Regulation of glutathione metabolism in Ehrlich ascites tumour cells

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Glutathione metabolism was studied in cancer cells during the growth of an Ehrlich ascites tumour. GSH, but not GSSG, content decreases when cell proliferation and the rate of protein synthesis in the tumour decrease. This change correlates with a decrease in the rate of GSH synthesis and an increase in glutathione peroxidase and glutathione *S*-transferase activities. Glutathione efflux from tumour cells seems to co-ordinate with the rate of GSH synthesis. Cysteine, and not methionine, promotes GSH synthesis in tumour cells. However, changes in the rate of GSH synthesis are not due to limitations in the supply of blood cysteine or to changes in the intracellular amino acid pool of the cancer cells. Our data suggest that changes in protein metabolism accompanying tumour growth *in vivo* can modulate glutathione content in cancer cells.

INTRODUCTION

GSH, the major non-protein thiol in mammalian cells, is involved in many cellular functions (Meister, 1983; Deneke & Fanburg, 1989). This tripeptide plays a role in protection against tissue damage produced by oxidative stress (Sies, 1986), radiation and chemotherapy (Arrick & Nathan, 1984; Mitchell & Russo, 1987). Modifications of GSH metabolism have been postulated as being useful in cancer therapy (Meister, 1983). Indeed, the introduction of agents that can either increase or decrease GSH concentrations in cells opened up the possibility of modulating the cellular response to different anticancer treatments (Meister, 1983; Mitchell *et al.*, 1989). Moreover, approaches to cancer treatment based on modulation of GSH concentrations in tumour cells must take into consideration the glutathione status and the rate of GSH synthesis in these cells. Indeed, elevation of intracellular GSH content has been associated with mitogenic stimulation (Shaw & Chou, 1986). GSH has been suggested as a potential regulator of protein synthesis (Kosower & Kosower, 1978), DNA synthesis and cell proliferation (Suthanthiran *et al.*, 1990). Moreover, some amino acid precursors for GSH synthesis have been shown to be essential in cancer metabolism, e.g. glutamine, which is the major respiratory fuel of tumour cells (Moreadith & Lehninger, 1984), methionine, which is essential for tumour cell growth *in vivo* as well as *in vitro* (Hoffman, 1985) and yields cysteine via transsulphuration (Greenberg, 1975), and cysteine, since certain human and rodent haematopoietic tumour cell lines are auxotrophic for cysteine when compared with non-tumour cell lines of similar origin (Glode *et al.*, 1981). However, the characteristics of glutathione metabolism in cancer cells and its relation to tumour growth *in vivo* have not been studied. In this paper, we report (a) changes in the glutathione status, (b) the rate of GSH synthesis during the growth of an Ehrlich ascites tumour in mice, (c) which amino acids are rate-limiting for GSH synthesis and (d) the activity of the enzymes involved in the glutathione redox cycle in tumour cells.

MATERIALS AND METHODS

Animals and tumour inoculation

Adult male mice OF1 from IFFA CREDO (Madrid, Spain) were used. The animals, fed *ad libitum* on a stock laboratory diet (Letica, Barcelona, Spain), were kept on a 12 h-light/12 h-dark

cycle with the temperature maintained at 22 °C. All experiments were carried out between 10:00 and 12:00 h. The Ehrlich ascites tumour was kindly provided by the Department of Pathology (Universidad de Valencia) and inoculated by intraperitoneal injection into the mice. Approx. 25×10^6 of viable cells were injected into each mouse. Nearly 100 % of the injected animals developed an ascites tumour. The mice died 15.1 ± 0.7 days ($n = 12$) after inoculation of the tumour.

Measurements related to growth of the Ehrlich ascites tumour *in vivo*, i.e. body weight, carcass weight, tumour volume, tumour cell density and rates of protein synthesis (see below), were measured as described by Lopes *et al.* (1989).

Isolation and incubation of tumour cells

Cells were collected from tumour-bearing mice and rapidly washed twice at 4 °C in Krebs/Henseleit bicarbonate medium (pH 7.4) without Ca^{2+} or Mg^{2+} and containing 0.5 mM-EGTA. We found that this washing procedure avoided intercellular adherence phenomena. For incubations, tumour cells (approx. 5–6 mg dry weight/ml) were suspended at 37 °C in Krebs/Henseleit bicarbonate medium (pH 7.4) and the additions indicated in the Tables. Glucose (5 mM) was always present, since many types of tumour cells, including Ehrlich ascites cells, actively metabolize glucose (Gold, 1974). The gas atmosphere was O_2/CO_2 (19:1). In all our experimental conditions, we determined cell membrane integrity by measuring Trypan Blue exclusion (cell viability was always greater than 95 %) and leakage of lactate dehydrogenase (always less than 3 % of the total activity measured in the incubation flask) from incubated cells.

To separate intracellular and extracellular compartments we used a variation of the technique described by Zuurendonk & Tager (1974) for isolated rat hepatocytes. For this purpose, samples of the cell suspensions were centrifuged through a layer of silicone oil (AR200/SF96:100, 6:1, v/v) (AR200 was from Serva, Heidelberg, Germany, and SF96:100 was from Klöckner, Duisburg, Germany) into a solution of HClO_4 (2 %) and glycerol (50 %).

Metabolite and enzyme assays

Enzyme activities were measured in cells obtained from tumour-bearing mice, washed twice at 4 °C in Krebs/Henseleit

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bicarbonate medium (pH 7.4) without Ca^{2+} or Mg^{2+} added and containing 0.5 mM-EGTA and then suspended and homogenized in 0.1 M-potassium phosphate buffer (pH 7.2) at 4 °C.

GSH was determined as described by Akerboom & Sies (1981). GSSG was measured by h.p.l.c. (Fariss & Reed, 1987). Unless otherwise stated, values of GSH and GSSG refer to the total values found in the incubation flasks. Glutathione peroxidase activity was determined as described by Flohé & Gunzler (1986), glutathione reductase as described by Akerboom & Sies (1981), ATP, glucose-6-phosphate dehydrogenase and lactate dehydrogenase were as described by Bergmeyer (1974), glutathione *S*-transferase was as described by Habig *et al.* (1974), cystathionase as described by Sturman *et al.* (1970) and γ -glutamyltranspeptidase as described by Tate & Meister (1974).

For calculations, we used: (1) a wet-weight/dry-weight ratio of 3.4 ± 0.3 ($n = 6$) obtained for tumour cells as described by Krebs *et al.* (1974) for adult rat hepatocytes; (2) a cell volume of $3.3 \pm 0.2 \mu\text{l}/\text{mg}$ dry wt. ($n = 5$) obtained as described by Roncero *et al.* (1986) for fetal rat hepatocytes ($^3\text{H}_2\text{O}$ was from The Radiochemical Centre, Amersham, Bucks, U.K.); (3) a ratio of protein weight/dry weight of 0.63 ± 0.03 ($n = 6$). These values did not change significantly during the growth of the tumour.

For amino acid analysis, whole arterial blood or the intracellular compartment were treated as described by Viña *et al.* (1981).

Measurement of protein synthesis

Fractional rates of protein synthesis were based on the flooding-dose method of Garlick *et al.* (1980). Animals were killed 10 min after intravenous injection of a mixture of L-[4- ^3H]phenylalanine (Nuclear Ibérica S.A., Madrid, Spain) and unlabelled phenylalanine (150 mM) to give 50 $\mu\text{Ci}/\text{ml}$; the amount administered was always 1 ml/100 g. Processing of the tissue samples, measurement of the protein content and of the specific radioactivity of the protein-bound and free L-[4- ^3H]phenylalanine were as described by Garlick *et al.* (1980).

Expression of results and statistical significance

The results are expressed as means $\pm$ S.D. for the indicated numbers of different experiments. The statistical significance of differences was assessed by Student's *t* test.

RESULTS AND DISCUSSION

Growth and glutathione status in tumour cells

We studied growth, glutathione metabolism and protein synthesis in the tumour over a period of 2 weeks between inoculation of the cells and severe cachexia in the host (Table 1) (see also Lopes *et al.*, 1989; Tisdale, 1991). Until day 4 after inoculation, the amount of ascitic tumour was very small and variable. Cell proliferation was rapid until days 9–10 and then began to decrease. The volume of ascitic fluid doubled from day 7 to day 14, but tumour cell density decreased significantly (Table 1). At 14 days after inoculation, the animals had lost approx. 25% of their initial body weight (Table 1). The fractional rate of protein synthesis in the tumour cells also fell from day 7 to day 14 as the rate of cell proliferation decreased (Table 1). This fall in protein synthesis has been attributed to decreases in both concentration and activity of the tumour-cell ribosomes (Lopes *et al.*, 1989). Behaviour similar to that described here has been previously observed in the same tumour by others (e.g. Lopes *et al.*, 1989).

As shown in Table 2, GSH concentrations in tumour cells changed from 2.8 $\mu\text{mol}/\text{g}$ at day 7 to 0.9 $\mu\text{mol}/\text{g}$ at day 14. In contrast, GSSG concentrations did not change significantly as the tumour grew (Table 2). Therefore the change in the

GSH/GSSG ratio from 70 (at day 7) to 30 (at day 14) is mainly due to a decrease in GSH content (Table 2).

In order to determine the possible reasons for the change in glutathione status, we measured the activities of glutathione reductase, glucose-6-phosphate dehydrogenase, glutathione peroxidase (selenium-dependent) and glutathione *S*-transferase, i.e. the main enzymes involved in the glutathione redox cycle (Table 2). We found that only the activities of glutathione peroxidase and glutathione *S*-transferase, i.e. enzymes that use GSH, increased from day 7 to day 14 (Table 2). On the other hand, glutathione reductase and glucose-6-phosphate dehydrogenase, i.e. enzymes that tend to regenerate GSH, did not change significantly when different stages of tumour growth were compared (Table 2). Thus these enzymic changes could explain, at least in part, the shift towards oxidation of the GSH/GSSG pair during tumour growth. However, one would have expected that the increase in glutathione peroxidase activity would be reflected by an increase in cellular GSSG content, but that is not the case (Table 2). The absence of changes in GSSG content *in vivo* could be explained by excretion of GSSG to the extracellular medium and subsequent degradation catalysed by γ -glutamyltranspeptidase. However, we could not detect any γ -glutamyltranspeptidase activity in the cancer cells at any stage of tumour growth; thus, if GSSG is released and degraded, other cells in the host's organs, e.g. kidney (see Meister, 1983), must be involved

Table 1. Growth of Ehrlich ascites tumours in mice

Determinations were as described in the Materials and methods section. Results are expressed as means $\pm$ S.D. for 10–12 different animals: * significantly different from day-7 group ($P < 0.05$). FSR, fractional rate of protein synthesis.

Parameter	Time after inoculation of the tumour (days)...	0	7	14
Animal wt. (g)		29.3 $\pm$ 0.4	35.0 $\pm$ 0.7	39.4 $\pm$ 1.1*
Carcass wt. (g)			28.7 $\pm$ 1.3	22.3 $\pm$ 0.9*
Tumour volume (ml)			5.7 $\pm$ 0.5	16.5 $\pm$ 1.1*
Tumour cell density ($10^{-6} \times$ cells/ml)			731 $\pm$ 95	460 $\pm$ 53*
FSR in tumour cells (%/day)			68.7 $\pm$ 5.6	32.2 $\pm$ 4.5*

Table 2. Glutathione status and glutathione-related enzyme activities in Ehrlich ascites tumour at different stages of growth in mice

Results are means $\pm$ S.D. for the numbers of observations shown in parentheses: * significantly different from day-7 group ($P < 0.05$).

	Time after inoculation of the tumour (days)...	7	14
GSH ($\mu\text{mol}/\text{g}$)		2.8 $\pm$ 0.4 (25)	0.9 $\pm$ 0.2 (24)*
GSSG ($\mu\text{mol}/\text{g}$)		0.04 $\pm$ 0.01 (15)	0.03 $\pm$ 0.02 (15)
GSH/GSSG		70.0	30.0
Glutathione reductase (units/g)		1.7 $\pm$ 0.4 (7)	2.0 $\pm$ 0.5 (7)
Glucose-6-phosphate dehydrogenase (units/g)		0.6 $\pm$ 0.06 (6)	0.5 $\pm$ 0.03 (6)
Glutathione peroxidase (units/g)		1.0 $\pm$ 0.3 (7)	2.6 $\pm$ 0.4 (7)*
Glutathione <i>S</i> -transferase (units/g)		1.7 $\pm$ 0.4 (7)	2.7 $\pm$ 0.3 (6)*

Table 3. Effect of *N*-acetylcysteine (NAC) or methionine on GSH concentration in Ehrlich ascites tumour at different stages of growth in mice

The initial GSH concentrations were $2.8 \pm 0.4 \mu\text{mol/g}$ ($n = 25$) for the day-7 group and $0.9 \pm 0.2 \mu\text{mol/g}$ ($n = 24$) for the day-14 group (see Table 2). Ehrlich ascites tumour cells were incubated, as described in the Materials and methods section, for 60 min. In all conditions tested cells were incubated in the presence of 5 mM-glucose, 5 mM-glutamine, 2 mM-glycine, 1 mM-serine. In these experiments only L-amino acids were used. The significance test refers for both groups to the comparison of GSH concentration in the absence and presence of NAC (* $P < 0.05$), and also refers to the difference between results for the day-14 group and day-7 group ($\dagger P < 0.05$). All values in the Table are means $\pm$ S.D. for five different observations.

NAC or Met added (mM)	Time after inoculation of tumour (days)...	GSH ($\mu\text{mol/g}$)			
		NAC added		Met added	
		7	14	7	14
0		2.7 ± 0.3	$0.7 \pm 0.3\dagger$	2.7 ± 0.3	0.7 ± 0.3
0.2		$3.6 \pm 0.4^*$	$0.8 \pm 0.3\dagger$	2.6 ± 0.1	0.7 ± 0.2
0.5		$4.1 \pm 0.6^*$	$1.2 \pm 0.2^{*\dagger}$	2.7 ± 0.3	0.9 ± 0.3
1		$4.9 \pm 0.5^*$	$1.6 \pm 0.3^{*\dagger}$	2.8 ± 0.2	0.8 ± 0.2
2		$4.7 \pm 0.6^*$	$1.8 \pm 0.5^{*\dagger}$	3.0 ± 0.4	1.0 ± 0.2

in the process. Blood GSH concentrations decreased from $0.4 \pm 0.04 \text{ mM}$ ($n = 5$) in normal mice to $0.2 \pm 0.1 \text{ mM}$ ($n = 4$; $P < 0.05$) in mice bearing the tumour for 7 days, and to $0.1 \pm 0.04 \text{ mM}$ ($n = 7$; $P < 0.005$) in mice bearing the tumour for 14 days. No significant differences were observed in GSSG concentrations between normal and tumour-bearing mice. The value obtained for GSSG in all cases was of $1.2 \pm 0.3 \mu\text{M}$ ($n = 6$). Thus changes in glutathione peroxidase activity shown in Table 2 are apparently not reflected by changes in GSSG content in tumour cells, the extracellular ascitic fluid or the blood, and cannot be used to explain the observed change in glutathione status accompanying tumour growth.

Finally, there is a correlation between intracellular glutathione content and cellular proliferation. Tumour cell density was lower at 14 days than at 7 days after inoculation of the tumour (Table 1). In parallel, GSH content was lower in tumour cells from mice bearing the tumour for 14 days than in those bearing it for 7 days (Table 2).

GSH synthesis in tumour cells

We studied whether changes in glutathione status in the tumour cells are related to changes in GSH synthesis and/or the rate of glutathione efflux. In previous studies we found that GSH synthesis from methionine is very active in hepatocytes (Viña *et al.*, 1978) and proceeds via the cystathionase pathway (Reed & Orrenius, 1977). We measured cystathionase activity in the Ehrlich ascites tumour and found that it was not detectable. Moreover, when tumour cells were incubated *in vitro* in the presence of a mixture of amino acid precursors for GSH synthesis (see Table 3) containing increasing concentrations of methionine (from 0.2 to 2 mM), we found that these cells do not synthesize GSH (Table 3). Tateishi *et al.* (1974) have suggested that cysteine is the limiting factor in the synthesis of GSH *in vivo*. Therefore we used *N*-acetylcysteine instead of methionine as cysteine source (Table 3). We used *N*-acetylcysteine and not free cysteine because the latter is rapidly oxidized in the cell suspension, giving rise to free radicals (Sáez *et al.*, 1982), and is toxic (Viña *et al.*, 1983). As

shown in Table 3, *N*-acetylcysteine at concentrations of 0.2 mM or above was effective in activating GSH synthesis in tumour cells. In the presence of this range of *N*-acetylcysteine concentrations, the rates of GSH synthesis were not altered by decreasing the concentration of the other three precursors (glutamine, glycine and serine) to 0.25 mM, 0.1 mM and 0.05 mM respectively. We compared different stages of tumour growth and found that the stimulation of GSH synthesis elicited by *N*-acetylcysteine was much lower in cells from mice bearing the tumour for 14 days than in those bearing it for 7 days. In fact, when maximal rates of GSH synthesis were studied (Table 4) we found that cells from mice bearing the tumour for 7 days synthesized 35.0 nmol of GSH/min per g, compared with 18.3 nmol of GSH/min per g synthesized by cells from mice bearing the tumour for 14 days. Thus there is an approx. 50% decrease in the rate of GSH synthesis between the two stages of tumour growth and, indeed, this could be the main factor responsible for the observed fall in GSH content accompanying tumour growth. Moreover, since cysteine appears to be the rate-limiting factor for GSH synthesis, a progressive decrease in the intracellular delivery of free cysteine could decrease GSH synthesis in tumour cells as the tumour grows.

Interestingly, the maximal rate of GSH synthesis in tumour cells from mice bearing the tumour for 7 days (35.0 nmol of GSH/min per g) was quite similar to that measured in isolated rat hepatocytes from methionine or *N*-acetylcysteine as cysteine sources (about 30.0 nmol of GSH/min per g; see Estrela *et al.*, 1988). However, this rate of GSH synthesis can be achieved in hepatocytes in the presence of 0.2 mM-methionine or *N*-acetylcysteine rather than the 1 mM described here for tumour cells. Thus cysteine requirements seem to be much higher in tumour than in normal cells. This probably reflects the high rate of protein synthesis and therefore cysteine consumption accompanying cell proliferation.

To ensure that the trans-sulphuration pathway that yields cysteine from methionine is not involved in regulating GSH synthesis in tumour cells, we incubated them in the presence of *S*-adenosylmethionine (1 mM) or homocysteine (1 mM). These are good precursors for GSH synthesis in hepatocytes (Viña *et al.*, 1978). However, we found that there is no GSH synthesis in tumour cells from these intermediates (results not shown). This fact is in agreement with the absence of any cystathionase activity in these cells mentioned above. Duthie *et al.* (1988), in the human hepatoma cell line HEP G2, also observed that cancer cells do not retain the cystathionine pathway which permits normal hepatocytes to utilize methionine for GSH synthesis.

It is important to emphasize that the absence of metabolic flux through the cystathionine pathway is not in controversy with the demonstrated methionine-dependence or methionine auxotrophy of cancer cells. This term describes the inability of cancer cells to grow and survive when methionine is absent (Hoffman, 1985; Djurhuus *et al.*, 1991). Indeed, methionine is utilized for protein synthesis, and, in addition, its conversion into *S*-adenosylmethionine is essential for cancer cells, since this intermediate is the predominant biological methyl-group donor in the cell and is in turn a precursor in polyamine synthesis (Hoffman, 1985). The present findings, however, are in agreement with the cysteine auxotrophy of human leukaemic fibroblasts reported by Glode *et al.* (1981).

Since GSH synthesis is ATP-dependent, changes in the intracellular ATP concentration in tumour cells could also be used to explain changes in the rate of GSH synthesis. We measured ATP contents in these cells and found a concentration of $3.7 \pm 0.3 \mu\text{mol/g}$ ($n = 6$) in cells from mice bearing the tumour for 7 days and $2.5 \pm 0.2 \mu\text{mol/g}$ ($n = 5$; $P < 0.05$) in mice bearing the tumour for 14 days. However, on the basis of the K_m of

Table 4. Rates of GSH synthesis and glutathione efflux in Ehrlich ascites tumour at different stages of growth in mice

The initial GSH concentrations were $2.8 \pm 0.4 \mu\text{mol/g}$ ($n = 25$) for the day-7 group and $0.9 \pm 0.2 \mu\text{mol/g}$ ($n = 24$) for the day-14 group (see Table 2). Ehrlich ascites tumour cells were incubated, as described in the Materials and methods section, for 60 min. Rates of GSH synthesis were calculated from total GSH content in incubations at 0, 30 and 60 min of incubation. Rates of glutathione efflux were calculated from contents of glutathione in extracellular medium at 0, 30 and 60 min of incubation. 'Amino acids' = 5 mM-Gln, 2 mM-Gly, 1 mM-Ser, 1 mM-*N*-acetylcysteine. In these experiments only L-amino acids were used. Glucose (5 mM) was present in all incubations. The significance test refers, for both groups, to the comparison of GSH concentration in the absence and presence of amino acids (* $P < 0.05$), and also to the difference between results for the day-14 group and day-7 group ($\dagger P < 0.05$). All values are means $\pm$ s.d. for five observations.

Additions	Rate (nmol/min per g)			
	GSH synthesis		Glutathione efflux (GSH + 2GSSG)	
	Day 7	Day 14	Day 7	Day 14
None	-1.7 ± 0.8	-3.3 ± 1.5	15.2 ± 3.2	$3.9 \pm 1.1\dagger$
Amino acids	$35.0 \pm 4.3^*$	$18.3 \pm 5.7^*$	$37.3 \pm 6.0^*$	$7.3 \pm 0.7^*\dagger$

Table 5. Amino acid concentration (μM) in whole blood from mice bearing the Ehrlich ascites tumour at different stages of growth

For details see the text. All amino acids are L-amino acids. The results are means $\pm$ s.d. for the number of mice shown in parentheses: * $P < 0.05$ compared with non-tumour-bearing groups. $\dagger P < 0.05$ compared with day-7 group.

Amino acid	Non-tumour bearing	Tumour-bearing	
		Day 7	Day 14
Glutamine	503 ± 123 (7)	364 ± 93 (15)*	379 ± 67 (7)*
Glutamate	128 ± 38 (7)	99 ± 38 (14)	79 ± 32 (5)
Glycine	337 ± 89 (7)	292 ± 137 (14)	384 ± 184 (6)
Serine	158 ± 24 (7)	124 ± 28 (15)*	138 ± 28 (6)*
Methionine	51 ± 12 (7)	34 ± 11 (15)*	28 ± 10 (5)*
Cyst(e)ine	18 ± 2 (6)	23 ± 7 (15)	31 ± 9 (6)* $\dagger$

γ -glutamylcysteine and GSH synthetase for ATP (Meister, 1983), the ATP concentration at 14 days is high enough to ensure a maximal rate of GSH synthesis.

We also studied the rate of glutathione efflux from tumour cells. As shown in Table 4, in the absence of amino acid precursors for GSH synthesis, we found an efflux of $15.2 \text{ nmol/min per g}$ in cells from mice bearing the tumour for 7 days. This rate was about 4 times lower in cells from mice bearing the tumour for 14 days. Besides, in the presence of amino acid precursors including 1 mM-*N*-acetylcysteine, the rate was $37.3 \text{ nmol/min per g}$ in cells from mice bearing the tumour for 7 days and about 5 times lower in cells from mice bearing the tumour for 14 days (Table 4). Thus when cells synthesize GSH at a lower rate, they also show a lower glutathione efflux. Aw *et al.* (1984), using isolated hepatocytes, reported that 1 mM-methionine decreased the rate of GSH efflux. However, in our experiments, glutathione efflux from cancer cells was increased in the presence of *N*-acetylcysteine (Table 4). Furthermore, we

found that in all measurements of glutathione efflux (Table 4), at least 98 % of the total glutathione (GSH + 2GSSG) was in the form of GSH. Our results suggest that when tumour cells synthesize GSH, there is a parallel glutathione efflux which contributes to keeping intracellular GSH concentrations at a level that is apparently co-ordinated with the stage of tumour growth.

Concentration of amino acids in tumour cells and in blood from tumour-bearing mice

Blood amino acid profiles have been used as an index of metabolic status (Oberholzer & Briddon, 1990). We measured possible changes in concentration of amino acids in blood of tumour-bearing mice. To correlate possible changes in blood concentration with intracellular changes in the tumour cells and thereby in glutathione metabolism, we also measured the intracellular pool of free amino acids.

Table 5 shows the blood concentration of the amino acids involved in GSH synthesis. In agreement with the idea that glutamine is the major respiratory fuel of tumour cells (Moreadith & Lehninger, 1984), its concentration in blood was lower in tumour-bearing mice than in controls. Of the other amino acids, serine and methionine concentrations were decreased in tumour-bearing mice compared with controls. This decrease in serine and methionine does not explain the decrease in GSH synthesis accompanying tumour growth, as shown in this paper. Moreover, cyst(e)ine (cysteine + cystine) concentration in blood increased significantly in mice bearing the tumour for 14 days compared with controls and with mice bearing the tumour for 7 days (Table 5). Cystine is taken up by cells through the x_c^- system (Cho & Bannai, 1990). However, cysteine is transported into the cell by the ASC system (Cho & Bannai, 1990), and therefore a defect in amino acid transport affecting this system should be reflected by changes in the blood amino acid profiles. However, blood concentrations of alanine and proline did not change significantly between controls and tumour-bearing mice (results not shown).

We also measured intracellular concentrations of amino acids in the cancer cells and found that the amino acid pool within these cells did not change as tumour growth progressed (results not shown). The most relevant fact is that we found that glutamine and cyst(e)ine were undetectable. A possible explanation for this is that glutamine is the major fuel used by the tumour cells (Moreadith & Lehninger, 1984) and that cyst(e)ine is rapidly used for protein synthesis (see, e.g. Estrela & Pallardó, 1990). The fact that, in spite of these very low (undetectable) concentrations of intracellular cyst(e)ine, the blood concentration of these amino acids does not decrease in tumour-bearing mice (Table 5) indicates that a limitation in their blood supply is not responsible for the decrease in GSH synthesis and content in tumour cells (Tables 2 and 4).

Glutathione content and protein synthesis in skeletal muscle and liver of mice at different stages of tumour growth

Skeletal muscle is by far the largest reservoir of structural proteins in the body, and therefore plays an essential role in providing amino acids for tumour growth (Tisdale, 1991). On the other hand, liver is the only or major site of production of many proteins and, besides, shows a high content of GSH (Deneke & Fanburg, 1989). Thus, both tissues are good candidates for the investigation of possible host-tumour correlations involving glutathione status and tumour growth.

Rennie *et al.* (1983) postulated that depressed protein synthesis is the dominant characteristic of muscle wasting and cachexia. Interestingly, as shown by Lopes *et al.* (1989), during the growth of an Ehrlich ascites tumour, the rate of protein synthesis in skeletal muscle falls very rapidly and in parallel with increases in

Table 6. Contents of GSH and GSSG and rates of protein synthesis in skeletal muscle (soleus) and liver of mice at different stages of tumour growth

For details see the text. The results are means $\pm$ S.D. for five to six observations: * $P < 0.05$ compared with non-tumour-bearing group (day 0). Abbreviation: FSR, fractional synthetic rate.

	Time after inoculation of the tumour (days)	Tissue contents	
		Skeletal muscle	Liver
GSH ($\mu\text{mol/g}$)	0	0.3 ± 0.05	6.7 ± 0.8
	7	0.3 ± 0.1	6.1 ± 0.5
	14	0.4 ± 0.03	$4.8 \pm 0.7^*$
GSSG ($\mu\text{mol/g}$)	0	0.04 ± 0.01	0.1 ± 0.05
	7	0.04 ± 0.02	0.1 ± 0.03
	14	0.03 ± 0.01	0.1 ± 0.05
FSR (%/day)	0	8.7 ± 0.3	78.2 ± 3.7
	7	$3.5 \pm 0.2^*$	$117.5 \pm 5.8^*$
	14	$2.0 \pm 0.2^*$	$132.7 \pm 7.0^*$

liver protein synthesis. We have reproduced these results and have measured the tissue concentrations of GSH and GSSG in skeletal muscle and liver (Table 6). GSH concentration in liver, when compared with controls, was only significantly lower in mice bearing the tumour for 14 days (Table 6). On the other hand, GSSG concentration in liver, when compared with controls, did not change significantly in tumour-bearing mice at any stage of tumour growth (Table 6). In addition, GSH and GSSG concentrations in skeletal muscle were similar in controls and in tumour-bearing mice (Table 6). Thus, although changes in GSSG contents have been previously suggested as potential regulators of protein synthesis in eukaryotes [see Estrela & Pallardó (1990) for a review], changes in glutathione status in tumour-bearing animals do not seem to be responsible for the reported changes in protein metabolism in host tissues. Moreover, GSSG content does not change in tumour cells as the tumour grows (Table 2), therefore variations in GSSG concentration are apparently not involved in the observed fall in protein synthesis in the tumour (Table 1).

Our results suggest that the observed decrease in GSH concentration and rate of synthesis in cancer cells accompanying tumour growth could be a consequence of the fall in protein synthesis affecting the enzyme activities synthesizing GSH. However, with the present results we cannot rule out the possibility that humoral factors may have direct effects on glutathione status in the tumour cells. As discussed by Lopes *et al.* (1989), food intake and protein metabolism in tumour-bearing animals can be profoundly affected by cytokines such as tumour necrosis factor or interleukins produced by macrophages in response to the invasion of the host by a tumour. Charters & Grimble (1988) have reported that injection of recombinant tumour necrosis factor α into rats produced similar effects to those demonstrated on protein metabolism by Lopes *et al.* (1989). Moreover, severe cachexia has been established in nude mice by inoculation of tumour cells transfected with the human tumour necrosis factor α gene (Ollif, 1988).

In conclusion, the relevant facts reported in this paper are: (1) GSH, but not GSSG, content in tumour cells decreases as the tumour grows; (2) cysteine promotes GSH synthesis in tumour cells, in contrast with methionine or any intermediate in the trans-sulphuration pathway; (3) cysteine and not glutamate or glycine is the rate-limiting factor for GSH synthesis in tumour cells; (4) there is a glutathione efflux from tumour cells which

seems to be co-ordinated with the rate of GSH synthesis; (5) the decrease in GSH content of tumour cells correlates with a decrease in the rate of protein and GSH synthesis but not with a lower supply of free cysteine.

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