

RETHINKING CYSTIC FIBROSIS PATHOLOGY: THE CRITICAL ROLE OF ABNORMAL REDUCED GLUTATHIONE (GSH) TRANSPORT CAUSED BY CFTR MUTATION

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Abstract—Though the cause of cystic fibrosis (CF) pathology is understood to be the mutation of the CFTR protein, it has been difficult to trace the exact mechanisms by which the pathology arises and progresses from the mutation. Recent research findings have noted that the CFTR channel is not only permeant to chloride anions, but other, larger organic anions, including reduced glutathione (GSH). This explains the longstanding finding of extracellular GSH deficit and dramatically reduced extracellular GSH:GSSG (glutathione disulfide) ratio found to be chronic and progressive in CF patients. Given the vital role of GSH as an antioxidant, a mucolytic, and a regulator of inflammation, immune response, and cell viability via its redox status in the human body, it is reasonable to hypothesize that this condition plays some role in the pathogenesis of CF. This hypothesis is advanced by comparing the literature on pathological phenomena associated with GSH deficiency to the literature documenting CF pathology, with striking similarities noted. Several puzzling hallmarks of CF pathology, including reduced exhaled NO, exaggerated inflammation with decreased immunocompetence, increased mucus viscoelasticity, and lack of appropriate apoptosis by infected epithelial cells, are better understood when abnormal GSH transport from epithelia (those without anion channels redundant to the CFTR at the apical surface) is added as an additional explanatory factor. Such epithelia should have normal levels of total glutathione (though perhaps with diminished GSH:GSSG ratio in the cytosol), but impaired GSH transport due to CFTR mutation should lead to progressive extracellular deficit of both total glutathione and GSH, and, hypothetically, GSH:GSSG ratio alteration or even total glutathione deficit in cells with redundant anion channels, such as leukocytes, lymphocytes, erythrocytes, and hepatocytes. Therapeutic implications, including alternative methods of GSH augmentation, are discussed. © 2001 Elsevier Science Inc.

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INTRODUCTION

Cystic fibrosis (CF) is a genetic disease afflicting nearly 30,000 persons in the United States and Canada, and an estimated 250,000 persons worldwide. The mutated gene is recessive, and there are hundreds of genetic mutations that can produce cystic fibrosis. The mutations all cause the ion channel created by the cystic fibrosis transmembrane conductance regulator (CFTR) protein to be either defective or absent altogether. More specifically, this protein creates an organic anion efflux channel in the cell

membrane, permeant to chloride and other larger organic anions, such as reduced glutathione [1,2]. CF patients typically die of respiratory failure due to profound lung injury secondary to chronic inflammation and chronic pathogen colonization of the lung, although several exocrine organs are negatively affected, including the pancreas and liver. In countries where these patients receive optimal care, average survival has risen to approximately 30 years.

Though the cause of CF pathology is understood to be the mutation of the CFTR protein, it has been difficult to trace the exact mechanisms by which the pathology arises and progresses from the mutation. For example, even when not facing pathogen challenge, inflammation is present in the youngest infants [3–6], and inflamma-

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tory mediators and cytokine markers appear to be constitutively elevated in CF patients [7–12]. When pathogens do begin to challenge the system, neutrophil infiltration in CF is especially high in response [13], and very young CF patients have been found to clear bacteria even without antibiotic intervention [14]. Over time, however, that capability decreases, and despite continuing high neutrophil infiltration rates, bacteria are no longer cleared. By age 2, most CF patients are consistently culturing at least one pathogen [15,16]. Indeed, lung cells expressing the mutant CFTR fail to undergo apoptosis in response to infection with *Pseudomonas aeruginosa* [17]. In many pulmonary diseases involving inflammation due to pathogen challenge, exhaled NO of patients is elevated [18–22]. In CF, exhaled NO is not elevated [19,22–27]. In pulmonary conditions involving high oxidant stress, extracellular levels of reduced glutathione (GSH) increase [28–30]. In CF, a systemic deficiency of extracellular GSH develops and progresses over time [31–33].

How are all of these puzzling phenomena related to a defective CFTR channel? We believe that abnormal transport of GSH caused by CFTR mutation, recently demonstrated by two research teams [1,2], is significantly related to the pathological puzzles mentioned above, as well as other aspects of cystic fibrosis disease. As will be detailed in a later section, CFTR mutation results in significantly diminished efflux of cellularly produced GSH into the extracellular milieu from certain cells without redundant channels to effect such efflux. The focus of this article will be on the role of this phenomenon in the pathogenesis of cystic fibrosis. We hypothesize that this transport abnormality will result in (i) normal levels of total glutathione in epithelia (those without anion channels redundant to the CFTR at the apical surface; however, cytosolic GSH:GSSG ratio may be diminished in these cells), (ii) a chronic and progressive extracellular deficit of GSH, and (iii) a similar deficit in cells with redundant anion channels, such as leukocytes, lymphocytes, erythrocytes, and hepatocytes. But perhaps most importantly in a theoretical sense, this transport abnormality is directly caused by the CFTR mutation, providing at least part of the missing linkage between the genetics and the pathology of CF. To explain this hypothesis, we must first step back and understand the importance of GSH in lung defense.

GSH AND LUNG DEFENSE

Reduced glutathione (GSH) is a ubiquitous tripeptide produced by plants and animals alike from the amino acids glutamine, glycine, and cysteine (with cysteine being the rate-limiting constituent). Its sulfur-hydrogen, or thiol, group is a potent reducing agent, and GSH can

be considered the one of the body's most important water-soluble antioxidants.

Antioxidant defenses in humans are comprised of both enzymatic and nonenzymatic defenses; some defenses operate intracellularly and others have additional extracellular functions. The antioxidant enzymes are glutathione peroxidase, superoxide dismutase, and catalase. There are two types of nonenzymatic antioxidants, the water-soluble and the fat-soluble. The water-soluble antioxidants include reduced glutathione and ascorbic acid; the fat-soluble include α -tocopherol and the carotenoids. The enzymatic antioxidants are lodged within the cellular membranes (though there is a free plasma glutathione peroxidase [34]); in contrast the water-soluble antioxidants (as free molecules) are present in the cytosol of the cells, though some, including GSH, are also present in the extracellular environment. The fat-soluble antioxidants are found within the lipid membranes. Each antioxidant defense system protects the cells from oxidative damage in its own sphere of action, and a deficiency in any category puts the cell at risk for oxidative damage. It should be noted that GSH is a precursor for the function of glutathione peroxidase, and that GSH, ascorbate, and α -tocopherol exist in an interdependent system, where normal levels of each in reduced form are dependent on normal levels of the others. As noted, in addition to its cellular functions, GSH is also present in extracellular epithelial fluids, such as the epithelial lining fluid (ELF) of the lung, blood plasma, semen, saliva, and so forth, where antioxidant action is similarly useful. In the extracellular milieu, GSH is capable of direct reduction without glutathione peroxidase. A generalized diagram of normal GSH system function and transport in a non-CF lung epithelium cell is given in Fig. 1.

GSH provides powerful antioxidant protection to body systems heavily exposed to reactive oxygen species (ROS), such as the lung [35,36]. For example, the normal level of extracellular GSH in the lung epithelial lining fluid (ELF) is 140 times the normal level of extracellular GSH in blood plasma, and it is probable that the lung, under oxidative stress, becomes a net importer of circulating GSH [28]. Glutathione deficiency in the ELF has been associated not only with cystic fibrosis, but also with such pulmonary diseases as acute respiratory distress syndrome (ARDS) [37], chronic obstructive pulmonary disease (COPD) [35], idiopathic interstitial pneumonia (IIP) [38], idiopathic pulmonary fibrosis (IPF—of nonsmokers) [39], idiopathic respiratory distress syndrome (IRDS) [40], and diffuse fibrosing alveolitis (DFA) [41]. A deficiency has also been found in the lungs of HIV-positive patients [42,43]. However, it would be wrong to view GSH only or even most importantly in terms of its antioxidant properties when considering its importance in lung defense.

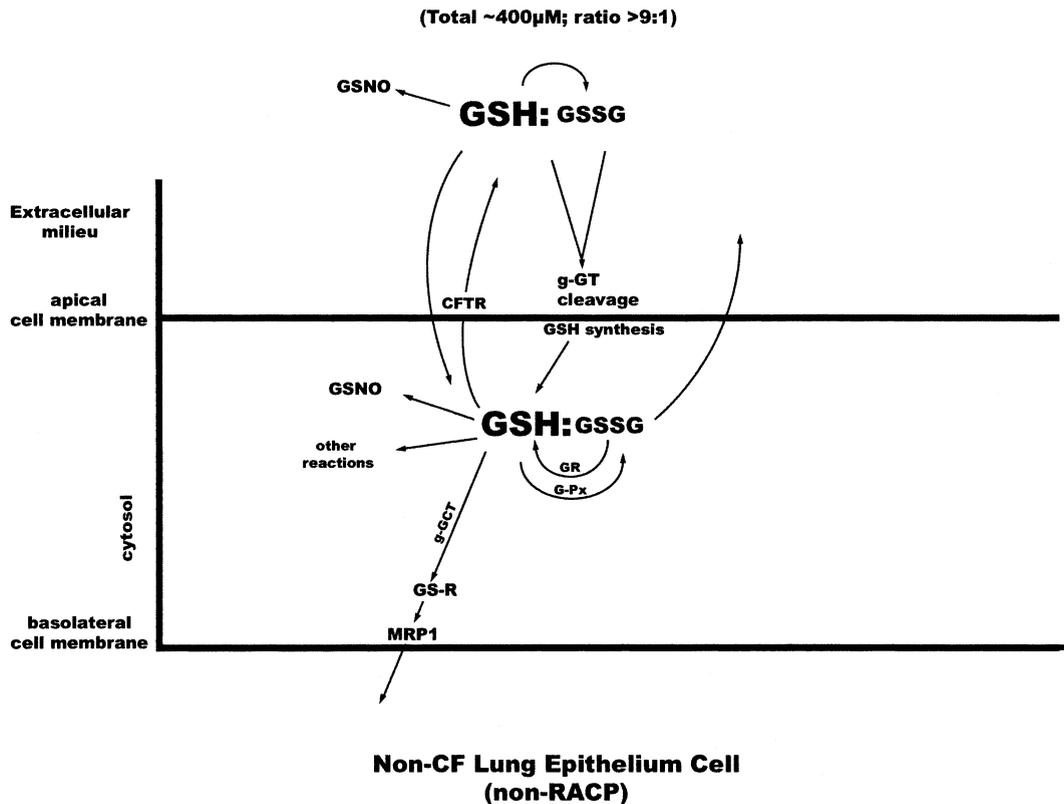


Fig. 1. Normal GSH system in non-RACP cell.

A second property of reduced glutathione that should not be overlooked is its promotion of mucolysis. Because of its chemistry, GSH, like N-acetylcysteine (NAC), is able to cleave disulfide bonds, which serves to reduce the viscoelasticity of mucus when the glutathione system is functioning normally [44,45]. Furthermore, reduced glutathione plays a role in establishing the volume of periciliary fluid, and also affects mucus hydration through its indirect relationship with epithelial sodium channels (ENaC), topics we will explore in a later section.

A third property of reduced glutathione is to regulate inflammation and immune response. This regulation is carried out in a number of ways, which will be discussed below. Generally speaking, however, the main mechanism of regulation is by redox potential. Since GSH is synthesized by virtually every cell in the body, a redox equilibrium is established within each of these cells, and the efflux of GSH therefrom establishes an extracellular redox equilibrium as well. Both intracellular and extracellular redox equilibria vary: extracellularly, the equilibrium varies by bodily system, as noted above; and intracellularly, the equilibrium varies by cell type. For example, the intracellular GSH level of monocytes is three times that of neutrophils [46]. The redox potential, expressed by the ratio GSH:GSSG (GSSG being glutathione disulfide, the product when reduced glutathione is

oxidized), influences many sensitive cellular systems and functions. Indeed, as we shall see, inflammation, immune response, and cell viability, among other things, are intimately tied to this ratio. If, as we will hypothesize in a later section, CF leukocytes and lymphocytes (along with other cells possessing anion channels redundant to the CFTR) are prone to chronic alteration of the GSH:GSSG ratio, immune response will be profoundly affected.

The clearest way to demonstrate the importance of a properly functioning GSH system is to detail what scientific research tells us about phenomena associated with GSH deficiency.

ASSOCIATIONS OF GSH DEFICIENCY

Given that GSH is one of the organic anions whose efflux depends on a functioning CFTR channel (or a channel redundant to the CFTR), we would expect CF persons to manifest a progressive systemic extracellular deficiency of GSH, with profound deficits in areas heavily exposed to ROS, such as the lung. This is, in fact, the case, as we will detail in a later section. We would also hypothesize that cells with anion channels redundant to the CFTR, such as leukocytes, lymphocytes, erythrocytes, and hepatocytes, may also develop chronic intra-

cellular GSH deficiency, or, at a minimum, chronically diminished GSH:GSSG ratio. (Let us call these cells RACP cells: cells with a Redundant Anion Channel Present.) Parallels can be drawn between the observed pathogenesis of CF and other pathological conditions associated with GSH deficiency [47,48]. The comparison is not perfect, because CF epithelia without redundant anion channels to the CFTR should display normal levels of total glutathione: indeed, it should prove difficult to lower that total level because of the missing or defective CFTR channel, even under natural conditions favoring such depletion, such as programmed cell death. (However, it is unclear whether there might also develop an alteration in GSH:GSSG ratio in such non-RACP cells, as well.) But in the extracellular compartment, and in cells with such redundant channels—especially immune system cells—the comparison should hold. We will discuss three main categories of known associations of GSH deficiency: impaired antioxidant capability, decreased mucolysis, and abnormal immune response, including decreased nitric oxide production and availability.

Impaired antioxidant capability

When a key antioxidant such as GSH is deficient in the extracellular compartment and in RACP cells, direct damage from oxidants increases greatly. In addition, glutathione deficiency in leukocytes (a type of RACP cell) has been shown to cause increased release of hydrogen peroxide [49]. Oxidant damage to lung epithelial cells from a diminished extracellular antioxidant screen reduces lung function, causing fibrosis and permitting greater adhesion of bacteria [35,36,50–52]. When GSH is deficient, other antioxidants may be consumed in higher-than-normal amounts to handle the increased oxidant burden. Thus, GSH deficiency is often accompanied by lower levels of α -tocopherol and ascorbate, and decreased activity of glutathione peroxidase, catalase, and superoxide dismutase, all of which are reversible when GSH level is normalized [53]. New research also points to the direct role of GSH in neutralizing HOCl, one of the deadliest oxidants to be found in the respiratory milieu, with the reaction being:



GSH deficiency also causes oxidant damage to the liver [55–65] and pancreas [66–68], resulting in impaired function of these organs, including reduced bile flow [57,62,64,69], and is associated with the onset of diabetes mellitus [70–76], rheumatoid arthritis [77], peripheral nerve damage [78], and myocardial injury [79]. GSH deficiency results in intestinal mucosal injury and chronic inflammation of the gut [80–85], decreased ab-

sorption of nutrients such as calcium [86], and general cachexia and growth retardation [87–90]. GSH deficiency also causes greater lipid peroxidation, which, in turn, causes cell damage [53,91–96]. As peroxidation of arachidonic acid yields especially damaging metabolites, it is noteworthy that CFTR knockout mice have been found to have 3-fold more arachidonic acid (AA) and 3-fold less docosahexaenoic acid (DHA) in the cell membrane than normal [97], and that the cytotoxicity of arachidonic acid is enhanced by GSH depletion [98]. The metabolism of arachidonic acid also entails the production of radicals by virtue of the lipoxygenase and cyclooxygenase pathways, further increasing oxidant burden. For example, 8-isoprostane in breath condensate of CF persons is elevated 3-fold above normal controls [99].)

Loss of GSH antioxidant capability in the extracellular milieu has secondary effects, as well. One such effect is oxidant-derived inactivation of the anti-protease system [100–102]. Such inactivation produces its own cascade of harmful effects due to unbound neutrophil elastase (NE). In the lung, these include, as Barbero notes in a review of the scientific literature, “cleavage of fibronectin, lung elastin, immunoglobulins and immune complexes, complement receptors in neutrophils, and other receptors on T-cells and B-cells. Furthermore, NE inhibits ciliary beating and stimulates mucus production from goblet cells and facilitates *P. aeruginosa* adherence. Finally, by cleaving receptors for interleukin (IL)-1 and IL-2 or the T-cell antigen receptor, it may hypothetically inhibit message transmission and immune recognition. Thus, besides destruction, [free NE] may lead to acquired immune suppression” [103]. GSH depletion has also been linked to increased levels of collagenase [104].

Other secondary effects of the loss of GSH antioxidant protection in the extracellular milieu include degradation of lung surfactant, both via oxidant damage and through the inability of the body to maintain appropriate levels of phosphatidylcholine under conditions of GSH deficiency [105–107]. This in turn may be related to the linkage between GSH depletion and reduced lung surface tension [40]. Though it is intriguing to note that GSH depletion also leads to free radical-induced respiratory muscle fatigue, which contributes to respiratory failure in patients with lung disease [108], and that more generalized skeletal muscle fatigue also results from GSH depletion [109], because MRP in muscle cells is usually not expressed at the membrane [110], it is not clear whether these findings would be pertinent to the case of CF or not. These cells should have normal levels of total glutathione, but it is possible that a diminished GSH:GSSG ratio in the cytosol of such cells might develop over time due to ever-increasing oxidant burden, leading to fatigue. However, it is relevant to the CF case

to note that GSH depletion, by increasing airway inflammation, has been hypothesized to lead to the development of asthma [111]. Furthermore, when GSH in the ELF is deficient, the body decreases enzymatic activity transforming GSSG back into GSH, and instead further oxidizes GSSG into chloramines, long-lived oxidants capable of profound injury to the lung [112,113]. (For illustration, chloramines are the agent of lung damage when ammonia and bleach are mixed.) Oxidation products also serve to inhibit the enzymatic activities of glutathione reductase, essential in recycling of cellular GSSG into GSH [114,115], and we would expect this inhibition in RACP cells where the cellular redox state has been chronically shifted in the direction of oxidation due to GSH depletion. Oxidation appears to decrease γ -glutamylcysteine transferase (γ -GCT) activity, as well [116], and this would affect cellular conjugation of cytotoxic substances. These secondary effects, by damaging the integrity of lung tissue, increasing epithelial permeability, and further disrupting the GSH antioxidant system, allow for increased adhesion of pathogens [117]. Regulation of neutrophil adhesion is also affected by GSH depletion [118]. GSH depletion also causes oxidation of protein thiols, protein denaturation and aggregation, increases in insoluble proteins [119], all of which can serve to initiate stress responses in cells, such as induction of heat shock protein 70 (HSP70) [120,121] and stress-activated protein kinases [122], as well as disruption of other types of cell signaling [123], repair processes [124], protein synthesis [125–127], and even mitochondrial energy production [128]. GSH depletion also causes other cell structure alterations, including increased DNA damage [129], loss of junctional and cytoskeletal integrity [130,131], and abnormal cellular bodies [132–134]. We would hypothesize that these cellular effects would be seen in the RACP cells of CF patients.

Impaired mucolysis

Extracellular GSH deficiency impairs mucolysis, as GSH facilitates cleavage of disulfide bonds in mucus, in much the same type of mucokinetic activity demonstrated by NAC [135]. Increased viscoelasticity of mucus further inhibits ciliary beating, and also allows for increased opportunity for bacterial colonization of the lung [136–140]. Interestingly, GSH depletion has also been shown to provoke mucin secretion by tracheal epithelial cells [141], and increased NF κ B due to GSH deficiency stimulates mucus oversecretion by epithelial cells in the presence of *Pseudomonas aeruginosa* [142]. However, since these epithelial cells are generally not RACP cells, these observations are only interesting if it is the case that despite their normal levels of total glutathione, CF epithelial cells have a significantly decreased GSH:GSSG

ratio. Given the high amount of oxidant stress in CF, in part due to chronic and progressive extracellular GSH deficit caused by the CFTR mutation, this alteration might occur. If so, then it is also interesting to note that GSH deficit also precludes IFN- γ inhibition of Na⁺ and fluid absorption [143,144], and perhaps NO regulation of ENaC, as well [145,146]. As noted above, oxidative stress due to GSH depletion leads to an increase in free NE, which also stimulates mucus secretion [147].

Abnormal immune response

Though GSH is well known for its antioxidant properties, and somewhat less well known for its mucolytic capability, it is not as widely known for the key role it plays in immune system regulation. Because RACP cells include leukocytes and lymphocytes, the literature in this area should be pertinent to the case of CF. The following discussion is not comprehensive, but rather serves to outline the broad parameters of the topic.

First, as noted in the introduction, the redox status of GSH is the primary mechanism by which this regulation takes place. GSH deficiency in immune system cells (which are RACP cells) triggers inflammation, and chronic GSH deficiency in these cells leads to chronic and exaggerated inflammation. Cellular GSH deficiency is related directly to increased transcription of NF κ B [148–155]. NF κ B codes for the inflammatory cytokines, and a deficit of either the water-soluble or fat-soluble antioxidants increases NF κ B activity, which is a critical early event in the pathogenesis of many lung diseases [156]. This mechanism is demonstrated in HIV-infected macrophages, wherein there is increased activity of NF κ B due to decreased GSH synthesis. Consequently, there is a progressive increase of inflammatory cytokines as the disease progresses (and as GSH continues to be diminished). Increasing the redox potential of infected macrophages correlates with a decrease in inflammatory cytokines, as well as a decrease in HIV replication [157–159]. The concept of cellular redox potential is particularly germane to immune cell function due to its influence on NF κ B activity. Diseases that have a chronic inflammatory component, whether affecting a single organ (e.g., hepatitis), or as systemic inflammation (as in AIDS), will manifest GSH deficiency either in the affected organ or systemically, respectively. These deficits can occur due to insufficient GSH synthesis or transport, or consumption in pathological oxidative reactions that outpaces replenishment.

Accordingly, the cytokine profile of GSH deficiency includes elevated levels of AP-1 [151,152,154,155], TNF- α and its by-products [150,160,161–163], IL-1-induced MCP-1 [151,164], and IL-8 [154,160,165]. GSH also appears to exert some regulation over HIF-1 α [153],

regulates chemokine receptor expression [166], and appears to increase levels of IL-1 α [167]. This cytokine profile leads to increased recruitment of neutrophils and macrophages [168,169], and, in general such a profile would produce chronic inflammation even in the absence of pathogen challenge.

However, under conditions of chronic GSH deficiency in RACP cells and in the extracellular milieu, the inflammation is for naught. GSH deficiency in neutrophils and macrophages decreases effectiveness of bactericidal action of these cells. There are several reasons for this: first, GSH deficiency in such cells produces premature apoptosis, both because GSH depletion is a necessary step in apoptosis, perhaps because GSH depletion is associated with the activation of sphingomyelinases [170], and because GSH deficiency allows for greater oxidant damage to these cells [49,171–182]. Second, GSH deficiency causes neutrophils to suffer microtubule damage, impaired release of lysosomal enzymes, depressed leukotaxis, and in general, decreased phagocytosis [49,183–185]. Third, premature apoptosis in a context of enhanced recruitment of neutrophils by cytokines causes increased spillage of toxic compounds and DNA into the extracellular milieu [186]. Such spillage promotes increased viscosity of secretions, increased oxidant and elastase burden, the establishment of a positive feedback loop to IL-8 production and further neutrophil recruitment, and, in general, an increased opportunity for pathogenic lung colonization [186]. Fourth, GSH appears to regulate the oxidative burst of neutrophils, with GSH deficiency leading to decreased burst activity [187]. The interplay of GSH and NO in bactericidal action will be discussed in a separate section below.

The immune response also becomes abnormal under conditions of chronic GSH deficiency because of an alteration of Th1/Th2 response. Depletion of GSH in antigen-presenting cells (APC, also a RACP cell) decreases interferon- γ (IFN- γ) and enhances Th2-associated humoral immunity responses at the expense of cell-mediated immune response [143,188,189]. This alters the ability of APC to function normally, in part because GSH reduction of disulfide bonds is necessary for proteolysis of the antigen [143]. This interference of signaling between the APC and the T cells prevents proper T-cell response [143,190,191]. Other cell signaling involving T cells may also be adversely affected [192]. As noted previously, IFN- γ signaling is dysregulated by GSH depletion [144]. Extreme depletion of GSH in APC as well as in T cells has been shown to diminish both T-cell proliferation and IL-12 production, as well as increasing apoptosis of T cells [143,191,193–203]. B cell and fibroblast proliferation also appears related to intracellular GSH level [204,205]. Activation and cytolytic activity of T cells and B cells is decreased with GSH

depletion [189,206–210]. Again, this appears to be the result of cell structure abnormalities due to GSH depletion [211]. Movement towards a Th2 response has been associated with a negative outcome in many diseases [212–215].

Decreased nitric oxide production and availability

NO, an important factor in cell signaling, pathogen killing, and smooth muscle relaxation, is profoundly affected by GSH system function. In the extracellular compartment, GSH deficiency will result in less extracellularly produced GSNO (S-nitrosoglutathione, which is produced both extracellularly and intracellularly), an important source of NO. In RACP cells (and perhaps non-RACP cells if depressed intracellular GSH:GSSG ratios were present in them), a variety of noteworthy consequences result. GSH deficiency has been shown to decrease iNOS expression and decrease levels of cellularly produced GSNO, which two phenomena may be interrelated [216–221]. NO and GSH appear to regulate cell energy metabolism [222,223], and NO stimulates enhanced expression of γ -glutamylcysteine synthetase, the rate-limiting enzyme for GSH synthesis, apparently to counteract NO cytotoxicity [224–227]. GSNO is not only a reservoir of readily accessible NO, it is also a reservoir of easily accessible GSH, which becomes important under conditions in which cells become depleted of GSH through cytoprotective activities. Indeed, there appears to be a balance between GSH and GSNO, which under conditions of GSH depletion, favors cleavage of GSNO [228]. The balance appears to determine whether NO will be cytotoxic or cytoprotective [229–231]. Depletion of GSH markedly increases cell susceptibility to the harmful effects of peroxynitrite (ONOO $^-$), leading to cytostasis and apoptosis [232–239]. In addition, under conditions of GSH depletion and resulting lower levels of GSNO (which is formed both intracellularly and extracellularly) and oxygen radical release from inflammatory cells, NO is readily transformed into nitrites and nitrates [240,241]. ONOO $^-$ has also been shown to inactivate glutathione reductase [115]. As NO is important in bactericidal action, lowered levels of NO significantly depress such action, leading to increased infection with organisms such as *Pseudomonas aeruginosa* [145]. GSH depletion reduces smooth muscle relaxation in response to NO, producing in the lung a tendency towards generalized bronchoconstriction [242,243]. NO also appears to regulate both the CFTR channel and a non-CFTR Cl $^-$ channel, and a decrease in NO leads to a decrease in conductance [244,245]. NO also appears to regulate ENaC sodium absorption via cGMP, with decreased NO leading to loss of the signal to downregulate sodium absorption [145,146]. Trans-epithelial potential

Table 1. Known Associations of GSH Deficiency Correlated with CF Pathology

Known associations of GSH deficiency	Present in cystic fibrosis pathology?
Oxidant damage to organs and tissues	Yes [248,249]
Oxidant damage to lungs	Yes [248]
Oxidant damage to liver	Assumed with cirrhosis [250,251]
Reduced bile flow in liver	Yes [252]
Oxidant damage to pancreas	Assumed with fibrosis [251,253]
Development of diabetes	Yes, about 20% [251,254]
Oxidant damage to intestines	Assumed with inflammation [255,256]
Impaired growth	Yes [254]
Development of cachexia	Yes [257]
Development of arthritis	Yes, about 20% [258,259]
Peripheral nerve dysfunction	Yes [260]
Oxidant damage to myocardium	Predisposition [261]
Greater lipid peroxidation	Yes [249,262]; especially damaging due to increased AA in CF [97], see also [98]
Diminished antioxidant shield	Yes [263]
Inactivation of antiprotease system	Yes [264]
Increased elastase damage	Yes [100, 264–267]
Increased mucus secretion	Yes [254]
Acquired immune suppression	Yes [268]
Inhibition of ciliary beat function	Conflicting studies
Decreased lung surfactant	Yes [269]
Respiratory and skeletal muscle fatigue	No respiratory muscle fatigue; skeletal muscle fatigue demonstrated [270,271]
Development of asthma and generalized bronchoconstriction	Yes, 10–40% of cases [254]
Increased production of chloramines	Yes [272, 273]
Decrease epithelial integrity	Yes [254,274,275]
Increased adhesion of pathogens	Yes [276]
Cell structure abnormalities	Yes [274,275,277–279]
Increased viscoelasticity of mucus	Yes [254]
Increased DNA load from increased neutrophil count	Yes, 40% of content [254]
Decreased mucolysis of disulfide bonds in mucus	Assumed
Increased sodium and fluid absorption, with decreased periciliary fluid volume/hydration	Yes [254]
Altered cytokine profile	Yes
Increased NF κ -b	Yes [10,11,280]
Increased AP-1	Yes [11]
Increased TNF- α	Yes [9,257,281]
Increased MCP-1	Unknown
Increased IL-8	Yes [3,4,7–9, 280, 282, 283]
Increased IL-1a	Yes [284,285]
Increased recruitment of neutrophils	Yes [3,7,265,286]
Decreased effectiveness of neutrophils	Yes [282,287–289]
Shift towards Th2 immune response, with concomitant decrease in IFN-gamma	Yes [290–292]
Abnormalities of T and B cell system, with decreased cytotoxicity	Yes [292–301]
Decreased iNOS expression	Yes [145,302–304]
Decreased GSNO levels	Yes [305]
Decreased NO	Exhaled NO lower [19,22–27]
Increased levels of nitrites and nitrates	Yes [27,306–308]; see also [309] regarding peroxynitrite/neutrophil relation]

difference, being regulated in part by sodium absorption, is thus seen to be regulated by iNOS, with decreased iNOS leading to increased potential difference [145]. Last, ciliary beat function is regulated by NO, with depletion of NO leading to impaired ciliary function [246].

OBSERVATION: THE LIST OF EFFECTS OF GSH DEFICIENCY RESEMBLES THE LIST OF KEY PATHOLOGICAL EVENTS IN CF

For those familiar with CF pathology, the previous inventory of known effects of GSH deficiency should

have seemed very familiar. For those unfamiliar with CF, we present Table 1, which identifies which known associations of GSH deficiency have also been found to be present in CF disease. We are not suggesting that GSH deficiency, but not other negative effects of CFTR mutation, produce each of the following pathological consequences. (To give but one example, pancreatic insufficiency in most CF patients leads to suboptimal levels of vitamins E and A [247], which are also important for antioxidant protection, though routine supplementation with water-miscible forms of these vitamins does signif-

icantly raise these levels.) Nevertheless, we argue that GSH deficiency may play an important role—sometimes causative, other times aggravating—in the development of these consequences, especially as there is no provision for correction of this deficiency in current CF treatment guidelines.

HYPOTHESIS: GSH DEFICIENCY IN CF IS A PRIMARY, NOT A SECONDARY EFFECT OF CFTR MUTATION, AND PLAYS A CRITICAL ROLE IN THE PATHOGENESIS OF CF

A question has arisen over whether GSH deficiency in CF is merely a by-product of increased oxidant stress and pathogen burden [313]. Recent research allows us to assert that GSH deficiency in CF, though it may be aggravated over time by higher oxidant stress in CF, is nevertheless caused in the first place by the CFTR mutation itself. For this hypothesis to be valid, certain empirical phenomena should be present. We will examine two such expected phenomena and assess the evidence confirming the presence of each.

CF cells without redundant anion transport channels at the apical surface will have markedly impaired GSH efflux

This impairment will lead to extracellular GSH deficiency, which should start out small and be progressive over time. To underscore that the CFTR mutation is at work, all other components of the GSH system should be functional in CF. Evidence:

Impaired GSH efflux from non-RACP CF cells. Linsdell and Hanrahan found that the CFTR effluxes GSH [1]. When the CFTR channel is chemically clamped, GSH efflux ceases in those cells without redundant anion transporter channels. Gao et al. replicated this finding using CF epithelial cells [2]. Gao and colleagues also noted that CF epithelial cells contain normal levels of total glutathione. It would be interesting to ascertain GSH:GSSG ratio in these cells as well, given the previous discussion. It should be noted that it is currently very difficult to ascertain cellular GSH:GSSG ratios, though it can be done [310]. (As a tangent, it may be that the normal level of total glutathione in non-RACP cells explains the puzzling lack of apoptosis in CF epithelial cells infected with PA [17], as GSH depletion is a crucial step in cellular apoptosis, as we have previously seen. Decreased cellular ability to be depleted of GSH may lead to decreased ability to appropriately undergo apoptosis.) Normal lung epithelial cells do express a redundant anion channel, MRP1, at the basolateral, but not at

the apical membrane [311]. MRP2/cMOAT is not present in lung epithelial cells [312]. Thus, absent a redundant anion channel at the apical surface, defective or absent CFTR would result in impaired GSH efflux from CF lung epithelial cells, as found by Gao et al.

Progressive extracellular GSH deficiency in CF. Hull et al. demonstrate that infants and young children with CF who are not infected have approximately the same levels of ELF glutathione as non-CF controls, though infants and children with CF who are infected have somewhat reduced ELF glutathione, which reduction does not reach statistical significance [313]. However, Hull et al. measured total glutathione, and it would be interesting to note whether the GSH:GSSG ratio was altered in either group. This ratio is somewhat easier to measure in extracellular fluid. A decrease in this ratio would be the first sign that the CFTR mutation-derived abnormal glutathione transport present in CF had begun to manifest itself. Tirouvanziam hypothesizes that the initial alteration in this ratio in CF infants would be precipitated by the high oxidative stress of birth [186,314]. As noted previously, several researchers have found upregulated markers of inflammation even in uninfected CF infants [3–6], which would be concordant with a birth-related alteration in the GSH:GSSG ratio. For CF patients beyond infancy, Brown et al. determine that plasma sulfhydryls significantly decrease with age in CF persons [47]. Examining CF patients ranging in age from 2–20 years, Brown et al. find that plotting age by plasma sulfhydryl level yields an r of -0.44 , with $p < .05$. FEV1 displayed a strong correlation with plasma sulfhydryl level in these CF patients, with an r of 0.52 , $p < .005$. Finally, Roum et al. examine adult CF patients and demonstrate a profound extracellular deficit of GSH, with ELF GSH levels 5–10% of normal levels, GSH plasma levels about 50% of normal, and a dramatically decreased GSH:GSSG ratio in both fluids (approximately 1:1, in contrast to normal ranges of 9–200:1) [48]. Roum et al. calculate mean ELF glutathione in CF persons and determine it to be approximately $78 \mu\text{M}$, but a careful examination of the data show there is an extreme outlier in the data skewing the mean. Median level is a more appropriate statistic in such a situation, and estimation reveals a median of approximately $40 \mu\text{M}$. Normal ELF glutathione levels range from $250\text{--}800 \mu\text{M}$, with a mean of approximately $429 \mu\text{M}$ for patients not under oxidative stress, and a mean of almost $800 \mu\text{M}$ for patients under oxidative stress. Though van der Vliet et al. [315] have used a new methodology for glutathione analysis showing a lower normal level of glutathione in the ELF of non-CF persons, we are confident that when they examine glutathione levels in the ELF of CF persons, the deficit percentage Roum et al. found will remain the

same. In conclusion, the data available appear to document a progressive extracellular deficit of GSH in CF persons.

All other components of GSH system present and normal. GSH synthesis in CF cells has been shown to be normal, and presence of a functioning glutathione enzyme system, including at least normal, or even elevated, levels of glutathione peroxidase, glutathione reductase, γ -glutamyl transpeptidase, and γ -glutamylcysteine transferase (in the absence of other genetic mutation involving these enzymes, such as the GSTM1-null genotype) has been demonstrated [48,249,313,316,317]. Thus, unlike diseases such as AIDS, where glutathione system dysfunction stems from decreased synthesis of cellular GSH, GSH deficiency in CF does not derive from abnormal synthesis of GSH. And unlike other genetic diseases of the glutathione system, the redox, transferase, and recycling systems are intact.

CF cells expressing MRP1 or MRP2 (redundant anion channels that would allow the efflux of GSH even in the context of CFTR channel mutation, i.e., RACP cells), may not be able to maintain normal levels of GSH over time in the context of progressive extracellular deficit of GSH, and may eventually enter a state of chronic depletion of cellular GSH, which would, at a minimum, be manifest in an abnormally low GSH:GSSG ratio. Levels of total glutathione may even drop below normal with advanced disease. Generally speaking, immune system cells express not only CFTR [318–320], but also MRP1, and thus would belong to this category [110,321,322]. Indeed, MRP1 has been shown to be upregulated in CF erythrocytes, and thus MRP-type transporters could also be expected to be upregulated in lymphocytes, leukocytes, and hepatocytes, which may account for increased drug clearance in CF patients [323]. (Though one study has found a lack of GS-X pump activity and expression in lymphocytes [324], other studies have found significant MRP expression therein [325].) Given that conjugates of GSH are the substrate for these pumps, it is possible that the severely depressed extracellular levels of GSH coupled with increased expression of MRP1 may together create a situation in which greater cellular efflux of GSH occurs. Indeed, in addition to GSH conjugates, MRP1 and MRP2 have also been shown to efflux GSH itself [326]. From what we now know about abnormal GSH transport caused by CFTR mutation, RACP cells may be some of the few cells able to respond to such a signal in the CF case [327,328]. Such efflux might lead to chronic depression of GSH levels within these cells, resulting in a diminished GSH:GSSG ratio and perhaps, with advanced disease, decreased levels of total glutathione. (A complicating factor is that peroxides have been shown to inhibit the activities of GS-X pumps through

direct oxidant damage, and peroxide formation is greatly increased in CF [329].) Chronic GSH depletion in immune system cells leads, as we have seen, to chronic and exaggerated inflammation coupled, paradoxically, with immunosuppression: a paradox that is a hallmark of CF pathology.

The evidence here is more sparse. Increased γ -glutamylcysteine transferase (γ -GCT) has been noted in the ELF of CF persons [313,317], indicating increased cellular activity to create GSH conjugates. Upregulation of MRP1 in CF erythrocytes has already been mentioned [323], denoting increased efflux of these conjugates. Increased γ -glutamyl transpeptidase activity in CF has also been observed, indicating increased cellular activity to cleave extracellular GSH and GSSG into components for cellular GSH resynthesis. This significantly increased level of γ -GT was found even in infants [313]. An increase in glutathione reductase (GR) levels has also been noted in CF [249,317], pointing perhaps to an increase of GSSG in the cytosol, and thus also an alteration in GSH:GSSG ratio. Studies have shown a dramatically decreased extracellular GSH:GSSG ratio in CF ELF, as noted above [48]. Are such alterations also present specifically in the immune system cells of CF persons? There is only one study of glutathione levels in CF RACP cells, and it does not report the GSH:GSSG ratio per se. Lands and colleagues found that total glutathione levels of the peripheral blood lymphocytes of CF persons, though not statistically different from controls, were, in general, somewhat depressed, especially in subjects with poor nutritional status [330]. This is prima facie evidence that total glutathione levels of immune system cells in CF persons can be depressed, though clearly more studies are needed on this topic. First, as noted above, it is currently under dispute to what degree lymphocytes manifest GS-X pump activity [325,331]. More to the point would be a study of leukocytes, but no such study now exists. Second, cellular GSH:GSSG ratios would be very important to know, if they could be obtained. For example, Lands et al. [330] find comparatively higher total glutathione in the lymphocytes of patients with the worst FEV1 scores than in those with the best scores. But in the absence of knowing the GSH:GSSG ratio, this finding is hard to interpret. Normal GSH:GSSG ratio is anywhere from 9:1 to 200:1 depending on compartment [34], but Roum et al. found that ratio in CF ELF to be reduced to approximately 1:1 [48]. If this depression was in fact both systemic and progressive in CF RACP cells, the signaling initiated by a chronic and significant alteration in GSH:GSSG ratio within immune system cells would certainly help explain the paradoxical abnormalities of CF immune response.

However, the mechanism of this alteration in GSH:GSSG ratio in CF immune system cells would still need

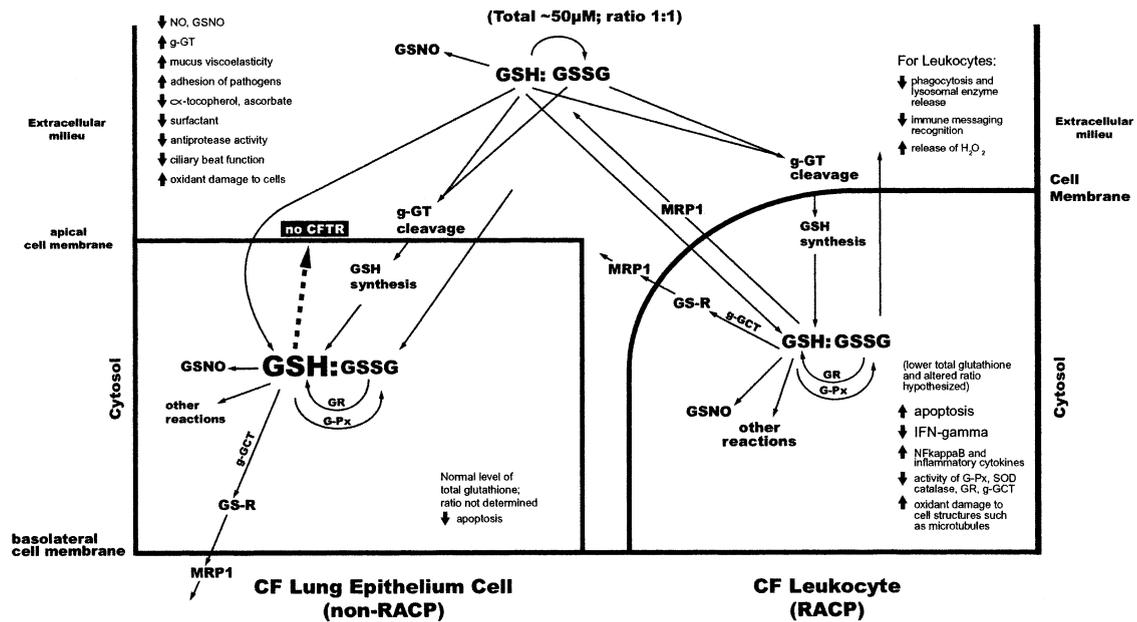


Fig. 2. Abnormal GSH transport in cystic fibrosis.

further elucidation, even if confirmed. Several possibilities exist. First, might it be a natural effect of high oxidant stress in CF? It might, but in normal persons, the activities of glutathione reductase, γ -GCT, γ -GT, and γ -GCS (γ -glutamylcysteine synthetase) are sufficient to normalize that ratio. As we have seen, all these enzymatic components of the GSH system are present in at least normal, and usually elevated levels in CF. Second, might it be that because the immune system cells normally draw upon extracellular stores of GSH to replenish and resynthesize their own cellular stores of GSH, which are severely depleted during the course of bactericidal activity by these cells [49,112,113,183,185,272], the lower-than-normal levels of ELF GSH in CF then compromise these cells' ability to maintain appropriate intracellular levels of GSH? Third, might the redundant anion channels of these cells allow for efflux of GSH, and might that efflux be somehow promoted by the extracellular redox ratio? The extracellular redox state has been shown to affect GSH efflux, but one study shows an oxidized state to be associated with inhibition of GSH efflux in pneumocytes where the channel of GSH efflux is the CFTR [332], while another shows increased GSH efflux from human lung epithelial cells under oxidative stress [333]. Only further research will clarify the mechanism(s) involved.

PUZZLES SOLVED

We do not claim that glutathione system dysfunction is the only initiator of pathology in CF. Neither do we

suggest that such dysfunction is the primary cause of CF pathology. We merely assert that it is an important part of the pathological picture in CF, sometimes causing certain pathological effects, sometimes aggravating pathology caused by other factors. A diagram summarizing this hypothesis can be found in Fig. 2. In the absence of all desirable empirical evidence, strong warrant for a theory can be gauged by the number of interesting puzzles solved by it. Listed below are the puzzles of CF pathology that are addressed by the theory of CFTR-derived GSH deficiency in CF.

1. Decreased exhaled NO in CF can be explained.
2. Constitutive and progressive inflammation in the absence of infection can be explained.
3. Lack of bactericidal action despite exaggerated inflammation can be explained.
4. Lack of apoptosis by PA-infected epithelial cells can be explained.
5. Greater viscosity of mucus in CF gains several additional explanations, including lack of GSH mucolytic action and premature neutrophil apoptosis due to GSH deficiency, among others.
6. The overall outline of CF pathology can, in general, be correctly predicted from the overall outline of the effects of GSH deficiency in non-CF cases.

THERAPEUTIC IMPLICATIONS

Should this theoretical framework be validated by further empirical research, therapeutic implications

present themselves. These therapies would not be cures, as only genetic therapy holds that promise, and these therapies could not substitute for other useful pharmacologic therapies, such as DHA supplementation [97]. Though our focus here is on exogenous GSH augmentation, it should be noted that any intervention that increases anion transport would also positively affect the GSH system situation in CF. For example, Forman and colleagues use a novel synthesized peptide to increase GSH and other anion transport, and this might also be a useful therapeutic approach [54]. Lenoir and his colleagues have experimented with the use of colchicine in CF to coax MRP expression at the apical surface of lung epithelia, which, if it could be accomplished, would have great therapeutic potential [334]. However, let us explore the possibility of direct exogenous modulation of GSH levels here.

First, cysteine supplementation has a role in CF care. This may help increase GSH levels in RACP cells. Given that cysteine is the rate-limiting amino acid for GSH production, provision of additional cysteine, usually through oral ingestion of a cysteine donor such as NAC, allows for increased cellular synthesis [149,335,336]. A case report of cysteine supplementation in a COPD patient notes dramatic improvement [337], and COPD is another pulmonary disease with generally depressed GSH levels. For CF patients whose genotypes afford them greater CFTR expression, increased GSH synthesis may be a fairly effective route of GSH augmentation, because greater GSH efflux from non-RACP epithelia in such genotypes is possible. Such milder mutations not only experience a slower decline in pulmonary function and greater life expectancy, but also suffer less frequently from CF-related liver cirrhosis or diabetes [338, 339]. Greater efflux of GSH may be part of the explanation for this. Also, other nutrients involved in the proper functioning of the GSH system, such as B6, magnesium, selenium, ascorbate, and E, should be examined for adequacy.

However, even more important in our opinion would be rectification of extracellular GSH levels by augmentation with GSH itself. Cysteine by itself will not produce a more normal extracellular GSH level in pancreatic insufficient CF persons (i.e., those not having mild mutations) because the CFTR defect will severely diminish GSH efflux from non-RACP CF cells, including lung epithelia. In addition to rectifying the extracellular deficit, exogenous GSH will also provide more substrate for γ -GT (which is significantly increased in CF [313]), which activity would increase GSH levels in any cells that were depleted of it. Furthermore, epithelial cells are capable of taking up GSH intact from the extracellular milieu [34]. Before turning the specific routes of exoge-

nous GSH delivery, a few parameters should be mentioned.

First, unless some type of time-release form of GSH could be developed, GSH would have to be administered about every 3–4 waking hours. In previous studies, elevation of ELF GSH through provision of exogenous GSH results in an initial peak increase at 30 min after dosage (when 600 mg are inhaled), followed by a gradual decline until baseline levels are reached after 2–4 h [340–347]. Period of elevation was also dose-dependent, as super-physiologic multigram doses have been found to extend that duration significantly [340,345]. Second, use of GSH-stripping substances, such as acetaminophen, alcohol, codeine, or morphine would have to be avoided by the patient. Third, as a vasodilator, GSH therapy might be contraindicated in CF patients prone to hemoptysis.

Fourth, GSH therapy might also be contraindicated in patients culturing *Burkholderia cepacia*, since GSH serves to decrease inflammation. When culturing *B. cepacia*, exuberant inflammation may be necessary to prevent fatal cepacia syndrome. The issue of whether use of an antioxidant such as GSH might serve to increase infection in CF patients is one that should be addressed at this point. Oxidant signaling is very important in immune response, and also in wound healing. It would not only be impossible, but it would be harmful to eliminate all oxidant signaling. However, in CF the antioxidant shield has been overwhelmed, in part, at least, because of abnormal GSH transport due to CFTR mutation. GSH levels are 5–20% of that found in a normal person. Pushing that percentage up above 50% of normal would not constitute introducing excessive amounts of antioxidants. Too high of an oxidant burden cripples immune system cells, as we have seen. By toning down, but not eliminating oxidant signaling, the immune system in CF should be made more effective [287]. In the only in vivo trial of GSH augmentation in CF persons, no trial subject developed any infectious symptoms or signs [347]. In vitro studies show that extracellular concentrations of GSH of up to at least 300 μ M do not inhibit bactericidal or phagocytic activity of neutrophils [327, 328]. Levels higher than that were not studied, but given that normal ELF levels range between 400 and 800 μ M, it is likely that inhibition does not occur at these higher levels, either, though this question can only be answered by further empirical investigation. Nevertheless, where potentially deadly *B. cepacia* is involved, more laboratory study is needed before GSH use can be contemplated.

Last, the younger the age at which the therapy is begun, the more likely that the CF patient will experience the desired effects of the therapy. Thus, children infected only with, for example, *Staph. aureus*, may find it pos-

sible to eradicate that bacteria using this therapy. However, older CF patients with greater lung damage culturing, for example, mucoid *Pseudomonas aeruginosa*, may not see similar results. Modes of GSH augmentation will now be discussed.

Intravenous administration

Intravenous GSH, with the GSH as a sodium salt, has been used to treat chemical or radiation poisoning, as well as to treat diabetes and Parkinson's disease [73, 348]. Intravenous GSH has been shown to raise not only blood levels of GSH, but also ELF GSH [340].

Oral administration

The oral ingestion of GSH has often been overlooked as an effective route of augmentation of extracellular GSH for a number of reasons. First, the unique CFTR-derived problem of GSH efflux is not present in other diseases, so simple provision of additional cysteine is sufficient to increase GSH levels in those other diseases. The second reason centers around the dispute over whether GSH is cleaved or destroyed in the digestive tract, or whether GSH can be taken up intact from the duodenum and jejunum and transported into the bloodstream. Fortunately, the number and sophistication of recent research articles demonstrating that GSH is taken up intact from the small intestine outweigh those denying that such uptake occurs [324,349–352]. These studies also show dose-dependent elevation of circulatory GSH, and tissue GSH, including the lung [324]. Remember that circulatory elevation of GSH does in fact lead to ELF elevation of GSH [340].

Inhalation

There have been eight *in vivo* studies of inhaled GSH, one a murine study and the other seven human studies [340–347]. Human subjects ranged in age from 4 years old on up to mature adulthood. Only one small *in vivo* study of seven subjects has specifically examined CF patients [347]. This study found that certain inflammatory markers significantly decreased after 3 d use of inhaled reduced glutathione. In addition, one *in vitro* study using CF sputum found that the addition of GSH caused the reduction of baseline O_2^- by approximately 90%, and reduction of PMN-induced burden by approximately 46% [353]. These two studies provide some encouragement that should a larger *in vivo* trial on CF patients be performed, at least some of the desired and predicted effects would be forthcoming.

Six of the seven studies used free acid GSH in an

isotonic saline solution (150 mg/ml). One study used pH-adjusted GSH sodium salt in isotonic saline solution [345]. All studies used pharmaceutical grade GSH (minimum 98% pure).

Bronchoconstriction was noted in one study [344], which was eliminated by the prior inhalation of salbutamol. Another approach to the bronchoconstriction problem is the use of pH-adjusted GSH sodium salt. Free acid solution GSH has a pH of approximately 2.7. Understanding that inhalation of low pH substances can induce lung irritation [354], the use of the sodium salt is worthy of investigation. In one *in vivo* study, a 2.4 g dose of inhaled GSH could be tolerated when the pH was adjusted to 7.0 by use of the sodium salt [345]. Other possibilities for modification include delivery by liposomes to lengthen duration of elevation [355–357], as well as the inhalation of a slower release crystalline form [340]. Maintenance of isotonicity of the solution is no doubt desirable as well [358]. No other safety issues surfaced in these studies. Oxidation of inhaled GSH to GSSG should occur, and indeed was found [347], but since all other components of GSH recycling remain intact in CF, with only GSH efflux from non-RACP cells affected, the extracellular GSH:GSSG ratio should normalize over time with consistent use because γ -glutamyl transpeptidase (γ -GT) will break down extracellular GSSG at the cell surface, recovering the component amino acids for use in cellular resynthesis of GSH [34, 135]. As we have noted, γ -GT is significantly increased in CF persons [313].

CONCLUSION

New research suggests that the CFTR defect associated with cystic fibrosis also causes abnormal GSH transport in non-RACP cells. Thus, the genetically defective CFTR appears to establish the foundation of a progressive GSH deficiency in the extracellular milieu and in RACP cells, including immune system cells. A diminished extracellular antioxidant shield, increased mucus viscoelasticity, and exaggerated inflammation coupled with ineffective immune response result. As inflammation becomes further amplified during the course of the disease, there is a progressive oxidative burden, which would cause further decrements in antioxidant defenses. There are no opportunities for the CF host to reestablish normal response without exogenous intervention, such as GSH augmentation.

The linkage between the genetics and the pathology of CF can thus be more clearly seen, as the known associations of GSH deficiency in these areas correlate well with known CF pathology, providing at least partial answers to several long-standing puzzles of CF pathology. Though abnormal GSH transport is not the sole or

perhaps even the primary cause of CF pathology, it plays an important role—a sometimes causative, sometimes aggravating role—that bears closer investigation. Theoretically, shifting extracellular GSH levels towards normal should provide significant ameliorative results, if this hypothesis is correct. Though more laboratory research is certainly needed to complete our understanding of the role of abnormal GSH transport in CF disease, it is also reasonable that clinical trials be contemplated, as well. Though not a cure for CF as the underlying defect remains, and though not a panacea for all CF-related ills for some of those ills are not caused by GSH deficiency, a therapy that appears safe, inexpensive, and has the potential to be significantly ameliorative should not be left unexplored for the CF patient who seldom sees the fourth decade of life.

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