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The importance of gamma-glutamyl transferase in lung glutathione homeostasis and antioxidant defense[1]

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1. Introduction

An extensive literature now supports a fundamental role for glutathione in the antioxidant protection of the lung and its gas exchange surface [1]. However, the physiological significance of lung glutathione turnover by the extracellular catabolic enzyme gamma-glutamyl transferase (GGT) has remained controversial. The function of GGT in the lung has been a major research focus in my laboratory. Our studies initially focused on normal rat lung both at different stages of development [2–4] and in different conditions of oxidant stress [5]. More recently, these studies have been complemented by experiments with a newly defined model of GGT deficiency, the GGT^{enu1} mutant mouse [6,7]. The cumulative information we have generated from this work in rat and mouse, that correlates the cellular sites of GGT gene expression with that of GGT protein and enzyme activity, supports a critical biological role for GGT in lung glutathione homeostasis and epithelial cell antioxidant defense.

Our initial studies in rat lung revealed key insights into epithelial cell biology, surfactant function, intercellular glutathione transport, the role of GGT in the lung response to oxidant stress, and the role of oxygen as a regulator of gene expression in the lung. The GGT^{enu1} mutant mouse provided a model in which to test some of these observations and to more specifically define the role of GGT in the lung. In particular, we find that the absence of GGT expression in the mutant mouse lung severely limits glutathione availability and induces oxidant stress in normal oxygen conditions in both a subset of epithelial cells and in the surfactant that bathes cells at epithelial surfaces. Injury in these GGT-deficient lung epithelial cells is accelerated in the presence of hyperoxia with GGT^{enu1} mutants dying more rapidly compared to normal mice [8]. Our study of the GGT^{enu1} mouse also led us to identify novel truncated GGT protein isoforms that are generated by alternative splicing of GGT mRNA. While the intact GGT protein is expressed on the plasma membrane, these truncated isoforms are retained in the endoplasmic reticulum (ER). Surprisingly, both the native GGT and one of its ER isoforms can mediate

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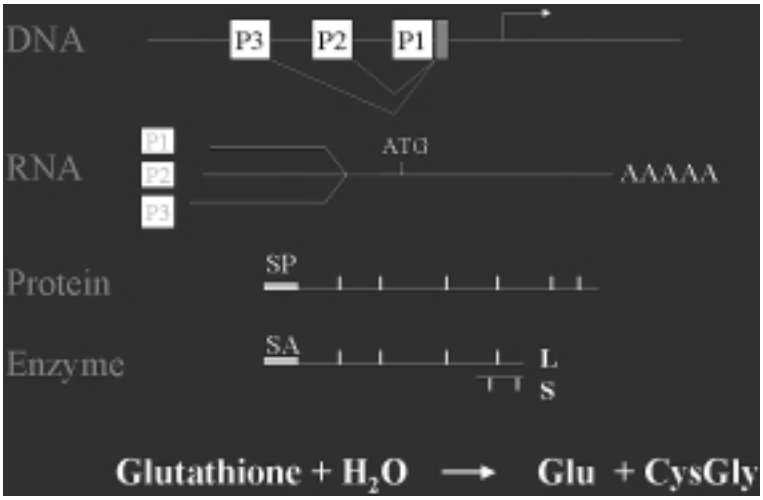

Fig. 1. Schematic of GGT gene. P1, P2, P3 denote alternative promoters and the corresponding mRNAs. SP marks signal peptide in primary translation product and vertical lines are glycosylation. SA is retained membrane signal anchor with L as large and S as small subunits after processing. GGT initiates the metabolism of glutathione.

an endoplasmic reticulum stress response demonstrating a new function for GGT [9]. Taken together, our results suggest that GGT expression is crucial for lung antioxidant defense and may serve a number of important homeostatic functions in lung epithelial cells. We believe that further investigation of lung GGT gene regulation and protein expression will allow us to identify new targets to design therapies for protecting both the developing and the mature lung and its epithelium against injury from oxidant stress.

2. The GGT gene and the lung

A summary of the GGT gene structure that is relevant to its expression in the lung is presented in Fig. 1. The rat and the mouse GGT genes are very similar in organization and regulated by multiple alternative promoters [10]. The three most proximal promoters, P1, P2 and P3 are utilized under different conditions in the lung and result in the transcription of three different mRNA species. Each transcript has a unique 5' untranslated region, corresponding with alternative promoter usage, followed by a common 5' region and coding domain (ATG denotes the start codon). The protein is translated as a single chain propeptide that is enzymatically inactive [Rebecca Hughey, personal communication, and 11]. GGT is a type II integral membrane protein as the uncleaved amino-terminal signal anchor (SA) orients the translocation of GGT into the ER, leaving the amino terminus in the cytosol and the glycosylated ectodomain with the active site residues in the lumen [12]. It is now apparent that GGT is a likely member of the family of "N-terminal nucleophile hydrolases" [11], as proposed in previous studies [13]. The new amino terminal threonine produced by autocatalytic cleavage of the GGT into a large (L) and small (S) subunit heterodimer is also the catalytic nucleophile for the enzyme. The heterodimer is stably associated, and the catalytic site for hydrolysis of glutathione includes residues from both subunits.

GGT is highly expressed on the surface of cells with secretory or absorptive activity, such as epithelia. GGT activity is also present in epithelial cell secretions [14]. A hydrophilic form resulting from proteolytic removal of the transmembrane domain in hepatocytes accounts for the GGT normally found in the blood [15], while the amphipathic form found in bile duct is due to its solubilization by the

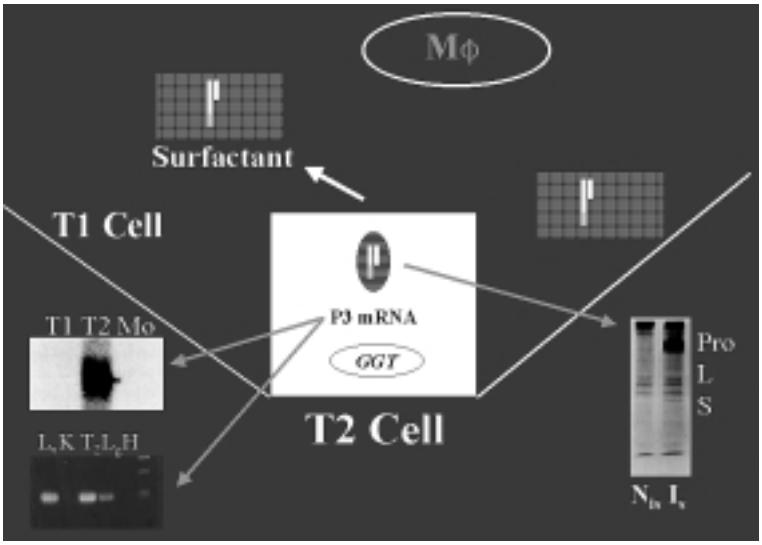

Fig. 2. Lung T2 cell GGT expression. Epithelial T2 and T1 cells and the alveolar macrophage (MO) are denoted and the results of Northern blot for GGT mRNA expression along with RT-PCR with P3 specific primers shown at left (Lv = liver, K = kidney, $T2$ = lung T2 cell, Lg = lung, H = heart). Protein immunoprecipitation is shown at right using nonimmune (Nis) or immune (Is) serum. Enzymatically active GGT heterodimer is shown in secretory lamellar body of T2 cell and in association with extracellular lung surfactant.

detergent action of secreted bile salts [16]. Our own work has shown that GGT in lung surfactant retains its membrane anchor and bilayer association consistent with a secretory mechanism for GGT release from alveolar epithelial type 2 cells [2]. GGT activity within epithelial secretions is postulated to regulate the turnover and the size of both intracellular and extracellular glutathione pools. The results of studies in two recent models of GGT deficiency, the $\mathrm{GGT}^{\mathrm{m1/m1}}$ mouse [17] and the $\mathrm{GGT}^{\mathrm{enu1}}$ mouse [6], have established the importance of this enzyme in mammalian glutathione homeostasis and cysteine supply. Our studies focused on the importance of GGT expression to glutathione homeostasis in the lung.

3. Alveolar epithelial type 2 cell GGT and surfactant

Controversy over the importance of GGT and glutathione metabolism in the lung stemmed from a deficit of knowledge about lung GGT expression. The level of GGT activity in the lung as a whole was known to be very low compared to other organs, especially the kidney. So glutathione metabolism in the lung was considered physiologically insignificant [18]. Studies focused solely on the presence of lung GGT enzyme activity or lung GGT protein suggested several different cell types [19–21] as potential GGT sources, including the flat alveolar epithelial type 1 (T1) cell [22]. Since the focus of my laboratory is lung alveolar epithelial cell biology, this observation suggested that GGT could serve as a new tool to investigate the process of differentiation as the cuboidal alveolar epithelial type 2 (T2) cell differentiates into the flat T1 cell. In order to study this process precisely, we integrated the expression of GGT mRNA [23] with that of protein and enzyme activity. Contrary to our working hypothesis, analysis of RNA from freshly isolated lung alveolar epithelial T1 cells, type 2 (T2) cells and alveolar macrophage cells (MN) revealed expression of GGT mRNA solely in the T2 epithelial cell (Fig. 2). PCR analysis with upstream primers targeting the unique 5' UT GGT mRNA regions showed that this

GGT Enzyme Source	Specific Activity (nmol/min/mg protein)
Kidney	1860 ± 435
Lung	6.8 ± 1.5
Heart	<0.1
T2 cell	15.0 ± 3.4
T1 cell	1.4 ± 0.6
Macrophage	1.6 ± 0.5
Lung Lavage	
Surfactant-enriched fraction	46.2 ± 5.6
Surfactant-depleted fraction	1.3 ± 0.2

Fig. 3. GGT enzyme activity. Values are specific activities in organs, lung cells and lung lavage fractionated into surfactant-enriched or depleted fractions.

T2 cell GGT transcript was derived from promoter 3, a promoter initially characterized from the rat liver [24]. Metabolic labeling experiments confirmed that T2 cells synthesize GGT protein and enzyme assays showed an enrichment of GGT activity in isolated T2 cells over that in whole lung (Fig. 3). This data provided firm evidence that GGT was expressed by the T2 cell.

However, we did detect a measurable level of GGT activity in the T1 cell and the alveolar macrophage, despite the absence of GGT mRNA expression. To explain this observation, we performed several additional experiments to show that the GGT activity in these cells could be derived from the T2 cell via surfactant, the lipoglycoprotein secretory product of the T2 cell. First, T2 cells could be stimulated to release GGT activity in parallel with surfactant lipid. Second, lung lavage fluid contains GGT activity that, upon centrifugation, partitions with the surfactant enriched fraction. The specific activity of GGT in this fraction is about 7-fold enriched over that of whole lung. Lastly, and in collaboration with Dr. Rebecca Hughey, GGT activity obtained from lung lavage fluid also partitions into the detergent phase of Triton X-114, like amphipathic GGT in renal brush border membranes [2]. GGT activity can be released from either the surfactant-enriched fraction or the detergent phase of Triton X-114 by the enzyme papain, which cleaves the GGT signal anchor and renders the protein hydrophilic [25]. Hence the GGT activity associated with lung surfactant results from the amphipathic protein.

These results suggested to us that lung surfactant can serve a novel function by acting as a vehicle to redistribute this amphipathic molecule throughout the entire gas exchange surface of the lung. Since surfactant lines the entire surface of the lung, this also implied that surfactant-associated GGT protein could have a broad impact on the biology of other lung cells, in addition to the T2 cell. For instance, glutathione is abundant in the lung lining fluid [18]. The presence of GGT activity suggests that glutathione turnover occurs in this pool. In fact, we showed that when the lung is exposed to an inhaled oxidant, there is a dramatic accumulation of surfactant-associated GGT activity [5]. Therefore, the response of the lung to this inhaled oxidant stress may involve an acceleration in the turnover of glutathione within this extracellular pool.

4. GGT ontogeny in lung epithelial cells

The ontogeny of GGT in the lung is a tale of two different epithelial cell types, the alveolar epithelial type 2 (T2) cell and the non-ciliated bronchiolar epithelial (Clara) cell [3]. GGT activity accumulates at

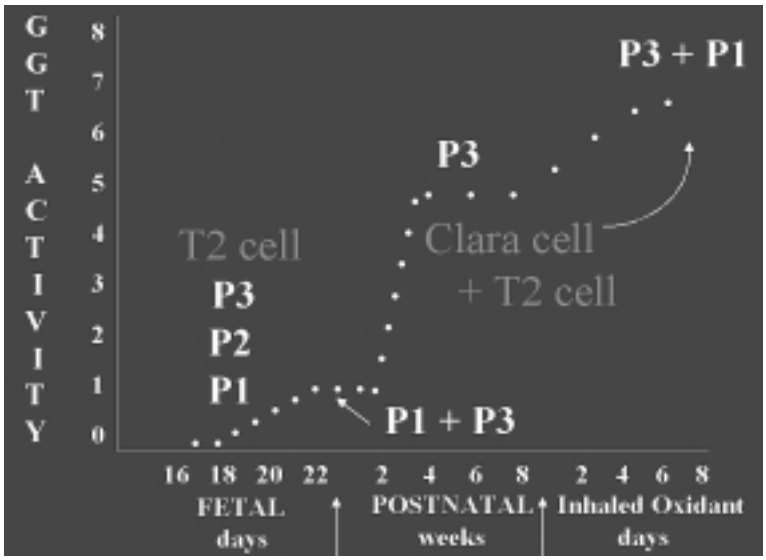

Fig. 4. GGT Ontogeny in Lung Epithelial Cells. Ordinate shows GGT activity (nmol/min/mg protein) and abscissa time during development. GGT ontogeny in specific lung epithelial cells is shown in grey. Activity of alternative promoters P1, P2, P3 is listed in grey.

two distinct periods during lung development (Fig. 4). The first occurs in the fetal lung, late in gestation, co-incident with the accumulation of surfactant phospholipids and several antioxidant enzyme activities. The level of activity, however, is only a fraction of that seen in the adult lung. This first peak results from activation of GGT gene expression in the alveolar epithelial type 2 cell (T2).

In fact, GGT ontogeny from the late fetal through the early postnatal periods is a feature solely of the T2 cell. This suggests that glutathione metabolism may be important in the alveolar region of the fetal lung and at the gas exchange surface of the perinatal lung. When we used PCR to examine the pattern of GGT promoter usage in the fetal lung [4], we found that GGT promoters 1 and 2 were active, in addition to GGT promoter 3. A change in this pattern of GGT promoter utilization became apparent almost immediately after birth with the disappearance of the GGT P2 mRNA within the first 24 hours of breathing oxygen. T2 cells, isolated from adult lung and returned to the level of hypoxia of the fetal lung, re-express GGT P2. In contrast, GGT promoter 1 remained active in the newborn lung, but was inactivated by day 10 after birth. GGT P1 can be re-activated if the adult lung is exposed to an inhaled oxidant [5]. The persistent usage of GGT promoter 3, under normal oxygen conditions, appeared to be due to its activation by oxygen. This differential regulation of the alternative GGT promoters in the lung suggested a mechanism to maintain lung GGT gene expression, and glutathione metabolism, over a wide range of oxygen concentrations. In addition, the change in the oxygen environment at birth may function to regulate the pattern of gene expression in the postnatal lung. Genes that are essential for postnatal development will be activated, and genes important for fetal lung development will be repressed. These data suggest that birth is a natural experiment with hyperoxia and that environmental oxygen could impact the course of postnatal lung development. However, the biological role of this regulation of the alternative GGT promoters remains obscure since the identical protein product is generated from all three promoters. Alternatively, promoter usage may be linked to mRNA function if sequences within the unique 5' untranslated regions of the GGT mRNA species can be shown to differentially regulate mRNA translation or stability in lung cells under different oxygen conditions.

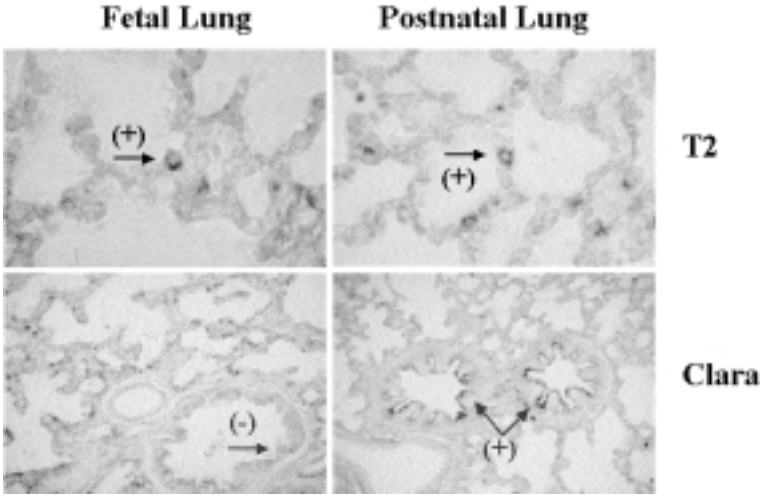

Fig. 5. Immunolocalization of GGT protein in lung parenchyma during development. GGT protein product (black color) localizes to alveolar T2 cell in rat lung at fetal day 21 and postnatal day 14 (black arrows), but to bronchiolar cells only in the postnatal lung (grey arrows).

The second peak of GGT activity occurs abruptly during the late postnatal period and raises the level of GGT activity to that seen in the adult lung. This peak results from activation of the GGT gene in an epithelial cell of the distal airways in the lung. This cell is the non-ciliated bronchiolar Clara cell and it becomes the primary cellular site of expression of GGT mRNA, protein (Fig. 5) and activity in the lung (3). This rise in GGT activity appears to correlate with an accumulation of GGT heterodimer in the lung, in contrast to the alveolar epithelial T2 cell where newly synthesized propeptide appears to accumulate in excess of heterodimer. It is possible that GGT protein serves a different function in these the two different epithelial cell types, perhaps related to the activation of GGT enzyme activity. Certainly, the bronchiolar Clara cell has a high demand for glutathione as a substrate for xenobiotic metabolism, and for antioxidant defense since it is located in small airways that are the primary sites for deposition of inhaled particulate matter. Expression of enzymatically active GGT heterodimer on its cell surface could ensure access to the pool of glutathione in the lung epithelial lining fluid. In support of this, GGT gene expression is highly induced in this epithelial cell following exposure of the lung to an inhaled oxidant gas, thereby increasing cellular access to extracellular glutathione. Taken together, these observations suggest that GGT expression is important for the bronchiolar Clara cell in the mature lung and imply two things: first, loss of lung GGT expression should make this cell particularly vulnerable to glutathione depletion and injury by oxidants; and second, since activation of Clara cell GGT expression is delayed until the late postnatal period, the bronchioles of the perinatal lung may be particularly susceptible to oxidants and inhaled particles.

5. Consequences of lung GGT deficiency in the GGT^{enu1} mouse

The GGT^{enu1} mutant mouse provided a model in which to test some of these observations and to determine if GGT expression was important in the lung. The GGT^{enu1} mouse is genetic model of GGT deficiency that was generated by randomly inducing point mutations in the mouse genome with ethylnitrosourea (enu) then selecting progeny for aminoaciduria. In the case of the GGT^{enu1} mouse, the

aminoaciduria was glutathionuria. This resulted from the nearly complete inactivation of GGT enzyme activity in the kidney. A systemic inactivation of GGT gene expression was suggested by the presence of growth retardation, premature mortality, infertility and cataracts [6]. The GGT^{enu1} phenotype was very similar to that of the $GGT^{m1/m1}$ mouse, a model of complete GGT gene inactivation produced by targeted mutagenesis of the mouse GGT gene [17]. The glutathionuria in the $GGT^{m1/m1}$ mouse was of a much greater magnitude than that in the GGT^{enu1} mouse and caused a deficiency in plasma cysteine content, systemic cysteine supply and glutathione content in many tissues. In contrast, a small residua of GGT activity 0.14% of normal persisted in the kidney of the GGT^{enu1} mouse (M. Joyce-Brady and R.P. Hughey, unpublished observation.) Hence the level of glutathionuria was less severe. Plasma cysteine content remained normal though a decrease in urinary excretion of taurine, a cysteine metabolite, suggested a relative deficiency in the supply of Cysteine or other sulfur-containing amino acids. Although the phenotype is similar in these two mouse models, the manifestations of GGT deficiency in the GGT^{enu1} mouse are less severe than that in the $GGT^{m1/m1}$ mouse.

In order to study the GGT^{enu1} mouse, we first had to characterize the site of the point mutation in order to develop a genotyping strategy to breed the mice in my laboratory. Since GGT is a single copy gene in the haploid mouse genome, and the phenotype suggested widespread GGT deficiency, we suspected that the point mutation was in the coding domain. We located the mutation in the first coding exon, where an AT-TA transversion caused a leucine codon (TTG) to be replaced by a stop codon (TAG). Introduction of this premature translation termination codon caused a secondary decrease in the steady state level of GGT mRNA in the kidney, a phenomenon known as a low RNA phenotype. In addition, premature termination of GGT protein synthesis produced a severely truncated oligopeptide lacking all of the amino acid residues required for enzyme activity. Together, these contributed to the loss of nearly all GGT activity. A tiny residua of GGT activity persisted in the kidney, which normally expresses GGT at very high levels. We proposed that this could result if some GGT protein synthesis was initiated at the downstream methionine codon, 117, which is also preceded by a Kozak consensus sequence. Despite the residual GGT activity, the kidney in the GGT^{enu1} mouse displays evidence of oxidant stress as heme oxygenase-1 mRNA is induced by 4-fold, and Cu, Zn SOD mRNA by 3-fold compared to that in normal kidney [7].

We then examined the GGT^{enu1} mouse lung for evidence of oxidant stress. To determine this, we assessed: 1) the pattern of lung GGT promoter usage, 2) the status of lung glutathione content and redox ratio, 3) the presence of a 3-nitrotyrosine signal in lung cells by immunohistochemistry, and 4) the response of these mice to an environment of $>$95% oxygen [8].

We used RT-PCR with GGT mRNA subtype specific primers to assess activity of the three GGT promoters. GGT promoter 3 was active in the normal mouse kidney and the lungs of normal and GGT^{enu1} mice and normal rat. GGT promoter P2 was only active in the normal mouse kidney. But GGT promoter 1 was active in normal mouse kidney and in GGT^{enu1} lung. Since GGT P1 promoter is activated by oxidant stress in normal lung, these results are consistent with oxidant stress in the GGT^{enu1} lung.

Total lung glutathione content, measured by the recycling method of Teitze, was only minimally decreased in homogenates of GGT^{enu1} compared to normal lung (Fig. 6). This likely reflected the adequacy of lung cysteine supply in the GGT^{enu1} mouse. However, the fraction of GGT^{enu1} lung GSSG (oxidized glutathione), assessed by HPLC in collaboration with Dr. Lou Ann Brown, was increased more than 3-fold. This suggested oxidant stress in sub-populations of lung cells, cells where glutathione homeostasis was likely dependent on lung GGT expression. Therefore we assessed glutathione content in specific lung cells and lung lining fluid.

	GGT^{enu1}	Normal
Lung (nmol/mg protein)	0.76 ± 0.15	0.80 ±0.12
%GSSG	31±2	9 ±1
Mϕ (nmol/10^6 cells)	0.88 ± 0.55	5.1 ±1.0
%GSSG	70 ± 6	10 ± 2
ELF (uM)	1639 ± 164	834 ±190
GSH/GSSG	3.3 ± 0.26	6.9 ± 0.2
Plasma (uM)	240 ± 52	61±2

Fig. 6. Glutathione Content. Glutathione content and redox ratio were determined in the lung, the macrophage, the epithelial lining fluid (ELF) and the plasma. The ELF values were normalized using the urea dilution method.

Glutathione content can be measured readily in the alveolar macrophage and the lung lining fluid using simple lung lavage. Total glutathione content in the GGT^{enu1} macrophage, determined by HPLC, was decreased by almost 6-fold compared to the normal macrophage and the GGT^{enu1} macrophage GSSG fraction was increased by 7-fold indicating intense oxidant stress. Macrophage glutathione content and the redox state of the cell were severely compromised by the absence of glutathione turnover in the alveolar lining fluid.

Interestingly, the concentration of glutathione in the GGT^{enu1} alveolar lining fluid was actually increased 2-fold over that in normal lung. But it was also more oxidized as the GSSG content increased by 4-fold. These data are consistent with a role for surfactant-associated GGT in the turnover of the extracellular glutathione pool in the lung. This result mimics the glutathionemia seen in the GGT^{enu1} plasma. Three questions arose from these studies. How did GSSG rise disproportionately over GSH? Is there a decrease in GSSG clearance over GSH in the GGT^{enu1} lung? Would epithelial cells in the GGT^{enu1} lung be protected against oxidants by this expanded glutathione pool?

Glutathione content in the mouse lung epithelium was not so simple to assess, particularly the non-ciliated bronchiolar epithelial cell population. A cell isolation procedure could only produce a partially pure population of these cells and might also perturb cellular glutathione content. Therefore, we assessed epithelial cell glutathione content in situ using an immunohistochemical technique in collaboration with Dr. Robert Marc at the Moran Eye Center of the University of Utah. Dr. Marc is an expert on the development and application of immunologic reagents to localize glutathione and its related amino acids in cells and tissues [26]. To validate this technique in the GGT^{enu1} mouse we first studied the liver.

Biochemical measure of total glutathione content in a homogenate of the GGT^{enu1} liver is decreased by 3-fold compared to that of normal liver [6]. The immunohistochemical method showed a decrease in glutathione signal intensity in the GGT^{enu1} liver compared to normals and the calibrated glutathione signal intensity in the GGT^{enu1} hepatocyte was decreased by 4-fold compared to the normal hepatocyte (175 uM vs 830 uM). Hence glutathione content correlated very well when assessed by these two different methods (Fig. 7).

This immunohistochemical technique was then applied to assess glutathione content in the lung, particularly epithelial cells in the bronchioles. Non-ciliated Clara cells are found in the bronchiole as

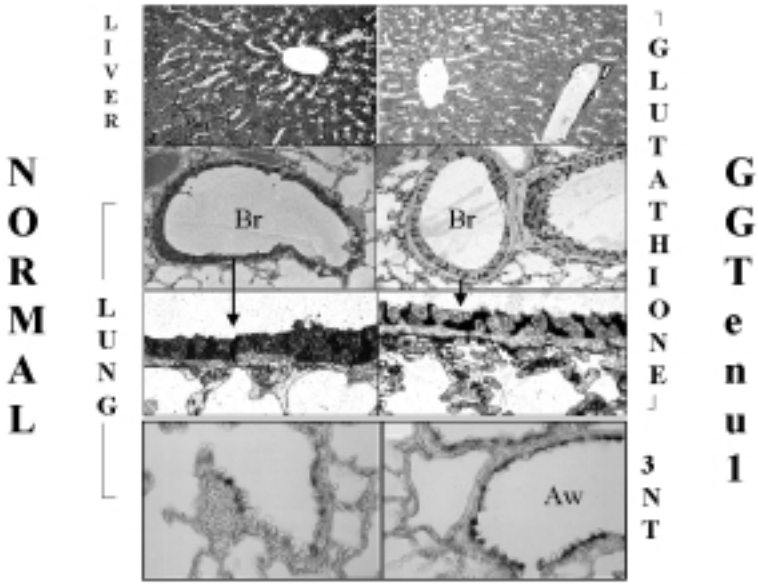

Fig. 7. Immunocytochemical localization of glutathione and 3-nitrotyrosine (3NT). The liver and the lung from normal (left) and GGT^{enu1} (right) mice was examined for glutathione content in the top three panels and 3-NT in the bottom panel. For glutathione, black signal denotes abundance and grey signal depletion of cellular glutathione content. A normal and GGT^{enu1} lung bronchiole (Br) is compared at low and high power in the second and third panels. Dense black signal localizes to ciliated epithelial cell in both. Glutathione signal is less dense in normal Clara cell but grey in GGT^{enu1} Clara cell indicating glutathione depletion. The 3 NT signal (black color) at the bottom shows sparse and weak signal in normal bronchiolar cells but dense and uniform signal in GGT^{enu1} bronchiolar cells.

well as a second epithelial cell type, the ciliated epithelial cell. In the normal lung, a glutathione signal was present in both types of epithelial cell, but it was more intense in the ciliated cell. The reason for this difference is not yet clear but it suggests a difference in glutathione utilization. In the GGT^{enu1} lung, an intense glutathione signal was still present in the ciliated cell but the signal was very weak in the non-ciliated (Clara) cell. Hence, the GGT deficient Clara cell is glutathione depleted. Alveolar epithelial type 2 cells express much lower levels of GGT than Clara cells and a difference in glutathione content between normal and GGT^{enu1} T2 cells was not evident by this technique.

To determine if glutathione deficiency in the GGT^{enu1} Clara cell was associated with oxidant stress, we probed for the presence of 3-nitrotyrosine. These modified tyrosine residues result from the interaction of tyrosine with peroxynitrite and are stable products [27]. A 3-nitrotyrosine signal (black color in lower panel of Fig. 7) was actually evident in some bronchiolar cells of normal lung but it was weak and sparse. This probably reflects the dynamic nature of cellular glutathione pools in bronchiolar cells. In contrast, the 3-nitrotyrosine signal was more intense and uniform in the GGT^{enu1} bronchiolar epithelium, consistent with oxidant stress. Specificity of the signal was assured by co-incubating the primary antibody with 10 mM nitrotyrosine which eliminated the nitrotyrosine signal. As a 3-nitrotyrosine signal was also evident in the alveolar macrophage, there was good correlation between loss of lung cell GGT expression with sites of decreased glutathione avaibility and oxidant stress.

To determine the consequences of change in epithelial cell redox state, we studied the response of the GGT^{enu1} mice to an environment of $>$95% oxygen and found that the GGT^{enu1} mice died more rapidly than the normal mice. Cellular injury and pulmonary edema developed more quickly in the GGT^{enu1} lung. This was quite evident when we compared normal and GGT^{enu1} lung after 96 hours in hyperoxia

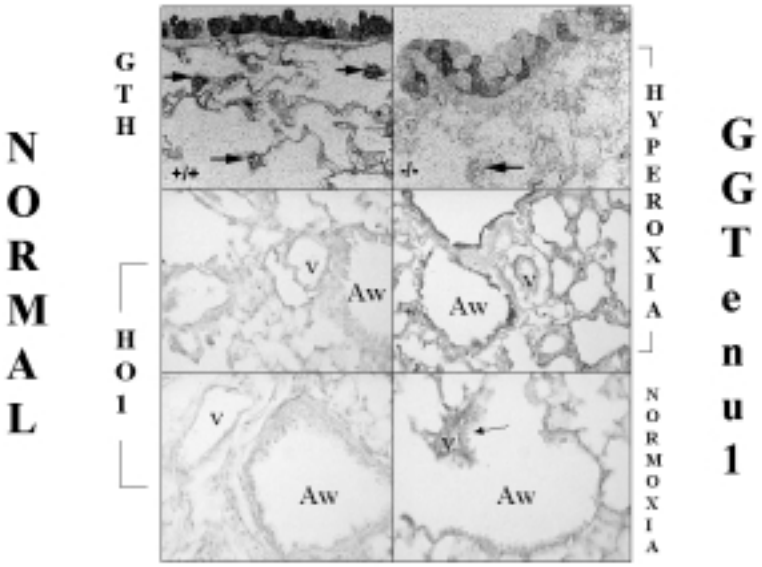

Fig. 8. Immunolocalization of glutathione (GTH) and heme oxygenase-1 (HO1). The lungs of normal and GGT^{enu1} mice were examined after a 96 hour exposure to hyperoxia. Dense black signal in top panel indicates abundant glutathione (GTH) in cells and lining fluid of normal lung but grey signal indicates depletion of glutathione from cells and lining fluid in GGT^{enu1} lung. In normoxia, heme oxygenase-1 expression (black signal) localizes only to vascular cells in GGT^{enu1} lung. In hyperoxia, HO-1 protein localizes only to vascular cells in normal lung but to vascular cells and the entire epithelial surface in the GGT^{enu1} lung. Severe lung injury and cell damage is now evident in the bronchiolar epithelium of the GGT^{enul} lung.

(in Fig. 8). GGT^{enu1} lung glutathione content was widely depleted by glutathione immunohistochemistry compared to the normal lung. In addition, heme oxygenase-1 protein expression was also widely induced throughout the entire epithelial surface of GGT^{enu1} lung but not in the normal lung. Interestingly, heme oxygenase-1 was already localized to vascular endothelial cells even in normal oxygen conditions but only in the GGT^{enu1} lung. So these cells were apparently under oxidant stress even prior to exposure to hyperoxia, and thus one would expect injury and pulmonary edema to develop more rapidly in the GGT^{enu1} lung. It is not yet clear whether this sensitivity resulted from loss of GGT in the plasma.

Taken together, our data supports an important role for GGT expression in the adult lung for antioxidant defense. Our present goals are three-fold: 1) to determine the consequences of GGT deficiency on lung epithelial cell and macrophage gene expression and function; 2) determine if GGT expression is important in the lung at birth under different oxygen conditions; and 3) begin to explore the mechanism by which GGT protects epithelial cells from oxidant stress. Of considerable interest is the question of whether GGT senses glutathione content or the cellular redox state, and whether it performs a function that is specific to epithelial cells.

6. New role for GGT in the endoplasmic reticulum

During our characterization of the point mutation that inactivated GGT gene expression in the GGT^{enu1} mouse, we identified four alternative splicing events in mouse GGT cDNA. Dr. Rebecca Hughey will discuss our detailed characterization of these events at this conference. I wish to use one of these events to introduce the concept that GGT may serve a new function in the epithelial cell. The first alternative splicing event that we found involved the insertion of 22 new bases into the mouse GGT cDNA. The

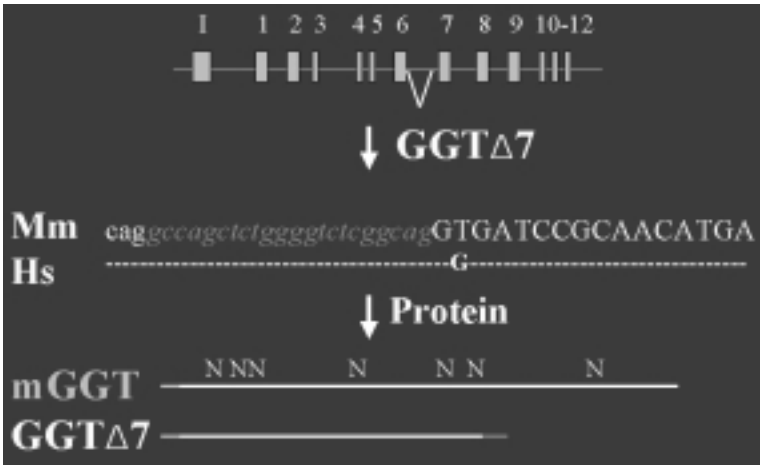

Fig. 9. Schematic of Alternative Splicing Event in GGTΔ7. Cartoon shows site of alternative processing event at GGT gene in exon 7. The 22 base insertion in GGT mRNA is shown in grey italics (boxed) for mouse (Mm) and single nucleotide difference in human GGT mRNA (Hs) is listed below. Normal mouse GGT protein and truncated GGTΔ7 protein isoform with its new C-terminus are compared at the bottom.

insertion caused a frame shift and introduced a premature stop codon into the GGT cDNA so that a new GGT protein was encoded. This protein was truncated within the large subunit and included 14 novel amino acid residues at the C-terminus. The loss if all residues from the small subunit predicted an absence of GGT activity.

Our initial impression was that ethylnitrosourea treatment introduced a point mutation in the GGT^{enu1} mouse that disrupted a constitutive splice site. However, a search of GenBank revealed that the same 22 base insertion was already described as a naturally occurring alternative splicing event in a human GGT cDNA [28]. Therefore, a mutation did not cause this event. Rather, cloning and partial sequencing of mouse GGT intron 7 revealed that the identical 22 base insertion sequence, preceded by a CAG, was shared between mouse and human GGT. This was an alternative splicing event that occurred within intron 7, so we called it GGTΔ7 (Fig. 9). The corresponding human GGT protein product was never characterized. Therefore, Dr. Hughey and I embarked on a collaborative effort to characterized this GGT protein isoform. The GGTΔ7 protein was a rather stable glycoprotein monomer that lacked GGT enzyme activity and was retained in the endoplasmic reticulum. This alternative splicing event was regulated in a developmental and tissue-specific fashion, suggesting that it served a physiologic function. When expressed in CHO cells, this isoform, as well as native GGT, mediated an endoplasmic reticulum stress response after the cells were challenged with a medium containing solely cystine, instead of a mixture of cysteine and cystine. Although we do not yet know the metabolic basis for this effect, the result suggests that GGT functions within the ER in a previously unknown fashion. This implies that GGT may actually serve a number of important homeostatic functions in the epithelial cell [9].

7. Questions raised by our research on lung GGT expression

The following is a summary of several questions about GGT gene expression that our work has raised and that should lead to new insights into the biologic functions of GGT. What is the biological role of alternative promoter usage in regulating lung GGT gene expression in the lung? Does it affect the

stability or the translational efficiency of different GGT mRNA species in lung epithelial cells? Does alternative GGT promoter usage impact the pattern of alternative GGT mRNA splicing within the coding region? Or is this alternative splicing pattern regulated by the cellular redox state or the cellular content of glutathione of lung epithelial cells?

Is there regulation of the autocatalytic activity that converts enzymatically inactive GGT propeptide into the active heterodimer? Such a mechanism could impact the rate of glutathione turnover which may prove important in the endoplasmic reticulum, at the cell surface or within epithelial cell secretions. In the lung, this could play a role in regulating glutathione turnover and the size of the glutathione pool in the lung lining fluid which protects the gas exchange surface against oxidants. It may also provide insight into different functions that GGT might serve in the Clara cell and the T2 cell.

Lastly, how does GGT protect lung epithelial cells from oxidant stress? Does it sense glutathione directly or, alternatively, the cellular redox state? How important is this function in alveolar epithelial type 2 cells in the perinatal lung before and after birth?

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