Regulation of the Human Cellular Glutathione Peroxidase Gene During In Vitro Myeloid and Monocytic Differentiation

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We have used the HL-60 and PLB-985 myeloid leukemia cell lines to examine the regulation of expression of the important intracellular antioxidant enzyme, glutathione peroxidase (GSH-Px), during phagocytic cell differentiation in vitro. Induction of differentiation along the monocytic pathway by phorbol ester results in an approximately twofold rise in enzyme activity and a parallel increase in the rate of Selenite (Se) incorporation into immunoprecipitable GSH-Px protein. Induction along the granulocytic pathway by dimethyl formamide (DMF) results in similar changes in steady-state enzyme levels and rates of GSH-Px protein synthesis. Steady-state levels of GSH-Px gene transcripts also increase more than twofold, approximately in parallel with the enzyme levels. Nuclear run-on transcription assays of GSH-Px mRNA synthesis show ratios of induced to uninduced transcript levels of 2.24 and 1.59 with phorbol myristate acetate (PMA) induction and DMF, respectively, in HL-60 cells, and ratios of 1.34 and 3.46 with PMA and DMF, respectively, in PLB-985 cells. Half lives of GSH-Px mRNA are unchanged or slightly shorter after differentiation of HL-60 cells, and slightly longer after induction of PLB-985. Overall, the present studies show that GSH-Px activity rises during in vitro-induced monocytic or granulocytic differentiation of myeloid cell lines and that the increased expression of the cellular GSH-Px gene occurs through complex mechanisms that include transcriptional up-regulation. This pattern contrasts with the nearly complete cotranslational regulation of GSH-Px expression by exogenous selenium.

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Cytosolic glutathione peroxidase (GSH-Px), EC 1.11.1.9, is a cytosolic enzyme, widely distributed in eukaryotic cells, that catalyzes the reduction of hydroperoxides by glutathione. GSH-Px reduces hydrogen peroxide as well as a wide range of organic peroxides derived from unsaturated fatty acids, nucleic acids, and other important biomolecules. For peroxides encountered under physiologic conditions, it is more active than catalase, which cannot reduce organic peroxides and has a higher Michaelis constant ($K_m$) for hydrogen peroxide. Thus, GSH-Px represents the major cellular defense against these toxic oxidant species. Such defense is particularly critical for phagocytes, which are subject to auto-oxidation by their own respiratory burst peroxide production.

Cellular GSH-Px, also termed GSH-Px-1, is the best characterized of a larger mammalian enzyme family that also includes plasma, phospholipid hydroperoxide, and GI glutathione peroxidases. In turn, this family is part of a larger class of selenoproteins, a unique group of prokaryotic and eukaryotic polypeptides incorporating the unusual amino acid selenocysteine. The first successful molecular cloning of a GSH-Px gene, the murine cellular GSH-Px, occurred almost fortuitously during the screening of cDNA species that were up-regulated during the induced differentiation of murine erythroleukemia cells. However, most research since then has focused on the effects of selenium supply on the expression of cellular GSH-Px genes in vitro and in vivo. We have previously used the human HL-60 myeloid cell line as a model system to study this relationship between selenium supply and the expression of the human cellular GSH-Px gene. However, HL-60 cells, as well as other myeloid cell lines such as PLB-985, also have the very important capacity to differentiate along at least three distinct pathways to produce monocytes, granulocytes, or eosinophils. Thus, these cell lines also provide an extremely useful model system in which to study human gene regulation during differentiation.

Treatment with phorbol esters induces a rapid differentiation along the monocyte-macrophage pathway, whereas treatment with dimethyl formamide (DMF) induces granulocytic differentiation. During differentiation of phagocytic cells in vivo, their abilities to ingest and destroy foreign particles matures, as does their ability to degrade the toxic products produced during the respiratory burst. We have previously shown that during differentiation of HL-60 cells to granulocytes, their ability to generate oxygen radicals also matures. As GSH-Px activity is an important component of the cellular antioxidant defense system, cellular GSH-Px activity might also be expected to change during phagocytic differentiation.

In this study, we have used the HL-60 and PLB-985 cell lines as model systems for the maturation of phagocytic cells. We have examined the expression and regulation of the human cellular GSH-Px gene (GPX1) during their induced, in vitro maturation along the granulocytic and monocytic pathways of differentiation. Levels of the selenoenzyme increase in conjunction with increases in steady-state mRNA levels and transcription rates for the GSH-Px gene. Thus, differentiation-induced expression of the human cellular GSH-Px gene is regulated at least in part at the transcriptional level, in contrast with its translational regulation by selenium.

MATERIALS AND METHODS

Cells. HL-60 cells were originally obtained from Dr. R. Gallo and PLB-985 cells from Dr. T. Rado. We have previously shown that GSH-Px is down-regulated by selenium deficiency in HL-60 cells. Thus, in the current studies, cells were maintained in maximally selenium-supplemented medium: RPMI 1640 with 10% heat-inactivated iron-supplemented calf serum plus sodium selenite (5 ng/mL) as well as insulin (5 μg/mL) and transferrin (5 μg/mL) (ITS)
premix; Collaborative Research, Waltham, MA). Each cell line was incubated with 100 mmol/L phorbol myristate acetate (PMA) or 80 mmol/L DMF to induce differentiation along the monocytic29 or granulocytic26 lineage pathways, respectively.

**GSH-Px activity.** GSH-Px enzymatic activity was monitored using an adaptation of the coupled peroxidase-reductase method of Beutler.29 Sample and reference cuvettes contained 10^6 cells, 0.05% Triton X-100, 0.2 mmol/L nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH), 2 mmol/L glutathione (GSH), and 1 mmol/L glutathione reductase in Dulbecco’s phosphate-buffered saline, pH 7.4. The oxidation of NADPH by t-buty1 hydroperoxide (added to sample cuvette only) was followed spectrophotometrically at 340 nm.28

**GSH-Px protein.** Levels of newly-synthesized GSH-Px protein were determined by autoradiography of sodium dodecyl sulfate (SDS)-polyacrylamide gels of immunoprecipitated, ^75-Se-labeled proteins, as previously described.36 Cells were first incubated for 2 hours at 37°C with 10 μCi of ^75-Se as sodium selenate in nitric acid, with an original specific activity of 750 to 1,000 Ci/g (from the University of Missouri Research Reactor Facility, Columbia). For PMA-induced cells, 5 μL of diisopropylfluorophosphate were added to ice-cold 96-well plates. After 5 minutes at room temperature, the medium was aspirated and 1.5 mL of triple-detergent buffer (50 mmol/L TRIS-HCl pH 8.0, 150 mmol/L NaCl, 0.02% sodium azide, 100 μg/mL phenylmethylsulfonyl fluoride, 0.1% SDS, 1% Nonident P-40, and 0.5% sodium deoxycholate) was added to each plate. After 20 minutes shaking at 4°C, the lyed cell suspension was transferred into a microfuge tube and spun at 14,000g for 10 minutes. SDS was added to the supernatant to a final concentration of 0.5% and the cell lysate was heated in boiling water for 5 minutes, then cooled on ice. Uninduced and DMF-induced cells were spun, washed, and resuspended directly in triple-detergent buffer, then further processed as above.

Immunoprecipitation used two rabbit antisera raised (by Berkeley Antibody Co, Richmond, CA) against synthetic peptide sequences from the GSH-Px polypeptide chain, one from residues 26 to 46 and the other from residue 174 to residue 192. Fifteen microliters of each antiserum, plus 20 μL of protein A-Sepharose CL-4B beads (Sigma Chemical Co, St Louis, MO) were added to the lysate. The mixture was incubated tumbling at 4°C overnight. The beads were subsequently pelleted, washed twice with washing buffer (50 mmol/L NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS) and once with 50 mmol/L HEPES at pH 7.8, then mixed with 30 μL SDS-gel loading buffer (50 mmol/L TRIS-HCl pH 6.8, 100 mmol/L dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), heated in boiling water for 3 minutes, and spun in a microfuge. The supernatant was collected for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), performed by standard techniques31 on 12% SDS-polyacrylamide gels. Band densitometry was performed by computer image analysis of the integrated optical density of autoradiograph bands, using ImageMeasure software (Microscience, Inc, Federal Way, WA).

The two rabbit antisera were characterized in preliminary experiments (not shown) that showed detection of a band on Western blots at the appropriate apparent molecular weight for GSH-Px protein.

To measure ^75-Se uptake into whole cells or total protein, cells were incubated with labeled selenious acid as above, washed, detergent-lysed as above, and ^75-Se content measured by scintillation counting of either whole or trichloroacetic acid-precipitated lysate.

**RNA analysis.** Total cell RNA was extracted from the cells by repeated ethanol precipitation from guanidine-HCl27 and analyzed on northern blots performed according to standard procedures.31 Control RNA from the cellular slime mold *Dictyostelium discoideum* was provided by Dr Jacobson. Hybridization analysis included a 600-bp EcoRI fragment containing most of the coding region and 3′-untranslated region of human GSH-Px. Procedures for sequential cycles of prehybridization, hybridization, washes, and filter stripping were performed as previously described.33 Equal loading of lanes was shown by examination of gels after ethidium bromide staining and by rehybridization with a 5.8-kb HindIII restriction fragment of rat 18S ribosomal DNA.28

Band densitometry was performed by computer image analysis of the integrated optical density of autoradiograph bands, as above, or by direct measurement of ^35S decay on a Betascope 603 (Betagen Co) blot analyzer. Data for bands of interest were normalized to the 18S rRNA signal to correct for small differences in lane loading.

**Nuclear run-on transcription assay.** Incubation of nuclei and subsequent RNA isolation were performed with modifications of previously described procedures.35 Nuclear run-on RNA was prepared from HL-60 cells that were uninduced or were induced 1 or 2 days with DMF or 1 day with PMA (as above). PMA-induced cells were harvested after trypsin treatment to release adherent cells. Uninduced and DMF-induced cells were harvested by centrifugation. All cells were washed with cold Hanks’ balanced salt solution and counted. Cell numbers ranged from 0.3 to 8.7 × 10⁸. Nuclei were isolated by incubating 5 minutes in lysis buffer (10 mmol/L TRIS-HCl pH 7.4, 10 mmol/L NaCl, 3 mmol/L MgCl₂, 0.01% NP40) on ice. Nuclei were pelleted (900g for 6 minutes at 4°C), and washed once with lysis buffer (as above) and once with lysis buffer without NP40. Nuclei were resuspended in 200 μL of nuclei suspension buffer (50 mmol/L TRIS pH 8.3, 40% (v/v) glycerol, 5 mmol/L MgCl₂, 0.1 mmol/L EDTA). Freshly prepared nuclei were incubated 30 to 45 minutes at 30°C with an equal volume of reaction buffer containing 10 mmol/L TRIS-HCl pH 8.0; 5 mmol/L MgCl₂; 250 mmol/L KCl; 0.8 mmol/L MmCl; 0.83 mmol/L each of adenosine triphosphate, cytidine triphosphate, and guanosine triphosphate; 250 μCi of ^35S-labeled polyribonucleotides, and 125 I-labeled selexin mRNA (3000 cpm) and 5′ adenine mRNA (3000 cpm). Nuclei were digested with DNase I and proteinase K and the ^35S-labeled RNA isolated by extraction in guanidine thiocyanate and phenol and then ethanol precipitation. Equal counts of incorporated label from each group (2 × 10⁷ counts per minute) were hybridized to saturating amounts of plasmid, with or without cDNA inserts, immobilized on nitrocellulose filters by slot blotting. Probes included the same GPH1 cDNA used for Northern blots, full-length cDNAs for gp91-phox and tumor necrosis factor alpha (TNFα) (obtained from American Type Culture Collection) as markers of myeloid and monocytic differentiation, and a 200-bp cDNA (obtained from Dr P. Dobner, University of Massachusetts Medical School, Worcester) for the 3′-untranslated region of the constitutively-expressed Kcrl tubulin gene,36 as well as a pBluescript (Stratagene, LaJolla, CA) plasmid-negative control. DNA for bands of interest were normalized to the signal for the tubulin mRNA to correct for nonspecific differences between preparations of nuclei.

**mRNA stability.** HL-60 cells, either unindifferentiated or exposed to PMA or DMF as above, were incubated with the adenosine analog 5,6-dichloro benzimidazole riboside (DRB) at 50 μmol/L for various time periods up to 8 hours, as previously described.37 Pilot dose-response experiments for DRB and ^1H-uridine incorporation showed that DRB concentrations from 20 to 50 μmol/L produced the same levels of inhibition (85% to 90%) of ^1H-uridine incorporation, with or without addition of differentiating agents. In the experimental studies, we used the higher concentration to further avoid any possibility of variation caused by different positions on the steeper portion of a dose-response curve. Total cellular RNA from each time point was extracted and the level of GSH-Px mRNA transcripts determined by northern blot analysis as above. Data normalized to the 18S rRNA signal to correct for small differences in lane loading.

Statistical analysis was performed using paired t-tests analyzing the differences between uninduced and maximally induced cells in each set of independent experiments.
RESULTS

We used the HL-60 and PLB-985 cell lines as model systems for the maturation of phagocytic cells. Both are myeloid cell lines in which uninduced cells have the morphologic and functional properties of promyelocytes. Treatment of uninduced HL-60 or PLB-985 cells with phorbol esters such as PMA induces differentiation along the monocyte-macrophage pathway, whereas induction with a variety of agents, including polar solvents such as DMF, results in granulocytic differentiation.

As shown in Fig 1, we first examined GSH-Px expression as enzyme activity in HL-60 (upper panels) and PLB-985 cells (lower panels) induced to differentiate with DMF (left panels) and PMA (right panels). PMA induction of HL-60 and PLB-985 differentiation along the monocytic pathway increases enzyme activity 1.8- to 3-fold. DMF induction of differentiation along the granulocytic pathway results in more modest, but still 1.2- to 2-fold, increases in GSH-Px activity. For both induction agents and cell types, the increases in enzyme activity were statistically significant (P ≤ .05) by paired t-tests analyzing the differences between uninduced and maximally induced cells in a set of independent experiments.

Table 1. 75Se Incorporation Into GSH-Px Protein During Induced Differentiation of HL-60 and PLB-985 Cells

<table>
<thead>
<tr>
<th>Induction Agent</th>
<th>Day</th>
<th>PMA</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.73 ± 0.40</td>
<td>2.39 ± 0.92</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.71 ± 0.81</td>
<td>4.72 ± 3.48</td>
</tr>
<tr>
<td>PLB-985</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.84 ± 0.82</td>
<td>1.49 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.94 ± 1.27</td>
<td>1.87 ± 0.96</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>ND</td>
<td>2.16 ± 0.91</td>
</tr>
</tbody>
</table>

Results are presented as mean ± SEM of ratios of densitometric units for induced cells relative to uninduced cells in autoradiographs of SDS-PAGE gels of immunoprecipitated GSH-Px protein in three independent experiments.

Abbreviation: ND, not determined.

In simultaneous assays, we also measured 75Se incorporation into immunoprecipitable GSH-Px. Table 1 presents a compilation of data from autoradiographs such as that illustrated in Fig 2 (note that the exposures of the autoradiographs are designed to show the relative change within each group; the baseline and induced values are not comparable between
Induction of GSH-Px mRNA expression in HL-60 cells. The autoradiograph shows Northern blots of total cellular RNA extracted from HL-60 cultures (either undifferentiated or exposed to the indicated inducing agents for the indicated number of days) and successively probed with cDNAs that are complementary to human GSH-Px or 18S rRNA, as indicated.

Fig 3. Induction of GSH-Px mRNA expression in HL-60 cells. The autoradiograph shows Northern blots of total cellular RNA extracted from HL-60 cultures (either undifferentiated or exposed to the indicated inducing agents for the indicated number of days) and successively probed with cDNAs that are complementary to human GSH-Px or 18S rRNA, as indicated.

Steady-state levels of GSH-Px gene transcripts, shown in Fig 3 and Table 2, also increase in response to induction of granulocytic or monocytic differentiation. The changes approximately parallel the induction of GSH-Px protein synthesis and are similarly somewhat greater in magnitude than the changes in enzyme activity, which are damped by the high baseline levels and relative stability of the enzyme molecules.

To further investigate the mechanism of this increase in GSH-Px transcripts, we examined rates of mRNA transcription and decay. Nuclear run-on transcription assays, such as the one illustrated in Fig 4, show a modest increase in the rate of GSH-Px mRNA transcripts after treatment of HL-60 cells (top panel) or PLB-985 cells (bottom panel) with either PMA (left column) or DMF (right column). The change is not as great as that noted for the genes encoding gp91-phox or TNFα, both of which undergo pronounced up-regulation during myeloid or monocytic differentiation. Quantitative measurement by densitometry of autoradiographs, with normalization to mRNA synthesis from the constitutive gene Kal tubulin, shows that the gene encoding GSH-Px undergoes transcriptional up-regulation in response to both in-
ducers in each cell line. HL-60 cells show ratios of induced to uninduced levels of GSH-Px transcription of 2.24 ± 1.05 with PMA induction and 1.86 ± 0.88 with DMF; ratios for PLB-985 cells are 1.34 ± 0.11 with PMA and 3.46 ± 0.16 with DMF. The up-regulation was statistically significant at P = 0.05, by paired t-test, for all cell and inducer combinations except HL-60 cells with DMF, which showed a trend at P = 0.12. Data are expressed as means ± SEM of four independent experiments for HL-60 and three experiments for PLB-985 (including, among others, those illustrated in Fig 4).

The slightly lesser change in transcription rates relative to steady-state mRNA levels could reflect a lack of quantitative comparability of the assays or the effect of changes in mRNA stability. To test the latter possibility, we examined levels of GSH-Px mRNA after incubation of HL-60 cells with DRB, a nucleotide analog that specifically inhibits initiation of transcription by RNA polymerase II and, hence, rapidly and virtually completely halts synthesis of most species of mRNA transcripts, but preserves production of ribosomal RNA. The calculated half-lives (expressed as means of duplicate experiments) of GSH-Px mRNA in uninduced and in PMA- or DMF-differentiated HL-60 cells are 3.8, 3.0, and 2.4 hours, respectively; GSH-Px transcript half lives in uninduced and in PMA- or DMF-differentiated PLB-985 cells are 2.8, 3.1, and 4.3 hours, respectively.

DISCUSSION

GSH-Px provides most eukaryotic cells with a primary defense against hydrogen peroxide and related toxic oxidants. Antioxidant defense becomes particularly important to differentiating phagocytic cells as the antimicrobial respiratory burst enzyme system matures and becomes capable of generating very high local concentrations of peroxide. Despite GSH-Px and other antioxidants, stimulated granulocytes eventually succumb to auto-oxidation, leading to termination of the respiratory burst or cell death. The present studies show that GSH-Px activity increases during in vitro induced monocytic or granulocytic differentiation of the HL-60 and PLB-985 myeloid cell lines and that the increased expression of the cellular GSH-Px gene occurs at least in part through transcriptional up-regulation.

GSH-Px belongs to an unusual group of prokaryotic and eukaryotic enzymes that contain the unusual amino acid selenocysteine within the active site. All incorporate the selenocysteine cotranslationally at a UGA codon that functions in other gene transcripts as a termination signal. Most research on GSH-Px gene expression has focused on the effects of exogenous selenium, both in vivo and in vitro, and has shown regulation at the levels of translation and mRNA stability. For example, studies of GSH-Px regulation in the liver of rats fed a selenium-deficient diet have shown the expected dramatic decrease in enzyme activity accompanied by a smaller but progressive decrease in steady-state GSH-Px mRNA levels, but without any significant decrease in transcription of the GSH-Px gene. Other studies have reported either little change or, more often, a marked decrease in liver GSH-Px mRNA levels with selenium deficiency. In the more controlled in vitro environment, human hepatoma cell lines exhibit a hierarchy of greater change in enzyme activity than steady-state mRNA, which is, in turn, more affected than the GSH-Px gene transcription rate. We have found similar results in selenium-depleted HL-60 cells, in which GSH-Px enzymatic activity falls 30-fold and immunoreactive protein levels correlate with enzymatic activity, but GSH-Px mRNA levels fell only 1.2- to 2.3-fold and the rate of gene transcription did not change significantly. Altogether, these data indicate that selenium controls GSH-Px expression in a variety of cell types by means of posttranscriptional mechanisms, primarily at the level of translational incorporation of selenocysteine, but also by effects on mRNA stability, such as a decline in mRNA half-life in the absence of translation.

Thus, our previous studies with the HL-60 cell line have indicated translational regulation by exogenous selenium, but the present data indicate that regulation during myelomocytic differentiation is a more complex process and includes a major contribution by transcriptional control. The human cellular GSH-Px gene appears to be subject to different mechanisms of molecular regulation, depending upon the conditions leading to the changes in gene expression. The less common translational regulatory mechanism derives from the requirement for the micronutrient selenium, which is needed to convert the unique phosphoserine-charged tRNA to selenocysteinyl-tRNA, in the synthesis of selenoproteins such as GSH-Px. However, during the coordinated regulation of the many genes involved in the terminal differentiation of a promyelocyte to a mature monocyte or granulocyte, transcriptional control is the more common rule, to which GSH-Px is no exception.

The magnitude of the change in GSH-Px transcription is less than that observed for genes such as gp91-phox, a component of the phagocyte oxidase that is expressed only upon induction of differentiation, and more like that of p22-phox, another oxidase component that is constitutively expressed and, therefore, shows proportionally less change when up-regulated during myeloid maturation. That is, the denominator for a ratio of gene expression for induced versus uninduced cells will always be greater for a constitutively transcribed gene, so the relative increase in expression will appear to be less than that for an entirely differentiation-dependent gene with a very low baseline level of expression. GSH-Px expression in these myeloid cell lines also undergoes some regulation by changes in mRNA stability. In PLB-985 cells, mRNA stability increases with differentiation, and thus, contributes to the elevation of steady-state levels of GSH-Px transcripts. In HL-60 cells, the half-life decreases upon induction, particularly with PMA, and probably limits the change in steady-state transcript level to the relatively modest increase observed. The paradoxical opposition of the changes in rates of transcription and degradation serve as a reminder that they are assayed with such different conditions and techniques that the data can be used to compare relative changes within a given measurement, but not for quantitative calculation of the resultant steady-state level of the mRNA product. Similarly, the rates of protein synthesis and levels of enzyme and transcript can not be compared with great precision. They are sufficiently concordant to indicate no
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major contribution of translational control (such as that seen with selenium regulation of GSH-Px expression), but this mechanism may play a minor role in the setting of induced differentiation.

In the larger context of cell physiology, the current study shows a lesser change in antioxidant defense relative to the well-established, major increase in the capacity for oxidant generation in developing phagocytes. This paradoxical discordance may play a physiologic role in the enhancement of intracellular microbialicidal activity and the limitation of extracellular tissue damage. Similar findings have been observed in mouse macrophages activated in vivo and tested for their ability to kill the intracellular parasite Toxoplasma gondii. The antioxidant protective enzymes, including GSH-Px as well as catalase and superoxide dismutase, protected both the parasite and the host cell from peroxide; killing was most effective in macrophages activated by lymphokines that enhanced peroxide generation, but not antioxidant defenses. Furthermore, limitation of neutrophil activity by auto-oxidation may serve as an important negative feedback mechanism in vivo. Consequently, the paradoxical constraint in the up-regulation of antioxidants could help control the level of damage to normal tissues during acute inflammation by causing the phagocytes to auto-oxidize, and thus, terminate respiratory burst function before release of excessive oxygen products that would damage neighboring cells.

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