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EFFECTS OF CULTURE CONDITIONS ON GLUTATHIONE CONTENT IN A549 CELLS

Gloria B. Post, Douglas A. Keller, Karen A. Connor, and D. B. Menzel

Departments of Pharmacology and Medicine, Comprehensive Cancer Center
Duke University Medical Center
Durham, North Carolina 27710

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Summary: The effects of varying culture conditions on glutathione content in A549 (human type II lung tumor derived) cells were examined. Parameters studied were growth time, serum concentration, and the presence or absence of a mixture of insulin, transferrin, and selenous acid. Glutathione content increased with serum concentration. When cells were grown with serum, glutathione increased sharply 24 hours after passage and decreased thereafter. Insulin, transferrin, and selenous acid had little effect on cell growth or glutathione content. Replacement of media with fresh media containing 10% serum did not prevent the growth dependent decrease in glutathione. These results demonstrate that glutathione content in A549 cells is strongly affected by culture conditions.

The tripeptide glutathione (GSH) can protect cells from reactive intermediates in several ways (1). It provides reducing equivalents, acts to destroy free radicals and peroxides, and forms S-substituted conjugates with metabolites of xenobiotic compounds to facilitate their elimination from the body or cell. Thus, the susceptibility of a particular cell to toxicity can be affected by the cell's glutathione content.

Studies of xenobiotic compound metabolism and toxicity are often performed in cultured cell lines. Cultured cells maintain the structural integrity which is lost in tissue homogenates and subcellular fractions, while allowing for more controlled experimental conditions than can be attained *in vivo* (2,3). Although there have been several earlier reports of variability in GSH levels in maintained cell lines (4,5,6), changes in GSH levels due to growth conditions have not usually been considered in planning or interpreting metabolism and toxicity studies.

Abbreviations used: FCS, fetal calf serum; GSH, glutathione; GSSG, glutathione disulfide; ITS, insulin, transferrin, and selenous acid Premix (Collaborative Research); NPSH, non-protein sulfhydryl; PBS, phosphate buffered saline.

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This report describes the effects of varying culture conditions on GSH content in a human lung (type II) tumor derived cell line, A549. The parameters examined were time after passage, serum concentration, and the presence of a combination of the growth factors, insulin, transferrin, and selenous acid.

Materials and Methods

A549 (human type II lung tumor derived) cells were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's Modified Eagles Media (Gibco) with dialyzed fetal calf serum (FCS, Gibco) as indicated. Insulin (5 mg/l), transferrin (5 mg/l), and selenous acid (5 ng/l) were added as ITS premix (Collaborative Research) as indicated. Cells were used in early passages, determined to be mycoplasma free, and cultured in monolayer in the presence of kanamycin sulfate (100 ug/ml, Gibco) in 5% CO_2 in a humid atmosphere at 37°. Cells were passed by trypsinization when approaching confluence.

For experiments in 2% or 10% FCS, cells were grown prior to passage in media containing the same FCS concentration to be used in the study. For studies in serum free media, cells were grown in 2% FCS and then replated at time 0 in media lacking FCS.

For determination of cell number and GSH content, cells were washed with PBS and removed by trypsinization. A hemocytometer was used for cell counting. For GSH analysis, cells from one 75 cm^2 flask were collected by centrifugation, washed with PBS and resuspended in 1 ml PBS. Protein was precipitated by addition of 50 ul 70% perchloric acid, followed by resuspension and centrifugation.

GSH analysis was performed by the high pressure liquid chromatography method of Reed et al. (7). The method involves reaction of GSH with iodoacetic acid (BDH Chemicals, Poole, England) to prevent GSH oxidation, followed by derivatization with 1-fluoro-2,4-dinitrobenzene (Aldrich) to form a colored product. Separations were performed on a 3-aminopropyl-Spherisorb column kindly supplied by Dr. D.J. Reed of Oregon State University. An Altex 420 gradient HPLC system equipped with a Spectra Physics SP4100 integrator was used. Sample injections were 100 ul, and effluent was monitored at 365 nm. The detection limit for both GSH and GSSG using this system was approximately 1 nmole.

Student's t test was used to determine statistical significance.

Results

Table 1 shows both cell number and glutathione content per 10^6 cells for A549 cells grown in 0%, 2%, or 10% FCS, with and without ITS. At each time point, GSH content was higher as serum concentration was increased. For example, at 24 hours, GSH levels for cells grown in 0%, 2%, and 10% FCS were 4.5 nmoles/10^6 cells, 24.2 nmoles/10^6 cells, and 120.8 nmoles/10^6 cells respectively. In both 10% and 2% FCS, GSH levels increased between 0 and 24 hours after passage and decreased at each time point thereafter. This effect was more pronounced in 10% than in 2% FCS. In 10% FCS, a 120% increase in GSH occurred between 0 and 24 hours, while in 2% FCS, a 31% increased was observed. In serum free media, the plating efficiency was reduced greatly, from 75%-80%

Table I

Effects of Time Since Passage, Serum Concentration, and ITS on GSH Levels in A549 Cells

Media Used:	0% FCS		0% FCS+ITS		2% FCS		2% FCS+ITS		10% FCS		10% FCS+ITS	
	Cells ($\times 10^6$)	GSH[a]	Cells ($\times 10^6$)	GSH	Cells ($\times 10^6$)	GSH	Cells ($\times 10^6$)	GSH	Cells ($\times 10^6$)	GSH	Cells ($\times 10^6$)	GSH
Hours After Passage:												
0	0.91± 0.01	15.8± 5.3	0.91± 0.01	15.8± 5.3	2.70± 0.12	18.5± 1.8[d,e]	3.30± 0.14	7.4± 2.1[d]	0.83± 0.02	41.0± 5.8[d]	1.13± 0.22	42.0± 1.3[d]
24	0.15± 0.07	4.5± 1.1[b,c]	0.28± 0.10	5.9± 1.5[b,c]	2.25± 0.09	24.2± 3.6[e]	1.93± 0.35	19.6± 4.3[e]	0.54± 0.12	120.8± 44.2	0.93± 0.28	92.2± 12.0
48	0.32± 0.07	8.5± 0.4[c]	0.34± 0.15	13.5± 5.3	3.02± 0.59	16.9± 3.7[d]	3.36± 0.63	15.6± 2.6	N.A.[f]	N.A.	N.A.	N.A.
72	—[g]—	——	—[g]—	——	4.00± 0.45	15.3± 2.7[d,e]	3.55± 0.16	12.7± 1.4[d,e]	1.01± 0.15	51.6± 8.0[d]	1.93± 0.74	31.4± 1.6[d]
96	——	——	——	——	6.54± 0.76	4.7± 1.2[d,e]	4.86± 0.68	7.8± 0.8[d]	1.60± 0.55	37.2± 0.9[d]	3.08± 0.94	6.8± 0.9[d]

[a] GSH expressed in nmoles/10^6 cells
[b] $p<0.05$ when compared to 0 hours in same media
[c] $p<0.05$ when compared to 2% FCS at same time after passage
[d] $p<0.05$ when compared to 24 hours in same media
[e] $p<0.05$ when compared to 10% FCS at same time after passage
[f] N.A. = Not analyzed
[g] Experiment terminated because cells lost attachment

in 2% or 10% FCS to 16% in serum free media. Time 0 conditions for cells grown in 0% FCS are equivalent to time 0 for 2% FCS, since cells were maintained in 2% FCS prior to passage. In serum free media, GSH content decreased between 0 and 24 hours, and then increased slightly between 24 and 48 hours.

ITS did not affect cell growth, except in the case of 0% FCS, where plating efficiency increased from 17% to 31% when ITS was present. However, the rate of growth between 24 and 48 hours was the same in serum free media with or without ITS. GSH content was higher in cells grown with ITS than in those grown without only at 72 and 96 hours in 10% FCS. However, the pattern of time dependent decrease in GSH was not altered by ITS.

Since GSH levels appeared to increase with increasing serum concentration and decrease with time after passage, the question arose as to whether the availability of GSH precursors in the media was responsible for the observed variations in GSH. To test this idea, cells were plated in media containing 10% FCS, which was replaced with fresh media at 24 hour intervals. Figure 1 illustrates that changing the media daily did not prevent the time dependent decrease in GSH.

Additionally, the observed variations in GSH level cannot be accounted for by oxidation of GSH to GSSG. GSSG was not detected in any cell sample, nor was any GSH or GSSG seen in the media in which the cells had been grown.

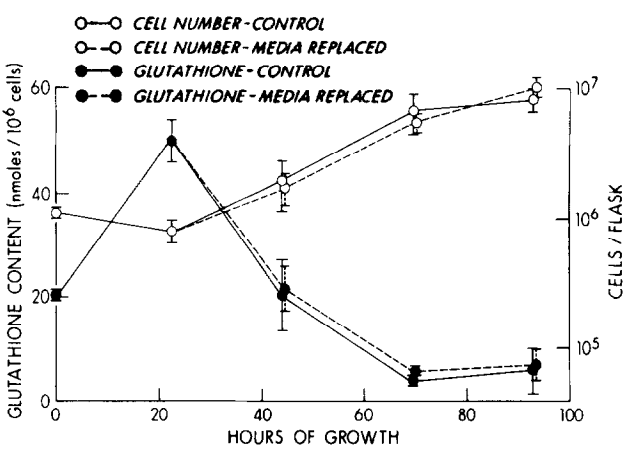

Fig. 1. Effects of changing media daily on growth and GSH content in A549 cells. Cells were grown in media containing 10% FCS, and media was removed and replaced with fresh media at 24, 48, and 72 hours. Values are mean ± S.D. for 3 flasks of cells.

Discussion

The results reported here demonstrate that GSH content in A549 cells varies with time after passage. GSH increased 24 hours after passage and decreased thereafter when cells were grown with FCS. Additionally, it was observed that serum concentration affects GSH levels in A549 cells. Although plating efficiency and rate of growth were similar in 2% and 10% FCS, GSH levels were much lower in 2% than 10% FCS, while in serum free media, GSH content was reduced further.

The combination of insulin, transferrin, and selenous acid sold as ITS by Collaborative Research has been claimed to reduce the serum requirements for many cultured cells. Our results show that for A549, growth is similar in 2% and 10% serum, and that ITS does not affect growth in these serum concentrations. Moreover, GSH content in cells grown in 2% FCS was much lower than in 10% FCS, regardless of the presence of ITS. Thus ITS did not influence the serum concentration related variations in GSH.

Fluctuations in non-protein sulfhydryl (NPSH) content, of which glutathione is the major component (8), in maintained cell lines have been observed previously. In Ehrlich ascites tumor cells, NPSH levels doubled within 9 hours after transplantation into a fresh host (4). Similarly, Chinese hamster ovary cell NPSH levels tripled within 4 hours after dilution of cell cultures with fresh media (6). Passage of A549 cells which are grown in monolayer could be considered analagous to transplantation to a fresh host or dilution with fresh media.

Harris and Teng (6) suggested that the rapid increases in cellular GSH after passage described above were due to an increase in the proportion of proliferating cells, which have a higher rate of protein synthesis than plateau phase cells. Our results do not reconcile with this theory, since the decreases in GSH content observed after 24 hours occurred during log-linear growth, during which the percentage of proliferating cells remains constant.

The time dependent decrease in GSH was not prevented by daily replacement of culture media with fresh media containing 10% FCS, the highest serum

concentration tested. The FCS used in these studies was dialyzed to remove free amino acids and other low molecular weight compounds, such as peptide growth factors. The amino acid precursors needed for de novo GSH synthesis were present in excess in the media. Thus the availability of GSH precursors in the media is unlikely to be responsible for variations in GSH content with time or serum concentration.

Large differences (greater than 10 fold in 10% FCS) in GSH content occurred with time during log phase growth. Thus, when reporting GSH levels, at least for A549 cells, it is important to mention the time after passage at which measurements were made. Sumner and Wiebel (9) reported GSH levels of 50.7 nmoles/mg protein in A549 cells. The cells were grown in 10% FCS and were used when approaching confluence (10). This data corresponds well to our value of 8 nmoles/10^6 cells at 96 hours after passage, when cells were nearly confluent, and is equivalent to 69 nmole/mg protein.

In conclusion, cellular GSH content is markedly affected by culture conditions. Serum concentration appears to be highly significant in determining GSH content. The mechanism of serum regulation of GSH is, however, unknown. Since the metabolism and toxicity of xenobiotic compounds can be mediated by the availability of GSH, knowledge of intracellular GSH levels is important for interpreting studies of the effects of these compounds. Thus, standardization of culture conditions is necessary when studying the effects of xenobiotic compounds on cultured cells.

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References:

1. Meister, A. (1981) Biochem. Soc. Trans. 10, 78-79.
2. Fry, J.R. and Bridges, J.W. (1977), in:Progress in Drug Metabolism,v.2. J.W. Bridges and L.F. Chasseaud, ed., pp.71-118, Wiley, London.
3. Tardiff, R.G. (1978) Ann. Rev. Pharmacol. Toxicol. 18, 357-369.
4. Harris, J.W. and Patt, H.M. (1969) Exptl. Cell. Res. 56, 134-141.
5. Ohara, H. and Terasima, T. (1969) Exptl. Cell. Res. 58, 182-185.
6. Harris, J.W. and Teng, S.S. (1972) J. Cell. Physiol. 81, 91-96.
7. Reed, D.J., Babson, J.R., Beatty, P.W., Brodie, A.B., Ellis, W.W., and Potter, D.W. (1980) Anal. Biochem. 106, 55-62.
8. Tietze, F. (1969) Anal. Biochem. 27, 502-522.
9. Sumnner, K.-H. and Wiebel, F.J. (1981) Toxicol. Letters 9, 409-413.
10. Weibel, F.J. and Singh, J. (1980) Arch. Toxicol. 44, 85-97.