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Altered metabolism of glutathione in cells transformed by oncogenes which cause resistance to ionizing radiations

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We measured glutathione (GSH) metabolism in normal NIH/3T3 fibroblasts, and in cells transformed by the oncogenes *sis*, *erb*B, *src*, *ras*, *dbl*, and *raf*. *erb*B, *src*, *ras* and *raf*, but not *sis* and *dbl* transformants, showed increased level of total and reduced GSH as compared with normal NIH/3T3 fibroblasts; oxidized GSH was elevated only in *src*- and *ras*-transformed cells. Increased total GSH content was associated with decreased activity of the synthetic enzyme γ-glutamylcysteine synthetase, and oxidized GSH level with increased activity of GSH reductase. These data suggest that GSH synthesis was selectively enhanced in cells transformed by specific oncogenes, with resulting down-regulation of its synthetic enzyme; alterations of GSH metabolism appeared to be peculiar of transformation by specific oncogenes, and not trivial epiphenomena of neoplastic transformation. Oncogenic transformants that presented elevated level of GSH were also those reported to be resistant to antineoplastic drugs and ionizing radiations, thus confirming a possible link between altered GSH metabolism and resistance to antineoplastic treatment.

Glutathione; Oncogene; Ionizing radiation; Radiation therapy; Neoplasm

1. INTRODUCTION

Glutathione (GSH), which predominantly exists in the reduced form, is involved in regulation of several cell functions, including aminoacid transport [1], enzyme activities, thiol–disulphide balance [2], synthesis of DNA precursors [3], and cell proliferation [4]. GSH-related enzymes, i.e. GSH peroxidase (GPX) and GSH-*S*-transferase (GST), are involved in free radical scavenging, peroxide reduction, and detoxification through formation of GSH-S conjugates [5,6]. High levels of GSH and related enzymes were observed in some cultures of transformed cells, and they were correlated with increased resistance to antineoplastic drugs and ionizing radiations [7–10]. Depletion of intracellular GSH, achieved with inhibitors of GSH synthesis, restored sensitivity to antineoplastic agents, and conversely, pharmacologically-induced increase of GSH was associated with increased resistance [11–14]; thus, study of GSH metabolism might provide useful information for clinical diagnosis, and for prediction of tumor responsiveness to antineoplastic treatment.

We recently noted that transformation by oncogenes involved in mitogenic signal transduction was associated with resistance to the killing effects of ionizing radiations, and we found a correlation between radioresistance and alterations of second messenger formation [15]. In order to further investigate the molecular mechanisms responsible for oncogene-associated resistance, we studied GSH metabolism in NIH/3T3 fibroblasts transformed by the oncogenes *sis*, *erb*B, *src*, *ras*, *dbl*, and *raf* which code for transforming protein interfering with signal transduction [16]: (i) *sis* codes for the BB-isoform of PDGF; (ii) v-*erb*B encodes a truncated form of the EGF receptor; (iii) the product of *src* is a non-receptorial tyrosine kinase associated with the inner layer of the membrane; (iv) *ras* codes for GTP-binding proteins (G proteins) involved in the transduction of mitogenic signals; (v) *dbl* is involved in GDP/GTP exchange at *ras*-like G-proteins; (vi) *raf* codes for a cytoplasmic serine/threonine kinase bearing homology of sequence and function with protein kinase C. Detailed study of GSH metabolism in transformed lines included analysis of: reduced GSH (r-GSH); oxidized GSH (GSSG); total GSH (i.e. GSH = r-GSH + GSSG); GSH-redox index (GSSG/r-GSH); GST; GPX; the synthetic enzyme γ-glutamylcysteine synthetase (γ-GCS); and GSH reductase (GR).

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Abbreviations r-GSH, reduced glutathione; GSSG, oxidized glutathione; GSH, total glutathione; GST, glutathione-*S*-transferase (EC 5.1.18); GPX, glutathione peroxidase (EC 1.11.1.9); GR, glutathione reductase (EC 1.6.4.2); γ-GCS, gamma-glutamyl cysteine synthetase (EC 6.3.2.2)

2. MATERIALS AND METHODS

GR, piruvate dehydrogenase and lactate dehydrogenase used for GPX and γ-GCS activity assay were from Boehringer and Soehne (Mannheim, Germany) as well as coenzymes and substrates. All other chemicals were supplied by Merck (Darmstadt, Germany). Normal NIH/3T3 fibroblasts, and their counterpart transformed by the oncogenes *erb*B, *ras*, *raf*, *src*, *sis*, and *dbl* were previously described [17]

2.1. *Glutathione determinations*

Cells ($4-10 \times 10^6$) were centrifuged in a microcentrifuge ($12,550 \times g$ for 15 min at 4°C). The precipitate was directly dissolved in 0.5 ml of 5% aqueous $HClO_4$ and the cells were lysed by sonication. Proteins were precipitated by centrifugation The supernatant was assayed for r-GSH and GSSG by HPLC as described by Reed and Fariss [18]. In this method, the GSH auto-oxidation and the thiol-disulfide interchange were prevented by rapid acidic extraction and addition of iodoacetic acid. The supernatant was neutralized by 2 M K_2CO_3 Then 50 μl of 300 mM iodoacetic acid, and 200 μl of 0.7 $MKHCO_3$ were added to 0.5 ml of extract. The resulting alkaline solution was incubated for 1 h at room temperature in complete darkness 0.1 ml of 5% (v/v) fluorodinitrobenzene dissolved in ethanol was added to the supernatant, which was then stored at 4°C overnight. The solutions were analyzed by liquid chromatography (Beckman Gold System equipped with an NEC/PC 8201H computer, a Shimadzu C-R6A chromatopac integrator, and a Biosil NH_2 90-5S BioRad column). The 2,4-dinitrophenyl derivatives were detected at 360 nm. The r-GSH and GSSG were quantified relative to standards by integration.

2.2. *Enzyme assay*

Cells ($4-10 \times 10^6$) were centrifuged in a microcentrifuge ($12,550 \times g$ for 15 min at 4°C). The precipitate was dissolved using 0.5 ml 150 mM Tris buffer containing 5 mM $MgCl_2$, 2 mM DTT at pH 7.4. Cells were lysed by sonication at 4°C. The homogenate was centrifuged at $15,000 \times g$ for 25 min. Except for GST, all enzyme activities were measured at 30°C by continuous optical tests based on the extinction change of pyridine nucleotides at 340 nm using a Kontron (Uvikon 710) spectrophotometer. GPX was assayed using the procedure of Paglia and Valentine [19], which uses H_2O_2 as substrate The measured activity was corrected for the occurrence of any spontaneous reaction in the absence of the enzymes. The activity of GR was determined using the method of Goldberg et al. [20] The activity of γ-GCS was assayed by Seeling's method [21]. GST was assayed with method of Habig et al. [22], using 1-chloro-2,4-dinitrobenzene as substrate. The formation of *S*-2,4-dinitrophenylglutathione was observed at 340 nm. All enzymatic activities were expressed in terms of μmol/min/mg of total protein. The protein concentration was determinated by Bradford's method [23]. Bovine serum albumin (Sigma Chemical Co) was used as the control standard

2.3. *Statistical analysis*

The significance of the disparities between the means observed in normal and transformed cells was evaluated using Student's *t*-test. A difference of $P < 0.05$ was considered significant.

3. RESULTS

Fig. 1 demonstrates that NIH/3T3 fibroblasts transformed by the oncogenes *erb*B, *ras*, and *src* exhibited a net increase of GSH as compared with normal cells: percent increase over GSH values obtained in normal cultures was of 91%, 120%, and 130%, respectively. *raf*-transformed fibroblasts showed a smaller, although significant, increase (61%) of GSH, whereas cell lines transformed by *sis* and *dbl* did not show appreciable variations of GSH as compared with normal cells. Detailed analysis of GSH metabolites revealed that r-GSH

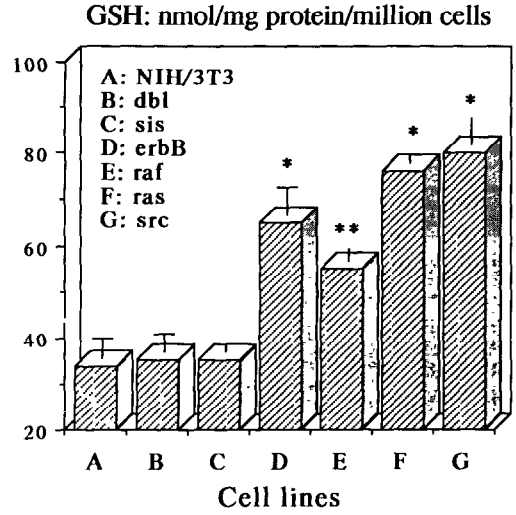


Fig. 1. GSH level in normal and transformed NIH/3T3 fibroblasts. Data are means ± S.E.M ($n = 5$, each experiment performed in duplicate samples). Significantly different from NIH/3T3 cells. *$P < 0.005$, **$P < 0.05$.

was increased in *erb*B, *ras*, *raf* and *src* transformants, while GSSG was significantly elevated only in *ras*-, and *src*-transformed cells (Table 1); GSH redox index, however, did not change in any of the cell line tested. In order to evaluate these changes of the GSH system, we simultaneously determined the specific activities of enzymes involved in GSH metabolism. Fig. 2A shows that cells transformed by the oncogenes *ras*, *src*, *erb*B and *raf* had lower level of total GST activity (from 38% to 55% decrease) as compared with normal NIH/3T3. Analysis of γ-GCS (i.e. the rate-limiting enzyme of GSH synthesis which is inhibited by negative feed-back) [24] revealed a similar pattern of decrease (Fig. 2B), that was significant for those transformants showing altered GSH metabolism. Unlike the latter two enzymes, GR (i.e. the enzyme responsible for GSSG reduction) specific activity significantly increased by about 50% in *ras*,

Table I

Level of GSH system components in normal and transformed NIH/3T3 cells

Cell line	r-GSH	GSSG	GSSG/r-GSH
NIH/3T3	30 ± 2	2.0 ± 0.5	0.066 ± 0 007
dbl	28 ± 3	2 1 ± 0.2	0 071 ± 0.007
sis	31 ± 2.5	2.0 ± 0.2	0.064 ± 0.005
raf	50 ± 5	2.5 ± 0.1	0.050 ± 0.002
*erb*B	56 ± 6*	2.5 ± 0.3	0.044 ± 0.009
ras	70 ± 2*	4.2 ± 0.3**	0.057 ± 0 004
src	70 ± 6*	5.1 ± 0.3*	0.071 ± 0 006

r-GSH and GSSG are expressed as nmol/mg protein/million cells. Data are means ± S E.M ($n = 5$; each experiment performed in duplicate samples). Significantly different from NIH/3T3: *$P < 0$ 005; **$P < 0.05$.

and *src* transformants (Fig. 2C), that exhibited the highest GSSG levels. We also measured the activities of selenium-dependent and independent GPX which are involved in the reduction of hydrogen peroxide, lipoperoxides, and hydroperoxides; however, the extremely low specific activities found in NIH/3T3 fibroblasts were well below the threshold of sensitivity of the described spectrophotometric method that we used [19], and no consistently reproducible results were obtained. Cells transformed by the oncogenes *sis* and *dbl* did not show alterations of GSH metabolism components (Table I, Figs. 1 and 2).

Analysis of the relationship between the components of the GSH system and GSH-related enzymes in normal and transformed cells, revealed a negative correlation between GSH level and γ-GCS specific activity (Fig. 3A) as well as a positive correlation between GSSG levels and GR specific activity (Fig. 3B); coefficients of correlation as determined by linear regression analysis were 0.911 and 0.913, respectively. A negative correlation between GSH level and GST specific activity was also noted, although the correlation coefficient was lower (0.776).

4. DISCUSSION

Our study is the first attempt to link alterations of GSH metabolism with single oncogene transformation in in vitro immortalized cell lines, and our results indicate specific correlation between oncogene-induced transformation and altered GSH metabolism. Variations of all components of GSH system and GSH-related enzyme activities were observed in *erb*B, *src*, *ras*, and *raf*, but not in *dbl* and *sis* transformants, thus indicating that these alterations were not trivial epiphenomena of neoplastic transformation, but rather specific oncogene-related features. The present results confirm those previously reported concerning elevated GSH in *ras* transformants [8,25,26], and extend the field of research to other oncogenes and to detailed analysis of GSH metabolism. Interestingly, the oncogenic transformants that presented elevated level of GSH were also those reported to be resistant to antineoplastic drugs and ionizing radiations [15], thus confirming a possible link between altered GSH metabolism and resistance to antineoplastic treatment. Elevated GSH content might favour DNA repair following drug-, and radiation-induced injuries, as evidenced by inhibition of DNA repair following treatment with a specific inhibitor of γ-GCS activity (buthionine sulphoximine) [12,13]. Concerning the origin of elevated GSH content in specific transformants, we hypothesized that oncogene-induced transformation was associated with increased GSH synthesis as evidenced by down-regulation of its synthetic enzyme, γ-GCS (Figs. 2B and 3A); consistently, recent data show acquired drug and radiation resistance by tumor cells following increased GSH synthesis [9].

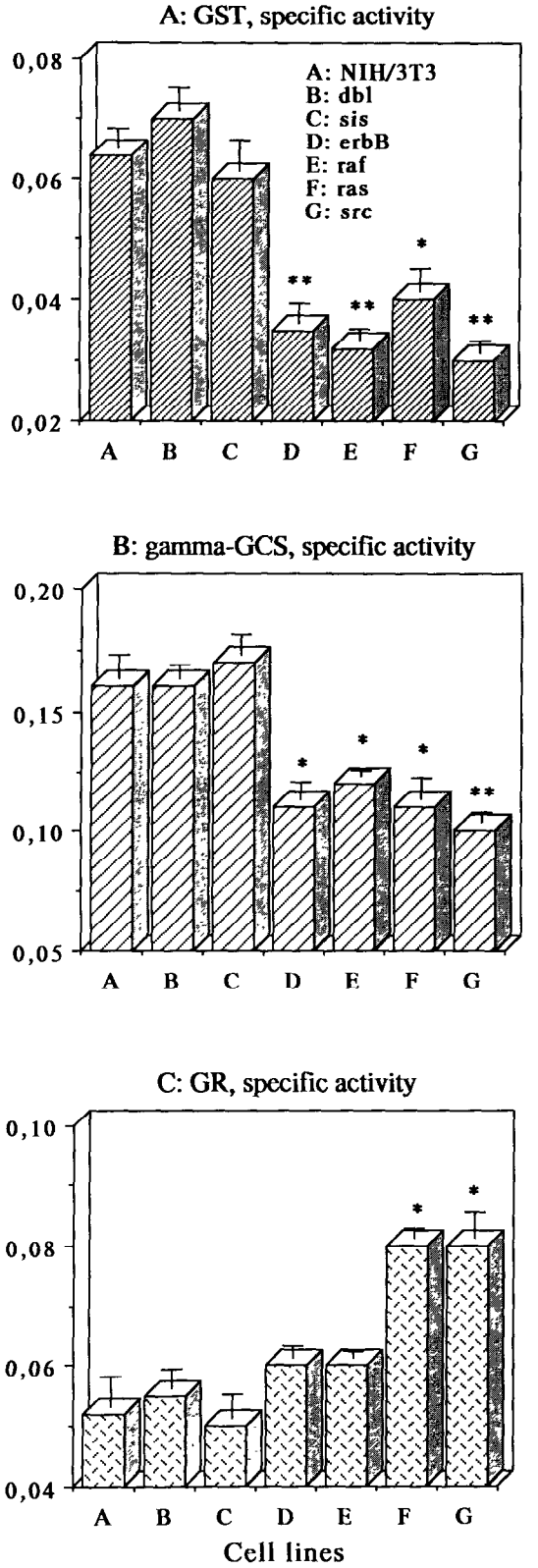
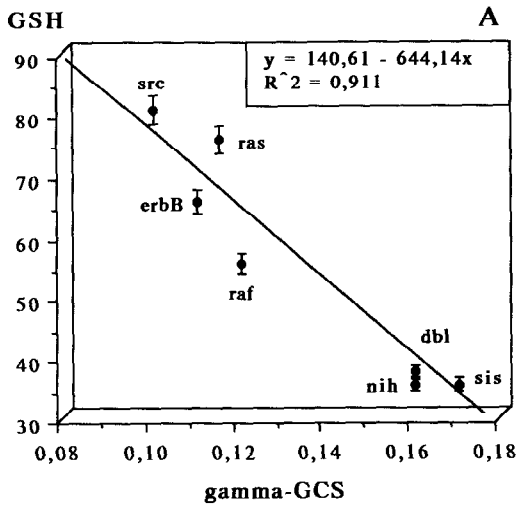


Fig. 2. Specific activity of GSH-related enzymes in normal and transformed NIH/3T3 fibroblasts. Specific activity of enzymes are expressed as micromoles per min. per mg of total protein Data are means ± S.E.M. (n = 5; each experiment performed in duplicate samples). Significantly different from NIH/3T3 cells: $^{*}P < 0.005$. $^{**}P < 0.001$.



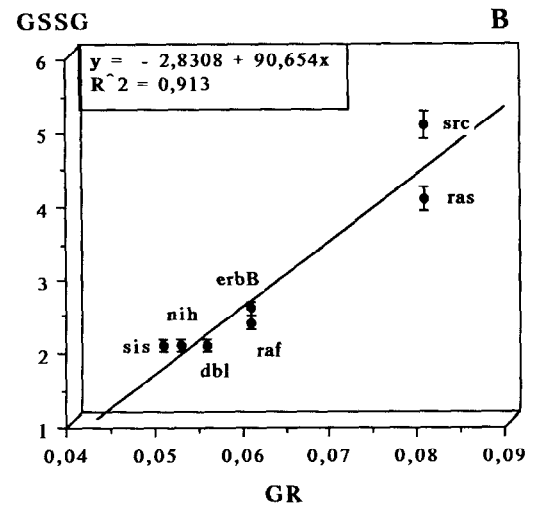


Fig. 3. Correlation between GSH and γ-GCS specific activity (A); correlation between GSSG and GR specific activity (B) in normal and transformed NIH/3T3 fibroblasts. GSH and GSSG are expressed as nmol/mg protein/million cells. Enzyme specific activities are expressed as μmol min mg of total protein.

Alteration of GSH content in neoplastic cells or in tumors was associated with reduction and detoxification processes which are in turn responsible for resistance to antineoplastic drugs and ionizing radiations [7–10]; in particular, increased GSH content was interpreted as an increased substrate demand for either GST or GPX, whose activities are involved in resistance to a wide range of chemotherapeutic drugs [7,13,27]. However, a confusing picture emerged from past studies as alteration of GSH level in drug-resistant tumor cell lines was associated with no variation, increase or decrease of GST activity [7–9,28,29]; other studies reported both increase and decrease [13] of total GST activity in tumor samples compared with normal tissue. Our results show a net decrease of total GST activity in those cell lines with high level of GSH (Fig. 2A), together with non-detectable GPX activity (not shown). The interpretation of specific GST decrease is complex: counter-regulation of GST activity by mixed disulphides formation which is dependent on r-GSH and GSSG level [30], might offer an explanation. Moreover, reduced GST activity might further increase GSH level owing to reduced loss of GSH-S-conjugates. It appears that detoxification and reduction processes, and resistance to antineoplastic treatment are uncoupled; therefore, GSH metabolism might be involved in resistance through other, unknown, mechanism(s).

The positive correlation between GSSG content, and specific GR activity (Figs. 2C and 3B) indicated efficient reduction of GSSG to r-GSH in oncogenic transformants; thus GSH-redox index was unaltered in *erb*B, *ras*, *src*, and *raf* transformants that exhibited elevated r-GSH and GSSG levels. Increase GR activity could then be interpreted as substrate-induced. Alteration of GR activity and GSSG level were also associated with resistance to antineoplastic agents [7], although no data were previously reported in oncogene-transformed lines.

In conclusion, our results demonstrate that oncogenes, whose products selectively interfere with specific passages of mitogenic signalling, cause profound alterations of GSH metabolism, thus proposing a link between oncogenic transformation, mitogenic signalling, GSH metabolism, and resistance to antincoplastic treatment.

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