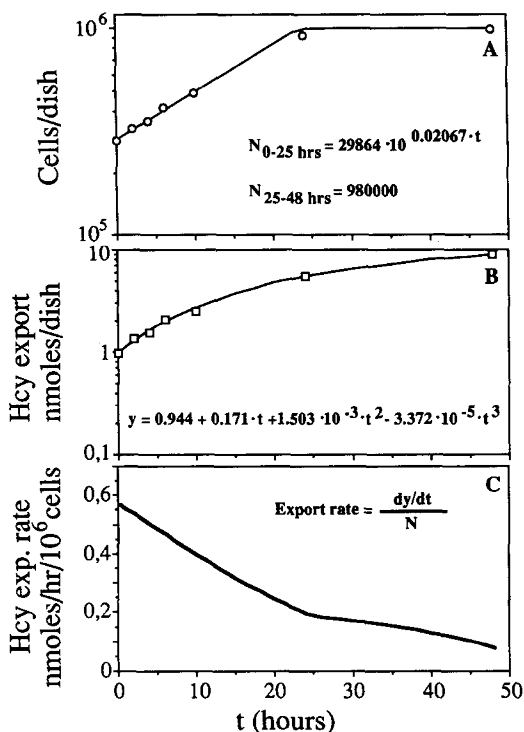

Fig. 1. Estimation of homocysteine export rate. The figure illustrates the treatment of the experimental data to obtain an estimate of the export rate of homocysteine. In this example Cl 8 fibroblasts were seeded and during exponential growth the homocysteine content of the medium as well as the cell number per dish, were determined (for details, see text). (A) Cell number as a function of time (t). The equations describing the curve are shown. (B) Accumulated amount of homocysteine in the medium as a function of time (t). The equation describing the curve are shown. (C) Export rate of homocysteine calculated from the data shown in (A) and (B).

Determination of extracellular homocysteine

The medium was mixed with perchloric acid to remove proteins, neutralized, and treated with dextran-coated charcoal to remove nucleosides and AdoHcy which would interfere with the homocysteine assay [4, 5]. Homocysteine was then determined as described previously [21].

Estimation of homocysteine export rate

The experimental design allowed us to determine the accumulated amount of homocysteine in the medium. Since the cells were proliferating during this accumulation, mathematical treatment of the data had to be performed to obtain an estimate for the specific export rate of homocysteine per unit of cell mass. The mathematical operations involved will be described in some detail below.

Number of cells. The number of cells (N) at any given time t in a population of dividing cells can be described by the equation

$$N = a \times 10^{bt}. \quad (1)$$

However, if mono-layer cells reach confluence, the cell number becomes fixed and

$$N = K, \quad (2)$$

K is a constant. In Fig. 1A is shown an example of fibroblasts in the late logarithmic growth phase, and the growth curve is described by equations as (1) and (2), determined by a curve-fitting computer program.

If cells are exposed to toxic agents, growth may decrease and deviate from Eqn (1), it can be described by a polynomial like

$$N = a_0 + a_1 t + a_2 t^2 + a_3 t^3 + \ldots + a_n t^n \quad (3)$$

where $a_0, a_1, a_2 \ldots a_n$ are constants $n = 0, 1, 2 \ldots$. The degree (n) of the polynomial and the constants are accurately determined applying a curve-fitting computer program.

Homocysteine export. The amount (y) of homocysteine accumulating in the medium is a function of time, i.e. $y = f(t)$. This function can again be described by a polynomial as above,

$$y = a_0 + a_1 t + a_2 t^2 + a_3 t^3 + \ldots + a_n t^n. \quad (4)$$

The differential dy/dt then gives the rate of accumulation in the medium. An example of homocysteine accumulation is shown in Fig. 1B where computer-analysis gave a trinomial as the equation best fitting the values obtained.

Homocysteine export rate. In a small time interval dt the homocysteine exported is dy, and the cell number responsible for this export is $n + dN$. If dt approaches zero, the cell number exporting dy approaches N. Then, the export rate (v) per cell mass can be expressed as

$$v = \frac{dy/dt}{N}. \quad (5)$$

Recording cell numbers and accumulation of homocysteine in the medium during exponential growth of monolayer cells, the homocysteine export rate per cell mass can be presented graphically as a function of time according to Eqn (5). An example using data from Cl 8 cells is shown in Fig. 1C.

Determination of glutathione

The frozen cells (−85°) were extracted with ice-cold 5% sulfosalicylic acid, scraped off the dish with

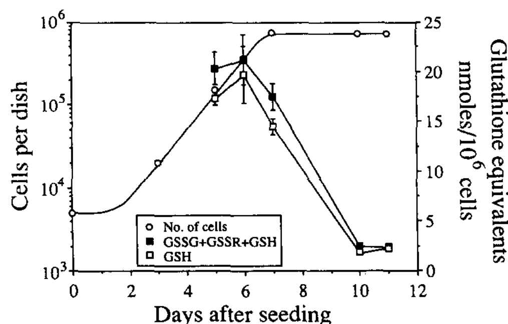

Fig. 2. Glutathione content of non-transformed C3H/10T1/2 Cl 8 cells as a function of growth state. Five thousand cells were seeded per dish (6.0 cm), and at the times indicated parallel dishes were removed for cell counting and determination of cellular glutathione. Results are expressed as equivalents of reduced glutathione and represent the average of four determinations ± SD.

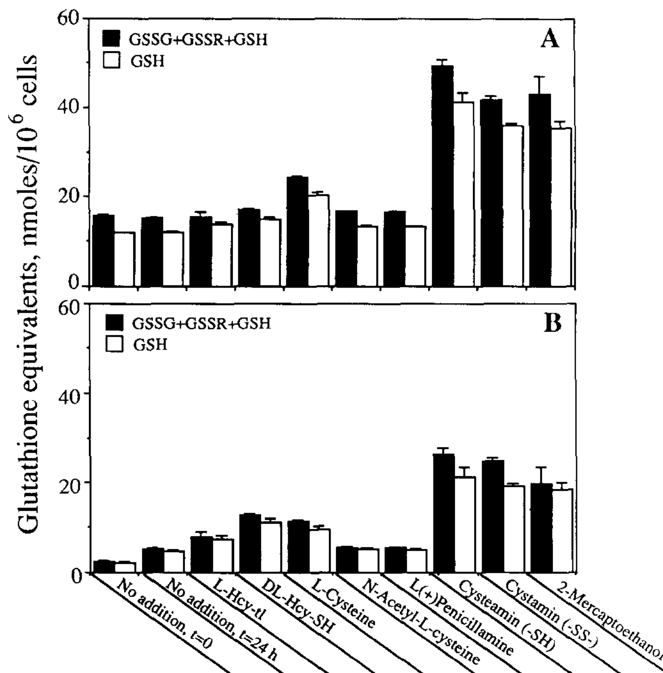

Fig. 3. Glutathione content of non-transformed C3H/10T1/2 Cl 8 cells after 24 hr exposure to different thiols. Ten thousand cells were seeded per dish (6.0 cm) and in mid-exponential growth or at confluence the medium was replaced by fresh medium containing either no additions or 100 μM thiol (or disulfide corresponding to 100 μM free thiol). (A) Treatment initiated in mid-exponential growth (22% confluence); (B) treatment initiated 3 days after reaching confluence. Results are expressed as equivalents of reduced glutathione and represent the average of four determinations ± SD.

a rubber policeman and the precipitated proteins removed by centrifugation.

GSH was determined in the acid extract by derivatization with monobromobimane (Kosower's reagent), and subsequent quantitation of the GSH-bimane derivative by HPLC as previously described [6]. After reduction with sodium borohydride the same procedure was used to determine total soluble glutathione (GSH + GSSG + soluble glutathione mixed disulfides, GSSR).

Determination of protein

Protein was determined according to Bradford [22] using the Bio-Rad Protein Assay Kit. Bovine γ-globulin was used as protein standard.

RESULTS

Effect of thiols on glutathione content

Exponential growing C3H/10T1/2 Cl 8 cells seem to have a rather constant level of glutathione, most of which is present in the reduced form. As the cells reach confluence the glutathione content decreases rapidly and remains at a low level (Fig. 2). We have previously shown [6] that Hcy-tl elevates glutathione in confluent Cl 8 cells, but not in exponentially growing cells. Figure 3 demonstrates the ability of several related thiols to increase glutathione content in both log-phase (Fig. 3A) and quiescent, confluent (Fig. 3B) Cl 8 cells. The most potent compound in this respect was cysteamine, which in contrast to homocysteine increased glutathione in both proliferating (3.2-fold) and confluent cells (5-fold). Cystamine, the oxidized form of cysteamine, induced glutathione as efficiently as the reduced compound. Similarly, cystine, the disulfide of cysteine had the same effect as the reduced compound (data not shown).

Notably, the reducing agents 2-mercaptoethanol (MSH) and dithioerythritol (DTE, data not shown) was among the most effective compounds in increasing glutathione levels, although not as effective as cysteamine, and DTE being less effective than MSH.

Effect of BSO on growth and glutathione content in C3H/10T1/2 Cl 8 cells

When BSO was added to exponentially growing cultures of C3H/10T1/2 Cl 8 cells, no toxic effects were observed during the first 24 hr of exposure up to 200 μM BSO. A slight growth inhibitory effect was observed at concentrations exceeding 20 μM 48 hr after BSO-addition, while 6 days after addition pronounced toxicity was observed at concentrations above 5 μM BSO (Fig. 4).

Figure 5 shows that 24 hr after addition of 5 μM BSO, intracellular glutathione content is reduced to 32.7% of control, while 20 μM BSO reduces glutathione to 5.5%.

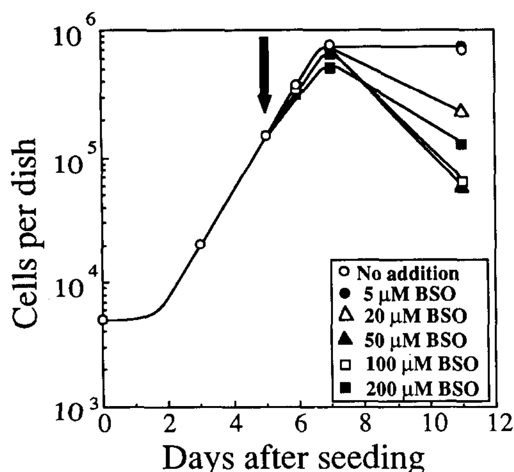

Fig. 4. Dose dependent effect of BSO on growth of non-transformed C3H/10T1/2 Cl 8 cells. Five thousand cells were seeded per dish (6.0 cm) and during exponential growth (arrow) the medium were replaced by fresh medium containing BSO at the concentrations indicated. Duplicate dishes were used for determination of cell number. The results are from a representative experiment.

Based on these findings, we chose 20 μM BSO as a concentration which would effectively deplete exponentially growing Cl 8 cells of glutathione with minimal toxicity during a 48-hr period.

Homocysteine export from glutathione depleted Cl 8 cells

Ten hours after addition of 20 μM BSO to exponentially growing Cl 8 cells, the glutathione content (both GSH and total glutathione) was depleted to 22%, and was further reduced to 4.7% of control level at 24 hr as shown in Fig. 6B. No toxic effects on cell growth were observed in the 48 hr duration of the experiment (data not shown).

Figure 6A shows that Cl 8 cells, under the experimental conditions used, excreted homocysteine at a decreasing rate during exponential growth, and that this rate was approximately constant or slowly decreased when the cells reached confluence. Notably, depletion of glutathione did not affect the homocysteine export from these cells.

Effect of BSO on growth and glutathione in R1.1. cells

Addition of BSO to exponentially growing cultures of R1.1. cells, produced slight growth inhibitory effects at concentrations above 20 μM BSO (Fig. 7). Four days after addition, the results demonstrated no growth inhibitory effects up to 20 μM BSO, 50 μM BSO had a slight inhibitory effect on growth, while concentrations exceeding 50 μM completely inhibited the proliferation of R1.1. cells. Furthermore, the effect on growth at high concentrations of BSO occurred after a lag period of about 24 hr (Fig. 7).

The glutathione content of R1.1. cells was initially only 20–25% of that observed in Cl 8 cells (data not shown), and slightly higher doses of BSO were required to achieve the same degree of glutathione depletion as in Cl 8 cells. Twenty-four hours after addition of 5 μM BSO to R1.1. cells, intracellular glutathione content was reduced to 49.6% of control, while 50 μM BSO reduced glutathione to 3.4% (data not shown).

Based on these findings, we chose 50 μM BSO as a concentration which would effectively deplete exponentially growing R1.1. cells of glutathione with minimal toxicity during a 48-hr period.

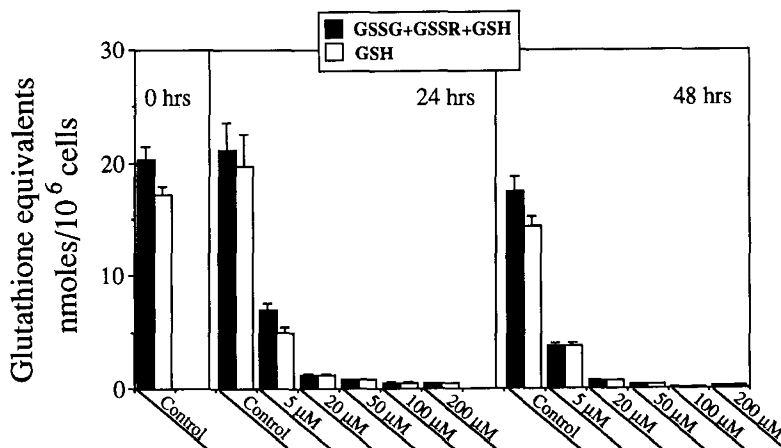

Fig. 5. Dose dependent effect of BSO on glutathione content in non-transformed C3H/10T1/2 Cl 8 cells. Five thousand cells were seeded per dish (6.0 cm) and during exponential growth the medium were replaced by fresh medium containing BSO at the concentrations indicated (as in Fig. 4), and the content of total and reduced glutathione determined 24 and 48 hr later. The samples are drawn from the same experiment as shown in Fig. 4. The results are expressed as equivalents of reduced glutathione and represent the average of four determinations ± SD.

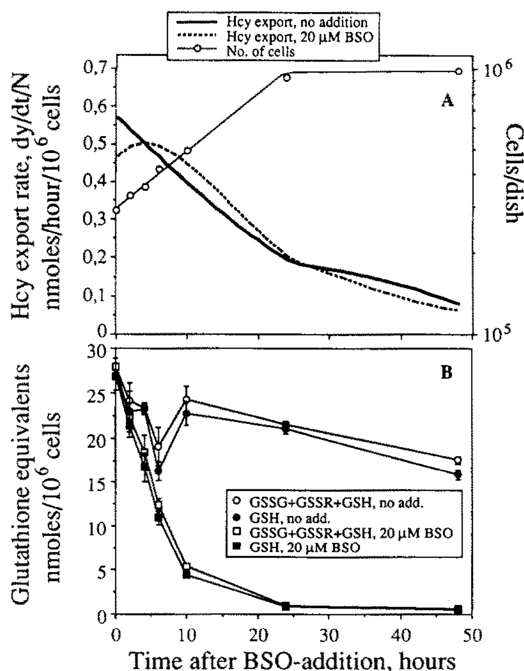

Fig. 6. Homocysteine export rate and glutathione content in non-transformed C3H/10T1/2 Cl 8 cells after treatment with BSO. One thousand five hundred cells were seeded per dish (6.0 cm) and in mid-exponential growth the medium was replaced by fresh medium containing either no additions or 20 μM BSO. At the times indicated, homocysteine in the medium and intracellular glutathione content as well as the cell number were determined. The homocysteine export rate was then estimated according to the method described in the text. (A) Homocysteine export rate and cell growth. Only the growth curve of the control cells is shown, since the BSO treated cells gave identical cell counts. (B) Reduced and total glutathione content. The results are expressed as equivalents of reduced glutathione and represent the average of four determinations ± SD.

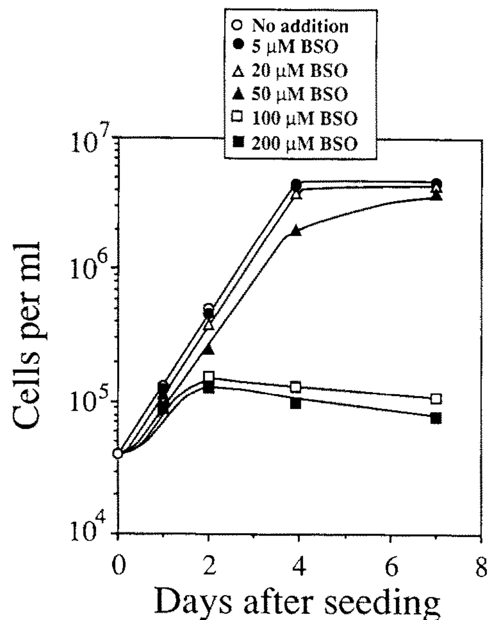

Fig. 7. Dose dependent effect of BSO on growth of R1.1. lymphoma cells. Forty thousand cells/mL were seeded in medium containing BSO at the concentrations indicated. At different times samples in duplicate were removed for determination of cell number. The results are from a representative experiment.

Homocysteine export from glutathione depleted R1.1. cells

The results shown in Fig. 8 confirmed the data with Cl 8 cells (Fig. 6B) showing that BSO was effective in depleting both the oxidized and the reduced form of glutathione (Fig. 8B). Twelve hours after addition of 50 μM BSO to exponentially growing R1.1. cells, the glutathione content was depleted to 6.9%, and was further reduced to 3.5% of control level at 24 hr (Fig. 8B).

After an initial decrease, the leukemic cells showed a marked increase in homocysteine export rate (Fig. 8A), contrasting the findings with Cl 8 cells (Fig. 6A). Beyond 12 h in medium supplemented with 50 μM BSO, there was a notable inhibition of cell growth which was accompanied by a moderate decrease in homocysteine export rate (Fig. 8A). It should be noted that the GSH content was reduced to less than 20% of control level at least 5 hr before the reduction in homocysteine rate occurred.

DISCUSSION

The reduction of glutathione content when monolayer cells stop proliferating (Fig. 2) is in accordance with other reports [23, 24]. The results confirmed our previous findings that Hcy-tl increases glutathione selectively in confluent cells [6], and the corresponding free thiol (Hcy-SH) seems somewhat more potent in this respect. This selectivity towards quiescent, confluent cells was confined to homocysteine only (Fig. 3). The biological implications of this observation are unclear.

Cysteine, but not Hcy-SH induced glutathione in proliferating cells, while both cysteine and Hcy-SH increased glutathione to the same extent in confluent cells, indicating that the increase was not a direct result of an increased amount of metabolic precursor stimulating glutathione synthesis, otherwise cysteine would be expected to be more effective than Hcy-SH. This was further supported by the findings that several other thiols, which are not metabolically related to the synthesis of glutathione, were far more potent in increasing the glutathione level. Both cysteamine and *N*-acetylcysteine have recently been shown to increase glutathione level several fold in chinese hamster ovary cells, and in agreement with our results, cysteamine was the most effective [13]. Thus, our observations do not add to previous reports on enhancement of glutathione level by cysteine and agents forming cysteine intracellularly [11, 12], since both cysteamine, MSH and DTE were far more potent in this respect than cysteine.

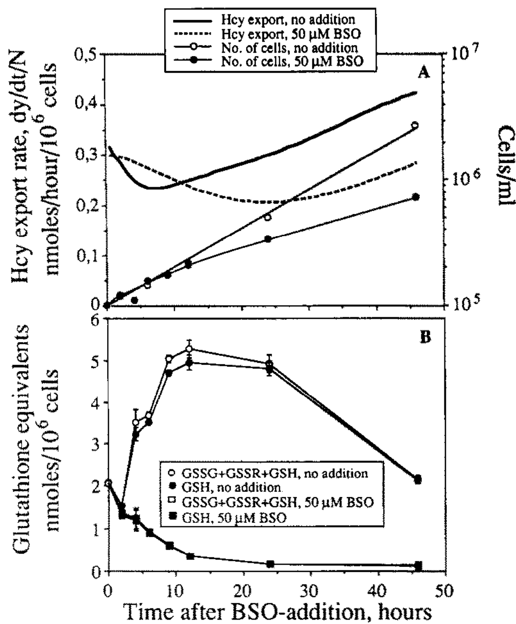

Fig. 8. Homocysteine export rate and glutathione content in R1.1. lymphoma cells after treatment with BSO. One hundred thousand cells/mL (in exponential growth) were seeded in medium containing either no additions or 50 μM BSO. At the times indicated, homocysteine in the medium and intracellular glutathione content as well as the cell number were determined. The homocysteine export rate was then estimated according to the method described in the text. (A) Homocysteine export rate and cell growth. (B) Reduced and total glutathione content. The results are expressed as equivalents of reduced glutathione and represent the average of four determinations ± SD.

However, several reports [13, 25, 26] have indicated a mechanism involving enhanced uptake of cysteine by thiols including MSH, cysteamine and *N*-acetylcysteine, thus leading to increased glutathione synthesis. It seemed therefore, that the effect on GSH level originally seen with Hcy-tl [6] was not specific for homocysteine as such, but rather related to the presence of thiol (or disulfide), possibly affecting uptake of cysteine.

The results shown in the present paper indicate that homocysteine is not a specific regulator of glutathione level. Still, since homocysteine is metabolically related to the synthesis of glutathione, the latter may well function as a mediator of homocysteine production. This possibility represents a novel aspect of this problem and was approached by depleting the cells of glutathione and evaluating the effect on intracellular homocysteine production, determined as export to the medium. BSO is an excellent tool in this respect since it is a specific, irreversible inhibitor of γ-glutamylcysteine synthetase, the enzyme catalysing the first step in glutathione synthesis [12]. However, since data on the cytotoxicity of this agent still seem incomplete [27], we performed detailed studies on the effect of BSO on GSH content and cytotoxicity towards two different mouse cell lines. The mouse fibroblasts (Fig. 4) were slightly more sensitive to BSO than the mouse lymphoma cells (Fig. 7).

From these studies we selected 20 μM BSO as an effective concentration in depleting glutathione in Cl 8 cells with minimal toxicity. In R1.1. cells the concentration had to be raised to 50 μM to obtain equivalent effect on the glutathione level. This concentration had a slight, but distinct toxic effect on the cells (Figs 7 and 8A).

Determination of the homocysteine export rate from Cl 8 cells gave no difference between control and BSO-treated cells (Fig. 6), indicating that depletion of glutathione did not influence the intracellular formation of homocysteine. This also indicates that BSO is a rather specific metabolic inhibitor which did not interfere with AdoMet dependent transmethylation reactions, since homocysteine formation and thereby the homocysteine export is a measure of the overall transmethylation rate in non-liver cells [28].

The results obtained with the lymphoma cells, R1.1., showed a different picture than the fibroblasts. A moderate reduction in homocysteine export rate (Fig. 8) was observed when R1.1. cells were depleted in glutathione, suggesting a regulatory effect of glutathione on homocysteine production in these cells. However, it should be noted that the GSH content was reduced to less than 20% of control level at least 5 hr before the reduction in homocysteine export rate occurred, and that a slight, but distinct reduction in growth rate was observed at the same time as the homocysteine export rate decreased. Taken together, these results might indicate that reduced homocysteine export was the result of a direct toxic effect of BSO on R1.1. cells, rather than an effect of glutathione depletion. This is in accordance with previous findings showing that homocysteine export was proportional to the growth rate [28].

Notably, these experiments revealed essential differences between the two cell lines with respect to their export of homocysteine. The fibroblasts showed a decreasing rate of homocysteine export during logarithmic growth (Fig. 6A), while the lymphoma cells showed a small, initial decrease, followed by a distinct increase in export rate throughout the rest of the exponential growth period. The reason for this difference is unclear, but it may be related to the fact that the Cl 8 cells are Met independent [17, 29] while the R1.1. cells are not able to utilize Hcy instead of Met.

It has previously been shown in our laboratory that only free homocysteine, and not protein bound homocysteine is available for export [8]. Furthermore it has been demonstrated for an exogenous supplied thiol like captopril that the protein binding probably via disulfide linkage, is enhanced following GSH depletion by BSO [30]. It is therefore remarkable that a drastic reduction of GSH content in Cl 8 cells did not decrease the amount of homocysteine available for export. This might suggest that physiological GSH concentrations are not required to prevent formation of disulfides between homocysteine and cellular proteins, or that the homocysteine

export is compartmentalized through a close coupling to the AdoHcy hydrolase reaction, as previously suggested [8].

In conclusion, the present results demonstrate that thiols, including homocysteine, increase glutathione levels in mouse embryo fibroblasts by a mechanism which is not known. Increased amount of metabolic precursors of glutathione synthesis from cysteine generating agents did not account for the observed increase in glutathione. The paper provides valuable data regarding toxicity and effect on glutathione of BSO towards two different mouse cell lines. These results are also important with respect to treatment of cancer with cytostatic drugs, the effect of which may be potentiated by decreasing the glutathione level [12, 16, 31].

The two cell types under investigation are shown to differ in their production of homocysteine during proliferation. Depletion of glutathione is shown to have no effect on the homocysteine production in the Cl 8 fibroblasts, while a moderate decrease can be observed in the lymphoma cells. The results add to data indicating that BSO is a specific metabolic inhibitor of GSH synthesis not interfering with AdoMet dependent transmethylation reactions.

The possible role of glutathione in mediating homocysteine production, and the mechanism of the thiol induced elevation of glutathione is under current investigation.

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