CALL FOR PAPERS: Biomarkers in Lung Diseases: from Pathogenesis to Prediction to New Therapies

Basal levels of glutathione peroxidase correlate with onset of radiation induced lung disease in inbred mouse strains

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Submitted 7 April 2014; accepted in final form 21 August 2014

Kunwar A, Haston CK. Basal levels of glutathione peroxidase correlate with onset of radiation induced lung disease in inbred mouse strains. Am J Physiol Lung Cell Mol Physiol 307: L597–L604, 2014. First published August 22, 2014; doi:10.1152/ajplung.00088.2014.—Biomarkers predicting for the radiation-induced lung responses of pneumonitis or fibrosis are largely unknown. Herein we investigated whether markers of oxidative stress and intracellular antioxidants, measured within days of radiation exposure, are correlated with the lung tissue injury response occurring weeks later. Mice of the eight inbred strains differing in their susceptibility to radiation-induced pulmonary fibrosis, and in the duration of asymptomatic survival, received 18 Gy whole thorax irradiation and were killed 6 h, 24 h, or 7 days later. Control mice were not irradiated. Lung levels of antioxidants superoxide dismutase, catalase, glutathione peroxidase (GPx), and glutathione, and of oxidative damage [reactive oxygen species (ROS) and 8-hydroxydeoxyguanosine (8-OHdG)], were biochemically determined. GPx was additionally measured through gene expression and immunohistochemical assessment of lung tissue, and activity in serum. ROS and 8-OHdG were increased postirradiation and exhibited significant strain and time-dependent variability, but were not strongly predictive of radiation-induced lung diseases. Antioxidant measures were not dramatically changed postirradiation and varied significantly among the strains. Basal GPx activity (r = 0.73, P = 0.04) in the lung and the pulmonary expression of GPx2 (r = 0.94, P = 0.0003) correlated with postirradiation asymptomatic survival, whereas serum GPx activity was inversely correlated (r = −0.80, P = 0.01) with fibrosis development. In conclusion, pulmonary oxidative stress and antioxidant markers were more affected by inbred strain than radiation over 7 days posttreatment. Lung GPx activity, and GPx2 expression, predicted for survival from lethal pneumonitis, and serum GPx for fibrosis, in this panel of thoracic irradiation; predictive biomarker; pneumonitis; fibrosis.

Late lung tissue responses of pneumonitis and fibrosis are the most serious dose-limiting side effects of thoracic radiotherapy, which is an essential treatment modality for a wide spectrum of malignant tumors affecting the thorax region (1). Whereas pneumonitis is an acute inflammatory response, fibrosis is characterized by progressive scarring of the lung, with vascular cell damage and collagen deposition in the interstitium (5, 13). If developing, pneumonitis and fibrosis have a considerable impact on patient morbidity and mortality. Biomarkers, whose levels pretreatment or in the presymptomatic posttreatment interval can predict for the occurrence of these radiotherapy side effects, are not yet available (6).

The pathogenesis of the radiation-induced lung diseases is not yet mechanistically understood (5, 13), but it is known that radiation therapy produces reactive oxygen species (ROS), which, if not neutralized, can result in the altered function or death of cells through oxidative damage to proteins, lipids, and DNA (2, 7, 20, 25, 35). Oxidative damage to DNA can be measured by production of 8-hydroxydeoxyguanosine (8-OHdG), which is the most abundantly generated DNA oxidation product following radiation exposure (36). To neutralize ROS and to prevent subsequent oxidative damage to DNA and other biomolecules, the cells have in place an endogenous antioxidant defense system comprised of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, and glutathione (GSH) (9). Whereas SOD catalyzes the conversion of highly reactive superoxide radicals to less reactive hydrogen peroxide, GPx and catalase aid in enzymatic detoxification of hydrogen peroxide. GSH not only directly scavenges ROS but is also required for the catalytic activity of GPx. These cellular antioxidants, however, may not be enough to overcome the primary oxidative damage caused by radiation exposure because of the excessive generation of ROS, a condition termed as oxidative stress (3, 18, 21). It is therefore possible that an individual’s susceptibility toward radiation-induced lung diseases could depend on how efficiently the lung cells are able to manage this primary injury (21).

Inbred strains of mice recapitulate the lung pathologies of pneumonitis and fibrosis, following exposure to whole thorax irradiation (10, 19, 22, 26, 27), and have been reported to vary in tissue intracellular antioxidant levels (33). In addition to differing in their fibrotic response to radiation exposure, we identified inbred mouse strains to vary in the time posttreatment to the onset of distress, or in asymptomatic survival time (22). Because it has been shown that asymptomatic survival time correlated with pneumonitis in mice (10), we have included this parameter in the current investigation. Inbred strains of mice are thus a resource with which to investigate whether variation in the oxidative stress or antioxidant response to radiation-induced primary injury influences susceptibility to the late lung tissue injury response of pneumonitis and fibrosis.
Herein we assessed whether the pulmonary levels of oxidative injury markers ROS and 8-OHdG, or the activity levels of endogenous antioxidants SOD, GPx, catalase, and GSH, measured during the acute radiation response, are strain dependent and, if so, whether these levels are predictive of the tissue response of pneumonitis or fibrosis in a panel of inbred strains.

**MATERIALS AND METHODS**

**Mice.** Female mice of eight inbred strains (C3H/HeJ, C57BL/6, A/J, AKR/J, 129S1/SvImJ, CBA/J, NZW/LacJ, and KK/HIJ) and fibrosis-resistant (C3H/HeJ, A/J, AKR/J, and CBA/J) responses to thoracic irradiation. Second, they collectively exhibit substantial variation in postirradiation survival time, which ranges from 10 to 14 wk in C3H/HeJ, AKR/J, CBA/J, and KK/HIJ mice, from 21 to 26 wk in C57BL/6, A/J, 129S1/SvImJ, and NZW/LacJ mice, and is independent of the development of fibrosis in these strains (22). All mice were handled according to guidelines and regulations of the Canadian Council on Animal Care, under a protocol approved by the Animal Care Committee of McGill University.

**Radiation treatment.** Eight-week-old mice, partially shielded with 3 cm of lead, received whole thorax radiation exposure (18 Gy; dose rate 0.54 Gy/min) using a Faxitron X-ray machine (14). After irradiation, the animals were housed under normal laboratory conditions, and groups of five mice per strain were killed at 6 h, 1 day, or 7 days after irradiation. The control mice of each strain were not treated and were killed at the 7-day time point.

**Serum and lung tissue collection.** After drawing >500 μl of blood through cardiac puncture, the mice were killed with an overdose of pentobarbital sodium, and the lungs were rapidly removed and rinsed with cold PBS. The left lobe from each mouse was perfused with 10% formalin buffered and preserved for immunohistochemical analysis. The right lungs were divided into aliquots for ROS estimation, gene expression, and biochemical assays. The blood was allowed to clot at room temperature for 1 h and centrifuged at 1,500 g for 10 min. The serum was collected and stored at –70°C until analysis. ROS estimation. A portion of right lung was cut in pieces and treated for 45 min at 37°C with 1 ml of 5 mg/ml DNase 1 (Sigma Chemicals) and 1 ml of 5 mg/ml collagenase D (Sigma Chemicals) in 3 ml of phosphate-buffered saline containing 1% albumin bovine serum (PBS/1% BSA). Cells were released by mincing lung tissue and filtering the resulting suspension through a nylon 70-mm cell strainer. The filtrate was treated with a solution of 150 mM NH4Cl, 10 mM NaHCO3, and 10 mM EDTA to lyse red blood cells (RBCs) and washed one time in PBS/1% BSA. Cells were counted with a hemocytometer and suspended to appropriate concentrations in PBS to be used for ROS estimation.

Intracellular levels of ROS were estimated using a cell-permeable oxidation-sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (DCFDA), whose fluorescence intensity increases after oxidation to dichlorofluorescein (DCF) by ROS. In brief, lung cells (1 × 106) in 100 μl of PBS were incubated with 5 μM of DCFDA (Invitrogen) for 30 min at 37°C and quantified for DCF fluorescence using a fluorescence plate analyzer (excitation at 480 nm and emission at 530 nm). The DCF fluorescence intensity was normalized with respect to appropriate control treated with probe without adding cells. The levels of intracellular ROS are represented as the mean fold increase in DCF fluorescence intensity.

**Assessment of antioxidant enzymes and GSH.** The remainder of the right lung was perfused each of three times with 1 ml of cold PBS to remove any trapped blood and edema and was then homogenized (10% wt/vol) in 100 mM Tris buffer, pH 7.4, containing 0.5% Triton X-100, 5 mM butylated hydroxytoluene, and 100 μM phenylmethylsulfonyl fluoride using a Tissuemizer (Fisher Scientific). The homogenate was centrifuged at 10,000 g for 5 min, and the supernatant, called tissue extract, was used for the estimation of oxidative damage marker, 8-OHdG, and antioxidants GPx, catalase, and GSH.

The activity levels of GPx, catalase, and SOD were determined using kits from Bio-vision and of total GSH using a kit from Sigma Chemicals (St. Louis, MO) according to the manufacturer’s instructions. The activity levels of antioxidant enzymes and GSH were normalized with respect to protein content in the lung homogenate, which was estimated using a Bio-Rad protein assay kit. Serum levels of GPx activity were measured with a Bio-vision kit as per the manufacturer’s instructions.

**8-OHdG assay.** Genomic DNA from the lung homogenate was isolated using a DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions with some modifications. DNase-free RNase (Sigma-Aldrich, St. Louis, MO) was added to the samples before addition of Buffer AL to digest the RNA as per the supplier’s recommendations. The RNA-free DNA (20 μg) thus obtained was converted to single-stranded DNA by incubating the samples at 95°C for 5 min and rapidly chilling on ice. After denaturation, the pH of denatured DNA was adjusted to 5.2 with 3 M sodium acetate (pH 5.2) and subjected to digestion with nuclease P1 (1 U) for 2 h at 37°C (28). Following this, pH of the reaction was adjusted back to 7.4 using 1 M Tris·HCl (pH 8.0), and the reaction mixture was incubated with 1 U of alkaline phosphatase for 1 h at 37°C and centrifuged for 5 min at 6,000 g. The supernatant obtained was used for 8-OHdG estimation using an Oxiselect oxidative DNA damage ELISA kit (Cell Biolabs, San Diego, CA) according to the manufacturer’s instructions. The levels of 8-OHdG were normalized with respect to per milligram of genomic DNA.

**Quantitative real-time PCR.** A portion of the right lung from each mouse was homogenized in 1.0 ml of Trizol reagent using a Tissuemizer (Fisher Scientific) and stored at –70°C until gene expression assessment by quantitative real-time PCR. Total RNA from the Trizol homogenate was isolated according to the manufacturer’s instructions, and 4 μg of total RNA were used for the synthesis of cDNA by reverse transcription using Superscript II RNase H- Reverse Transcriptase (Invitrogen, Carlsbad, CA). Quantitative real-time PCR assays were performed using an Applied Biosystems International Prism 7500 Sequence Detection System using the TagMan Gene expression assays Mm00665767_g1 for Gapdh, Mm00492427_m1 for Gpx2, Mm00656767_g1 for Gpx1, Mm01286848_gH for Gpx4, GAPDH (gapdh, assay Mm99999915_g1) was used as the reference gene as in prior studies (14), and data analysis was also as in a prior study (14).

**Immunohistochemistry.** Immunohistochemical detection was completed on paraffin tissue sections (5 μm thick). These sections after dewaxing in xylene and rehydration in graded alcohols were boiled in antigen unmasking solution (Vector Laboratories) for 50 min to expose antigenic determinants and blocked with 4% goat serum. Afterward sections were incubated with primary anti-GPx1 (Abcam), anti-GPx2 (Abcam), or anti-GPx4 (Cayman Chemical) antibodies, followed by biotinylated goat anti-rabbit or anti-rat secondary antibody (Vector Laboratories). Sections were labeled using avidin-biotin-alkaline phosphatase and red alkaline phosphatase substrate kits (catalog no. SK5100; Vector Laboratories) as per the manufacturer’s instruction. Finally, sections were counterstained with methylene blue, mounted with nonaqueous media.

**Statistical analysis.** The results are presented as means ± SE (4–5 mice/strain at a time point). The statistical significance of the variability among the means of treatment groups was determined by one-way ANOVA followed by Tukey’s posttest, P < 0.05. The correlation analysis of biochemical parameters with disease phenotypes was performed using the Pearson’s rank correlation test, and P values <0.05 were considered as statistically significant. The radiation-induced lung disease phenotypes of average fibrosis score and

**AJP-Lung Cell Mol Physiol • doi:10.1152/ajplung.00088.2014 • www.ajplung.org**
asymptomatic survival time for the eight strains were taken from a previous report (22).

**RESULTS**

**Acute oxidative damage response in eight inbred strains.** To determine whether whole thorax irradiation induces a strain-dependent oxidative damage response in the lungs of exposed mice, we treated mice of eight strains with 18 Gy and assessed lung tissue up to 7 days later. As shown in Fig. 1, A and B, radiation treatment produced a significant increase in ROS and 8-OHdG levels, although the time to the peak response was strain dependent. At each of the four time points (control, 6 h, 24 h, and 7 days), the extent of oxidative damage varied significantly \((P < 0.0001)\) among the strains, and measures of 8-OHdG agreed well with those for ROS.

To assess the predictive value of these assays for lung disease correlation, analyses were completed. The values of 8-OHdG at day 7 were suggestively correlated with asymptomatic survival time \((r = -0.64, P = 0.086)\), and no other measures were significantly correlated with extent of radiation-induced fibrosis \((P > 0.15)\) or asymptomatic survival time, apart from 8-OHdG at day 7 \((P > 0.3, \text{data not shown})\).

**Acute antioxidant response in eight inbred strains.** To determine whether whole thorax irradiation induces a strain-dependent antioxidant response in the lungs of exposed mice, specific parameters were measured in lung homogenates taken from the eight strains over the time course. In contrast to the oxidative damage response, thoracic irradiation did not profoundly alter lung tissue levels of SOD, GPx, or total GSH in the majority of strains, as shown in Fig. 2, A, B, and C. Tissue catalase levels were affected by radiation in six of eight strains (Fig. 2D). Each of GPx, total GSH, and tissue catalase activity, was, however, significantly dependent on strain \((P < 0.0001)\) at the control, 6-h, 24-h, and 7-day time points, as were the levels of SOD \((P < 0.04)\) at all times except 24 h. Because the values of GSH and GPx in KK/HJ mice appeared to differ from those of the other strains, the analysis of variance was repeated with this strain removed. The significant strain dependence in GPx values was retained, except for levels measured at 6 h postirradiation. For GSH, the strain dependence, without KK/HJ mice, was significant only at 24 h and 7 days postirradiation.

Given this strain dependence, we assessed the correlation of the antioxidant measures to the tissue injury response and identified basal (control tissue) levels of GPx to be significantly correlated with asymptomatic survival time in the eight inbred strains \((r = 0.73, P = 0.04)\) as shown in Fig. 3. The correlation of pulmonary GPx and asymptomatic survival time was also evident, and comparable to that at baseline, at each of 6 h, 24 h, and 7 days postirradiation. Tissue levels of catalase, SOD, and total GSH were not significantly correlated with extent of radiation-induced lung disease measured as fibrosis \((P > 0.25, \text{data not shown})\) or as asymptomatic survival \((P > 0.11, \text{data not shown})\).

To evaluate whether basal GPx activity in the serum was reflective of that in the lung, and in turn correlated with lung disease, measures in this fluid were completed. As shown in Fig. 4A, the activity of GPx in the serum was not significantly correlated with that of the lung \((P = 0.24)\). Serum GPx activity was, however, significantly negatively correlated with extent of radiation-induced pulmonary fibrosis in this panel of strains \((r = -0.80, P = 0.01)\) as shown in Fig. 4B. Serum levels of GPx were not significantly correlated with extent of radiation-induced lung disease measured as asymptomatic survival \((P > 0.62, \text{data not shown})\).

**GPx expression.** Because at least four different GPx family members (GPx1–4), of different functionality are expressed in lung (31), we investigated whether their expression was predictive of radiation-induced lung disease. The expression of GPx1–4 in the lungs of unirradiated control mice was evaluated with RT-PCR and by immunohistochemistry. GPx3 was not assessed by immunohistochemistry, since it is known to be...
secreted by lung cells (12, 31). In agreement with a previous report (31), the pulmonary expression of GPx1 and GPx3 was abundant compared with that of GPx2 and GPx4 in each of the eight strains analyzed (Fig. 5A). Significant ($P < 0.0002$) strain-dependent variability was observed in the basal expression of each of GPx1, GPx2, and GPx3 (Fig. 5A). These data were supported by immunohistochemical staining as shown in Fig. 5B and supporting information. Analyses to correlate the mRNA expression of GPx1–4 to lung disease indicated that the expression of GPx2 was significantly ($r = 0.94$, $P = 0.0003$) correlated with asymptomatic survival time in the eight strains (Fig. 6).

**DISCUSSION**

In present study using murine models of radiation-induced lung disease, we demonstrated that the duration of asymptomatic survival time before the lung response of pneumonitis, with or without fibrosis, following thoracic radiation exposure, was significantly correlated with pulmonary GPx, both by activity and mRNA expression levels. Second, the radiation-induced pulmonary fibrosis response of the lung was correlated with the basal serum GPx activity. Neither the asymptomatic survival time nor the fibrosis response was predicted by the levels of other intracellular...
antioxidants SOD, catalase, or GSH within 7 days of thoracic irradiation in this panel of mice.

The pulmonary activity of GPx, which showed little change with time but significant variability among the strains, positively correlated with the lung disease phenotype of postirradiation asymptomatic survival time. The correlation between the GPx activity and asymptomatic survival time, although significant at all evaluated time points, was not improved postradiation exposure compared with the basal activity; thus, baseline measures of this enzyme appear adequate for prediction of tissue radiation response. Second, because baseline GPx activity in the serum was correlated with tissue fibrosis in these mice, serum levels of this enzyme may be a useful biomarker, although they were not predictive of the pulmonary activity of the same enzyme. These findings are supported by one clinical report wherein the lower levels of RBC GPx activity, both pretreatment and after radiotherapy, were observed in lung cancer patients developing pneumonitis compared with those in patients who did not develop pneumonitis following radiotherapy (21).

The association of higher GPx activity with the better postirradiation survival time, and reduced fibrosis, suggests a protective function for GPx against the onset of radiation-induced lung pathologies. In line with this observation, we have previously demonstrated that the postradiation treatment with a organoselenium compound, 3,3'-diselenodipropionic acid, which increased the pulmonary GPx activity, significantly delayed the onset of pneumonitis in C3H/HeJ mice (14). In another such study, Liu et al. (15) showed that the administration of quercetin liposomes (known to activate antioxidant enzymes) 2 h before and for 4 wk after 16 Gy whole thorax irradiation increased the lung GPx level and decreased radiation-induced fibrosis in mice. Finally, mice deficient in nuclear factor-E2-related factor 2, a redox-sensitive transcription factor that regulates antioxidant response element-dependent genes, including GPx2 (28), succumb earlier than wild-type mice to lung disease postthoracic radiotherapy (32) in a response that may be related to tissue GPx levels, although this has not been shown.

Among the four GPx isoforms expressed in the lung, the basal expression levels of GPx2, specifically, were correla-
tive of lung disease in these mice.

In this work, the potential radiation response biomarkers assessed were taken from components of the oxidative stress response as classically defined. In addition to this prooxidant antioxidant definition, Jones (11) has redefined oxidative stress to include “a disruption of redox signaling and control.” Additional pathways included in this definition of the oxidative stress would be those through which peroxiredoxin and thioredoxin enzymes function (11). There are six mammalian peroxiredoxin enzymes that function to degrade hydroperoxides to water, and their cellular levels may be an indication of the oxidative stress response (23, 24). Mathew et al. (17) have reported the expression of

![Fig. 3. Scatter plot of asymptomatic survival time of mouse strains post-18 Gy whole thorax irradiation on the mean pulmonary GPx activity levels of unirradiated control.](http://ajplung.physiology.org)

![Fig. 4. Scatter plots of mean pulmonary and serum GPx activity levels of unirradiated control mice (A) and average fibrosis score of mouse strains post-18 Gy whole thorax irradiation on the mean serum GPx activity levels of unirradiated control (B). P value indicates significance of correlation by Pearson’s rank correlation test.](http://ajplung.physiology.org)
Fig. 5. Pulmonary expression of GPx1, GPx2, GPx3, and GPx4 in unirradiated mice of eight inbred strains. A: the fold change in mRNA expression of each GPx gene in the right lungs of mice, relative to that of a reference gene (Gapdh), is presented (means ± SE, n = 5). Line above the groups indicates significance by one-way ANOVA. B: representative lung tissue sections immunostained with anti-GPx1, anti-GPx2, and anti-GPx4; >200 magnification.
peroxiredoxin-1 to be increased in the lung within weeks of irradiation. The thioredoxin antioxidant system is comprised of three thioredoxin reductase enzymes and two of thioredoxin that, through reductase activity, serve to defend against oxidative stress (16). Although not evaluated here, components of this expanded definition, acting individually or in composite, and possibly with the innate GPx activity, may also be predictive of radiation response in the lung.

Among the remaining antioxidants assayed, catalase, SOD, and GSH showed strain-dependent variability in their pulmonary levels over the time following thoracic irradiation, but none was significantly correlated with asymptomatic survival time or the lung disease phenotype of fibrosis, and thus are not suggested by these data to be useful predictive biomarkers. In support of this finding, RBC catalase and GSH measures were independent of the pneumonitis response in radiotherapy patients (21), although high SOD was associated with pneumonitis in this group of patients.

Regarding the strain dependence of the biomarkers, we are not aware of other studies documenting this variability for lung tissue, although multiple groups have reported inbred strain background to have a major effect on GSH levels in the liver (8, 30, 34, 37) and the kidney (37), and on liver catalase levels (30). Indeed GSH activity levels in the liver differing by threefold over a panel of strains have been reported (37), which is similar to the range in values measured here for the lung. The specific genetic differences among inbred strains that result in varied GSH levels are unknown at present, but recent studies have identified candidate genes for this trait based on association studies in mice (8, 37). Finally, early levels of the primary radiation injury of ROS generation or oxidative stress were not strongly correlated with the later lung disease in mice. In the lungs of the mice of this panel, this early radiation response was not indicative of the later pathological response.

In conclusion, our studies showed that, among oxidative stress and antioxidant biomarkers assayed, higher control levels of pulmonary GPx activity and of GPx2 expression in the lung correlated with better survival of mice exposed to thoracic irradiation, whereas greater levels of serum GPx were associated with sparing of a fibrosis response.

GRANTS
This work was supported by funding from the Canadian Institutes of Health Research to C. K. Haston. A. Kunwar was supported by the Richard and Edith Strauss Postdoctoral Fellowship in Respiratory Medicine.

DISCLOSURES
No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS
REFERENCES


