Decreased expression of haem oxygenase-1 by alveolar macrophages in idiopathic pulmonary fibrosis

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ABSTRACT: Haem oxygenase (HO)-1 is an oxidative stress responsive protein that may be involved in the pathogenesis of interstitial lung disease.

HO-1 expression in alveolar macrophages from bronchoalveolar lavage was investigated in 24 patients with idiopathic pulmonary fibrosis (IPF), 16 with sarcoidosis, 14 with hypersensitivity pneumonitis (HP) and 13 controls. Using immunocytochemistry, HO-1 expression in macrophages was scored semiquantitatively from 0–3 according to increasing intensity. The mean score of 100 macrophages was calculated. Macrophages were cultured and levels of interleukin (IL)-12 and IL-18 in the culture supernatants were measured by ELISA.

The mean score of HO-1 was significantly lower in IPF (67) than in sarcoidosis (105), HP (106) or controls (106). There was no significant difference between sarcoidosis, HP and controls. The score of HO-1 correlated positively with the lymphocyte percentage in sarcoidosis and HP. Positive correlations were found between the score of HO-1 and the release of IL-12 and IL-18 by macrophages in IPF.

The expression of haem oxygenase-1, a critical defender against oxidative stress, is decreased in macrophages of idiopathic pulmonary fibrosis patients compared with those with granulomatous lung disorders. This supports the hypothesis of an oxidant–antioxidant imbalance in the pathogenesis of idiopathic pulmonary fibrosis.

KEYWORDS: Alveolar macrophage, cytokine, haem oxygenase-1, interstitial lung disease

Idiopathic pulmonary fibrosis (IPF) is a chronic and fatal lung disease of unknown cause characterised by progressive fibroblast proliferation, destruction of the alveolar architecture and a relentless decline in pulmonary function [1]. The precise pathogenetic mechanisms of IPF are yet to be determined. A number of studies have suggested that an oxidant–antioxidant imbalance plays a role in the progression of pulmonary fibrosis in animal models and also possibly in human IPF [2]. There is ample evidence suggesting increased oxidative stress in patients with IPF. Alveolar macrophages (AMs) and neutrophils, which dominate in the lower respiratory tract of patients with IPF, are capable of inducing oxidant-mediated injury to lung parenchymal cells [3, 4]. Superoxide radicals and hydrogen peroxide are released spontaneously by bronchoalveolar lavage (BAL) cells of IPF patients [3]. High levels of myeloperoxidase associated with epithelial injury were found in the alveolar epithelial lining fluid (ELF) and BAL fluid (BALF) of patients with IPF [3]. Oxidative modification of mitochondrial

DNA has been observed in lung epithelial cells of IPF patients [5]. Oxidised BALF proteins, characterised by oxidation of methionine residues or carbonylation, are increased in patients with IPF [6, 7]. There is also an increase in 8-isoprostane, a biomarker of oxidative stress, in the BALF of IPF patients [8].

Conversely, there is a deficiency of cellular antioxidants in IPF patients [9–12]. Glutathione, a major component of the lung antioxidant defence system, is decreased in the ELF, both in the bleomycin model of lung fibrosis and in IPF patients [9]. Antioxidants and detoxification enzymes, including thioredoxin, glutaredoxin, manganese superoxide dismutase (MnSOD), catalase and glutamate cysteine ligase (γ -glutamyl cysteine synthetase, the rate-limiting enzyme in glutathione synthesis), show a diminished expression in patients with IPF [10–12]. These data support the hypothesis of an oxidant–antioxidant imbalance in the pathogenesis of IPF.

Haem oxygenase (HO), the rate-limiting enzyme in the catabolism of haem, is responsible for the

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STATEMENT OF INTEREST

None declared.

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TABLE 1 Demographics of the study population

	IPF	Sarcoidosis [#]	HP [†]	Controls
Subjects (M/F) n	24 (20/4)	16 (7/9)	14 (8/6)	13 (8/5)
Age yrs	67±2***	48±4*+ [‡]	61±4***	37±3
Nonsmokers n	14	16	10	13
Current smokers n	0	0	0	0
Ex-smokers n	10	0	4	0
VC % pred	72±4**	80±6	70±7**	99±4
TLC % pred	68±3***	79±4	78±5	97±4
FEV1/VC %	77±2	74±2	75±2	85±5
PaO₂ mmHg	71±2***	77±2	73±4**	90±2
PA-aO₂ mmHg	30±3***	21±2	32±5**	11±1

Data expressed as mean±SEM, unless otherwise stated. IPF: idiopathic pulmonary fibrosis; HP: hypersensitivity pneumonitis; M: male; F: female; VC: vital capacity; % pred: % predicted; TLC: total lung capacity; FEV1: forced expiratory volume in one second; PaO₂: arterial oxygen tension; PA-aO₂: alveolar–arterial oxygen tension difference. [#]: according to chest radiographical staging, six sarcoidosis patients had stage I, six patients had stage II and four patients had stage III disease; [†]: six were budgerigar fanciers, seven were pigeon breeders and one had humidifier lung. *: p<0.05, **: p<0.01 and ***: p<0.001 (when compared with controls); +: p<0.001 when compared with IPF; [‡]: p<0.01 when compared with HP. 1 mmHg=0.133 kPa.

degradation of haem to biliverdin, free iron, and carbon monoxide (CO). Biliverdin is subsequently converted to bilirubin through the action of the cytosolic enzyme biliverdin reductase, and free iron is sequestered by ferritin [13]. At present, three isoforms of HO have been identified as HO-1, HO-2 and HO-3. HO-1, the inducible form of HO, is thought to be an oxidative stress responsive protein, whereas HO-2 and HO-3 are constitutively expressed [13]. HO-1 has been implicated in a number of conditions and diseases associated with lung injury. HO-1 seems to be upregulated in smokers, asthma, acute lung injury, cystic fibrosis and lung transplant rejection, whereas it is downregulated in severe chronic obstructive pulmonary disease [14–19]. Since an oxidant–antioxidant imbalance contributes to the pathogenesis of lung fibrosis, HO-1, one of the critical defenders against oxidative stress, may be important in fibrotic lung diseases.

The aim of the present study was to investigate the expression of HO-1 in AMs recovered by BAL in patients with IPF, sarcoidosis and hypersensitivity pneumonitis (HP). Potential correlations between the expression of HO-1 and the production of pro-inflammatory cytokines were also explored. For this purpose, interleukin (IL)-12 and IL-18 were chosen, since they are involved in regulating the balance between T-helper cell (Th) type 1 (Th1) and Th type 2 (Th2) profiles [20, 21]. IPF is thought to be associated with a Th2 profile [22], whereas sarcoidosis and HP are Th1 diseases [23, 24].

METHODS

Patients

In the present study, 24 consecutive patients with IPF, 16 patients with active pulmonary sarcoidosis, 14 with chronic HP and 13 control subjects were investigated (table 1).

No patient was receiving treatment with corticosteroids and/or immunosuppressants at the time of BAL. Written informed consent was obtained according to institutional guidelines.

The 24 IPF patients were diagnosed according to American Thoracic Society/European Respiratory Society criteria [1]. Of these, seven had a surgical biopsy with histological evidence of usual interstitial pneumonia (UIP).

The 16 sarcoidosis patients were diagnosed on the basis of compatible clinical and radiographical features, histological evidence of noncaseating granuloma and/or an increased CD4/CD8 ratio of BAL lymphocytes, and the exclusion of other granulomatous disorders [23]. All of the sarcoidosis patients showed manifestations of disease activity as evidenced by recently developed or worsening symptoms, and/or chest radiographical evidence of progressive disease and/or deterioration of lung function tests.

The 14 HP patients fulfilled the following diagnostic criteria: 1) a history of exposure to organic antigens; 2) clinical signs and symptoms consistent with HP; 3) radiographical features and/or functional abnormalities characteristic of interstitial lung disease; 4) evidence of serum precipitins against one or more organic antigens; and 5) increased lymphocytes in the BALF. All 14 HP patients presented with the chronic form of insidious onset. On high-resolution computed tomography, all HP patients showed widespread and dominant ground-glass densities, with only minor reticulation and no honeycombing. Late-stage cases with extensive fibrosis were not investigated in the present study.

TABLE 2 Bronchoalveolar lavage fluid cell differentials

	IPF	Sarcoidosis	HP	Controls
Total cells				
n × 10 ⁶	15.0±2.4	17.0±2.6*	25.3±3.7***, #	6.9±1.0
n × 10 ⁴ ·mL ⁻¹	8.1±1.1	12.0±1.4*	19.3±3.5***, ##	5.8±0.9
Macrophages				
%	76.3±3.3	52.9±5.4***	23.0±2.4***, ###	92.1±0.9
n × 10 ⁴ ·mL ⁻¹	6.2±1.0	5.8±0.7	4.5±1.0	5.3±0.8
Lymphocytes				
%	14.0±2.6	43.7±5.1***, ##	64.8±2.7***, ###	6.8±0.9
n × 10 ⁴ ·mL ⁻¹	1.3±0.4	5.8±1.0***, ##	12.7±2.6***, ###	0.4±0.09
Granulocytes				
%	9.7±1.5***	3.7±0.8 [†]	11.8±2.2***	1.2±0.3
n × 10 ⁴ ·mL ⁻¹	0.7±0.1	0.5±0.1***, ##	2.1±0.5***, #	0.07±0.02
Neutrophils				
%	7.0±1.2***	2.5±0.6	8.5±2.0***	1.1±0.3
n × 10 ⁴ ·mL ⁻¹	0.5±0.09**	0.3±0.08***, [†]	1.6±0.5***	0.07±0.02
Eosinophils				
%	2.5±0.5***	0.9±0.3	2.6±0.7***	0.08±0.03
n × 10 ⁴ ·mL ⁻¹	0.2±0.03***	0.1±0.05**	0.4±0.08***	0.001±0.00

Data expressed as mean±SEM. IPF: idiopathic pulmonary fibrosis; HP: hypersensitivity pneumonitis. *: p<0.05, **: p<0.01 and ***: p<0.001 when compared with controls; #: p<0.05, ##: p<0.01 and ###: p<0.001 when compared with IPF; [†]: p<0.05 when compared with HP.

TABLE 3 Semiquantitative score of haem oxygenase-1 expression in bronchoalveolar lavage macrophages

Score	Staining intensity
0	No staining
1	Weak staining in the cytoplasm
2	Moderate staining in the cytoplasm, nucleus still visible
3	Strong staining in the cytoplasm, nucleus not visible

In total, 13 patients underwent diagnostic bronchoscopy and showed no evidence of lung disease. They had normal BAL cytology and served as controls.

BAL procedure

BAL was performed during fiberoptic bronchoscopy according to established guidelines [25]. Sterile isotonic saline solution was instilled into the right middle or left ligular lobe in 20 mL aliquots to a total volume of 100–200 mL, with immediate aspiration by gentle suction after each aliquot. A volume of >50% was retrieved. The BAL cell differentials of the four study groups are shown in table 2.

Immunocytochemical analysis

Immunocytochemistry was used to investigate the expression of HO-1 (Stressgen Biotechnologies Corp., Victoria, BC, Canada) in AMs from BAL. The peroxidase–antiperoxidase method was applied as previously described [26]. To evaluate the percentage of HO-1 positive cells, 500 macrophages were counted under a light microscope. In addition, HO-1 expression by AMs was scored semiquantitatively. The score for each AM counted ranged 0–3 according to increasing intensity (table 3). The mean score of 100 AMs was calculated for each patient and ranged 0–300. All analyses, including immunocytochemical grading, were performed blind by two investigators. The interobserver correlation was good. The Spearman's r -value for the correlation between the two counts was 0.89 ($p<0.01$).

Culture of AMs

AM cultures were performed as previously described [21], in patients with IPF ($n=12$), sarcoidosis ($n=8$) and HP ($n=4$). The AMs from BAL were incubated for 24 h with 1 mL RPMI 1640 medium (Seromed; Biochrom KG, Berlin, Germany) alone, or RPMI 1640 medium with 100 ng·mL⁻¹ lipopolysaccharide (LPS; Sigma-Aldrich, Munich, Germany). The culture supernatants were centrifuged and stored at -80°C.

ELISA assay for cytokines

The concentration of IL-12p70 and IL-18 in the culture supernatants was quantified using commercially available human ELISA kits (R&D systems, Inc., Minneapolis, MN, USA and Medical and Biological Laboratories Ltd, Nagoya, Japan), with a sensitivity of 0.5 pg·mL⁻¹ and 12.5 pg·mL⁻¹, respectively. The concentrations of these cytokines were expressed as pg·mL⁻¹ per 10⁶ AM after correction for the proportion of AMs.

Total bilirubin and ferritin analysis

The supernatants were analysed using ELISA kits for total bilirubin (Beckman Coulter, Fullerton, CA, USA) and for ferritin (Immulite; Siemens Healthcare Diagnostics, Tarrytown, NY, USA), with sensitivities of 0.1 mg·mL⁻¹ and 0.4 ng·mL⁻¹, respectively, using the manufacturers' instructions.

Statistical analysis

Data are expressed as mean $\pm$ SEM. To compare the four study populations (IPF, sarcoidosis, HP and control subjects), the data were analysed using Kruskal–Wallis one-way ANOVA by ranks. Two groups were compared using the unpaired t -test for parametric data. The correlations of different parameters were analysed by Spearman rank. A p -value <0.05 was considered statistically significant.

RESULTS

Expression of HO-1

HO-1 immunostaining was observed mainly in BAL AMs, occasionally in epithelial cells, but not in other BAL cells including lymphocytes, neutrophils, eosinophils, mast cells and plasma cells.

The score (mean $\pm$ SEM) of HO-1 positive AMs was significantly lower in patients with IPF (67.4 ± 5.0) than in those with sarcoidosis (105.4 ± 7.1 , $p<0.01$), HP (105.6 ± 5.4 , $p<0.01$) or control subjects (105.9 ± 5.2 , $p<0.01$; fig. 1). The percentage of HO-1 positive macrophages was also significantly decreased in patients with IPF (56 ± 3) compared with those with sarcoidosis (76 ± 3 , $p<0.01$), HP (74 ± 3 , $p<0.01$) and control subjects (86 ± 2 , $p<0.01$). There was no difference in the score or percentage of HO-1 positive macrophages between sarcoidosis and HP patients or controls.

IL-12 and IL-18 release from BAL macrophages in IPF and granulomatous lung disorders

Since the number of BAL samples with sufficient cells for AM culture was small, the patients with sarcoidosis ($n=8$) and HP ($n=4$) were grouped together as granulomatous lung disorders

FIGURE 1. Semiquantitative score of haem oxygenase (HO)-1 expression in alveolar macrophages in bronchoalveolar lavage fluid, in patients with idiopathic pulmonary fibrosis (IPF), sarcoidosis, hypersensitivity pneumonitis (HP) and controls. **: $p<0.01$.

FIGURE 2. Spontaneous (□) and lipopolysaccharide-stimulated (■) production of a) interleukin (IL)-12 and b) IL-18 from bronchoalveolar lavage (BAL) alveolar macrophages (AM) in patients with idiopathic pulmonary fibrosis (IPF; n=12) and granulomatous lung disorders (GLD; sarcoidosis and hypersensitivity pneumonitis; n=12). *: p<0.05; **: p<0.01.

(GLD) to allow statistically meaningful comparisons. After 24 h of culture, the spontaneous production of IL-12 (mean±SEM) from BAL macrophages was significantly lower in IPF than in GLD (0.7 ± 0.07 versus 7.5 ± 2.9 pg·mL⁻¹ per 10⁶ AM, $p<0.05$). Similarly, the level of IL-18 was significantly lower in IPF than in GLD (55.2 ± 10.7 versus 188.7 ± 44.7 pg·mL⁻¹ per 10⁶ AM, $p<0.01$; fig. 2).

The LPS-stimulated production of IL-12 and IL-18 was significantly higher than the spontaneous release in both groups ($p<0.05$ and $p<0.01$, respectively). The LPS-stimulated production of IL-12 and IL-18 was also significantly lower in IPF than in GLD (IL-12: 6.5 ± 3.2 versus 33.8 ± 13.7 pg·mL⁻¹ per 10⁶ AM, $p<0.05$; IL-18: 123.1 ± 24.2 versus 358.8 ± 81.7 pg·mL⁻¹ per 10⁶ AM, $p<0.01$; fig. 2).

Correlations between HO-1 expression and BAL cell differentials

In IPF patients, there were only weak correlations between the score of HO-1 positive AMs and the percentage of neutrophils ($r=0.40$, $p=0.052$) or eosinophils ($r=0.33$, $p=0.111$) in the BALF, statistically of borderline or no significance. In

FIGURE 3. Correlation of scores of haem oxygenase (HO)-1 expression in macrophages with percentage of lymphocytes from bronchoalveolar lavage (BAL) in granulomatous lung disorders (sarcoidosis n=16, hypersensitivity pneumonitis n=14). $r=0.38$, $p<0.05$.

sarcoidosis and HP patients, the score of HO-1 positive AMs showed a significant correlation with the percentage of lymphocytes ($r=0.38$, $p<0.05$) in the BALF (fig. 3).

Correlations between HO-1 expression and cytokine release from BAL macrophages

In IPF patients, positive correlations were found between the score of HO-1 positive AMs and the spontaneous production of IL-12 and IL-18 in supernatants of AM cultures ($r=0.58$, $p<0.05$ and $r=0.60$, $p<0.05$, respectively; fig. 4). No further correlations were found between HO-1 expression and cytokine production either in IPF or in GLD patients.

Correlations between HO-1 expression and lung function

The score of HO-1 failed to show any correlation with the lung function values of the patients with interstitial lung disease.

Total bilirubin and ferritin in the macrophage culture supernatants

Total bilirubin was significantly lower in the AM culture supernatants of IPF patients than in sarcoidosis and HP patients (3.6 ± 0.4 versus 9.9 ± 2.1 mg·mL⁻¹ per 10⁶ AM, $p<0.05$), whereas the ferritin concentration was not different between IPF and granulomatous lung disorders (170 ± 34 versus 189 ± 35 ng·mL⁻¹ per 10⁶ AM, $p>0.05$; fig. 5).

DISCUSSION

The present study showed that the expression of HO-1 by AMs is reduced in patients with IPF. In these patients, the score of HO-1 expression in macrophages correlated positively with IL-12 and IL-18 release from AM *in vitro*. There was no difference in the AM HO-1 expression between patients with GLD (sarcoidosis and HP) and the control subjects. The present results are in agreement with an immunohistochemical study which showed that HO-1 expression was higher in newly diagnosed sarcoidosis than in UIP patients [27]. The present study's limitation was due to the causality of the decreased HO-1 expression in IPF patient AMs not being determined.

FIGURE 4. Correlations of spontaneous release of a) interleukin (IL)-12 and b) IL-18 with score of haem oxygenase (HO)-1 expression in alveolar macrophages (AM) from bronchoalveolar lavage (BAL) in patients with idiopathic pulmonary fibrosis. a) $r=0.58$, $p<0.05$; b) $r=0.60$, $p<0.05$.

HO-1, as an important mediator of cytoprotection against oxidative injury, may be implicated in the pathogenesis of IPF. Basal expression of HO-1 has been described in hamster fibroblasts and was associated with resistance to hyperoxia [28]. In a murine model of pulmonary fibrosis, HO-1 was found to be one of the most differentially expressed genes as revealed by cDNA array analysis, and increased HO-1 protein expression was also observed [29]. A potential salutary effect of HO-1 in the pathogenesis of pulmonary fibrosis has been proposed. TSUBURAI *et al.* [30] reported that adenovirus-mediated transfer of the HO-1 gene can attenuate the intensity of bleomycin-induced pulmonary fibrosis in a murine model. Conversely, zinc-deuteroporphyrin (Zndtp), a chemical inhibitor of HO activity, has recently been shown to be protective in a bleomycin-induced lung fibrosis model by decreasing total lung collagen and the degree of extracellular matrix deposition and increasing glutathione levels [31]. How can the different effects of the two studies be explained, one showing a protection against bleomycin-induced fibrosis by HO-1 overexpression [30], the other a similar protection by HO inhibition [31]? First, the antioxidative properties that are intrinsic to the metalloporphyrin Zndtp itself may act to restore the alveolar antioxidant capacity. Secondly, the role of HO-1 in tissue injury

FIGURE 5. The concentration of a) total bilirubin and b) ferritin in the alveolar macrophage (AM) culture supernatants of patients with idiopathic pulmonary fibrosis (IPF; $n=12$) and granulomatous lung disorders (GLD; sarcoidosis and hypersensitivity pneumonitis; $n=12$). *: $p<0.05$; #: $p>0.05$.

is complex and is determined by its local concentration and temporal prominence [16, 32]. In the study by TSUBURAI *et al.* [30] HO-1 protein expression was induced before bleomycin treatment, whereas, in the study by ATZORI *et al.* [31], Zndtp was given after the initial inflammatory phase. This may account for the reduced fibrosis in both the overexpression study and the inhibitory study, suggesting that HO may play different roles in the early inflammatory phase and in the later fibroproliferative phase of bleomycin-induced lung toxicity. However, it is important to note that the bleomycin model does not completely correlate with human IPF. The bleomycin model results from acute lung injury leading to early fibrosis, whereas clinically diagnosed IPF patients are mostly in the late fibrotic stage, with little evidence of inflammation.

The role of the observed downregulation of HO-1 in the pathogenesis of IPF may be explained in two ways: it may either serve as an adaptive mechanism to protect the tissue against harmful effects of haem degradation products, or it may indicate that loss of protection against oxidative stress is involved in the pathogenetic mechanisms of IPF. There is more evidence to support the second assumption. Similarly to HO-1, the expression of MnSOD, an important superoxide radical-scavenging antioxidative enzyme, has been shown to be increased in inflammatory disorders and GLD but to be low in the fibrotic lesions of IPF lungs [12]. Likewise, other

antioxidants and detoxification enzymes including catalase, glutamate cysteine ligase, glutaredoxin and thioredoxin show similar changes in opposite directions, *i.e.* upregulation in acute inflammation models or diseases and downregulation in chronic fibrosis models or diseases [2, 10–12].

Although the role of inflammation in the pathogenesis of IPF remains controversial, evidence supports an imbalance of the Th1 (interferon (IFN)- γ , IL-12 and IL-18) and Th2 (IL-4 and IL-5) cytokine profiles in favour of the Th2 profile, which may contribute to the accumulation of collagen [22]. Sarcoidosis and HP are characterised by a Th1-type immune response which leads to exaggerated inflammation, setting the stage for granuloma formation and only rarely for the development of irreversible remodelling of the lung tissue and fibrosis [23, 24]. IL-12 and IL-18 are key cytokines involved in regulating the balance between Th1 and Th2 profiles. The results of the present study showed that both the spontaneous and LPS-stimulated production of IL-12 and IL-18 from BAL macrophages was significantly higher in sarcoidosis and HP patients than in IPF patients. This is consistent with the assumption that sarcoidosis and HP are Th1 diseases and IPF is a Th2 disease [22–24].

Recently, IL-12 has been shown to attenuate bleomycin-induced murine lung fibrosis by an IFN- γ dependent manner [33]. In the present study, the score of HO-1 expression in AMs failed to correlate with the production of IL-12 and IL-18 in patients with sarcoidosis and HP. However, in IPF patients, low HO-1 expression in AMs correlated with low spontaneous production of IL-12 and IL-18, indicating that the switch to the Th2 pattern was most profound in those IPF patients with the lowest HO-1 expression. The involvement of HO-1 in the regulation of inflammation has been shown in a number of diseases and animal models. Both HO-1 and CO can decrease the LPS-induced expression of pro-inflammatory cytokines, including tumour necrosis factor- α , IL-1 β and macrophage inflammatory protein-1 β , and augment the expression of the anti-inflammatory cytokine IL-10 [34, 35]. IL-10 mediated protection against LPS-induced septic shock in mice can be significantly attenuated by co-treatment with the HO inhibitor zinc-protoporphyrin [35]. Exogenous administration of CO to mice or macrophages leads to a decrease in the production of IL-6 [36]. To summarise, HO-1 is involved in the inflammatory modulation of lung diseases, the major effect being down-regulation of pro-inflammatory cytokines. Conversely, transforming growth factor- β 1, an important pro-fibrotic cytokine, is able to downregulate HO-1 in the rat lung [37]. Diminished expression of HO-1 is probably associated with a cellular micro-environment that promotes fibrosis.

Although the mechanisms underlying the cytoprotective function of HO-1 remain unclear, the haem degradation products including bilirubin, CO and iron-induced ferritin seem to be mediators of the effects of HO-1 activation [13]. In asthmatic patients, exhaled CO and sputum bilirubin levels have been shown to be elevated in agreement with increased HO-1 staining [15, 38]. Increased ferritin staining in AMs was observed in cystic fibrosis lungs compared with normal lungs, and the number of ferritin-stained macrophages correlated with the amount of HO-1 staining [17]. Inhaled CO had suppressive effects on fibrosis in bleomycin models of lung injury [39]. Bilirubin is also regarded as a powerful antioxidant substance *in*

vitro [40] and a very effective physiological antioxidant *in vivo* [41]. Bilirubin is not only an important antioxidant in serum, but may also play an important role in pulmonary fibrosis. This was suggested by a recent study where hyperbilirubinaemia was shown to attenuate bleomycin-induced pulmonary fibrosis [42]. In the present study, total bilirubin was significantly lower in the AM culture supernatants of IPF patients than in those of sarcoidosis and HP patients, similar to the changes observed for the HO-1 expression in AM.

In conclusion, the present study showed that haem oxygenase-1, an important antioxidant, is depleted in bronchoalveolar macrophages of idiopathic pulmonary fibrosis patients. Given the fact that murine models have been reported suggesting that overexpression of haem oxygenase-1 attenuates bleomycin-induced lung injury [29], the current study translates the findings of murine models into the human realm. This has not been previously reported but is necessary to advance research in this field. The findings support the hypothesis of an oxidant-antioxidant imbalance in the pathogenesis of idiopathic pulmonary fibrosis. Targeting haem oxygenase-1 expression in the lungs of patients with idiopathic pulmonary fibrosis may be a new therapeutic approach, but further research is warranted to elucidate the complex beneficial or harmful role of haem oxygenase-1 in interstitial lung disease.

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