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Effects of oxygen on the antioxidant responses of normal and transformed cells

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Abstract

Basal antioxidant defense levels are often aberrant in tumor cells; however, less attention has been given to differences in the way that normal and transformed cells respond to changes in oxidative stress. This study evaluated differences in the responses of various normal and transformed cell lines to different oxygen tensions. Exposure to hyperoxia generally failed to induce either the activity of GSH peroxidase (GPx) or the manganese-containing form of superoxide dismutase (MnSOD) after 48 h, although at 605 mm Hg oxygen, small inductions of MnSOD activity were observed in adult lung fibroblasts and amelanotic melanoma. Exposure to 605 mm Hg O_2 for 48 h was inhibitory to GPx activity. MnSOD activity was strongly induced in virally transformed WI-38 cells by treatment with the herbicide paraquat or inhibition of GSH synthesis with BSO. In normal cells GSH concentration was proportional to ambient oxygen tension. Tumor cells exhibited greater GSH concentrations at low oxygen tensions than normal cells but were unable to increase GSH in response to elevation of oxygen tension. These results reveal differences in tumor and normal cell responses to changes in ambient oxygen tension and show that MnSOD activity is inducible when an appropriate stimulus is applied.

Keywords: Oxygen; Oxygen toxicity; Glutathione; Glutathione peroxidase; Superoxide dismutase; Cancer; Fibroblasts; Epithelial cells

Introduction

Oxygen free radicals and other reactive oxygen species (ROS)[2] are believed to play a fundamental role in neoplastic transformation [1–4], and to increase the incidence of metastasis of some types of tumors [5,6]. Both tumor and normal cells contain antioxidant defenses that remove ROS. The superoxide dismutases (SOD) remove superoxide radicals ($O_2^{\cdot-}$) and produce hydrogen peroxide. Catalase and peroxidases remove H_2O_2 and produce water. Glutathione peroxidase (GPx) can also remove peroxidation products formed by the reactions of lipids with ROS. Cells also contain a number of nonenzymatic antioxidant defenses such as tocopherol, carotenoids, ascorbate, and glutathione [7,8] that remove free radicals by reacting directly with them, break chain oxidation reactions [9], and dissipate energy from singlet oxygen [10,11]. The equilibrium between oxidant generation and removal is ultimately manifested in the cellular redox environment [12].

Although exceptions can be found [13,14], antioxidant defense levels are often aberrant in tumor cells [1,3,15]. For example the activities of both copper/zinc and manganese-containing forms of SOD are relatively low in many types of tumor cells as compared with the normal cells from which they arose [1,5,16]. The importance of the loss of SOD, when it occurs, is not well understood; although, the manganese-containing form of superoxide dismutase (MnSOD) has been observed to suppress the transformed phenotype in some types of tumor cells [17,18]. Other antioxidant enzyme activities also tend to be diminished in some

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[2] Abbreviations used: BSO, L-buthionine-*S*,*R*-sulfoximine; GPx, glutathione peroxidase; GSH, glutathione; GSSG, oxidized glutathione; Lidru, Laboratory for Investigative Dermatology, The Rockefeller University; MnSOD, manganese-containing form of superoxide dismutase; SOD, superoxide dismutase. ROS, reactive oxygen species.

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types of tumor cells [3]. Additionally, malignant transformation is frequently associated with an elevation of cellular glutathione concentration [19].

The rate of oxidant generation has been reported to be greater in transformed cells than in normal cells [20–22]. However, the type of oxidants being generated and the mechanisms of generation remain unclear. Antioxidant enzymes such as SOD are inducible in normal cells, and increased exposure to O_2^- would be expected to stimulate SOD activity [23,24]. The relatively low levels of SOD observed in some tumor cells may suggest that the ability of these cells to respond to O_2^- is impaired or, alternatively, that the steady-state level of O_2^- is lower in tumor cells with low SOD activity even if the total oxidant concentration is elevated. The activities of other antioxidant enzymes are also frequently diminished in tumor cells as compared to normal cells [15,25]. Whether these decreases are a result of decreased substrate concentration or impaired capacity to respond to oxidative stress is unclear. There are currently few studies that have compared the stress responses of normal and tumor cells in a systematic way.

The present study was conducted to evaluate the capacity of normal and tumor cells to respond to oxidative stress. Specifically, we determined the activities of the manganese-containing form of SOD and GP× at 5, 20, and 95% oxygen. We also examined cellular GSH concentration at 6 different oxygen tensions. Although hyperoxia is a form of oxidative stress the underlying cause of oxygen toxicity may arise from causes other than superoxide. Therefore, we also compared the effects of hyperoxia to those induced by the superoxide-generating herbicide, paraquat, in a virally transformed cell line.

Methods

Cell lines

Lines established in our laboratory were maintained under 5% O_2 during the initial outgrowth and when cultures were expanded. Commercially obtained cell lines were established at 20% oxygen; however, upon receipt, these were transferred to a 5% O_2 environment until used. Human diploid embryonic lung fibroblast cells WI-38 were obtained from Dr. Vincent J. Cristofalo of the Lankenau Center for Medical Research (Wynnewood, PA). The human skin fibroblast culture used in this study was established from skin specimens taken from individuals we are following in a longitudinal aging study. The fetal skin fibroblast culture was obtained from the National Institute of Aging Cell Repository at the Institute for Medical Research (Camden, NJ). The keratinocyte culture was established from a human skin biopsy using the method described by Eisinger et al. [26] and melanocytes were obtained from Biowittaker (Walkersville, MD). A summary of the cell lines used in this study is presented in Table 1. All cell lines were at ≤ 40% lifespan completed when the experiments were performed.

Table 1
Cell lines examined

Cell line	Sex	Tissue		Age
WI-38	F	Lung	Fibroblast	12 FW*
AG04431	F	Skin	Fibroblast	15 FW*
AG02258	F	Lung	Fibroblast	46
Lidru 162	F	Skin	Fibroblast	94
Keratinocytes	F	Skin	Keratinocytes	36
Melanocytes	F	Skin	Melanocytes	34
SCC	F	Metastasized to lung	SCC	32
Lidru 80	F	Skin	Unpigmented Melanoma	58
Lidru 81	F	Skin	Pigmented Melanoma	49
C32TG	M	Skin	Amelanotic Melanoma	53

* FW, fetal weeks.

Cell culture

Fibroblasts and tumor cells were grown in Dulbecco's modified Eagle's medium [DMEM, 1 g/liter glucose: Gibco Laboratories, Grand Island, NY; see Ref. 27]. Keratinocytes were grown in keratinocyte medium (Biowittaker), which consisted of MCDB 153 supplemented with basic fibroblast growth factor and bovine pituitary extract. Melanocytes were cultured in a special melanocyte medium according to the directions of the manufacturer (Biowittaker). This medium was essentially identical to keratinocyte medium except that it was supplemented with phorbol 12-myristate 13-acetate (PMA). Immediately before use, the media was supplemented with L-glutamine (2 mm) (Flow Laboratories, Rockville, MD) and fetal bovine serum (10% vol/vol) (KC Biologicals). Antibiotics were not used. Cultures were grown at 37°C in 175 cm^2 sealed polystyrene flasks (Falcon Labware, Oxnard, CA) containing 90 ml medium [0.54 ml/cm^2; Ref. 28]. The cells were released from the plastic with trypsin (0.25%) (Flow Laboratories) in Ca^{2+}- and Mg^{2+}- free Hanks' balanced salt solution (M.A. Bioproducts, Walkersville, MD). After suspension in medium containing 10% FBS, the cells were counted and inoculated into appropriate vessels at a density of 10^4 cells/cm^2. A Coulter counter (Coulter Electronics, Hialeah, FL) was used for cell counts. We monitored for mycoplasma contamination by the methods of Schneider and Stanbridge [29] and by incubation of cells and media on selective agar (Flow Laboratories). All cultures were free of mycoplasma contamination by these criteria.

Maintenance of gaseous phase

To study the effects of ambient oxygen tension on antioxidant responses, cultures were maintained under gas mix-

Table 2
Oxygen tensions used in study

% Oxygen	Partial pressure	Actual* after 48 h	Actual % oxygen
0	0	8	1.1
5	38	36	4.7
20	152	129	17.0
35	266	227	29.8
50	380	323	42.5
95	722	605	79.6

* After correction for CO_2 and water vapor.

tures that contained 5% CO_2, various percentages of oxygen (5, 20, or 95%), and nitrogen to balance. Until enough cells were obtained to seed flasks at all of the required oxygen tensions, the initial stock cultures were grown without shaking in a sealed atmosphere of 5% CO_2, 5% O_2, and 90% N_2. At each subcultivation, flasks were equilibrated with analyzed and certified standard gas mixtures (Matheson Gas Products, East Rutherford, NJ) both before and after seeding. The cultures used for enzyme analysis were then transferred to one of three incubators (Heraeus, Tekmar Co., Cincinnati, OH) maintained electronically at the percentage oxygen tension used to equilibrate the flask. Cultures used for analysis of glutathione were transferred to six different incubators equilibrated to the appropriate oxygen tensions. Each incubator was equipped with a Clark oxygen electrode and a CO_2 thermal conductivity detector to maintain the desired percentages of atmospheric oxygen and carbon dioxide. Incubators were calibrated each day by withdrawing a sample of gas and measuring the partial pressure of oxygen and carbon dioxide with a blood gas analyzer (Model 113; Instrumentation Laboratory, Inc., Lexington, MA).

Design and exposure times

All cultures were seeded and permitted to attach to the flasks and grow for 96 h under a gaseous phase that contained 5% CO_2, 5% O_2, and 90% N_2. Culture flasks were then equilibrated with gas mixtures that contained the desired O_2 tension and transferred to incubators that also were maintained at the desired oxygen tension. Cultures used in enzyme studies were transferred to 5, 20, or 95% O_2 and harvested 48 h later. At that time the amount of oxygen remaining in the flasks was diminished due to metabolism and through the plastic of the culture vessel (see Table 2). We have observed that glutathione concentration is strongly affected by the growth state of cells. Therefore we permitted cells to grow for a shorter period of time in the cultures that were analyzed for glutathione; this ensured that cultures remained subconfluent at harvest. Cells used to analyze GSH concentration were seeded and allowed to grow at 5% O_2 for 72 h before transfer to different oxygen tensions. All of the cultures were analyzed 48 h after transfer to the different oxygen tensions examined (0, 5, 20, 35, 50, and 95% O_2, for final O_2 tensions see Table 2). In some studies 500 μM paraquat or 30 μM buthionine sulfoximine was added to culture media 72 h after cells were seeded. These cultures were maintained in a 20% oxygen environment and were harvested 48 h after addition of the chemicals to the medium.

Superoxide dismutase activity

Superoxide dismutase activity was determined by a modification of the positive assay of Misra and Fridovich [30]. Cells were suspended in 16.6 mM potassium phosphate buffer, pH 7.8, sonicated for 15 s, and then centrifuged at 20,000g for 30 min. The supernatants were dialyzed overnight against 50 mM potassium phosphate buffer, pH 70.0. A 100-μl aliquot of homogenate (containing between 100 and 200 μg protein) was added to a clean cuvette that contained 0.25 ml riboflavin (100 μM in 16.6 mM potassium phosphate buffer, pH 7.8); to this was added 1.65 ml of a 0.24 mM solution of o-dianisidine. The absorbance of the samples was determined; the cuvettes were then illuminated 2 min with two 15-W Sylvania black lights. The absorbance was read again immediately following illumination. The difference in absorbencies before and after illumination minus a blank was proportional to SOD activity. In order to determine MnSOD activity, homogenate was added to a reaction mixture that contained 1.5 mM KCN about 5 min prior to illumination. This amount of KCN is sufficient to eliminate at least 2 units of Cu/Zn SOD. This is far greater than the total SOD activity of most samples. We observed that the dianisidine assay was sensitive to the protein content of samples; this effect was linear at low protein concentrations, but undetected at higher protein concentrations. Therefore, an amount of bovine albumin equal to the average protein content of the samples was routinely added to all of the blanks. Also, the addition of cyanide (to distinguish SOD-2) significantly increases the background change in absorbance; however, the color change observed with 1 unit SOD-2 (minus the appropriate blank) was identical with or without cyanide. Hence, one unit of activity was taken to be the amount of SOD that yields a color change above background equal to the change observed in the blank used for total activity.

Glutathione (GSH + GSSG)

The level of total glutathione was determined by the cycling method of Tietze [31]. In this procedure, homogenate (deproteinized with 10% perchlorate and neutralized with K_3PO_4), GSSG reductase, NADPH and 5,5′-dithiobis-(2-nitrobenzoic acid) were mixed in a cuvette and the color change was monitored at 412 nm. A standard curve was constructed with known amounts of GSSG and used to estimate the concentration of glutathione present in the homogenates. Also, an attempt was made to determine GSSG concentration by first eliminating GSH with 2%

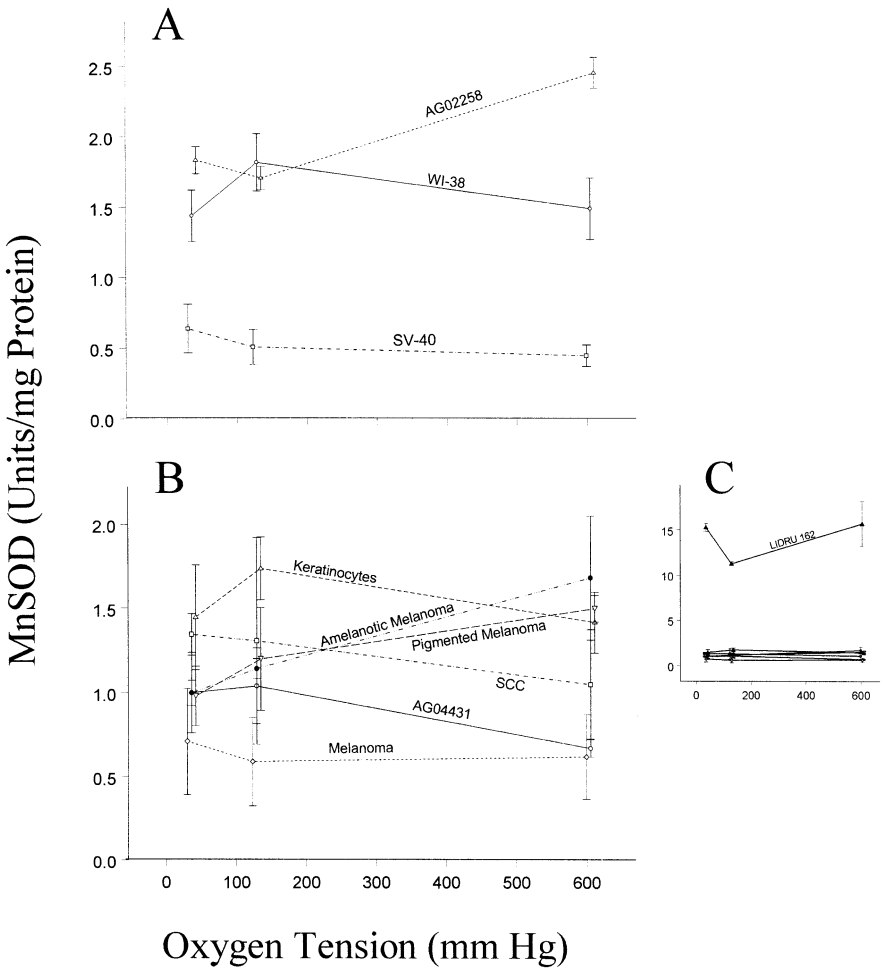

Fig. 1. MnSOD activity in normal and transformed cells. All points are the average of at least two experiments. Cells were seeded at 10,000 cells/cm^2 and plated under a gas mixture that contained 5% O_2. After 96 h, the gas phase of the flasks was replaced with one that contained 5, 20, or 95% O_2. All cultures were harvested 48 h after transfer to different oxygen tensions. The final oxygen tensions are presented in Table 2. (A) Cells derived from lung tissue. (B) Cells derived from skin tissue. (C) Same as B, but this figure includes LIDRU 162. This graph was presented separately to allow better resolution of lines in part B.

2-vinylpyridine according to the method of Griffith [32] and then repeating the above assay. However, the concentration of GSSG was low and often below the detection limits of the assay.

GSH peroxidase (GPx) activity

This assay couples the oxidation of GSH by GSH peroxidase to the oxidation of NADPH by GSH reductase. The assay mixture was modified from [33] and [34]. The assay mixture contained 50 mM Tris HCl, pH 7.6, 1 unit/ml GSSG reductase, 0.25 mM GSH, 0.2 mM NADPH, and 3 mM KCN to increase the stability of the assay mixture. The reaction was initiated by addition of 50 μl of a 12 mM H_2O_2 solution. The loss of NADPH was monitored at 340 nm. Although units of GPx activity are usually reported as μM NADPH/min, due to the relatively low GPx activity in fibroblasts, we report units of activity as nmol NADPH/min.

Statistics

Data were compared using analysis of variance (ANOVA). In all cases, $P \leq 0.05$ was inferred to indicate significance.

Results

Manganese SOD (MnSOD)

We examined MnSOD activity in 11 normal and transformed cell lines derived from lung and skin. MnSOD activity was largely unaffected by ambient oxygen tension during the period of exposure used in this study (Figs. 1A and B). The activity of AG02258 observed at an O_2 partial pressure of 605 mm Hg was significantly greater than the activity present in cells maintained under O_2 partial pressures of 36 mm Hg (ANOVA, $P = 0.009$) or 129 mm Hg

(ANOVA, $P = 0.002$). Additionally, hyperoxia stimulated MnSOD activity in the amelanotic melanoma line which also exhibited significantly greater activity at an O_2 partial pressure of 605 mm Hg than at oxygen tensions of either 31 mm Hg (ANOVA, $P = 0.004$) or 129 mm Hg oxygen (ANOVA, $P = 0.02$); no other significant increase was detected in any of the lines maintained under hyperoxic conditions.

The greatest MnSOD activity was observed in the adult human skin fibroblast line (Fig. 1B insert). The observed MnSOD activity in this lines was significantly greater than the SOD activity observed in any other line at all oxygen tensions (for all comparisons, $P \leq 0.00001$). There were no significant differences between adult and fetal lung fibroblasts at any of the oxygen tensions examined. MnSOD activity in fetal lung fibroblasts (WI-38) was greater than that observed in SV-40-transformed WI-38 cells in cultures maintained under O_2 tensions of 36, 129, or 605 mm Hg (ANOVA, $P = 0.00005$, $P = 0.00003$, and $P = 0.00001$, respectively). Although we attempted to use suitable transformed and parent lines for our comparisons, this type of comparison was not possible for melanomas and melanocytes because MnSOD activity was extremely low or below the limits of detection in melanocytes (data not shown). The most probable reason for this is that phorbol ester was included in the melanocyte medium (see Discussion). As compared to keratinocytes, MnSOD activity was lower in melanoma cells maintained under oxygen partial pressures of 36, 129, and 605 mm Hg (ANOVA, $P = 0.0003$, $P \leq 0.000001$, and $P = 0.0001$, respectively), and in pigmented and amelanotic melanoma lines as well as SCC maintained under an O_2 tension of 121 mm Hg (ANOVA, $P = 0.017$, $P = 0.006$, and $P = 0.04$, respectively).

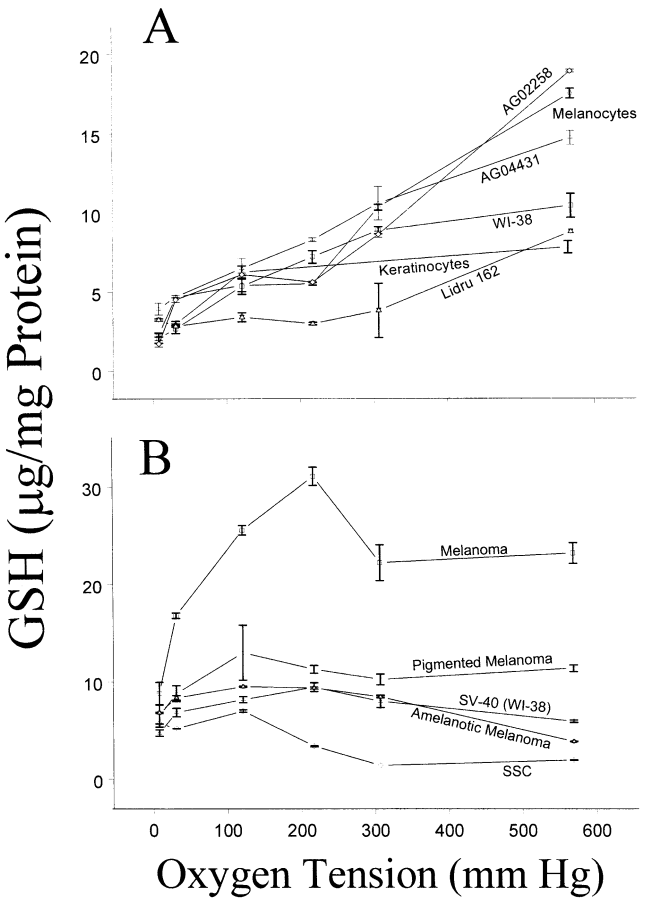

Fig. 2. Effects of ambient oxygen tension on total glutathione concentration in normal and transformed cells. Cells were seeded at 10,000 cells/cm^2 and plated under a gas mixture that contained 5% O_2. After 72 h, the gas phase of the flasks was replaced with one that contained 0, 5, 20 35, 50, or 95% O_2. All cultures were harvested 48 h after transfer to different oxygen tensions. The final oxygen tensions are presented in Table 2. (A) Normal cell responses. (B) Transformed cell responses.

Total glutathione

Large volumes of medium surround cells in culture. Because cell membranes are partially permeable to oxidized glutathione (GSSG), it exists only at very low concentrations in normal cells. Nearly all of the glutathione present in cells is in the reduced form (GSH). For the remainder of this discussion we will refer to total glutathione as GSH. GSH concentration was determined at six different oxygen tensions (8, 36, 129 227, 323, and 605 mm Hg; it was determined only at oxygen partial pressures of 36, 129, and 605 mm Hg in keratinocytes). Fig. 2A depicts the GSH responses of normal cells. In all cases, GSH concentration was stimulated by elevation of ambient oxygen tension. The least pronounced effects were those observed in adult skin fibroblasts and skin keratinocytes, which exhibited increased GSH levels only at relatively high ambient oxygen tensions. Both of these cell lines had a relatively low concentration of GSH. In contrast, transformed cells tended to exhibit relatively high GSH levels at lower oxygen tensions (Fig. 2B). GSH concentration rose as ambient oxygen tension was increased, until the oxygen concentration reached 121–217 mm Hg (15–28%, depending on the line). No further increases in GSH concentration were observed in any of the transformed lines when oxygen tension was increased to levels exceeding 227 mm Hg (Fig. 2B). In fact, GSH concentration decreased in most of the lines when they were exposed to an ambient oxygen tensions of 323 mm Hg or greater. These results suggest that at lower oxygen tensions the cellular redox environment of the transformed cell lines that we examined is more chemically reduced than in normal cells maintained under similar conditions.

Effects of paraquat on MnSOD

As noted above, the reasons for low MnSOD activity in transformed cells, when it occurs, are unclear. It is possible that cells expressing low MnSOD activity have lost their ability to respond to changes in the level of environmental stress. Most of the transformed cell lines we examined failed to increase their MnSOD activity in response to

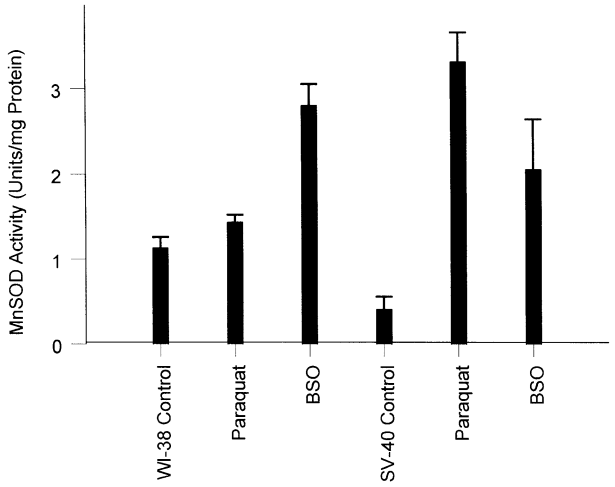

Fig. 3. MnSOD activity in WI-38 (fetal lung fibroblasts) and SV-40-transformed WI-38 cells treated with paraquat (methyl viologen) or buthionine sulfoximine (BSO).

changes in oxygen tension; however, normal cells also failed to increase their MnSOD under similar environmental conditions. This suggested that the stimulus was insufficient to stimulate a change in MnSOD activity. As a further test of the capacity of normal and transformed cells to respond to oxidative stress we treated WI-38 cells and an SV-40-transformed WI-38 line with the superoxide-generating herbicide paraquat (0.5 mM for 48 h). This concentration of paraquat was only mildly toxic as seen by the fact that it inhibited growth by only 10% in both normal and transformed cells as compared to the complete cessation of growth caused by a 48-h exposure to 605 mm Hg oxygen. As seen in Fig. 3 , paraquat treatment stimulates a very large and highly significant (ANOVA, $P < 0.00001$) increase in MnSOD activity in the SV-40-transformed WI-38 cells. Paraquat also stimulated an increase in the parent line but this change was much smaller than observed in the transformed cells. This suggests that both WI-38 cells and SV-40-transformed WI-38 cells can respond to a superoxide-producing stimulus by increasing their MnSOD activity.

Effects of BSO on MnSOD

We further investigated the effects of transformation-associated differences in the redox environment observed on MnSOD activity by inhibiting GSH synthesis. Cells were treated 48 h with 30 μM BSO. This concentration of BSO decreased GSH concentration by about 90% after a 48-h exposure, although the rate of the decrease appeared to be approximately twice as fast in the parent line (WI-38). Surprisingly, we observed no effects on growth from this treatment. Fig. 3 shows that treatment of WI-38 cells and SV-40-transformed WI-38 cells with 30 μM BSO for 48 h resulted in a significant increase in MnSOD activity in both WI-38 cells and SV-40-transformed WI-38 cells (ANOVA, $P < 0.0001$ and $P < 0.00001$, respectively).

GSH peroxidase (GPx)

GPx activity is strongly modulated by Se concentration in the medium. About 13 nM Se was present in DMEM with 10% serum. No Se is added to either the keratinocyte or melanocyte medium; however, enough was present in the pituitary extract to prevent any decrease in GPx activity with continued subcultivation. There were no statistical differences in the adult and fetal lung fibroblasts or between SV-40-transformed WI-38 cells and the parent WI-38 line. AG02258 did exhibit statistically lower GPx activity when maintained under an O_2 partial pressure of 605 mm Hg than the SV-40-transformed cells (ANOVA, $P = 0.013$). Keratinocytes and the adult skin fibroblasts exhibited greater GPx activity at all oxygen tensions examined than any of the other skin-derived normal or tumor cells (ANOVA, $P \leq 0.00001$ in all cases). Adult skin (Lidru 162) also exhibited greater activity than keratinocytes at all three oxygen tensions examined (ANOVA, $P \leq 0.0001$ in all cases). As noted above, the Se concentration probably differed between keratinocyte medium and DMEM; whether this accounted for differences in GPx activity between keratinocytes and other types of cells that were grown in DMEM was not determined. With the exception of pigmented melanoma, GPx tended to decrease at least slightly at the highest oxygen tension in all of the lines (Fig. 4) . In several cases the magnitude of the change was statistically significant. Specifically, GPx activity was significantly lower in cells maintained at 605 mm Hg as compared to cultures maintained at 31 mm Hg in WI-38 cells (ANOVA, $P = 0.025$), Lidru 162 (ANOVA, $P = 0.00017$), melanocytes (ANOVA, $P = 0.025$), amelanotic melanoma (ANOVA, $P = 0.0019$), and SCC (ANOVA, $P = 0.0058$). Similarly GPx was decreased at 605 mm Hg as compared to cultures maintained at 129 mm Hg in WI-38 (ANOVA, $P = 0.038$), Lidru 162 (ANOVA, $P = 0.0009$), amelanotic melanoma (ANOVA, $P = 0.00079$), and melanoma (ANOVA, $P = 0.021$). In other lines the observed decrease was not statistically significant; however, we infer that taken together, these data suggest that hyperoxia diminishes GPx activity.

Discussion

We compared the effects of ambient oxygen tension on the activities of GPx, and MnSOD in five transformed and six normal cell lines that were maintained under ambient oxygen tensions of 35, 129, and 605 mm Hg for 48 h. MnSOD activity was elevated in adult lung fibroblasts and amelanotic melanoma cells exposure to an O_2 partial pressure of 605 mm Hg for 48 h but was unaffected in all of the other lines examined. Hyperoxia decreased GPx activity in all but one line. Total glutathione concentration was determined in cultures maintained at oxygen partial pressures of 8, 36, 129, 227, 323, and 605 mm Hg for 72 h. Whereas normal cells exhibited a roughly linear increase in GSH

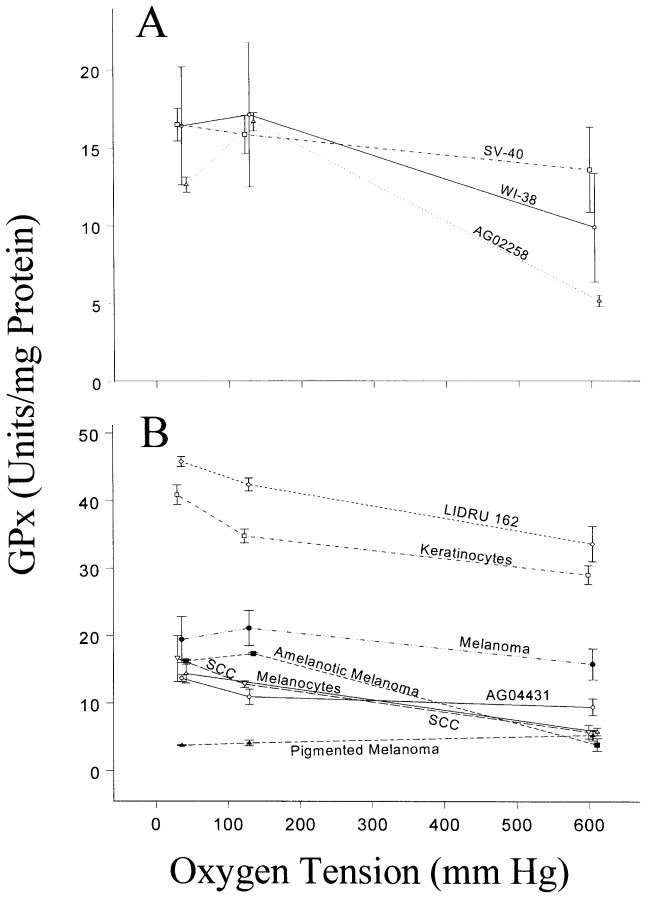

Fig. 4. Glutathione peroxidase (GPx) activity in normal and transformed cells. Cells were seeded at 10,000 cells/cm^2 and plated under a gas mixture that contained 5% O_2. After 96 h, the gas phase of the flasks was replaced with one that contained 5, 20, or 95% O_2. All cultures were harvested 48 h after transfer to different oxygen tensions. The final oxygen tensions are presented in Table 2. (A) Cells derived from lung tissue. (B) Cells derived from skin tissue.

concentration that correlated with increased ambient oxygen tension, tumor cells tended to exhibit greater GSH concentrations at low oxygen tensions than was observed in normal cells and failed to increase GSH concentration at ambient oxygen tensions greater than 227 mm Hg. MnSOD activity was induced in virally transformed cells by treatment with the herbicide paraquat or BSO, an inhibitor of GSH synthesis. These results demonstrate differences in tumor and normal cell responses to changes in ambient oxygen tension and clearly show that MnSOD activity is fully inducible in transformed cells when an appropriate stimulus is applied.

Changing ambient molecular oxygen concentration has frequently been used as a method of applying a controlled level of oxidative stress to cells, but the precise nature of the resulting oxidative stress has never been clearly defined and has remained a subject of intense controversy for more than a century. Halliwell [35] suggested that oxygen toxicity might arise from an increased propensity of ground-state oxygen to absorb energy to form more reactive singlet $^1O_2$, from increased superoxide or hydrogen peroxide generation, or as a result of diverting metabolic pathways.

If oxygen toxicity arises from increased $O_2^{\cdot-}$ production, it might be expected that SOD activity and particularly the mitochondrial form (MnSOD) would increase as an adaptive response. Two of the lines we examined did exhibit a modest, albeit significant, increase in MnSOD activity when placed under hyperoxia. What is clear, however, is that most lines fail to respond during a 48-h exposure to hyperoxia by increasing their MnSOD activity. In intact organisms high concentrations of O_2 are toxic and can be lethal after relatively short exposures, but the effect of hyperoxia on SOD activity in adult animals is usually observed only after prolonged exposures during which animals are gradually adapted to hyperoxic conditions [35–37]. This could suggest that the capacity of cells to respond to increased rates of $O_2^{\cdot-}$ generation by increasing SOD activity is slow or limited. This was clearly not the case in the cell cultures we examined since stimulation with a superoxide-generating herbicide for 48 h strongly stimulated MnSOD activity. It is unlikely that paraquat was simply more effective at increasing oxidative stress since it was only mildly toxic to growth, while exposure to an oxygen tension of 605 mm Hg completely blocked growth in cells. Yet, paraquat increased MnSOD activity in SV-40-transformed WI-38 cells and in the parent WI-38 line, while culture under an oxygen tension of 605 mm Hg for a similar period did not. These results suggest that the effects of paraquat were a result of increased $O_2^{\cdot-}$ while the effects of hyperoxia may stem from the production of other types of oxidants.

Another difference between the effects of paraquat and hyperoxia is also indicated by the fact that paraquat had relatively little effect on GSH concentration or GPx activity (data not shown) while both of these parameters were strongly modulated by hyperoxia. The fact that hyperoxia stimulated MnSOD activity in two of the lines suggests that it can stimulate $O_2^{\cdot-}$ production; however, the results presented here and in several other studies [38,39] fail to support the view that oxygen toxicity is principally the result of increased $O_2^{\cdot-}$ production. It should also be noted that subsequent studies with different adult lung fibroblast and amelanotic melanoma lines revealed no change in MnSOD activity when the cells were transferred from 5% O_2 to and 95% O_2. This suggests that the increase in MnSOD activity at high oxygen tensions observed in two of the lines used in this study may have been specific to them. In any case, increasing oxygen tension was a generally ineffective means of increasing MnSOD activity.

As discussed above many types of tumor cells exhibit relatively low MnSOD activity, but it seems unlikely that this is the result of impaired capacity to respond to stimulation with $O_2^{\cdot-}$. In addition to our results with SV-40-transformed WI-38 cells, paraquat has also been observed to induce SOD activity in melanoma cells [40], mouse lymphoma [24], and Kirsten sarcoma virus-transformed rat kidney cells [41], indicating that SOD activity is inducible in

multiple types of transformed cells. These observations demonstrate that increased rates of O_2^- production stimulate MnSOD activity in tumor cells. This would seem to suggest that in tumor cells that exhibit low MnSOD activity, the rate of O_2 generation is correspondingly lower than in the normal cells that exhibit greater SOD activity.

Interestingly, tumor cells are frequently reported to produce large amounts of oxidants [20–22]. The types of ROS include O_2^- as well as H_2O_2 [22]. The reason that steady-state levels of oxidants can in some cases increase without a corresponding increase in MnSOD is unclear. The mechanism of free radical generation in tumor mitochondria has been reported to be similar to that found in normal cells [42]. It is unlikely that oxidant generation in tumor cells is compartmentalized to locations outside of mitochondria, because mitochondrial aconitase activity (which is sensitive to O_2^-) declines in transformed cells exhibiting increased O_2^- [43], which is indicative of intramitochondrial production of O_2^-. Unfortunately, methods for the specific detection of O_2 in intact cells have not been perfected and the lower SOD activity seen in tumor cells that exhibit higher levels of oxidant production may indicate that oxidants other than O_2^- are increased in these cells.

Tumor cells are frequently observed to exhibit elevated levels of GSH [19,44–52]. This is particularly true of melanomas, which exhibit many times the level of GSH that is observed in healthy tissue [19,44]. The transformed cells we examined exhibited elevated GSH concentration in hypoxic and normoxic oxygen ranges. In most cases a plateau in GSH concentration was reached as the ambient oxygen tension approached 20%. None of the transformed lines examined exhibited an increase in GSH concentration at oxygen tensions exceeding 35%. In most cases, GSH concentration decreased as ambient oxygen concentration was increased to above 35%. In contrast, normal cells exhibited a roughly linear increase in GSH concentration that was proportional to ambient oxygen tension. As previously reported [38], adult skin fibroblasts were relatively unresponsive to changes in ambient O_2 at lower concentrations and exhibited no large increase in GSH concentration until cultured under $>50\%$ O_2. It is clear from our observations that both the basal levels of GSH and the capacity to adjust GSH in response to stress are altered in the transformed cells examined.

The higher GSH concentration observed in tumor cells maintained under low oxygen tensions may permit them to survive oxidative stress even when SOD activity is low. For example, elevated GSH has been observed to confer resistance to oxidant generators such as menadione [53]. It is also possible that the elevated GSH concentration observed in the transformed lines may account for the lower MnSOD activity observed in these lines. This is supported by the observation that inhibition of GSH synthesis stimulated MnSOD activity in the SV-40-transformed line almost as much as treatment with paraquat. This is consistent with the hypothesis that the elevated GSH concentration observed in tumor lines maintained at lower O_2 tensions might to some extent suppress MnSOD activity.

The observation that GPx activity was lower under hyperoxic conditions indicates that this form of oxidative stress inactivates the enzyme. It should be noted that paraquat had no effect on GPx activity (data not shown), again suggesting that the effects of hyperoxia differ from the effects of paraquat (presumably O_2^- generation). The amount of Se in the DMEM with the serum lot used for this study was 13 nM. The Se concentration not determined for keratinocyte/melanocyte media but is low. Although none is added when the media are prepared there will be traces present in the pituitary extract used as a source of growth factors. Thus, keratinocytes and melanocytes were probably exposed to concentrations of Se that differed from the amount in the media used for other cells. Nevertheless, the tendency for GPx activity to decrease under hyperoxia was not the result of using less than saturating concentrations of Se in the medium. In other studies, we determined that addition of Se in concentrations ranging from 30 to 3000 nM makes no difference in the effects of oxygen on GPx activity; i.e., hyperoxia always inhibits the enzyme (unpublished).

Finally it seems appropriate to address the question of consistency since we presented data from only one cell line for each cell type studied. We previously examined several of the “normal” cell types used in this study in great detail. For example, we have shown that the difference in MnSOD activity that exists between fetal and postnatal skin fibroblasts was apparent in 8 fetal and 24 postnatal lines [54] and that these differences are unaltered by exposure to different ambient oxygen tensions [39,55]. We have also demonstrated that other fetal and adult lung fibroblast lines exhibit responses similar to those reported here when they are exposed to different ambient oxygen tensions [38]. Additionally, we examined other keratinocyte and melanocyte lines (over a smaller range of oxygen tensions) and obtained results similar to those presented here (unpublished). Thus, we can state with great confidence that the effects observed in the various nontransformed lines is highly reproducible in many different cell lines. Although we have studied the effects of oxygen tension less extensively in transformed cells, the patterns of change (or lack of change) associated with increased oxygen tension were essentially the same in all of the transformed lines examined. Although we expect exceptions to be found, the consistency of the patterns we observed between different transformed cell lines suggests that similar patterns of change will be detected in other similar transformed lines.

The results of this study demonstrate that increasing ambient oxygen tension stimulates changes in cellular GSH concentration; however, the patterns of changes in GSH concentration in response to variations in ambient oxygen tension differ in the normal and transformed cells that we examined. Hyperoxia inhibited GPx but generally failed to stimulate MnSOD activity. Conversely, MnSOD activity

was induced in virally transformed WI-38 fibroblasts and the parent WI-38 cells by paraquat. This suggests that different mechanisms are involved in the toxicity of O_2 and paraquat. The steady-state level of $O_2^{\cdot-}$ of transformed cells with low MnSOD activity may simply be lower than in cells that exhibit greater activity. The relatively greater GSH concentration observed in tumor cells at lower oxygen tensions may also influence the capacity of these cells to increase MnSOD activity.

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