Inactivation of Glutathione Peroxidase Activity Contributes to UV-Induced Squamous Cell Carcinoma Formation

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Abstract

Cutaneous squamous cell carcinomas (CSCC) are a common malignancy of keratinocytes that arise in sites of the skin exposed to excessive UV radiation. In the present study, we show that human SCC cell lines, preneoplastic solar keratoses (SK), and CSCC are associated with perturbations in glutathione peroxidase (GPX) activity and peroxide levels. Specifically, we found that two of three SKs and four of five CSCCs, in vivo, were associated with decreased GPX activity and all SKs and CSCCs were associated with an elevated peroxide burden. Given the association of decreased GPX activity with CSCC, we examined the basis for the GPX deficiency in the CSCCs. Our data indicated that GPX was inactivated by a post-translational mechanism and that GPX could be inactivated by increases in intracellular peroxide levels. We next tested whether the decreased peroxidase activity coupled with an elevated peroxidative burden might contribute to CSCC formation in vivo. This was tested in Gpx1+/− and Gpx2+/− mice exposed to solar-simulated UV radiation. These studies showed that Gpx2 deficiency predisposed mice to UV-induced CSCC formation. These results suggest that inactivation of GPX2 in human skin may be an early event in UV-induced SCC formation. [Cancer Res 2007;67(10):4751–8]

Introduction

Cutaneous squamous cell carcinoma (CSCC) is a common and sometimes fatal malignancy arising from transformed keratinocytes of the epidermis. Although excessive exposure to UVA/UVB irradiation is considered to be the main etiologic factor in the development of CSCC, there is mounting evidence that infection sometimes fatal malignancy arising from transformed keratinocytes of the epidermis. Although excessive exposure to UVA/UVB irradiation is considered to be the main etiologic factor in the development of CSCC, there is mounting evidence that infection sometimes fatal malignancy arising from transformed keratinocytes of the epidermis. Although excessive exposure to UVA/UVB irradiation is considered to be the main etiologic factor in the development of CSCC, there is mounting evidence that infection
GPx2 protein expression in...tumor promoters (14); and...EGF receptor phosphatase resulting in constitutive EGF receptor phosphorylation and activation of this pro-proliferative signaling pathway to adducts of proteins, lipids, and DNA (21);...tert-butylhydroperoxide (700 μmol/L final concentration), H₂O₂ (500 μmol/L final concentration), the catalase inhibitor aminotriazole (5 μmol/L final concentration; ref. 29), the peroxidase mimetic eosin (100 μmol/L final concentration; ref. 30), and the antioxidant rt-α-tocopherol (vitamin E; 1 mmol/L final concentration; ref. 22).

RNA isolation and real-time PCR. Total RNA was isolated using Trizol reagent (27) and single-strand cDNA was synthesized (3). Real-time PCR used Platinum SYBR Green qPCR SuperMix UDG (Invitrogen), reactions were amplified on a Rotor-Gene real-time PCR machine, and the data were analyzed using the Rotor-Gene (version 6) software (Corbett). The following primers were used: GPX1, ACAGATGTGCCTGAACACTT (forward) and TCGATGTCAATGGTCTGGAA (reverse); GPX2, TAAGTGGCTCAGCCTCTCTC (forward) and GTGCAAGACGAGCTGGA (reverse); GPX3, ACAGGAAGAGCTGACCCAT (forward) and CTCTGTTCTCGTTTCCCA (reverse); GPX4, CAGTGGACGAAAGC-GAAGT (forward) and CTGCTTCCCCAAGTTGTTAC (reverse) and Actin, GGACCTGACTGACTACCTCA (forward) and AGCTTCTTAAATGGTCAG (reverse). PCR amplifications were done under the following conditions: denaturation at 94°C for 5 s, annealing at 55°C for 10 s, and extension at 72°C for 15 s.

Tissue collection. In the mouse studies, epidermal sheets were isolated from wild-type (WT) or Gpx1⁻/⁻/Gpx2⁻/⁻ DKO mice (5) as described (23–25). For human tumor studies, resected tumors were taken from patients (with consent) and the classification of the lesions [i.e., solar keratoses (SK) or SCC] was made by the staff pathologist. The epithelial sheets were removed (23, 27), and GPX activity assays (Bioxytech GPX-340, Oxis International), Western blots, and real-time PCR were done.

Antibody production and Western blotting. Isoform-specific rabbit polyclonal antibodies against the COOH-terminal peptides of human GPX1 (IEMPDAISQG) and human GPX2 (CDIKRLLKVAI) were generated. Peptides were synthesized and coupled to keyhole limpet hemocyanin (Auspep). Rabbit polyclonal antisera were then generated (Institute of Medical and Veterinary Sciences, Adelaide, South Australia, Australia) and tested for cross-reactivity against purified GPX1 and GPX2 peptide (data not shown). Antisera were frozen down in aliquots and used in Western blots as crude antisera at a 1:1,000 dilution. For Western blotting, 20 to 40 μg of protein samples were fractionated by SDS-PAGE on a 12% acrylamide gel. For tissue samples, epithelial/tumor sheets were isolated following dispase digestion (23, 27). The samples were then placed directly into 200 μL reducing buffer [7% glyceral, 0.5 mmol/L EDTA, 1 mmol/L phenyl-methylsulfonyl fluoride, 2% SDS, 10 mmol/L DTT, 60 mmol/L Tris-HCl (final pH 6.8)] and boiled for 5 min before freezing at −80°C. For tissue culture cells, the cells were trypsinized and placed directly into reducing buffer, boiled for 5 min, and frozen at −80°C. Immunodetection protocols have been described previously (31, 32), and normalization for loading equivalence is provided by immunodetection of α-tubulin levels (T9026; 13,000; Sigma-Aldrich) or amido black staining.

Peroxide activity and peroxide level measurement. The GPX activity assay measures GPX1 and GPX2 activity. Briefly, epithelial sheets or cells were homogenized in sample buffer [50 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 1 mmol/L mercaptoethanol] and spun at 2,000 x g for 10 min (4°C). The supernatant was then collected and frozen at −80°C until used in the GPX assay. The GPX assay measures NADPH disappearance in the presence of glutathione reductase and GSH using tert-butylhydroperoxide as substrate (BioxyTech). In experiments testing the ability of peroxides to inactivate GPX, we used an in vitro reconstitution assay comprising purified RBC GPX (G4013; Sigma-Aldrich) at a final concentration of 50 milliunits/mL in the GPX assay buffer. To this was added Pro⁷⁹⁹Leu polymorphism that is associated with reduced GPX activity (19, 20); (c) peroxide levels are increased following acute exposure to damaging levels of UV irradiation and tumor promoters; (d) peroxides are generated as intermediates during the metabolism of known carcinogens; (e) peroxides are precursors to adducts of proteins, lipids, and DNA (21); (f) the production of the highly reactive hydroxyl radical following UV exposure is a direct consequence of increased peroxide levels (22); (g) certain organic peroxides (e.g., benzoyl peroxide and tert-butylhydroperoxide) are known tumor promoters (14); and (h) peroxides inhibit the EGF receptor phosphatase resulting in constitutive EGF receptor phosphorylation and activation of this pro-proliferative signaling pathway (13). These data would support the hypothesis that GPX enzymes are tumor protective. However, data implicating disturbed peroxide metabolism with human cancers have not been reported to date. In the present study, we examine the regulation and expression of GPX isoforms in human skin and examine whether inactivation of GPX activity may be a contributory factor in the development of UV-induced CSCC.

Materials and Methods

Tissue culture. Normal human keratinocytes (HEKs) were isolated and cultured from neonatal foreskins following circumcision as described (23–25). The maintenance and culture of the epidermal SCC cell line Colo-16 and the tongue-derived SCC cell line SCC25 have also been described previously (26, 27). Due to a deficiency of selenium in the culture medium, the SCC25 and Colo-16 cells were grown in medium supplemented with 50 mmol/L sodium selenite (28). In some instances, cells were treated with tert-butylhydroperoxide (700 μmol/L final concentration), H₂O₂ (500 μmol/L final concentration), the catalase inhibitor aminotriazole (5 μmol/L final concentration; ref. 29), the peroxidase mimetic eosin (100 μmol/L final concentration; ref. 30), and the antioxidant rt-α-tocopherol (vitamin E; 1 mmol/L final concentration; ref. 22).

Figure 1. GPX1 and GPX2 expression is increased in SCCs in vitro and in vivo. RNA was isolated from (A) proliferating (P) or confluent (C) HEKs, Colo-16, or SCC25 cells or from (B) normal epidermis (skin), one patient, preneoplastic SKs (three patients), or neoplastic SCCs (seven patients). Quantitative real-time PCR was done for the expression of GPX1, GPX2, GPX3, and GPX4 isoforms. All samples are normalized for actin expression. Columns, mean of triplicate determinations for each sample; bars, SE. Western blot analysis of GPX1 or GPX2 protein expression in (C) confluent cultures of HEK, Colo-16, or SCC25 cells or (D) in vivo in foreskin, SKs, or SCC. Expression levels visualized by chemiluminescence. The expression level for α-tubulin is shown to allow for loading inequalities in (A) and by amido black staining in (B). The numbers in (D) correspond to the same numbered samples in Figs. 2 and 3.
770 μmol/L tert-butylhydroperoxide for differing times and the GPX activity was subsequently measured. A similar set of experiments was also conducted using HEK extracts as the GPX donor.

Intracellular peroxide levels in cultured keratinocytes or the SCC cell lines Colo-16 and SCC25 were estimated using either dihydrorhodamine 123 or 5,6-chloromethyl-2,7’-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA; Molecular Probes) using a FACS Calibur (Becton Dickinson). Total peroxide levels in normal or tumor epithelial tissue were estimated by measuring horseradish peroxidase–mediated oxidation of phenol red in tissue samples as described (33).

KO mouse strains and UV induction protocol. We have previously generated Gpx1–/– and Gpx2–/– mice (C57Bl/6J and 129S background; ref. 5). The mice were housed under gold lighting (GEC F40GO tubes) that does not emit UVB radiation. The solar-simulated UV (SSUV) radiation source and protocol have been described (34). For the first week, mice received a daily dose of 0.5 × minimal erythemal daily dose (MED), and for the next 19 weeks, 1.0 × MED daily. At this time, the daily SSUV dose was increased weekly to a final dose in weeks 23 to 27 of 2.5 × MED. Tumor promotion was then accelerated by the topical application of 0.1 mL 0.01% (v/v) croton oil (Sigma-Aldrich)/acetone solution twice weekly for 10 weeks. Tumors began to appear at weeks 35 to 36, and the croton oil applications were discontinued. Each mouse was examined for tumor appearance, and tumors of diameter greater than ~1 mm were mapped and counted weekly. At the end of the study, pieces of tumor were collected routinely for histopathologic analysis.

Significance of the differences between the genotypes was assayed by the Mantel-Haenszel log-rank assay for tumor incidence and by the balanced ANOVA assay and the Kruskal-Wallis test (34).

**Results**

**GPX2 mRNA is selectively induced in SCCs in vitro and in vivo.** Analysis of mRNA expression levels for GPX1, GPX2, GPX3, and GPX4 by real-time PCR using RNA derived from proliferative or nonproliferative/differentiated cultures of HEKs, SCC25, or Colo-16 cells indicated that only GPX2 was consistently and significantly increased in the SCC cell lines (Fig. 1A). This elevation was independent of whether the cells were proliferative/undifferentiated or nonproliferative and differentiated. GPX1 mRNA expression was slightly elevated in the Colo-16 cells, whereas GPX3 mRNA expression was elevated in the SCC25 cells and confluent HEKs but not in the Colo-16 cells (Fig. 1A). GPX4 mRNA was modestly elevated in the Colo-16 cells and, to a lesser extent, in the SCC25 cells (Fig. 1A). We next determined whether our in vitro findings could be validated in patient lesions. We took three precancerous SKs and seven CSCCs from patients and found that, in all three SKs and in six of seven SCCs, there was a significant and selective increase in GPX2 mRNA (Fig. 1B). A modest increase in GPX1 and GPX4 mRNA was also evident in most lesions (Fig. 1B). These data...
indicate that the GPX2 mRNA is expressed in keratinocytes and that its expression is inducible. These data also show that preneoplastic and neoplastic skin lesions are associated with altered expression of GPX isofoms.

**GPX activity is reduced in SCCs in vitro and in vivo.** To examine GPX protein expression levels, we focused on GPX1 and GPX2 because earlier studies have shown that GPX3 is a secreted isoform (6) and GPX4 has a very restricted substrate preference (8, 9). For these reasons, GPX3 and GPX4 are unlikely to contribute to the intracellular peroxidase activity of HEKs and SCC cells. Western blot analysis of GPX1 and GPX2 expression in HEKs and SCC cell lines indicated that both GPX1 and GPX2 protein were present and that there was a modest increase in GPX1 expression in the cancer cell lines compared with normal keratinocytes (Fig. 1C). The induction of protein expression in the cell lines was clearly not as great as for the mRNA induction, which suggests that GPX1 and GPX2 may be subject to complex transcriptional, posttranscriptional, translational, and/or post-translational processing. In patient SCCs, the levels of protein expression more closely reflected the mRNA expression profiles. GPX1 was not noticeably induced in preneoplastic SKs but was profoundly elevated in SCCs compared with foreskin samples (Fig. 1D). Similarly, GPX2 protein expression was low in skin and SKs but was elevated in three of four SCCs (Fig. 1D). Because the assays used only the epithelial component of the tumors, we can exclude the effects of any underlying interstitium. It should be noted that the SCC and SK sample numbering in Figs. 2, 3, and 4 relates to the same patient sample throughout. We next examined whether the changes in mRNA and protein expression, observed in vitro and in vivo, were reflected by alterations in GPX activity. Figure 2A shows that the GPX activity in SCC25 and Colo-16 cells was significantly reduced (approximately 75% and 50%, respectively) relative to the HEK control. Similarly, we found that two of three SKs and four of five SCCs had significantly reduced GPX activity compared with normal epidermis (Fig. 2B). Conversely, one of three and one of five SKs and SCCs had elevated GPX activity, respectively (Fig. 2B). These data indicate that, although GPX1 and GPX2 mRNA and protein are frequently induced in SCCs, this is not reflected in a corresponding increase in GPX activity. Indeed, CSCC patients 1, 3, and 5, in which GPX1 and GPX2 protein was most profoundly induced, had the most reduced GPX activity. Similarly, the GPX activity in two of three SKs is significantly less than in normal epidermis, yet the protein level remains similar. These data, plus the expression/activity data for the CSCCs, clearly show that GPX activity does not correlate with protein expression levels. It could be argued that the low levels of GPX1 and GPX2 protein expression in the epidermis or SKs indicate that they do not contribute to the GPX activity measured in the normal epidermis. However, we have found that GPX activity in WT mouse epidermis is 426 ± 41 milliunits/mg protein, whereas in Gpx1−/− × Gpx2−/− DKO mice (5) the activity is reduced to 23 ± 40 milliunits/mg protein. These data convincingly show that the combined activity of GPX1 and GPX2 is likely to account for almost all the GPX activity observed in the epidermis and that the inactivation of GPX is due to a profound

![Figure 4](https://example.com/figure4.png)

**Figure 4.** GPX2 deficiency predisposes mice to UV-induced SCC formation. WT (+/+; n = 16), Gpx1−/− (n = 13), and Gpx2−/− (n = 14) mice were subjected to SSUV protocol as described in Materials and Methods. Tumor incidence rates (A) or tumor multiplicity (B) was plotted as a function of time. *, P < 0.05, significantly different from WT mice. Statistical differences in tumor incidence between WT and Gpx mice were assessed by a Mantel-Haenszel test. Statistical differences in tumor multiplicity between WT and Gpx mice were assessed by the balanced ANOVA and Kruskal-Wallis test. C, gross appearance of tumors on the back of a mouse at the termination of the study. D, histologic section of a tumor at the end of the study. H&E stained. Magnification, ×12.
Figure 5. GPX enzyme activity is peroxide sensitive. A. HEKs were treated with 500 μmol/L H2O2, 700 μmol/L tert-butylhydroperoxide (TBH), or 5 mmol/L aminotriazole (AT) for 3 h. B. Colo-16 cells were left untreated (control) or were treated with 100 μmol/L ebselen or 1 mmol/L dl-α-tocopherol (Vit. E) for 48 h. GPX activity was then estimated. Columns, mean of triplicate samples from at least two experiments; bars, SE. *, P < 0.05, activities that are significantly different from the control group. A and B, numbers above the columns, relative peroxide level (percentage of control) for each condition as estimated by FACS determination of dihydrorhodamine 123 fluorescence. C, HEK extract was used as a source of endogenous GPX activity. To this 780 μmol/L tert-butylhydroperoxide was added for the times shown and then the GPX activity was measured. Columns, mean of triplicate determinations from two experiments; bars, SE. D, purified RBC GPX was used as source of GPX activity in a reconstitution assay in the presence of 780 μmol/L tert-butylhydroperoxide for the times shown. Columns, mean of triplicate determinations from two experiments; bars, SE.

A significant increase in progressive average tumor multiplicity in the Gpx2−/− mice (P < 0.0001) compared with WT control. In contrast, this analysis showed no significant difference (P = 0.16) between WT and Gpx1−/− mice. Analysis of the time from the start of the trial to the time when the tumors reach 1 cm in diameter showed there was no significant difference in the growth rates of tumors between the WT (48.1 ± 10 weeks) and Gpx1−/− (51.7 ± 14.6 weeks) and Gpx2−/− mice (52.4 ± 9.5 weeks). These data unequivocally show that Gpx2 deficiency predisposes mice to UV-induced SCC formation.

Intracellular peroxides regulate GPX activity. If GPX deficiency is a causative event in UV-induced SCC formation, then it becomes important to understand the mechanism underlying GPX inactivation in SCCs. Previous reports have shown that the exposure of keratinocytes to exogenously added peroxides can result in a reduction in GPX activity (29). If true, this would suggest that the reduction in GPX activity we see in the majority of SCCs in vitro and in vivo may be a direct result of the elevation in intracellular peroxide levels. To test whether this was plausible, we incubated HEKs with agents that are known to increase peroxide levels (i.e., H2O2, tert-butylhydroperoxide, and aminotriazole; Fig. 5A). These experiments were designed to test whether the elevation of peroxide levels in a normal keratinocyte could lead to the inhibition of GPX activity and thus leave cells vulnerable to oxidative damage. All of these treatments resulted in elevated peroxide levels (Fig. 5A). Neither H2O2 nor aminotriazole caused a reduction in GPX activity. However, the organic peroxide tert-butylhydroperoxide produced a profound reduction in GPX activity (Fig. 5A). In a further set of experiments, we treated the GPX-deficient Colo-16 cell line with the peroxidase mimetic ebselen or the antioxidant vitamin E (Fig. 5B). We found that treatment of Colo-16 cells with ebselen or vitamin E resulted in a reduction in peroxide levels that was accompanied by an increase in GPX activity.

decrease in the specific activity of the GPX1 or GPX2 isozymes. Thus, we show (a) that SCCs in vitro and in vivo possess significantly less GPX activity than their nontransformed counterparts, (b) that the reduction of GPX activity is not due to reduced GPX protein expression and, (c) that peroxidase activity is subject to post-translational regulation.

Reduced GPX activity is associated with elevated peroxide levels. We determined whether GPX deficiency would result in pathologic increases in the total intracellular peroxide burden in SCCs. Formal examination of this question indicated that the reduced GPX activity observed in SCC cell lines was associated with elevated peroxide levels compared with normal HEKs (Fig. 3A). Moreover, when we examined the peroxide levels in SCCs, we found that all the lesions were associated with increased peroxide levels (Fig. 3B). If one compares the same SCC patient sample number between the figures, it becomes very clear that GPX activity is inversely related to peroxide burden (Fig. 3C). Thus, reduced peroxidase activity is associated with an increased peroxidative burden in the patient lesions.

GPX2 deficiency predisposes mice to UV-induced SCC formation. To determine whether GPX deficiency could be a causative factor in UV-induced SCC formation, we examined the ability of chronic low-dose UVA and UVB exposure to induce SCC in WT mice or Gpx1−/− or Gpx2−/− mice (Fig. 4A–D). We selected low-dose UVA and UVB irradiation protocols similar to those implicated in CSCC formation in humans (i.e., SSUV irradiation; ref. 34). Analysis of tumor incidence by the Mantel-Haenszel test showed a highly significant increase in the tumor incidence in the Gpx2−/− mice (P < 0.001; n = 14) compared with the WT control mice (n = 16; Fig. 4A). In contrast, there was no significant difference (P > 0.05) in tumor incidence between Gpx1−/− mice (n = 13) and WT control mice (Fig. 4A). Similarly, balanced ANOVA and the Kruskal-Wallis test for tumor multiplicity revealed a significant increase in progressive average tumor multiplicity in the Gpx2−/− mice (P < 0.0001) compared with WT control. In contrast, this analysis showed no significant difference (P = 0.16) between WT and Gpx1−/− mice. Analysis of the time from the start of the trial to the time when the tumors reach 1 cm in diameter showed there was no significant difference in the growth rates of tumors between the WT (48.1 ± 10 weeks) and Gpx1−/− (51.7 ± 14.6 weeks) and Gpx2−/− mice (52.4 ± 9.5 weeks). These data unequivocally show that Gpx2 deficiency predisposes mice to UV-induced SCC formation.
activity (Fig. 5B). These data clearly show (a) that GPX activity can be modulated directly/indirectly by conditions that alter peroxide levels, (b) that the reduced GPX activity observed in SCC cells can be reversed by reducing the peroxidative/oxidative burden of the cell, (c) that the elevated peroxidative burden observed in the SCC cells could contribute to the reduction in GPX activity observed in the SCCs in vitro or in vivo, and (d) that GPX1/GPX2 may be subjected to redox-sensitive post-translational regulation.

We next examined whether the ability of organic peroxides to reduce GPX activity was a direct or indirect effect of tert-butylhydroperoxide addition. Figure 5C shows that the addition of tert-butylhydroperoxide to HEK extracts did not result in GPX inactivation (Fig. 5C). However, if the HEK extracts were preincubated with tert-butylhydroperoxide for 60 min, there was a significant reduction in GPX activity (Fig. 5C). These data are consistent with peroxide-mediated inactivation being mediated by an indirect mechanism. Peroxides are chemically unstable, and so we examined whether the indirect inhibition of GPX activity could be due to the nonenzymatic degradation of tert-butylhydroperoxide to a reactive intermediate. To test this, we repeated the experiments in Fig. 5C using purified GPX1 protein rather than a HEK extract. These experiments gave similar results (Fig. 5D) to those obtained using the HEK extracts. These data clearly show that GPX activity can be inactivated indirectly by organic peroxides and that this inhibition can result from the nonenzymatic degradation of organic peroxide.

Discussion

**GPX deficiency and UV-induced CSCC formation.** The present study has shown that human CSCCs are associated with frequent reductions in GPX activity combined with a concomitant elevation in intracellular peroxide levels. In addition, our data indicate that GPX2 inactivation acts as a predisposing factor for UV-induced CSCC formation in vivo. These data indicate that GPX2 inactivation should join a growing list of genetic and biological defects affecting important regulatory pathways that have been implicated in the formation of CSCCs. These defects commonly target regulatory molecules involved in the p16-cyclin D-RB-E2F axis (26, 35–37), the EGFrec-ras-extracellular signal-regulated kinase pathway (3, 35, 38), the p53-DNA repair pathways (39, 40), or telomerase activity (35). The present study now provides the first direct evidence that disruptions to peroxide and ROS metabolism are direct contributors to UV-induced SCC. Such a conclusion is entirely consistent with earlier studies, which showed that elevated peroxide levels and peroxide-derived reactive intermediates were associated with UV-induced skin cancers (14, 21) and that antioxidants, such as vitamin E, can inhibit UV-induced CSCC formation (41, 42). Although UV irradiation is known to induce elevations in ROS and the ROS-dependent 7,8-dihydroxyguanine DNA adduct in skin and CSCC, there is growing acceptance that this is predominantly due to the UVA wavelength component of UV irradiation (35, 43–45). This suggests that the contribution of GPX2 deficiency to UV-induced CSCC may be restricted to the effects of UVA wavelengths. This would also suggest that, although the GPX2 enzyme affords protection from UVA damage, it may not be protective against UVB-induced effects (43) on CSCC formation. Interestingly, UVA and UVB are known to modulate local and systemic immune function (41, 45). Thus, the predisposition of Gpx2-deficient mice to UV-induced carcinoma formation may be attributable to defects in the keratinocytes as well as other cell types. This certainly is the case with inflammation-induced intestinal carcinomas in Gpx1/Gpx2-deficient mice (5).

The finding that the GPX2 enzyme plays a specific role in protecting the epidermis from UV-induced CSCC formation raises the obvious question of what is the function of GPX2 in the epidermis. The weight of evidence would suggest that differences between GPX isoform functions are likely to be dictated by differences in substrate specificity or subcellular location of the enzyme. GPX1 is localized to the cytosol and mitochondria, whereas GPX2 is restricted to the cytosol (4, 15), suggesting that differences in subcellular localization are not responsible for the GPX2-specific functions. In contrast, there is no data relating to the identification of endogenous substrates for GPX2 or GPX1. We do
know they are both capable of transforming H$_2$O$_2$ or model organic peroxide substrates, but the identification of the physiologically or pathologically relevant substrates for GPX2 is unknown. Clearly, the identification of such substrates will give important insights into pathologically relevant substrates for GPX2 is unknown. Clearly, the role of GPX2 as a tumor protective enzyme in skin.

**Mechanism of post-translational inactivation of GPX.** Because GPX inactivation is associated with CSCC formation, it is of considerable interest to identify how the GPX enzymes are inactivated in CSCC. In this regard, we know that GPX inactivity is not due to mutations in the GPX2/GPX1 transcripts (data not shown) nor to selenium deficiency (all cells cultured in Se$^{2-}$-containing medium). We also showed that GPX transcription and translation is disrupted in SKs and CSCCs but does not contribute to the inactivation of GPX activity in SKs and CSCCs. In contrast, the inactivation of GPX activity observed in CSCCs and SCC cell lines clearly involves a post-translational mechanism and is likely to be oxidation/peroxidation dependent. For example, it has been previously reported that GPX activity in keratinocytes can be reduced by exogenous organic peroxides but not by inorganic peroxides (29). Moreover, cysteine-containing and selenocysteine-containing enzymes are generally considered to be vulnerable to electrophilic attack (46, 47). Our data extend these earlier observations by showing that elevations in organic peroxide levels can decrease GPX activity and, conversely, that reduction of intracellular peroxide levels can increase endogenous GPX activity. This reversibility would allow for prophylactic pharmacologic interventions in high-risk patient groups (e.g., immunosuppressed patients) to try and prevent CSCC formation. In this regard, the earlier reports that vitamin E can inhibit UV-induced SCC formation are entirely consistent with the results of the present study and also support the thesis that an increased peroxidative burden may contribute to UV-induced SCC formation (41, 42).

Of particular relevance to the *in vivo* situation is the observation that the reduced GPX activity *in vivo* can be reversed by agents that reduce the oxidative/peroxidative burden. We believe this reversibility is likely to be at the level of nascent protein synthesis for the following reasons. GPXs have been reported to exist in three forms: (a) a reduced active form, (b) a reversibly inactive form, or (c) an irreversibly inactive oxidized form (48). Therefore, the incubation of the cancer cells with ebselen or vitamin E would provide a reducing environment in which the oxidation and irreversible inhibition of newly synthesized (preexisting irreversibly inhibited) GPX1 or GPX2 would be prevented. This would seem to be the case, *in vitro* and *in vivo*, because if the inhibition of the GPX activity were reversible, then it would be reversed under the reducing conditions used in the assay. This implies that the inactivation of GPX activity observed in the SCC cells is most likely to be irreversible in nature and that the only way to recover activity is for newly synthesized GPX protein to be made in a reducing environment (i.e., in the presence of antioxidants).

Taken together, our data support a model (Fig. 6) in which normal keratinocytes are able to maintain intracellular peroxide levels via GPX. However, if peroxides are elevated, in response to stressors, the elevated peroxides then induce GPX2 mRNA (data not shown) or, if sufficiently high, stimulate apoptosis. In this way, the cells are protected from peroxide-mediated macromolecular damage. However, if the stress stimuli occur in a cell with defective apoptotic responses (e.g., p53 mutation), then the elevated peroxide levels could lead to the inactivation of GPX activity and the further elevation in damaging reactive intermediates. In this way, it can be seen that an overwhelming carcinogenic insult may lead to a cycle of events in which the protective enzymes may be chronically “inactivated” by an overwhelming peroxidative burden. This would suggest that defects in apoptosis (e.g., p53 mutation) are an early event in keratinocyte transformation followed by disruption to peroxide metabolism in preneoplastic lesions (e.g., SKs). This latter event would make cells vulnerable to peroxidative/oxidative damage and subsequent genetic and epigenetic damage. Such a sequence of events is observed in nontumorigenic p53-deficient HaCaT cells, which can be fully transformed following exposure to oxidative stress (49).

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**References**

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