The effect of glutathione on HL-60 treated with dimethylsulfoxide, butyric acid or 12-*O*-tetradecanoylphorbol-13-acetate

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HL-60 promyelocytic leukemic cells can be induced to differentiate into granulocytes or macrophages. Reduced glutathione lyses undifferentiated HL-60 cells but has minimal effect on their differentiated counterparts. The addition of reduced glutathione to HL-60 promyelocytic leukemic cells retards cell growth and lyses cells. HL-60 cells can be induced to differentiate into granulocytes with dimethylsulfoxide butyric acid or into macrophages with 12-*O*-tetradecanoylphorbol-13-acetate. After treatment of HL-60 cells with these inducing agents the HL-60 cells become unresponsive to the effects of glutathione.

Differentiation *Glutathione* *HL-60 promyelocytic leukemia* *Cell volume* *Lysis*

1. INTRODUCTION

The administration of glutathione (GSH) to rats bearing aflatoxin B-induced liver tumors or ethionine-induced liver tumors prolonged the survival of the tumor-bearing rats [1,2]. If we assume that GSH affected the liver cell directly, a tissue culture model system could be set up to study the effects of GSH on the cell in culture. The measurement of the effects of GSH on cells is facilitated by using cells which grow in suspension culture as opposed to cells growing in monolayers. For this reason, HL-60 promyelocytic leukemia cells were used as a model system instead of liver cell monolayers to study the effects of high doses of extracellular GSH. HL-60 promyelocytic cells can be induced to differentiate into granulocytes by the addition of dimethylsulfoxide (DMSO) or butyric acid (BA), or differentiated into macrophages by the addition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [3,4]. Therefore, a model system exists containing cells in the cancerous phase and in the differentiated stage of the same cell line. In these experiments HL-60 cells prior to treatment with differentiating agents were shown to be sensitive to lysis by GSH while after treatment with these agents the HL-60 cells become unresponsive.

2. MATERIALS AND METHODS

2.1. *Cell culture and reagents*

HL-60 cells were obtained as a generous gift of Robert C. Gallo and were maintained in continuous suspension culture in RPMI medium 1640 (GIBCO, Grand Island NY) supplemented with 20% heat-inactivated fetal calf serum and antibiotics [3]. The cells were maintained in logarithmic culture at 37°C in a 5% CO_2 atmosphere and passed every 4 days at 2.5×10^5 cells/ml.

A stock solution of reduced glutathione (GSH 20 mg/ml) was freshly made daily and used primarily at 200 μg/ml. Dimethylsulfoxide (DMSO, 1.2%) and butyric acid (BA, 0.6 mM) were added to induce granulocytes to differentiate. After 0–3 days the cells were reacted with GSH and the cell volume was measured. TPA was stored at 10^{-5} M and used at 10^{-8} M.

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2.2. *Cell count and volume measurement*

The electronic cell volume was measured on a Coulter H4 Channelyzer and the electronic cell count was made simultaneously on a Coulter counter, model ZBI equipped with a 70 μm diam. and 84 μm long orifice on the aperture tube (Coulter Electronics, Hialeah FL). To obtain reproducible results for electronic cell volume analysis and counting, the cells were removed from culture, dispersed and immediately fixed with an equal volume of filtered 3% glutaraldehyde in Dulbecco's phosphate-buffered saline (NaCl 8 g/l, MgCl 0.1 g/l, KCl 0.2 g/l, $NaHPO_4 \cdot H_2O$ 2.16 g/l, KH_2PO_4 0.2 g/l, $CaCl_2$ 0.1 g/l) resulting in a final fixation concentration of 1.5% [4–6]. Cell viability determination was made by mixing HL-60 cells with 0.4% Trypan blue and counting 200 cells microscopically. The volume histogram obtained was integrated and the areas designated as small (channels 12–40), medium (channels 41–70), and large (channels 71–100) sized cells were defined.

2.3. *Cytolysis assay*

To determine the susceptibility to lysis by GSH, about 4×10^6 HL-60 cells were labeled with 50 μCi ^{51}Cr (sodium chromate, ICN, Irvine CA) for 45 min, washed 2-times and placed in a 24-well dish (1 ml/well) at ~2.5×10^5 cells/ml [7,8]. Samples were measured in quadruplicate. GSH (200 μg/ml) and $CuSO_4$ (50 μM) were added to the cells and incubated for 6 h. The cells were centrifuged and the supernatant was counted in a Nuclear Chicago Gamma Counter. To obtain the total counts, the cells were frozen and thawed 3 times while the background count was obtained by incubating the cells without GSH for 6 h. The specific ^{51}Cr release was obtained by the following formula:

$$\% \text{ specific cytotoxic release} = \frac{\text{experimental cpm} - \text{background release cpm}}{\text{total release cpm} - \text{background release cpm}}$$

2.4. *NBT reaction*

The ability of granulocytes to reduce nitroblue tetrazolium (NBT) provides a system to measure human myeloid differentiation in vitro. NBT reduction was assayed by incubating 2×10^6 cells/ml for 25 min at 37°C with 2% NBT desolved in Dulbecco's PBS containing 200 ng/ml freshly diluted TPA. The cells containing intracellular blue–black deposits were determined to be positive and were counted by phase microscopy. The macrophage formation was assessed morphologically by the cells adhering to the plate, and sending out pseudopods. Many cells adhere but did not transform into macrophages.

3. RESULTS AND DISCUSSION

The addition of GSH to HL-60 cells has been shown to lyse cells by the following assays: cell counts, Trypan blue exclusion, cell volume and ^{51}Cr release [10]. Correlated with these parameters there was a decrease in population cell size and the appearance of a debris peak measured by a Coulter H_4 system. This was a rapid test to measure the effects of GSH on HL-60 cells (fig.1A). Using a Coulter H_4 system the decrease in cell growth and decrease in cell volume were shown to be maximum when testing the following concentrations of GSH: 20 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml, 300 μg/ml, 400 μg/ml, 800 μg/ml, 2000 μg/ml [9]. The effects of GSH on cells incubated with DMSO or BA were measured using similar assay procedures as these used for the undifferentiated cells. It was found that the cell volume changes which were observed in undifferentiated cells did not occur when various doses of GSH between 50 μg and 800 μg were added to the HL-60 cells which were incubated with BA, DMSO, or TPA. Therefore we used for the studies reported 200 μg GSH/ml the level which gave the maximum response on uninduced HL-60 promyelocytic cells. The addition of GSH to BA-stimulated or DMSO-stimulated HL-60 cells between day 1 and 4 does not significantly increase the amount of smaller cells or debris suggesting the cells have become unresponsive to the GSH effect. Fig.1 shows at day 3 the change in cell volume of control cells while BA-treated or DMSO-treated cells show only minor changes in volume.

The addition of TPA to HL-60 cells results in the gradual adherence of HL-60 cells to the plastic substrate followed by morphological transformation into macrophages. During the time GSH was added to TPA-treated cultures over 90% of the cells were in suspension programmed to develop

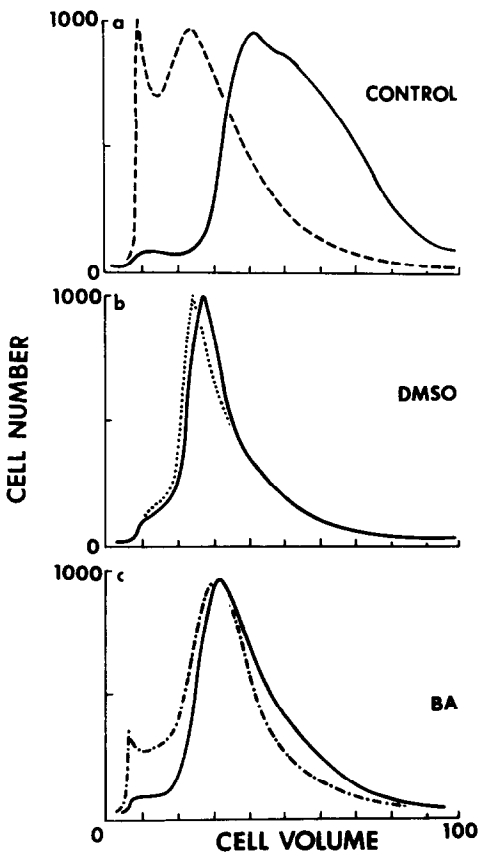

Fig.1. The effect of GSH on the cell volume distributions of differentiating HL-60 cells. About 2.5 × 10^5 cells were induced to differentiate with 1.2% DMSO or 0.6 mM BA. After 3 days, the cells were diluted to 2.5 × 10^5 cells/ml and 200 μg/ml GSH was added. After 6 h the cells were fixed with 3% glutaraldehyde and counted with a Coulter H_4 Channelyzer system. The dotted and dashed lines indicate the cell volume after GSH was added. A large shift in volume occurs with control cells but does not occur with BA- or DMSO-induced cells. The smaller volume of the BA- and DMSO-induced population prior to the addition of GSH is the result of a volume decrease which occurs as the promyelocyte differentiates into more mature myeloid cells. The cell volume was calibrated using 9.69 μm, 9.96 μm and 10.07 μm diam. Coulter spheres. The following calibration equation was derived: vol. (μm^3) = 12.33 × channel number. About 50000 cells were counted in each distribution. The NBT reaction yielded the following percentages of differentiated cells at the 3rd day: BA, 10%; DMSO, 23%; control, 1%; and at the 4th day BA, 32%; DMSO, 26%; control, 1%.

into macrophages. The time-dependent cell cycle effects of TPA on HL-60 cells were studied using flow cytometry, cell volume and cell counts. It was found that TPA blocks HL-60 cells which are in suspension or adherent in the G_1 and G_2 states [4–6]. The cells in suspension traverse through the cell cycle and gradually adhere to the tissue culture dish; 6 h after TPA is added, < 10% of the cells adhere but after 2 days almost all the cells have become adherent, some transforming into macrophages.

Due to the adherence of G_1 cells and the transient block of G_2 cells, the suspension population's cell volume increases [6]. The increased number of larger cells resulting from TPA treatment is shown in table 1 with the mean, mode and percentage large-sized cells being greater than those in the control. As shown in fig.1 the addition of GSH to HL-60 control cells decreases the mean and mode of the population while increasing the percentage of small-sized cells. This transition to smaller sized cells is inhibited if TPA is added simultaneously with GSH. If TPA is added 1 h prior to GSH, the changes are reduced further (fig.2). This suggests that TPA modifies the cells in a manner which inhibits the GSH-mediated lysis of HL-60 cells.

The ^{51}Cr release assay was used to test the data obtained by cell volume. The addition of GSH to cells which were incubated with BA, DMSO or TPA showed a reduction of ^{51}Cr release suggesting the cells were not as responsive to GSH-mediated lysis. In a representative experiment (table 2) GSH was added to 3-day-old BA- or DMSO-stimulated HL-60 cells or simultaneously with TPA, resulting in less ^{51}Cr released compared to control cells.

The mechanism by which GSH lyses cells is unknown but appears to involve peroxide as catalase (100–3000 units/ml) was shown to eliminate the GSH lysis response [10]. The oxidation of GSH might generate oxygen radials which can be converted into H_2O_2 by a superoxide dismutase. H_2O_2 generated by either glucose oxidase or by xanthine and xanthine oxidase, though a presumed superoxidase dismutase on the cell surface showed a decrease in cell volume and cell growth similar to that observed with GSH. Other reducing agents containing sulfhydryl groups (i.e., cysteine or dithriothreitol) also generated a lysis of HL-60 cells, but not to the same extent as GSH (not shown).

Table 1

The effect of GSH and TPA on HL-60 cells

Sample	Mean (channel)	Mode (channel)	% Small (channels) 12–40	% Medium (channels) 41–70	% Large (channels) 71–100
TPA and GSH added simultaneously					
Control	59	49	10	66	24
TPA	66	60	4	56	40
GSH	33	12	71	25	4
GSH and TPA	47	30	43	40	17
TPA added 1 h prior to GSH					
Control	59	49	10	64	26
TPA	64	56	6	58	36
GSH	31	19	76	21	3
GSH and TPA	55	54	23	51	26

The addition of GSH to HL-60 cells decreases the cell volume. The simultaneous addition of GSH and TPA retards the decrease in cell volume. A greater retardation of the GSH effect occurs if the TPA is added 1 h prior to GSH. These results are shown in the table by changes in the percentages of small cells and the value of the mean and mode. The mean was determined by integrating between channels 12 and 100 and the percent cells in each region was determined by integrating the distribution between 12 and 100. All samples were measured on cells remaining in supernatant 6 h after the addition of GSH. The addition of TPA to HL-60 cells increases the cell volume due to the selective adherence of G_1 cells. Over 90% of the cells remain in the supernatant 6 h after the addition of TPA

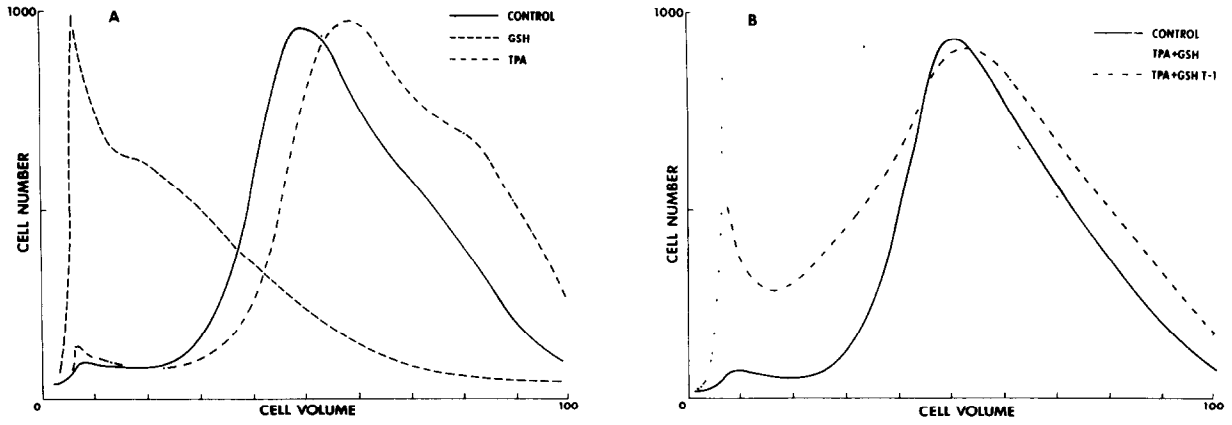

Fig.2. The effect of GSH on TPA-induced HL-60 cells. About 2.5×10^5 cells were induced to differentiate into macrophages with TPA. (A) The addition of TPA results in larger cells due to small-sized G_1 cells adhering to the flask. The addition of GSH to HL-60 cells results in cell lysis and smaller sized cells and particles. (B) The addition of TPA and GSH (TPA + GSH) simultaneously results in a reduction of the cell volume changes. The addition of TPA 1 h prior to the addition of GSH (TPA + GSH T-1) results in a greater reduction of cell volume changes than the addition of GSH alone. The values are quantitated in table 1. All samples were taken 6 h after the addition of GSH and each histogram represents over 50000 counts. The cell volume was calibrated by the following equation: cell volume (μm^3) = $12.33 \times$ channel number.

Table 2

^{51}Cr release from HL-60 cells after GSH treatment

Sample	Cpm (mean ± SD)[a]	Specific release (%)
Control	6665 ± 965	
Control-background	302 ± 10.7	25
Control-GSH	1897 ± 134	
DMSO-total	4095 ± 134	
DMSO-background	230 ± 32.6	7
DMSO–GSH	504 ± 41.4	
BA-total	3030 ± 121	
BA-background	278 ± 136	4
BA–GSH	401 ± 14.7	
TPA-control	4456 ± 180	
TPA-background	215 ± 11.6	9
TPA–GSH[b]	606 ± 36	

[a] Means of 4 sample points
[b] TPA and GSH added simultaneously

^{51}Cr (50 μCi) was added to 10^6 cells. After 45 min, the cells were washed twice and placed in a 24-well dish at ~2.5 × 10^5 cells/ml. Control, DMSO-total, BA-total, TPA-control were derived by freeze-thawing the sample 3-times. The following reagents were added: GSH, 200 μg/ml; DMSO, 1.2%; BA, 0.6 mM; TPA, 10^{-8} M

HL-60 cells contain myeloperoxidase which may react with H_2O_2 in the presence of chloride ion to yield cell lysis [12]. It is conceivable that cells incubated with TPA, BA or DMSO either change their internal biochemistry to dispose of H_2O_2 or change their extracellular membrane to disperse, or not form H_2O_2. The major site of action of TPA appears to be the cell membrane [13]. It has been reported that TPA induced changes on the cell membrane which include an increase in phospholipid synthesis, membrane transport, and changes in the type of membrane glycopeptides [13–15]. The elimination of the GSH-mediated lysis in the presence of TPA may be due to this modification of the membrane of TPA. The specific toxicity of 200 μg/ml might be related to a delicate balance in which GSH can generate H_2O_2 at one concentration and can protect the cell at a saturated concentration.

An intracellular GSH redox cycle has been postulated to protect proteins and cell membranes against peroxides and free radicals [16]. Extracellular GSH is believed to be transported into the cell by membrane-bound γ-glutamyl transpeptidase (γ-GT) as γ-glutamyl amino acids [17–19]. Extracellular GSH is oxidized to GSSG (oxidized glutathione) by γ-TP on the cell surface. Unlike intracellular GSSG which can be converted back to GSH by GSH reductase, the extracellular GSSG undergoes hydrolytic transpeptidations leading to γ-glutamyl amino acids and H_2O_2. The γ-glutamyl amino acids can be transported into the cells while H_2O_2 is believed to occur as cysteinyl glycine undergoes spontaneous oxidation. Thus, there may be a relationship between the γ-glutamyl cycle translocation of GSH and external oxidation of GSH to GSSG [20,21]. The addition of extracellular GSH may effect this balance and the cells' ability to disperse H_2O_2.

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