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Oxidants/antioxidants in idiopathic pulmonary fibrosis

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Introductory article

The effect of oral N-acetylcysteine on lung glutathione levels in idiopathic pulmonary fibrosis

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Idiopathic pulmonary fibrosis (IPF) is characterized by an increased oxidant burden and by a deficiency of glutathione, a major antioxidant, in the lung epithelial lining fluid (ELF). Therefore, a rational therapeutic approach is to reverse the imbalance between oxidants and antioxidants in the lung by enhancing the antioxidant screen. With this background, the aim of our study was to evaluate oral N-acetylcysteine (NAC) as a strategy to augment lung glutathione levels in patients with IPF. Concentrations of total glutathione in bronchoalveolar lavage fluid (BALF) were quantified spectrophotometrically, before and following oral therapy with 3 × 600 mg NAC per day for 5 days, in 17 nonsmoking patients with biopsy-proven IPF. The volume of ELF recovered by BAL was determined using the urea method. Pretherapy, total glutathione levels in ELF in IPF patients were significantly less than normal (187 ± 36 vs 368 ± 60 μM), in contrast to levels in BALF (0.99 ± 0·12 vs 1.18 ± 19 μM). Following therapy with oral NAC, glutathione levels in BALF were 1.54 ± 0.24 μM (a significant increase compared to pretherapy), whereas the increase in ELF levels (319 ± 92 μM) did not reach significance. The therapy was well-tolerated, and all routine clinical and bronchoscopic parameters remained unchanged. It is thus feasible and safe to augment deficient lung glutathione levels in patients with IPF; thereby, potentially augmenting pulmonary antioxidant protection. (*Eur Respir J 1994;7*:431–6)

Idiopathic pulmonary fibrosis (IPF) is thought to arise as a response to persistent lung injury and inflammation.[1] The early response to injury to the alveolar epithelium and/or the vascular endothelium results in the influx of neutrophils to the interstitium and airspaces which may persist.[2] The hypothesis that the condition is immunologically mediated arises from the mononuclear phase of the disease in which the recruitment of monocytes/macrophages and lymphocytes in the lungs occurs, presumably in response to an as yet unidentified antigen or immunological target. These cells are thought to release fibrogenic cytokines such as tumour necrosis factor alpha, transforming growth factors alpha and beta, and eicosanoids such as leukotrienes B_4 and C_4 which recruit and activate fibroblasts and stimulate deposition of connective tissue.[3] Treatment strategies for pulmonary fibrosis, whether old or new, can therefore be categorised in terms of the stage of the fibrotic response in the lung to which they are targeted – whether to the inflammatory/immune response or tissue injury, to the subsequent release of eicosanoids and cytokines, or the consequent connective tissue deposition (fig 1).[4]

The treatment described by Meyer and coworkers[5] is based on a strategy to prevent tissue injury, and hence further amplification of the inflammatory response in the lungs, by attempting to correct the oxidant/antioxidant imbalance which is proposed as a mechanism of tissue injury in IPF.[1]

The need for new treatments in IPF is compelling. Clinical studies indicate that the medium time from diagnosis to death is 3–5 years,[6–8] although those patients with an associated connective tissue disorder appear to have a less aggressive form of the condition.[9] The traditional treatment for IPF has been with corticosteroids as non-specific anti-inflammatory agents which produce an objective response in only 10–20% of cases.[10] In those who do respond to corticosteroids this treatment has shown only a modest influence on the fatal course of the disease.[6 10 11]

The hypothesis that the condition is immunologically

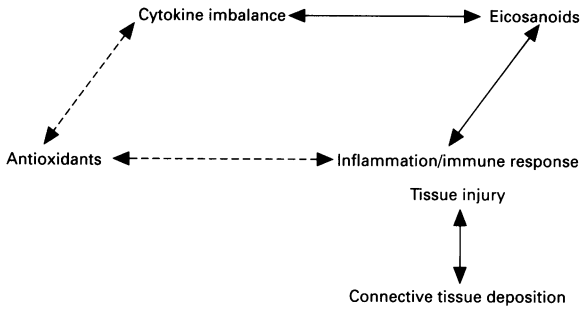

Figure 1 Major steps in the pathogenesis of pulmonary fibrosis, interconnected by double headed arrows to indicate that each of these processes mutually regulates the other. The putative steps which may be influenced by antioxidant therapy are shown by the broken arrows. Modified from ref 4.

mediated has led to the use of several non-specific immunosuppressive agents such as azathioprine,[12] cyclophosphamide,[13] penicillamine,[14] and cyclosporin.[15] Most studies of the use of these agents have involved small uncontrolled clinical trials; no definite evidence of a survival advantage has been reported following these treatments and their use is often limited by side effects.[16] Indeed, in a randomised controlled trial in which prednisolone alone was compared with a combination of cyclophosphamide and low dose prednisolone in 43 patients with IPF, although improvement or clinical stability persisted in more patients treated with corticosteroids plus cyclophosphamide (eight of 20) than in those treated with prednisolone alone (five of 22), there was no survival advantage of the combined regimen (fig 2).[13]

Some features have been identified that are more likely to be associated with objective responses to treatment. In the study by Turner-Warwick and coworkers[10] patients who responded to treatment were younger women with a short history and well preserved lung function at the time of diagnosis. More recently Schwartz *et al*[17] confirmed that men with more severe symptoms and smoking are associated with a poor prognosis. Thus, in a recent survey of treatment for IPF in the UK 55% of patients were given corticosteroids while only 10% were given other immunosuppressive agents and 34% of patients received no treatment.[18] A further treatment option in younger patients is, of course, lung transplantation.[19]

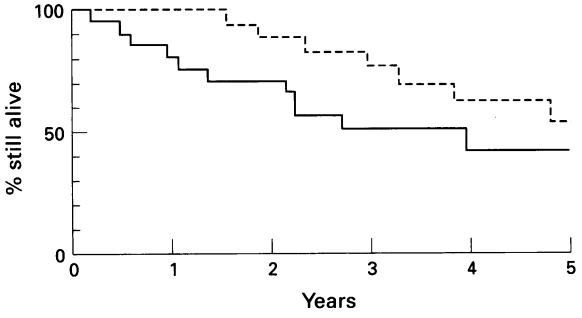

Figure 2 Survival curves for patients with IPF receiving prednisolone only (——, n=22) and cyclophosphamide plus prednisolone (- - - -). Modified from ref 13.

Oxidant/antioxidant imbalance in IPF

As a result of this rather bleak progress with conventional immunosuppressive therapy, there has been some interest in other proposed mechanisms in the pathogenesis of IPF which may be amenable to treatment. An oxidant/antioxidant imbalance in the lower respiratory tract has been proposed as the mechanism of the lung injury in a number of inflammatory lung conditions including IPF.[1] An increased oxidant burden in the lungs in IPF is thought to arise from the accumulation of inflammatory cells in the lower respiratory tract,[20] including activated alveolar macrophages and neutrophils which show an exaggerated release of oxygen radicals.[21] Oxidants have been implicated in the epithelial injury which is characteristic of this condition[1]; however, oxidants may also be released by the target cells.[22] Studies showing that increased amounts of reactive oxygen intermediates (ROI) are spontaneously released by inflammatory cells in the lungs of patients with IPF[23,24] also indicates that these ROIs react with the excessive amounts of myeloperoxidase present in the epithelial lining fluid of patients with IPF to form the highly toxic hypohalide ion.[23]

The mechanisms responsible for the increased release of ROIs by alveolar leucocytes in patients with IPF are not known. Immune complexes, however, which are present in the lungs of patients with IPF,[24,25] may be involved since they are a potent stimulus for ROI production by macrophages, neutrophils, and eosinophils.[26]

Other potential oxidants which may be involved in the tissue injury in IPF are the nitrogen centred radicals. More evidence is needed to support this possibility, which, if confirmed, may lead to a potential therapeutic role for nitric oxide synthetase inhibitors in pulmonary fibrosis. In addition to their direct injurious effects, oxidants may also upregulate cytokine production possibly through activation of the transcriptional factor for cytokine genes NFκB.[27] Hence, oxidants may promote fibrosis indirectly through the enhanced release of cytokines.

Antioxidants

The epithelial lining fluid forms an interface between the underlying epithelial cells of the respiratory tract and the environment. It provides a first line of defence against inhaled oxidants or those released from inflammatory cells in the airspaces. The epithelial lining fluid contains a number of antioxidant species in the form of low molecular weight antioxidants such as ascorbic acid, glutathione, and uric acid; metal binding proteins such as ceruloplasmin and transferrin; antioxidant enzymes such as superoxide dismutase and catalase; and sacrificial reactive proteins such as albumin.[27,28]

GLUTATHIONE

Reduced glutathione (GSH) and its redox enzymes comprise a major antioxidant system in the lungs. By sacrificing its thiol(-SH) groups GSH protects cells and thiol-containing enzymes from the toxic effects of oxidants.[29] Glutathione peroxidase has a major role in detoxifying hydrogen peroxide to water and, in doing so, utilises GSH (fig 3). Thiols such as glutathione are

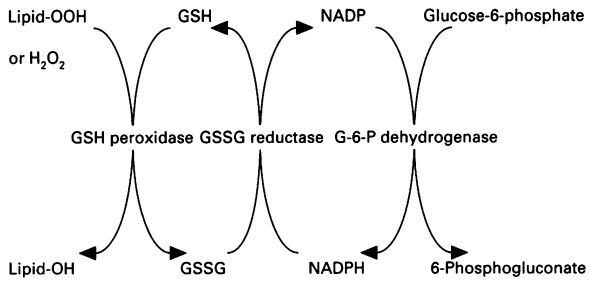

Figure 3 The glutathione redox systems.

able to scavenge the hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl).[30]

Glutathione is present in approximately 100 times higher concentrations in epithelial lining fluid than in plasma,[31] and is also an important intracellular antioxidant in lung cells.[32] Cantin and coworkers[33] reported a fourfold relative deficiency in reduced glutathione (GSH) in the epithelial lining fluid of patients with IPF (97 (18) μM) compared with normal subjects (429 (34) μM) (fig 4). These data have important implications for the ability of the lungs to protect themselves against oxidant-induced injury.

Both extracellular and intracellular GSH are depleted by oxidants[32]; this is associated with detachment and lysis of airspace epithelial cells in vitro.[34] In addition, the oxidant-mediated cytotoxic effect of lung inflammatory cells obtained from patients with IPF is enhanced in the presence of epithelial lining fluid from patients with IPF.[21] Furthermore, in vivo and in vitro models of oxidant-induced increased airspace epithelial permeability – a feature characteristic of patients with IPF[35] – have been shown to be critically dependent on GSH levels in the lung and epithelial lining fluid.[36 37] Indeed, evidence from animal studies suggests that a reduction

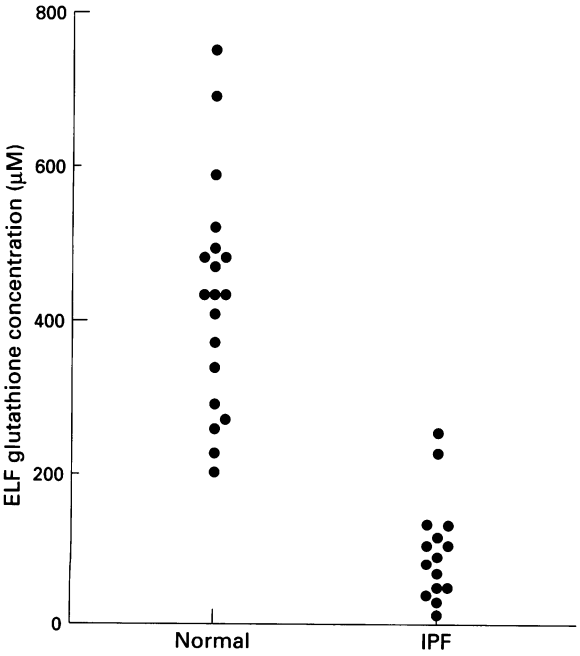

Figure 4 Glutathione (GSH plus GSSG) concentrations in the epithelial lining fluid (ELF) in normal subjects and patients with IPF. Modified from ref 33.

in lung GSH per se, by inhibition of GSH synthesis with buthionine sulfoximine can induce increased airspace epithelial permeability.[37]

Concentrations of GSH normally found in the epithelial lining fluid (500 μM) can suppress mitogen-induced fibroblast proliferation,[38] an event which may be a critical component of the interstitial changes leading to pulmonary fibrosis. However, the levels of GSH in the epithelial fluid do not correlate with the severity of the disease, nor is there a GSH deficiency in the plasma or alveolar macrophages of patients with IPF,[33] suggesting that the GSH deficiency in the epithelial lining fluid is not the result of a generalised decrease in GSH synthesis.

Evidence is therefore accumulating to implicate a deficiency of GSH in the epithelial lining fluid in the pathogenesis of IPF. The logical therapeutic approach which follows from this is to augment the GSH levels in the lower respiratory tract in patients with IPF by supplying excess cysteine for GSH synthesis in an attempt to prevent oxidative damage to lung cells. Over the past 20 years there has been considerable interest in N-acetyl-L-cysteine as a therapeutic agent in lung diseases.[39] This drug has been widely prescribed in Europe as a mucolytic agent, but it has also been used as a precursor of GSH to augment depleted levels of liver GSH when given intravenously in cases of paracetamol poisoning,[40] so recognising its antioxidant potential.[30]

N-acetylcysteine

Although N-acetylcysteine is a good antioxidant, when taken by mouth it does not appear in the plasma and therefore does not reach the epithelial lining fluid[41 42] since it is deacetylated in the gut and metabolised in the liver.[43] Cysteine, the product of this deacetylation, is a precursor in the biosynthesis of glutathione.[29]

Several workers have shown an increase in plasma cysteine concentrations following oral administration of N-acetylcysteine.[41 42] However, the subsequent metabolism to GSH, and hence the appearance of GSH in plasma, is more variable[44] since it may depend on individual variations in the metabolism of GSH in the liver. Thus, when given as a single daily dose of 600 mg for five days, although plasma levels of GSH were higher than in a control group of subjects, this effect was transient[42] and peaked at variable time points after the dose of N-acetylcysteine in different individuals.

N-ACETYLCYSTEINE IN IPF

The study of Meyer and coworkers[5] was designed to test the ability of N-acetylcysteine to correct the GSH deficiency in the epithelial lining fluid of patients with IPF. The rationale for this study came from preliminary data by the same investigators which showed, in a small group of patients with fibrotic lung disorders of various types, that N-acetylcysteine could increase GSH levels in bronchoalveolar lavage fluid.[45] The study group consisted of 17 patients with biopsy proven IPF, six of whom were receiving treatment with prednisolone.[5] The treatment regimen consisted of a fairly high dose of N-acetylcysteine, 600 mg three times daily. An initial

parallel group study by Bridgeman and coworkers[42] suggested that there is only a transient increase in GSH levels in the epithelial lining fluid up to six hours after dosing with a lower dose of 600 mg daily for five days[42]; however, a subsequent study by the same group did not confirm these findings.[44] In this study a higher dosage regimen of 1800 mg N-acetylcysteine/day increased plasma GSH levels over five days in a parallel group study in patients under investigation for bronchial carcinoma, but failed to produce a persistent increase in GSH levels in either epithelial lining fluid or in lung tissue.[44]

One of the problems of measuring GSH and other components in bronchoalveolar lavage fluid is the complex and almost certainly variable dilution of the solute of interest produced by the lavage procedure. Various methods have been employed to obtain data on the levels of solutes in bronchoalveolar lavage fluid.[46] Presentation of a solute simply as its concentration in bronchoalveolar lavage fluid ignores probable differences in dilution between subjects. These problems are highlighted by the study of Meyer and colleagues,[5] who, like many workers, have tried to calculate the volume of the epithelial lining fluid using an endogenous marker, usually urea or albumin.[47 48] In the study by Meyer *et al* the volume of epithelial lining fluid recovered was quantified by the same urea method, which assumes that the concentration of urea in epithelial lining fluid is the same as that in plasma, and that the concentration of urea is not influenced by the lavage procedure, nor the disease itself.[48] Urea almost certainly does move into the airspaces during the lavage procedure[49] and may also vary in IPF. Indeed, Cantin and colleagues,[33] who originally described glutathione deficiency in the lower respiratory tract in patients with IPF, suggested that the apparent increase in calculated epithelial lining fluid recovered from patients with IPF, which was reported both in their study and in the study by Meyer *et al*,[5] was likely to be due to the increased permeability of the alveolar/capillary barrier which has been shown to occur in this condition.[35] Thus, part of the apparent GSH deficiency may be due to dilution of epithelial lining fluid by plasma, which may account for the apparent discrepancy between the decrease in GSH in epithelial lining fluid which was not present when measured in bronchoalveolar lavage fluid in the study by Meyer *et al.*[5] This contrasts with the almost twofold increase in GSH in epithelial lining fluid in cigarette smokers[31] who also have an increased permeability of the alveolar/capillary barrier.[50] However, the increase in GSH in the epithelial lining fluid of smokers may result from a different mechanism involving transcriptional upregulation of the gene for gamma-glutamylcysteine synthetase in airspace epithelial cells which is the rate limiting enzyme for GSH synthesis.[29] It is possible that a similar mechanism may operate in reverse to downregulate gamma-glutamylcysteine synthetase in the epithelial cells in IPF.

One further concern over the apparent GSH deficiency in the epithelial lining fluid in the study by Meyer and colleagues is the younger age of the normal subjects (28 (1) years) compared with the patients with IPF (62 (2) years) since it is known, at least in plasma, and presumably epithelial lining fluid, that thiol levels decrease with age.[51]

The problem of measuring GSH in epithelial lining fluid or bronchoalveolar lavage fluid is compounded in this study when the results of treatment with N-acetylcysteine are considered. Following administration of N-acetylcysteine the level of GSH in the bronchoalveolar fluid, which was not significantly different from control levels in patients with IPF, is said to increase. However, the mean GSH level in the epithelial lining fluid did not increase to "normal" levels, neither was the increase significant.

The increase in GSH levels in bronchoalveolar lavage fluid was measured as total GSH, since the relative increases in reduced and oxidised glutathione were not measured. One potential hazard of increasing GSH levels in the epithelial lining fluid in an environment of increased oxidants released from activated airspace leucocytes, such as occurs in patients with IPF, is the potential to increase the levels of oxidised GSH (GSSG), which is toxic in high concentrations.[52] Furthermore, thiols can also react with reactive oxygen intermediates to form potentially toxic thiyl and/or oxysulphur radicals[53] which could then react with thiol-containing enzymes and proteins rendering them inactive.

Antioxidant therapy in IPF

Are we therefore in a position to recommend clinical trials of antioxidant therapy as a novel treatment for IPF and, in particular to use oral N-acetylcysteine to augment GSH levels in the lower respiratory tract? Several problems remain to be resolved. Firstly, although there is substantial circumstantial evidence for an increased oxidant burden in IPF, there is still a lack of convincing proof that oxidants have a central role in the pathogenesis of this condition, which is also true of other lung conditions in which oxidants have been implicated.[54] Perhaps the only way of proving this hypothesis will be a clinical trial in IPF of the efficacy of antioxidant therapy alone, or in combination with steroid therapy, with outcome measures of clinical, radiographic, and respiratory functional improvement.

There is also a need for a reliable marker of oxidant/antioxidant balance which can be easily repeated in such a trial. Logistically such a marker would be more easily measured in blood than in bronchoalveolar lavage fluid. Candidates for such a biochemical marker are the products of lipid peroxidation, which are, however, considered to be non-specific as a measure of oxidant stress.[55]

Borzi *et al*[56] found significantly raised levels of serum copper-zinc superoxide dismutase (CuZnSOD) in patients with IPF compared with healthy control subjects which could potentially be used as a marker of disease activity. The increase in enzyme levels may be due to release following the degranulation of neutrophils, as suggested by a very strong correlation between serum levels of CuZnSOD and polymorphonuclear elastase, a marker of neutrophil activity as well as disease activity. This suggests that neutrophils are the main source of the CuZnSOD in serum. A recent publication by the same investigators[57] confirmed that higher serum levels of superoxide dismutase in IPF are of CuZnSOD which is located in the cytoplasmic granules of the neutrophil, and not the manganese-SOD dependent form which is not located in the cytoplasmic

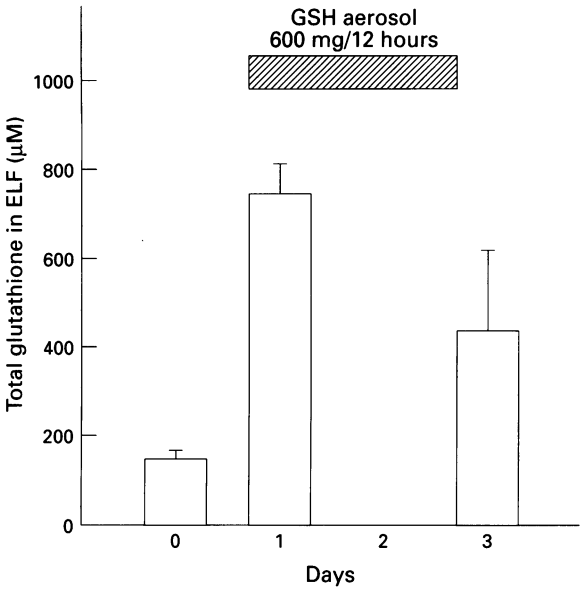

Figure 5 Effect of aerosolised reduced glutathione (GSH) on total glutathione concentrations in epithelial lining fluid (ELF).

granules but in the mitochondria which do not degranulate upon stimulation.[58] These data support the hypothesis that the increased level of CuZnSOD in the serum of patients with IPF is due to neutrophil activation and active secretion rather than release from damaged cells. The high serum CuZnSOD levels could also lead to increased levels of hydrogen peroxide which may contribute to the deficiency of GSH in the lower respiratory tract in patients with IPF. Preliminary evidence of a decrease in the total antioxidant potential in the plasma is also worthy of further study as a marker of oxidant stress.[59]

From the evidence presented above, the most logical antioxidant to target as a therapeutic intervention in IPF is glutathione. However, there is still some doubt over whether the reported GSH deficiency in epithelial lining fluid can be explained, at least partly by the methodological problems of quantifying the level of GSH in epithelial lining fluid. There are various ways of attempting to augment GSH levels in lung epithelial lining fluid. The study by Meyer and coworkers[45] has explored the possibility of using high dose oral N-acetylcysteine with only limited success due to the low bioavailability of N-acetylcysteine. Other studies of oral N-acetylcysteine are no more encouraging, producing either no,[41] or only a transient, increase in GSH levels in the epithelial lining fluid,[42] and no significant change in GSH levels in lung tissue,[44] which may be as important, if not more so, than levels in the epithelial lining fluid. Nebulised GSH held some promise of increasing GSH levels in epithelial lining fluid since oral administration of GSH does not enhance GSH levels due to gastric degradation by peptidases. Borok and coworkers[52] were able to raise GSH levels in the epithelial lining fluid from 140 (13) μM to normal levels (270 (14) μM) one hour after two doses of GSH aerosol (600 ng) separated by 12 hours in patients with IPF (fig 5). However, the effect was transient and was not present after three days. In addition, GSSG levels also rose although there was a transient decrease in spontaneous superoxide anion release by alveolar macrophages. Other potential techniques of increasing intracellular GSH levels include the infusion of methyl/ethyl esters of GSH[60] and low doses of diethylmaleate[61] which enhance membrane transport of GSH.[60] However, no firm evidence is available in vivo on the enhancement of intracellular GSH by these agents. Cysteine delivery systems such as L-2-oxithiazolidine-4-carboxylate are also incorporated by the cell and metabolised to GSH[62,63] and can be shown to protect lung cells against oxidant injury.

Conclusions

Our knowledge of the regulation of GSH in lung cells and epithelial lining fluid is rudimentary. We do not know the source of GSH in epithelial lining fluid nor the reason for the apparent concentration of GSH in epithelial lining fluid with plasma.[31] Resident lung cells such as epithelial cells and macrophages contain GSH as an important intracellular antioxidant, which not only protects the cell against oxidative damage but is critical for normal cell function.[29] GSH levels in epithelial lining fluid may be augmented from plasma, and both airspace epithelial cells and alveolar macrophages may secrete GSH or, perhaps, absorb GSSG, reduce it, and thereafter release GSH. Regulation of intracellular GSH is under the control of a number of enzymes including gamma-glutamylcysteine synthetase and gamma-glutamyl transpeptidase.[29,31] The glutathione redox system also involves a number of redox enzymes including glutathione peroxidase and reductase. Recent evidence suggests that the gene for gamma-glutamylcysteine synthetase may be oxidant sensitive, mediated by oxidant activation of the AP-1 transcriptional factor.[64] Further studies to elucidate the regulatory mechanisms of the genes for the enzymes involved in GSH synthesis and redox systems therefore offer the potential for genetic manipulation of GSH homoeostasis in conditions such as IPF where depletion of GSH is thought to play a part in its pathogenesis.

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LEARNING POINTS

* **Corticosteroids produce an objective response in only 10–20% of cases of IPF.**
* **Cyclophosphamide combined with corticosteroids produces no significant survival advantage over corticosteroids alone in IPF.**
* **There is a fourfold deficiency in glutathione levels in bronchoalveolar lavage fluid of patients with IPF compared with normal subjects.**

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