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A DECREASE IN GLUCOSE 6-PHOSPHATE DEHYDROGENASE ACTIVITY AND mRNA IS AN EARLY EVENT IN PHORBOL ESTER-INDUCED DIFFERENTIATION OF THP-1 PROMONOCYTIC LEUKEMIA CELLS

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Summary

Redox modification of regulatory proteins implicates the glutathione redox system (GRS) in the control of gene expression. Glucose-6-phosphate dehydrogenase (G6PD) provides reducing equivalents for the GRS, and it has been suggested that high levels of G6PD in preneoplastic lesions are directly related to neoplastic transformation. We have used THP-1 human promonocytic leukemia cells, an established model of induced macrophage differentiation, to test an important corollary of this hypothesis, viz., that a decrease in G6PD activity should accompany the loss of the transformed phenotype. Phorbol 12-myristate 13-acetate (PMA) arrests the constitutive cycling of THP-1 and induces a phenotype that approaches normalcy. We measured the specific activities of the GRS enzymes, G6PD, glutathione peroxidase, and glutathione reductase during the early stages of phorbol ester-induced differentiation of THP-1 cells. We observed an 80% decrease in G6PD activity and an increase in the apparent K_M for glucose 6-phosphate. In contrast, glutathione peroxidase (GPX) activity increased, while glutathione reductase (GR) activity remained essentially constant. The reduction in G6PD activity, preceding the loss of the transformed phenotype, is accompanied by a fourfold decrease in steady-state levels of G6PD mRNA. These findings are consistent with the hypothesis that high levels of G6PD are causally related to neoplastic transformation.

Key Words: G6PD, promonocytic leukemia, THP-1, differentiation, phorbol ester, redox modification

Glucose-6-phosphate dehydrogenase (G6PD) catalyzes the first reaction of the hexose monophosphate pathway (HMP). The HMP generates reducing equivalents (NADPH) for

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a variety of biosynthetic reactions and the glutathione redox system (GRS), as well as phosphopentoses for nucleotide biosynthesis. The GRS plays a critical role in the scavenging of active oxygen, the maintenance of intracellular protein sulfhydryl groups, the detoxification of xenobiotics and the acquisition of multidrug resistance in transformed cells (1). Higher levels of G6PD activity have also been demonstrated in multi-drug resistant variants of leukemic cells (2). G6PD is considered a housekeeping enzyme, but its activity is variable and shows adaptive regulation in certain tissues: among human cell types, the variation in G6PD activity is more than 50-fold (3); in rats, the enzyme is under nutritional and hormonal control (4,5,); and steady-state levels of G6PD mRNA vary in response to the carbohydrate content of the diet (6).

Several regulatory proteins including the transcription factors AP-1 (7), human glucocorticoid receptor (8), chicken progesterone receptor (9), OxyR of *E. coli.* (10), and NF-κB (11) as well as the iron response element-binding protein (12) and protein kinase C (13) are known to undergo reversible redox modification. Given the importance of these molecules in signal transduction and gene expression, their modulation by redox modification implicates the GRS in the control of gene expression, and provides a plausible mechanism by which deregulated GRS activity can alter gene expression. While over-expression of G6PD is likely to drive nucleotide biosynthesis, excess NADPH can create a prooxidant state due to its ability to reduce hydrogen peroxide univalently in an iron-catalyzed Fenton reaction (14).

It has been suggested that the high G6PD activities observed in some preneoplastic lesions are causally related to neoplastic transformation (15-18). A role for increased G6PD activity in cell proliferation is supported by the observation that both epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) up-regulate G6PD activity (19). We have tested a corollary of this hypothesis, viz., that a decrease in G6PD activity should accompany the loss of the transformed phenotype, by measuring the specific activities of G6PD during the phorbol ester-induced cytostasis and differentiation of human promonocytic leukemia cells, THP-1. THP-1 cells grow as nonadherent promonocytes, but differentiate to macrophage-like cells upon treatment with phorbol 12-myristate 13-acetate (PMA) (20). PMA, a potent activator of protein kinase C (PKC), arrests THP-1 proliferation and induces the expression of several macrophage characteristics (21,22). PMA-differentiated THP-1 cells therefore constitute a tractable system for investigating changes in the levels of G6PD during the early stages of the differentiation process that leads to the loss of the transformed phenotype.

Methods

Cell culture and preparation of lysates. THP-1 cells were obtained from American Type Culture Collection (Rockville, MD) and maintained as described previously (22). For PMA differentiation, triplicate samples of 1.4×10^7 cells in 20 ml medium were incubated in 85-mm tissue culture dishes, with or without 100 *nM* PMA (Sigma). A stock solution of PMA (333 μg/ml) in ethanol, stored at -20°C, was diluted in ethanol, and this working dilution was added to the growth medium to a final concentration of 100 *nM*. The final concentration of ethanol in the medium was 0.1%. The cells were incubated for 3 hours at 37°C, after which control cells (nonadherent) were harvested by centrifugation, washed with Hanks' balanced salt solution (HBSS), and lysed by repeated pipetting in 0.7 ml of cold lysis buffer (0.05 *M* Hepes, pH 7.5 containing 20 *mM* KCl, 0.1% Triton X-100, and 100 μ*M* phenylmethylsulfonyl fluoride. The adherent, PMA-treated cells were rinsed with cold HBSS and lysed *in situ* in the same volume of lysis buffer. Cell lysates were centrifuged at 12,000 x *g* for 30 min at 4°C and the supernatants were used for enzyme assays and protein determination. All assays were

performed on freshly prepared lysates kept at 0°C, and used within 2 h after harvesting of cells.

Enzyme assays and G6PD kinetics. Glucose-6-phosphate dehydrogenase was assayed by the method of Olive and Levy (23) with minor modifications. Final concentrations of reagents were as follows: 0.1 *M* Hepes, pH 7.3; 2.55 *mM* $MgCl_2$; 0.3 *mM* $NADP^+$; and 2.0 *mM* G-6-P. The reaction was started by adding 50 μl of homogenate, and the reduction of $NADP^+$ was measured as the increase in absorbance at 340 nm. One unit of enzyme converts 1 μmole of $NADP^+$ to NADPH + H^+ per min at 25°C. Specific activity is expressed as milliunits of G6PD per mg protein. Results are the means of triplicate assays ± SD.

The apparent Michaelis constant (K_M) of the enzyme from control and PMA-treated cells was estimated from Lineweaver-Burk plots of $1/v_o$ *vs* 1/[G-6-P].

Glutathione peroxidase activity was measured as the non-cyanide-inhibitable decomposition of hydrogen peroxide (24). Final concentrations of reagents in the reaction mixture were: 0.05 *M* Hepes buffer, pH 7.5; 0.16 *mM* NADPH; 1.0 *mM* sodium azide; 0.4 *mM* EDTA; 1.0 *mM* reduced glutathione; 0.2 *mM* H_2O_2; 4 μg of yeast glutathione reductase (Sigma); and 50 μl of cell homogenate in a total volume of 1.0 ml. The reaction was started by the addition of homogenate and the oxidation of NADPH was followed spectrophotometrically at a wavelength of 340 nm at 25°C for 2 min. The non-enzymatic oxidation of NADPH was measured without homogenate and this reaction rate was subtracted from that with homogenate in order to determine the true enzymatic activity. One unit of enzyme is the amount required to oxidize 1 μmole GSH/min which is equal to 0.5 μmole NADPH oxidized per min. Specific activity is expressed as milliunits of GPX/mg protein.

Glutathione reductase activity was measured by the method of Carlberg and Mannervik (25). Final concentrations of reagents in the reaction mixture were: 0.1 *mM* NADPH; 1.3 *mM* glutathione disulfide (GSSG); and 0.1 *M* sodium phosphate, pH 7.2. The reaction was started by adding homogenate, and the decrease in absorbance at 340 nm followed for 2 min at 25°C. One unit of GR catalyzes the oxidation of one μmole of NADPH per min. Specific activity is reported as milliunits of enzyme per mg protein. Student's *t*-test was used to determine the significance of differences between mean specific activities of G6PD, GPX, and GR.

Protein determination. Protein concentration in detergent-containing homogenates was determined by the bicinchoninic acid (BCA) assay (Pierce, Rockford IL)(26).

Northern blot analysis of G6PD mRNA. Total cellular RNA was extracted from control and PMA-treated cells by the guanidine isothiocyanate-cesium chloride (GuSCN-$CsCl_2$) ultracentrifugation method (27). Non-adherent cells (< 3 h of PMA treatment) were harvested by centrifugation, rinsed with Dulbecco's phosphate-buffered saline (PBS) and lysed by the addition of 4 *M* GuSCN. Adherent cells (≥ 3 h of PMA treatment) were rinsed with PBS, and lysed *in situ* with 4 *M* GuSCN. The DNA was sheared by 10 strokes in a Dounce homogenizer, after which the lysate was layered on a 5.7 *M* $CsCl_2$ cushion and centrifuged for 18 hours at 100,000 x *g* in a Beckman SW40 rotor at 15°C. The RNA pellet was recovered and dissolved in 10 *mM* Tris-HCl, pH 7.5, with 0.1 *mM* EDTA, ethanol precipitated and quantitated. To quantitate G6PD mRNA, 20 μg per track of total cellular RNA was electrophoresed in denaturing formaldehyde agarose (1.4%) gels and transferred to hybridization membranes (GeneScreen*Plus*, New England Nuclear, Boston, MA) by capillary blotting. The RNA-bound membranes were pre-hybridized for 1 h, and then hybridized with

^{32}P-labeled (28) *G6PD* cDNA probe. The probe used was the 1.5 kb EcoRI-EcoRI fragment of clone pGD-P-25A (29; American Type Culture Collection). Following hybridization, the filters were washed free of unbound probe, autoradiographed, and quantitated by video densitometry. Equal loading of RNA was confirmed by UV fluorescence of the ethidium bromide-stained gel.

Results

Enzyme activity

Fig. 1 shows the specific activities of G6PD and GPX, and GR in untreated controls and in THP-1 cells exposed to PMA for 3 h (PMA/3 h). The change in G6PD activity was dramatic: There was an 80% decrease in specific acitivity in PMA-treated THP-1 cells (31.36 ± 0.78 SD mU/mg protein, n = 3) at 3 h post-treatment compared with untreated controls (6.2 ± 1.98 SD, n = 3). The difference between the mean G6PD activities for control and PMA-treated cells was highly significant ($P < 0.01$) Conversely, the specific activity of GPX increased by 75% during this time, from 41.62 mU/mg protein ± 6.35 (mean ± SD, n = 3) to 72.73 ± 16.8 (mean ± SD, n = 3), but this increase was not statistically significant ($P < 0.4$); the specific activity of GR in PMA-treated cells (21.45 ± 3.11 SD, n = 3) was similar to that of controls (22.03 ± 1.74). By 3 h post-treatment, more than 95% of the cells had adhered firmly to the substratum.

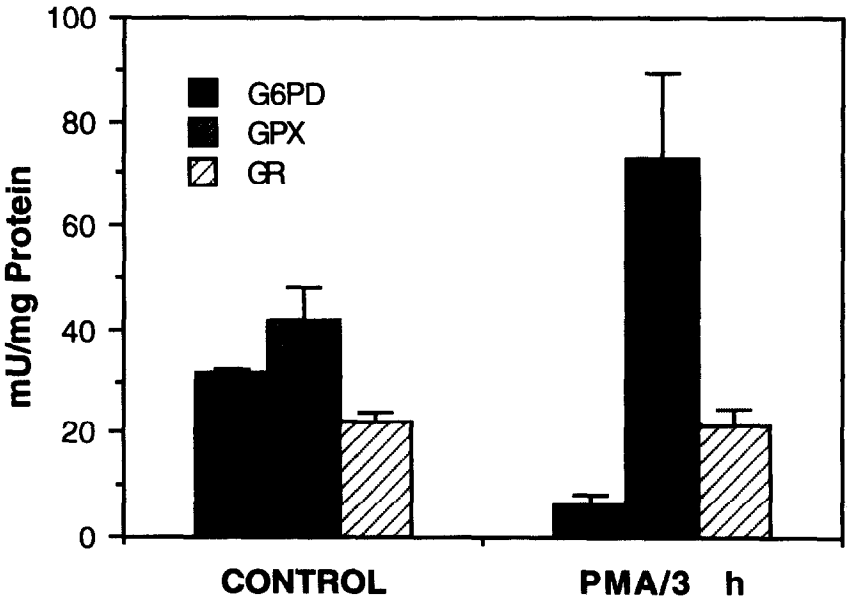

Fig. 1
Specific activities of G6PD, GPX and GR in control and PMA-treated THP-1 cells at 3 h post-treatment (PMA/3 h). THP-1 cells were treated with 100 *nM* PMA for 3 h at which time adherence was more than 95% complete. Control cells were harvested and lysed in lysis buffer. Adherent PMA-treated cells were washed with HBSS and lysed *in situ*. Cell lysates were centrifuged at 12,000 x *g* and the supernatants were assayed for enzyme activity. Results are the mean specific activities ± SD of triplicate assays. Legends for bars are shown as an inset.

Kinetics

Lineweaver-Burk plots of kinetic data for G6PD from control and PMA-treated cells are shown in Fig. 2. Apparent K_M of the enzyme from PMA-treated cells (9.5 x 10^{-5} *M* G-6-P) is approximately three times that of the control enzyme (3.4 x 10^{-5} *M*). This change is suggestive of a decreased affinity of G6PD from PMA-treated cells for the substrate.

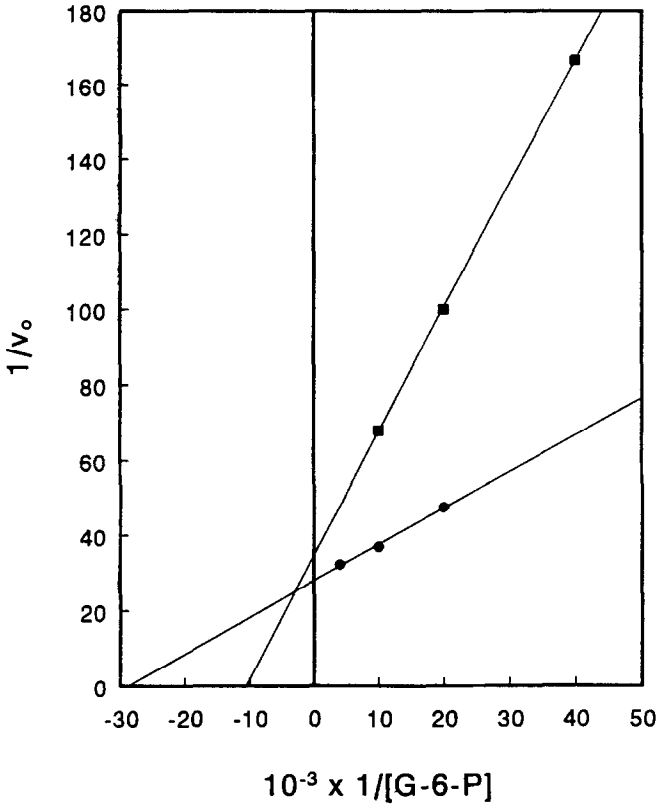

Fig. 2

Lineweaver-Burk plots of $1/v_o$ vs 1/[G-6-P] for G6PD from control THP-1 cells (●) and from THP-1 cells treated with 100 *nM* PMA for 3 h (PMA/3 h, ■). Initial velocities (v_o) of G6PD were measured at different molar concentrations of G-6-P. The x-intercept is $-1/K_M$. Apparent K_Ms for G6PD from controls and PMA/3 h cells are 3.4 x 10^{-5} *M* and 9.52 x 10^{-5} *M*, respectively.

Steady-state levels of G6PD mRNA

Fig. 3 shows the relevant portion of the Northern blot of G6PD mRNA (Top), the densitometric quantitation of the same blot (Middle), and the ethidium bromide stained gel (Bottom) before blotting showning equal loading. The G6PD probe hybridized with a single 2.4 kb mRNA transcript whose level increased slightly between times 0 and 1 h after PMA treatment and then declined dramatically by 3 h. Results for PMA-treated cells are presented in Fig. 3B as fold changes in the steady-state level of G6PD mRNA in control THP-1 cells. By 3 h, there was a 4-fold decrease in the steady-state level of G6PD mRNA relative to controls.

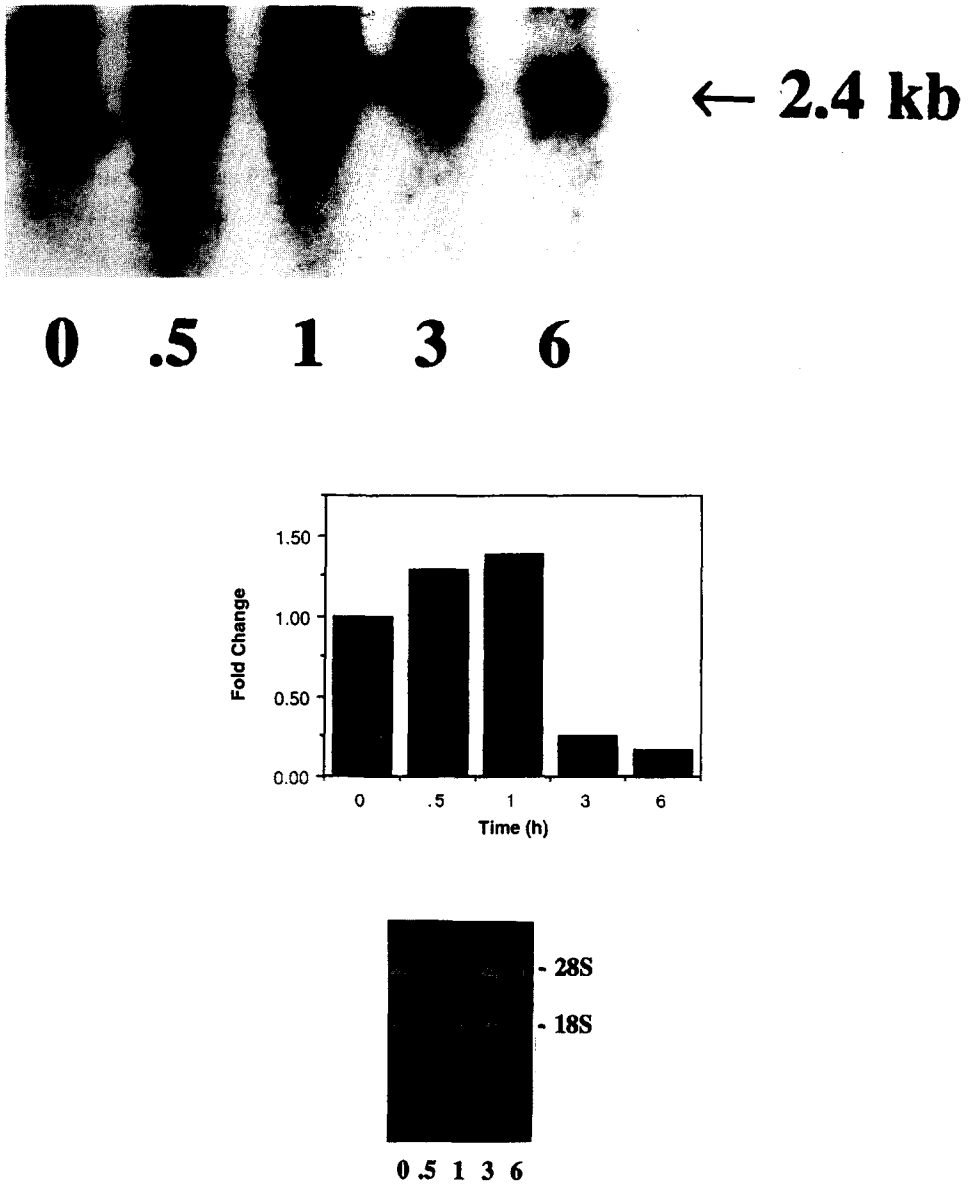

Fig. 3

(Top) Northern blot of G6PD RNA from control and from THP-1 cells treated with 100 nM PMA (PMA/3 h). 20 μg per track of total cellular RNA was electrophoresed in a denaturing formaldehyde agarose gel and transferred to a GeneScreen *Plus* membrane. The membrane was hybridized with a ^{32}P-labeled *G6PD* cDNA probe. The probe identified a single 2.4 kb transcript. Numbers below the tracks represent hours post-treatment. **(Middle)** Densitometric quantitation of the Northern blot of G6PD mRNA shown in **(Top)**. Results for PMA-treated cells are presented as fold changes in the steady-state level of G6PD mRNA in control THP-1 cells. **(Bottom)** Ethidium bromide stained gel before blotting showning equal loading of RNA.

Discussion

We have shown previously that cytoadherence is one of the earliest observable morphological changes that signal the commitment of PMA-treated THP-1 cells to macrophage differentiation (22). Cells that do not adhere do not differentiate. Therefore, in our experiments, cytoadherence was diagnostic of the onset of differentiation. A significant decrease in G6PD activity during the early stages of the process leading to the loss of the transformed phenotype is persuasive, though not conclusive, evidence that high levels of G6PD activity are causally related to the continuous cycling and deregulated gene expression of THP-1 cells. The differential response of the three enzymes assayed in this investigation suggests that the decrease in G6PD activity was physiologically significant and not part of a generalized reduction of enzymatic activity in response to PMA treatment. The increase in GPX activity, although not statistically significant, may be an adaptive response to the pro-oxidant state induced by PMA treatment, because GPX scavenges hydrogen peroxide. Furthermore, a decrease in G6PD, an increase in GPX, and a constant level of GR) suggests that the three enzymes are not coordinately regulated during PMA induced differentiation of THP-1. The stability of intracellular G6PD under conditions of PMA treatment in culture is not known. PMA is known to induce high fluxes of active oxygen, and G6PD is one of several enzymes known to be destabilized by oxidation (30). It is, therefore, possible that the decrease in G6PD activity is caused by redox modification of the mature enzyme. However, the dramatic decrease in G6PD mRNA by 3 h post treatment parallels the loss of enzyme activity, and suggests that decreased synthesis of the enzyme is one of the early events of PMA-induced differentiation of THP-1 cells. The more than three-fold increase in the K_M for G-6-P may account in part for the observed decrease in specific activity, and suggests that G6PD is altered in some way, perhaps covalently, by redox modification by a species other than H_2O_2. In erythrocytes under oxidant stress, glucose is metabolized preferentially by the HMS. The enzymatic changes which underlie this re-routing cannot be due to differential gene expression and must result from alterations in the catalytic activities of pre-existing proteins. Such modification of existing protein may explain the absence of a correlation between G6PD levels and tumor progression when the enzyme is detected immunohistochemically (17). Varying the activity of a constant pool of enzyme molecules is an established mechanism of cellular regulation.

The genes encoding the GRS enzymes play a potentiating function by virtue of the fact that their products maintain the conditions required for the expression of other genes. Their "housekeeping" appellation tends to obscure this role. By virtue of its participation in the GRS, the potential importance of G6PD in the regulation of gene expression warrants an examination of this enzyme that goes beyond its familiar role in intermediary metabolism. The large pool of putative human *G6PD* mutations may include regulatory or catalytic variants associated with over-abundant enzyme activity, and these should be studied in relation to their potential for affecting gene expression. We are currently investigating the possibility that THP-1 G6PD is interconverted between variably-active (bound vs free) forms, as has been reported for rat renal cortical cells (19). We are also screening selected inhibitors of HMP activity to determine whether such agents can directly induce differentiation of THP-1 and similar systems. We are also evaluating these agents for their ability to activate G6PD or up-regulate *G6PD* expression.

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