Expression of glucocorticoid receptors α and β in steroid sensitive and steroid insensitive interstitial lung diseases

L Pujols, A Xaubet, J Ramirez, J Mullol, J Roca-Ferrer, A Torrego, J A Cidlowski, C Picado

Background: Sensitivity to glucocorticoids may be related to the concentration of glucocorticoid receptors α (GRα) and β (GRβ). A study was undertaken to assess GRα and GRβ expression in steroid insensitive interstitial lung disease (idiopathic pulmonary fibrosis (IPF)) and steroid sensitive interstitial lung diseases (sarcoidosis and cryptogenic organising pneumonia (COP)).

Methods: Lung tissue was obtained from control subjects and from patients with IPF, sarcoidosis, and COP. Pulmonary function tests were carried out at the time of lung biopsy and every 3 months. GRα and GRβ expression was evaluated by both competitive RT-PCR and immunohistochemistry. Data are presented as median and 25–75th percentile.

Results: GRα mRNA expression (10^5 cDNA copies/μg total RNA) was higher in patients with steroid sensitive interstitial lung diseases (10.0; 7.8–14.9; n = 11) than in patients with IPF (4.4; 3.2–6.6; n = 19; p<0.001). GRβ expression was at least 1000 times lower than that of GRα and did not differ between the three groups. A negative correlation was found between GRα mRNA levels and the fibrotic pathology score of the tissue (r = −0.484, p<0.01) and a positive correlation was found between GRα mRNA levels and improvement in forced vital capacity (r = 0.633; p<0.01) after treatment of patients with glucocorticoids. Immunoreactivity for GR protein was also higher in patients with sarcoidosis and COP than in those with IPF.

Conclusion: The variable response of some interstitial lung diseases to steroid treatment may be the result of differences in the expression of GRα.
for diagnosis of interstitial lung disease from 30 untreated patients.

**Idiopathic pulmonary fibrosis**

Lung tissue was obtained from 19 patients (12 men) of mean (SD) age 61 (3) years. Three were smokers (36 (2) packs), 10 were non-smokers and six were ex-smokers. The diagnosis of IPF was made following the criteria established by the American Thoracic Society (ATS)/European Respiratory Society (ERS) statement. Surgical lung biopsy was performed to diagnose IPF because patients did not fulfill all the clinical criteria established by the ATS/ERS statement. The indications for surgical biopsy in the 19 patients with IPF included in the study were: presence of ground glass opacification on HRCT scan (n = 5), suspicion of associated lung cancer (n = 3), fibroepithelial bronchoscopy not performed (n = 6), presence of lymphocytes >15% or eosinophils >20% in BAL fluid (n = 5). Histological analysis of the biopsy specimens showed that all patients had a usual interstitial pneumonia. None of the patients had other causes of interstitial lung disease such as drug toxicity, environmental exposure, or collagen vascular diseases.

**Sarcoidosis**

Lung tissue was obtained from five patients (two men) of mean (SD) age 55 (7) years. Four were non-smokers and one was an ex-smoker. All patients were in radiographic stage III. Surgical lung biopsy was indicated because transbronchial lung biopsy was negative and patients did not have any extrapulmonary involvement that made it possible to obtain histological confirmation of the disease.

**Cryptogenic organising pneumonia**

Lung tissue was obtained from six patients (three men) of mean (SD) age 56 (6) years. Four were non-smokers and two were ex-smokers. All the patients had clinical and radiological manifestations that suggested the diagnosis of COP. Surgical lung biopsy was indicated because transbronchial lung biopsy was negative.

The presence of dyspnoea was graded as follows: grade 0 = no dyspnoea; grade 1 = breathlessness on hills or after two flights of stairs; grade 2 = breathlessness after one flight of stairs; grade 3 = shortness of breath after walking on level ground; grade 4 = shortness of breath at rest or on minimal exertion. Shortness of breath was present in 16 out of 19 patients with IPF (six with grade 1, eight with grade 2, and two with grade 3). All the patients with sarcoidosis had shortness of breath at diagnosis (one with grade 1, three with grade 2, and one with grade 3). Shortness of breath was present in all the patients with COP (one with grade 1, four with grade 2, and one with grade 3).

Pulmonary function test findings at the time of the lung biopsy are shown in Table 1. Clinical examination, chest radiography, and pulmonary function tests were carried out every 3 months or when there was any clinical evidence of disease progression. Progression of the disease was defined as follows: (1) increase in the degree of dyspnoea, and/or (2) fall in forced vital capacity (FVC) and/or lung carbon monoxide transfer factor (TLCO) >15% with respect to the initial pulmonary function testing. The response to glucocorticoid treatment was defined as an improvement in FVC and/or TLCO of >15% and/or an improvement in the degree of dyspnoea. Patients who did not show any improvement or deterioration were considered stable.

The study was approved by the ethics committee of our institution and informed consent was obtained from all subjects.

**Histopathological analysis**

Lung biopsies were analysed and graded for 11 pathological abnormalities according to the method described by Watters et al. Five were cellular abnormalities (alveolar desquamation, alveolar septal inflammation, inflammatory airway narrowing, obstructive pneumonitis, and lymphoid nodules) and six were fibrotic abnormalities (alveolar septal fibrosis, cystic changes, obliteratorative airway narrowing, smooth muscle hypertrophy, thickened pulmonary arterioles, and cuboidalisation of alveoli). Each abnormality was graded according to the extent and intensity of change present as absent, mild, moderate, or severe. An inflammatory score, a fibrotic score, and an index of overall pathological derangement of the lung structure (total pathological score) were calculated on the basis of the sum of the inflammatory and fibrotic scores.

**Immunohistochemistry**

Immunohistochemistry was performed in 4 μm thick sections from tissue samples embedded in OCT (Tissue-Tek, Zoeterwoude, The Netherlands) using the Dako EnVision+ System kit, peroxidase (Dako Corporation, Carpinteria, CA, USA). Briefly, sections were fixed in cold acetone for 10 minutes, permeabilised in 0.5% saponin for 30 minutes, washed in phosphate buffered saline (pH = 7.4) and incubated with either polyclonal anti-GR antibody 57 or polyclonal anti-GRβ antibody BShGR13 diluted in Dako’s antibody diluent (0.05 M Tris-HCl, pH 7.2–7.6, containing 1% bovine serum albumin) for 30 minutes. Endogenous peroxidase was inactivated with 0.03% hydrogen peroxide. After washing in phosphate buffered saline, sections were incubated with the peroxidase labelled polymer conjugated to goat anti-rabbit immunoglobulin for 30 minutes. Sections were then washed in phosphate buffered saline and the peroxidase staining was visualised with hydrogen peroxide and 3,3’-diaminobenzidine chromogen solution. Finally, sections were counterstained with Gill’s haematoxylin, ethanol-dehydrated, and mounted in non-aqueous permanent mounting medium. Negative control of the immunohistochemical reaction was performed by replacing the primary antibody with antibody diluent.

The immunostained tissue sections were counted blindly by a pathologist using an Olympus microscope (×400 magnification). The following cell types were counted in the immunohistochemical analysis: mononuclear cells

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Pulmonary function test results in patients with interstitial lung diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IPF (n = 19)</td>
</tr>
<tr>
<td>FVC (%)</td>
<td>66.6 (3.8)</td>
</tr>
<tr>
<td>FVC (l)</td>
<td>2.4 (0.1)</td>
</tr>
<tr>
<td>FEV1/FVC (%)</td>
<td>0.8 (0.0)</td>
</tr>
<tr>
<td>TLC (l)</td>
<td>46.8 (2.7)</td>
</tr>
<tr>
<td>TLC (ml/min/ALv)</td>
<td>1.5 (0.1)</td>
</tr>
</tbody>
</table>

Data are presented as mean (SE).

IPF, idiopathic pulmonary fibrosis; COP, cryptogenic organising pneumonia; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; TLC, carbon monoxide transfer factor.
including lymphocytes, plasma cells and histiocytes, fibroblasts, epithelial cells, and alveolar macrophages. A minimum of 100 cells were counted for each cell type.

**Reverse transcriptase polymerase chain reaction (RT-PCR)**

Total RNA from lung tissue was reverse transcribed to complementary DNA (cDNA) using SuperScript II RNase H- reverse transcriptase. GRα and GRβ cDNAs were measured by competitive PCR in which known amounts of an exogenous DNA (competitor or internal standard) were co-amplified in competition with the target cDNA in the same test tube, according to methods previously reported in detail elsewhere.20–21

**Statistical analysis**

GR mRNA and protein data are presented as median and 25–75th percentiles. GRα or GRβ mRNA is expressed as 10^5 copies GRα cDNA or 10^2 copies GRβ cDNA per μg total RNA. Results from immunohistochemical analysis are expressed as percentage GR positive cells. The Kruskal-Wallis test was used to compare GR expression in the three groups and the Mann-Whitney U test was used for between group comparisons. The differences in age between controls and patients were compared using independent sample t tests and the differences in the smoking status between controls and patients were compared using the Fisher test. Spearman rank correlation was used when analysing relationships between data. Statistical significance was set at \( p < 0.05 \).

**RESULTS**

**Response to glucocorticoid treatment**

**Idiopathic pulmonary fibrosis**

Seventeen of the 19 patients were treated with prednisone at a dose of 1 mg/kg daily for 1 month. The dose was then tapered by 10 mg every 2 weeks to 10 mg/day or every other day. Two patients did not receive any treatment. Contact was lost in five of the 17 glucocorticoid treated patients. Two patients died due to progression of the disease 12 months after the diagnosis. Another patient presented a significant increase in the degree of dyspnoea but he was unable to perform pulmonary function tests because he underwent a laryngectomy for cancer. Serial clinical evaluation and pulmonary function tests were available from nine patients for a follow up period of 24.7 (4.2) months (range 6–42 months). In six patients there was a deterioration in functional parameters (FVC, Tlc) and in the degree of dyspnoea, one remained stable, and two improved. On the whole, there was a deterioration in functional parameters despite glucocorticoid treatment. To determine more fully the changes in pulmonary function tests over the follow up period the changes in pulmonary function tests were divided by the duration of follow up for each patient (table 2).

**Cryptogenic organising pneumonia**

Patients with COP (n = 6) were treated with prednisone at a dose of 1 mg/kg daily for 1 month. The dose was then tapered by 10 mg every 2 weeks to 10 mg/day or every other day.Serial pulmonary function tests and clinical evaluation were available in four patients for 20 (4.9) months (range 12–32 months). All of them showed a significant improvement in functional parameters and the degree of dyspnoea (table 2). Another patient had a resolution of pulmonary infiltrates after glucocorticoid treatment. Contact was lost with one patient.

**Histopathological findings**

Inflammatory, fibrotic, and total scores were higher in patients with IPF and with steroid sensitive interstitial lung diseases (sarcoidosis and COP) than in control subjects (table 3). No significant differences were found in any of these scores between patients with IPF and those with steroid sensitive interstitial lung diseases.

**Expression of GRα and GRβ mRNAs**

There was a higher expression of GRα mRNA (×10^5 GRα cDNA copies/µg total RNA) in patients with steroid sensitive interstitial lung diseases (10.0; 7.8–14.9; n = 11) than in patients with IPF (4.4; 3.2–6.6; n = 19; p < 0.001). Although not statistically significant, there was a tendency to a higher expression (p = 0.06) of GRα mRNA in patients with steroid sensitive interstitial lung diseases than in control subjects (6.0; 4.7–13.3; n = 12). GRβ mRNA expression in patients with IPF was also not significantly different from that of control subjects (p = 0.07, fig 1). It is worth noting that the

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**Table 2** Changes in pulmonary function tests over the follow up period

<table>
<thead>
<tr>
<th></th>
<th>IPF (n = 9)</th>
<th>Sarcoidosis (n = 5)</th>
<th>COP (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (range) follow up (months)</td>
<td>24.7 (6–42)</td>
<td>17 (10–24)</td>
<td>20 (12–32)</td>
</tr>
<tr>
<td>FVC (% change from initial assessment)</td>
<td>−10.6 (4.2)</td>
<td>12.2 (6.6)</td>
<td>30.2 (10.1)</td>
</tr>
<tr>
<td>FVC (% change/month)</td>
<td>−0.4 (0.4)</td>
<td>0.8 (0.5)</td>
<td>1.5 (0.3)</td>
</tr>
<tr>
<td>Tlc (% change from initial assessment)</td>
<td>−3.9 (10.2)</td>
<td>19 (12.2)</td>
<td>54.5</td>
</tr>
<tr>
<td>Tlc (% change/month)</td>
<td>−0.2 (1.2)</td>
<td>1 (0.8) †</td>
<td>4.5‡</td>
</tr>
</tbody>
</table>

Data are expressed as mean (SE). Percentage change per month in FVC and Tlc were calculated by dividing the change in FVC in Tlc by the follow up period in months for each patient.

IPF, idiopathic pulmonary fibrosis; COP, cryptogenic organising pneumonia; FVC, forced vital capacity; Tlc, carbon monoxide transfer factor.

\*n = 7; †n = 3; ‡n = 1.
control group was younger (p<0.001) and had many more smokers (p<0.05) than the patient group. However, no correlation was found between GRα mRNA expression levels and age (r = −0.018; p = 0.910; n = 42) or smoking status (p = 0.604; n = 42). In all the lung tissues analysed the expression of GRβ mRNA was at least 1000 times lower than that of GRα. In addition, no significant differences in GRβ mRNA expression (×10^6 GRβ cDNA copies/µg total RNA) were found between patients with steroid sensitive interstitial lung diseases (1.0; 0.3–1.7; n = 11) and either patients with IPF (0.8; 0.2–1.3; n = 19) or control subjects (0.8; 0.2–3.2; n = 12). There was no correlation between GRβ mRNA expression levels and age (r = −0.218; p = 0.165; n = 42) or smoking status (p = 0.320; n = 42).

No correlation was found between GRα mRNA levels and the tissue inflammatory score, and a negative correlation was found between GRα mRNA levels and the tissue fibrotic score (r = −0.484; p<0.01). A positive correlation was also found between GRα mRNA levels and changes in FVC (r = 0.633; p<0.01; n = 18) after treatment of patients with glucocorticoids in the follow up study (fig 2). The correlation between GRα mRNA levels and changes in TLCO was not statistically different (r = 0.345; p = 0.083; n = 11).

**Immunohistochemistry of GRα and GRβ**

Because of limitations in tissue availability, immunohistochemistry could not be performed in all subjects. Immunohistochemistry was carried out in eight control lung tissues, 12 IPF tissues, and six sarcoidosis + COP tissues. Positive staining for GR was observed in mononuclear cells (lymphocytes, histiocytes and plasma cells), fibroblasts, epithelial cells, and alveolar macrophages. With the exception of alveolar macrophages, the positive immunostaining for all cell types disappeared after incubation of tissue sections with antibody 57 preabsorbed with the peptide antigen (fig 3). The positive immunostaining observed in alveolar macrophages was therefore considered non-GR specific. In both control and pathological lungs the abundance or GR positive staining was mononuclear cells > fibroblasts > epithelial cells (fig 4).

Interestingly, patients with steroid sensitive interstitial lung diseases showed higher immunoreactivity for each of these cell types than patients with IPF, which was significant for total cells (the sum of mononuclear cells, fibroblasts and epithelial cells; p<0.05, figs 4 and 5). No correlation was found between total cell GR immunoreactivity and age (r = −0.204; p = 0.318; n = 26) or smoking status (p = 0.334; n = 26).

Low immunoreactivity for GRβ was observed in all tissues regardless of the tissue type, with occasional positive staining in mononuclear cells, fibroblasts, and epithelial cells from bronchioles.

**DISCUSSION**

To test the hypothesis that the different responses of some interstitial lung diseases to steroid treatment might be due to differences in the regulation of the GR, we assessed the expression levels of both GR isoforms (GRα and GRβ) in lung tissue from control subjects and patients suffering from steroid insensitive interstitial lung disease (IPF) and steroid sensitive interstitial lung diseases (sarcoidosis and COP). The main findings of our study are: (1) GRα mRNA and GR protein expression were higher in lung biopsy specimens obtained from patients with steroid sensitive interstitial lung diseases than in biopsy specimens from patients with IPF; (2) there was a direct correlation between the improvement in FVC and basal GRα mRNA expression levels; and (3) in all tissues examined GRβ mRNA expression was more than

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**Table 3** Inflammatory, fibrotic, and total scores from healthy and pathological lungs

<table>
<thead>
<tr>
<th>Lung type</th>
<th>N</th>
<th>Inflammatory score</th>
<th>Fibrotic score</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>2.0 (0.5–3.0)</td>
<td>1.0 (0.0–4.0)</td>
<td>2.0 (1.0–7.0)</td>
</tr>
<tr>
<td>IPF</td>
<td>18</td>
<td>7.0 (4.7–10.5)**</td>
<td>8.5 (5.7–14.0)**</td>
<td>15.5 (10.7–23.5)**</td>
</tr>
<tr>
<td>Sarcoidosis + COP</td>
<td>11</td>
<td>7.0 (4.0–8.0)**</td>
<td>5.0 (2.0–10.0)*</td>
<td>11.0 (7.0–18.0)**</td>
</tr>
</tbody>
</table>

Results are expressed as median and 25–75th percentile. 
N, number of subjects; IPF, idiopathic pulmonary fibrosis; COP, cryptogenic organising pneumonia. 
*p<0.05, **p<0.01, ***p<0.001 compared with controls (Mann-Whitney U test).
had higher immunoreactivity than in the IPF tissues. For sarcoidosis the highest GR immunoreactivity was shown in mononuclear cells. In fact, in both control and pathological lungs fibroblasts were less immunoreactive for GR than mononuclear cells. In line with this, immunohistochemical analysis showed that the fibrotic score of the tissue and basal GR mRNA levels may be partly explained by the fibrotic process of the tissue. We could therefore speculate that the expression of GR may have been upregulated in the sarcoidosis + COP lung tissues by local tissue specific stimuli. In keeping with this, stimuli such as cyclic adenosine monophosphate, lipopolysaccharide, interleukin (IL)-1β, or IL-6 have been shown to upregulate the expression of GR in different cell lines.25-28 A number of proinflammatory molecules have been shown to be overexpressed in COP and sarcoidosis.29 Increased expression of Th1 cytokines (interferon-γ, IL-2, IL-12, and IL-18) has been reported in sarcoidosis, whereas overexpression of Th2 cytokines (IL-4, IL-5, and IL-13) has been found in IPF lung tissue.30 However, whether any tissue specific cytokine, chemokine, or other stimuli released within the sarcoidosis + COP lung upregulates the expression of GR is currently not known.

In spite of the limitation that functional studies during the follow up period were not available for all patients, we found a direct correlation between basal levels of GR mRNA expression and the sensitivity of the patients to glucocorticoid treatment. These findings concur with previous studies reporting a direct correlation between hormonal sensitivity and cellular receptor levels.11 12

Since we have reported that fibroblasts—the predominant cell type in IPF lung—express lower amounts of GR than mononuclear cells, we could speculate that their increase in numbers, in parallel with progression of the disease, would lead to a progressively reduced sensitivity to glucocorticoids. Regardless of the exact mechanism of glucocorticoid resistance in patients with IPF, our findings ultimately suggest that the poor response of patients with IPF to glucocorticoid treatment may be explained, at least in part, by the limited presence of GR.

The role of the GR in the response of interstitial lung diseases to glucocorticoids has barely been investigated. No previous studies have distinguished between GRα and GRβ isoforms. Early studies using [3H]-prednisolone binding reported a decreased number of GR in BAL cells from patients with IPF compared with control subjects.26,27 In agreement with our results, Ozaki et al30 found a direct correlation between the number of [3H]-prednisolone binding sites per cell in BAL cells from patients with IPF and the responsiveness of patients to glucocorticoids.

Three studies have assessed GR expression in patients with sarcoidosis and healthy subjects. Anderson et al30 could not find any significant difference in GR mRNA expression in...
to glucocorticoids. Since the beneficial effects of glucocorticoids are mediated through activation of the GR receptor, our results suggest that the different expression of the GR isoform may determine hormone sensitivity in interstitial lung diseases.

In summary, we report that the expression of GRβ mRNA and protein was significantly higher in patients with sarcoidosis + COP than in those with IPF. A negative correlation was found between GRβ mRNA levels and the tissue fibrotic score, and a positive correlation was found between GRβ mRNA levels and the sensitivity of the patients to glucocorticoid treatment. IPF is a steroid insensitive condition while sarcoidosis and COP are diseases that usually respond to glucocorticoid treatment. Since the beneficial effects of glucocorticoids are mediated through activation of the GRβ receptor, our results suggest that the different responses of some interstitial lung diseases to glucocorticoid therapy may be due to differences in the expression of the GRβ isoform. Although differences in the expression of GRβ have been reported in some steroid insensitive diseases, our study suggests that GRβ does not play a relevant role in determining hormone sensitivity in interstitial lung diseases.

**Figure 5** Representative image of GR immunohistochemistry in lung tissue from (A) a control subject, (B) a patient with IPF, (C) a patient with steroid sensitive interstitial lung disease, and (D) a negative control (one tissue section incubated with antibody diluent instead of primary antibody). Magnification ×400.

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**REFERENCES**

Glucocorticoid receptors in the lung


LUNG ALERT

Bronchial basal cell and epithelial repair

Understanding the mechanisms of epithelial repair provides important insights into airway diseases such as asthma and COPD. The airway epithelial structure differs between the larger conducting airways and bronchioles in which, characteristically, basal cells are absent. In the distal airway Clara cells are absolutely necessary for epithelial repair but this is not so in tracheobronchial tissue. Here, the relative contribution to repair from Clara and basal cells is controversial.

This study explores the role of basal cells as a progenitor population in mouse bronchial epithelium of ablated Clara cells. Basal cells, identified by CK14 and GSI-B4 immunoreactivity, renewed bronchial epithelium after ganciclovir mediated depletion of Clara cells in CCtk transgenic mice and after naphthalene induced airway injury. Naphthalene is a toxin which specifically targets Clara cells. Following injury, basal cells were proliferative and adopted an intermediate morphology and molecular phenotype between true basal and Clara cells. In addition, the multipotential differentiation capacity of these cells was confirmed by an elegant lineage analysis in bitransgenic K14/R5 mice in which basal, Clara, and ciliated cell populations were found to have arisen from CK14 expressing progenitors.

The authors have used multiple methodologies to establish the stem cell niche of epithelial basal cells in the mouse bronchus, providing evidence that they function as a transient amplifying cell in response to injury. If applicable to humans, this work implicates bronchial basal cells as contributory to the pathobiology of chronic lung diseases, and renders these cells as possible therapeutic targets. Bronchial basal cells are a worthy focus of further translational research.

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