

PREVIOUSLY, we found that inflammatory mediators modulated the number and binding affinity of glucocorticoid receptors (GR) in human bronchial epithelial cell lines. In this study we investigated whether smoking and chronic obstructive pulmonary disease (COPD), both characterized by airway inflammation with increased levels of inflammatory mediators, affect GR characteristics in cultured human bronchial epithelial cells (HBEC). A statistically significant difference was found between the dissociation constant ( $K_d$ ) values in HBEC from smoking ( $K_d = 0.98 \pm 0.08$  nM;  $n = 6$ ) and nonsmoking controls ( $K_d = 0.76 \pm 0.10$  nM,  $P = 0.03$ ;  $n = 5$ ), but no significant difference was found between the mean number of binding sites. Our results are the first indication that cultured HBEC from smokers possess GR with a lower binding affinity. This may result from the inflammation found in the airways from smokers. Furthermore, these results provide further evidence that the bronchial epithelium may be an actual target for inhaled glucocorticoid therapy.

**Key words:** Glucocorticoid receptors, Human bronchial epithelial cells, Chronic obstructive pulmonary disease, Smoking

## Glucocorticoid receptor expression in human bronchial epithelial cells: effects of smoking and COPD

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## Introduction

The bronchial epithelium has long been regarded as a passive barrier to protect the underlying tissue.<sup>1</sup> Now, bronchial epithelial cells are also known to play an active role in airway inflammation. They are able to produce various inflammatory mediators including cytokines and eicosanoids.<sup>2,3</sup> Bronchial epithelial cells recovered from asthmatic patients show increased gene expression for GM-CSF, IL-6 and IL-8.<sup>3</sup> Furthermore, increased expression of endothelin-1, TNF- $\alpha$ , IL-1 $\beta$  and MCP-1 has been demonstrated.<sup>4,5</sup>

Glucocorticoids are widely used in the treatment of inflammatory pulmonary diseases, e.g. bronchial asthma and chronic obstructive pulmonary disease (COPD).<sup>6</sup> Recent studies showed that glucocorticoids are able to inhibit the release of some bronchial epithelial cell-derived cytokines.<sup>3,7</sup> These effects of glucocorticoids combined with the above-mentioned role of bronchial epithelial cells in airway inflammation, suggest that glucocorticoid therapy may suppress airway inflammation, at least partially, by modulating the function of bronchial epithelial cells. This hypothesis is supported by the observation that the greater part of inhaled glucocorticoids precipitate

on the epithelium of the larger airways.<sup>8</sup> A prerequisite for steroid responsiveness is the presence of specific glucocorticoid receptors (GR).

In a recent study we identified and characterized specific GR in two SV-40/adenovirus-transformed human bronchial epithelial cell lines, BEAS 2B and BEAS S6.<sup>9</sup> We also demonstrated that inflammatory mediators such as IL-1 $\beta$  and LPS may modulate the number and the binding affinity of GR in BEAS 2B cells. Similar results were found by other investigators in human T cells, other cell lines and murine macrophages.<sup>10–12</sup> These results indicate that inflammatory processes may influence the response of the bronchial epithelium to glucocorticoid therapy via locally produced cytokines.

Here, as an extension of our findings in cell lines, we report on the identification and characterization of specific GR in cultured human bronchial epithelial cells (HBEC). From the above-mentioned *in vitro* findings concerning the effects of inflammatory mediators on GR, it may be expected that inflammation *in vivo* may also affect GR characteristics. Therefore, we also studied whether in cultured HBEC from smokers and COPD patients, in whom general inflammation has been described,<sup>13</sup> the number or binding affinity of GR is altered.

**Table 1.** Clinical characteristics of subject groups

| Subjects                 | Number | Age (years) | Smoking (pack-years) | FEV (% of predicted) |
|--------------------------|--------|-------------|----------------------|----------------------|
| Nonsmoking controls      | 5      | 71 ± 2      | 0                    | 87 ± 6               |
| Smoking controls         | 6      | 59 ± 3      | ≥35                  | 90 ± 4               |
| Nonsmoking COPD patients | 3      | 67 ± 1      | 0                    | 68 ± 6               |
| Smoking COPD patients    | 5      | 60 ± 3      | ≥35                  | 52 ± 3               |

Data are shown as mean ± SEM.

## Materials and Methods

### Subjects

A total of 19 subjects were entered into the study (Table 1). Of the subjects eight fulfilled ATS criteria for COPD.<sup>14</sup> COPD patients were nonallergic with complaints of chronic cough and sputum production. Chronic cough means that coughing occurs on most days during at least three consecutive months in two consecutive years. One-second forced expiratory volume (FEV<sub>1</sub>) was measured in all subjects before entry into the study. COPD patients suffered from major airway obstruction, that is, the FEV<sub>1</sub> was <75% of predicted. No reversibility of airway obstruction in these eight patients was obtained after inhalation of 0.5 mg terbutaline. Controls (*n* = 11) were subjects who denied any symptoms of asthma or COPD, and had normal lung function parameters. The COPD and control group contained five and six smoking individuals, respectively. From the nonsmoking controls one individual stopped smoking 12 years ago. Smoking individuals were current smokers with a smoking history of at least 35 pack-years.

### Isolation and culture conditions of HBEC

Bronchial tissue was obtained from patients undergoing surgery for lung cancer and used immediately for culture of HBEC by a cell culture method described previously.<sup>15</sup> Briefly, bronchial tissue distant from the tumour was cut into pieces and incubated either overnight at 4°C or 1 h at 37°C in 0.1% protease XIV (Sigma, St Louis, MO). Subsequently, epithelial cells were gently scraped from the tissue samples, washed twice in culture medium and plated onto 35-mm dishes at a density of  $2.5 \times 10^5$  cells/dish. HBEC were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F12)(GIBCO, Paisley, UK), with supplements as described previously.<sup>15</sup> Cells were characterized as epithelial cells by immunofluorescence staining using a mouse monoclonal antibody directed against a number of human cytokeratins (CK-1; DAKOPatts, Glostrup, Denmark). At least 99% of the isolated cells stained positive for cytokeratin.

When dishes were confluent, HBEC were passaged to 75 cm<sup>2</sup> flasks and used for experiments after confluence. Twenty-four hours before performing GR binding experiments the medium was replaced by a basal medium of DMEM/F12 (1:1) with penicillin G sodium and streptomycin sulphate, but without hydrocortisone or other supplements to prevent influence of endogenous steroids on the number and affinity of GR.

### Cell lines

BEAS 2B is a SV40/adenovirus transformed human bronchial epithelial cell line, which was kindly provided by Dr J. Lechner (Inhalation Toxicology Research Institute, Albuquerque, NM).<sup>16</sup> Cells were maintained in a complete keratinocyte growth medium (KGM) containing bovine pituitary extract, EGF, penicillin G sodium and streptomycin sulphate (GIBCO).

The COS-1 cell line was used as a negative control for GR Western blot experiments. The COS-1 cell line, derived from the kidney of a male adult African green monkey (American Type Culture Collection, Rockville, MD) was cultured in DMEM with 5% FCS.

### Preparation of cytosol

Flasks containing approximately  $10 \times 10^6$  cells were washed twice with cold phosphate buffered saline (PBS) and HBEC were scraped in 0.5 ml of buffer A (40 mM Tris-HCl pH 7.4, 1 mM EDTA, 10% (v/v) glycerol, 10 mM dithiothreitol, 10 mM Na<sub>2</sub>MoO<sub>4</sub>) supplemented with 0.6 mM phenylmethylsulphonyl fluoride (PMSF), 0.5 mM bacitracin and 0.5 mM leupeptin. The cells were lysed by freezing/thawing three times and the homogenate was centrifuged for 10 min at  $400\,000 \times g$ .<sup>17</sup> The supernatant was collected and stored at -80°C. Protein concentrations were determined according to the method of Bradford<sup>18</sup> and ranged from 1 to 2 µg/µl. Cytosol preparations were used for both Western blotting and binding assays.

## Western blotting

Samples (20  $\mu$ l cytosol containing 1  $\mu$ g/ $\mu$ l protein) were mixed with 5  $\mu$ l of 5  $\times$  sample buffer (1  $\times$  sample buffer = 50 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 10 mM dithiothreitol, 0.001% (w/v) bromophenol blue), boiled for 2.5 min and separated on a 7% SDS-PAGE gel. After electrophoresis the gels were blotted to nitrocellulose membrane in blotting buffer (16.5 mM Tris-HCl, pH 8.3, 150 mM glycine and 20% v/v methanol). The membranes were blocked for 1 h at RT with 1% block solution (Boehringer, Mannheim, Germany) and incubated for 1 h at room temperature with a commercially available GR polyclonal rabbit antibody, no. 57 (PA1-511; Affinity Bioreagents, Neshanic Station, NJ), diluted 1:250 in 0.5% block solution. Membranes were washed twice with PBS/Tween 0.1% and twice with 0.5% block solution, then incubated with horseradish peroxidase-conjugated goat anti-rabbit-IgG (Sigma) for 1 h at RT. After 4  $\times$  15 min washes (PBS/Tween 0.1%) proteins were detected with Boehringer-Mannheim's Chemiluminescence Western Blotting kit and membranes were exposed to X-ray film.

## Steroids

$^3$ H-labelled dexamethasone (1,2,4,6,7 [ $^3$ H] dexamethasone; specific activity 81 Ci/mmol) was obtained from Amersham (Buckinghamshire, UK). Nonradioactive dexamethasone was kept in a stock solution of 2  $\times$  10 $^{-3}$  M in ethanol (Duchefa bv. Haarlem, the Netherlands).

## Binding assays

GR numbers and  $K_d$  values were determined according to established methods.<sup>19</sup> Six serial doubling dilutions (50  $\mu$ l) were prepared in duplicate in PBS to final  $^3$ H-labelled dexamethasone concentrations of 32, 16, 8, 4, 2, and 1 nmol/l, respectively. For measurements of nonspecific binding, parallel dilutions of  $^3$ H-labelled dexamethasone plus a 100-fold molar excess of nonradioactive dexamethasone were prepared. To each of the 24 tubes 150  $\mu$ l of the cytosol preparation was added and the mixture was incubated overnight at 4°C. Binding equilibrium was reached at all concentrations after incubation overnight at 4°C. Subsequently, 160  $\mu$ l aliquots of each incubation mixture were transferred to albumin (0.1%) coated tubes, 750  $\mu$ l of protamine dihydrochloride solution (0.5 mg/ml) was added and tubes were centrifuged for 15 min at 4000 rpm. From the remaining incubation mixture 20  $\mu$ l was used to establish the exact concentration of  $^3$ H-labelled dexamethasone. Tubes containing protein pellets were washed three times with 1 ml of incubation buffer (50 mM Tris-HCl pH 7.4, 1.5 mM EDTA, 1.5 mM DTT,

10% glycerol) and pellets were solubilized in Soluene-350 (Packard, Meriden, CT). Thereafter, samples were transferred to scintillation vials and radioactivity was determined by liquid scintillation counting. Specific binding was calculated as the difference between the totally bound radioactivity and the nonspecifically bound radioactivity at each glucocorticoid concentration. Nonspecific binding was calculated from the aliquots containing the 100-fold molar excess non-radioactive dexamethasone, assuming that nonspecific binding was nonsaturable and linearly related to the concentration of free glucocorticoid.

## Analysis

Binding curves were constructed from increasing concentrations of  $^3$ H-dexamethasone. GR number and  $K_d$  values were determined by Scatchard analysis of these data. Data were expressed as mean fmol/mg protein specific binding  $\pm$  the standard error of the mean (SEM). The Wilcoxon Matched-Pair Signed-Ranks test was used to assess the equality of GR number and  $K_d$  distributions in HBEC from patients with COPD and controls. A *P*-value of less than 0.05 was considered significant.

## Results

### Expression of glucocorticoid receptor protein in normal HBEC

Cytosolic fractions were isolated from HBEC, BEAS 2B and COS-1 cells, and GR levels were examined by Western blotting. The GR polyclonal rabbit antibody

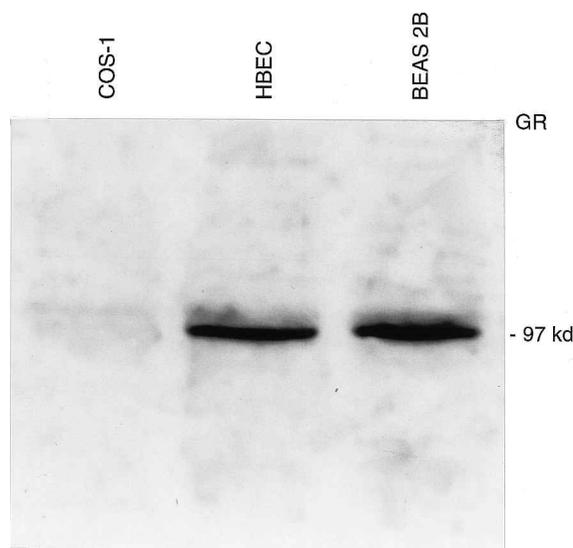


FIG. 1. Western blot of 20  $\mu$ g protein illustrating immunoreactive GR levels in cultured human bronchial epithelial cells (BE) and in the cell line BEAS 2B. One representative experiment out of three is shown. The COS-1 cell line (20  $\mu$ g) was used as a negative control.

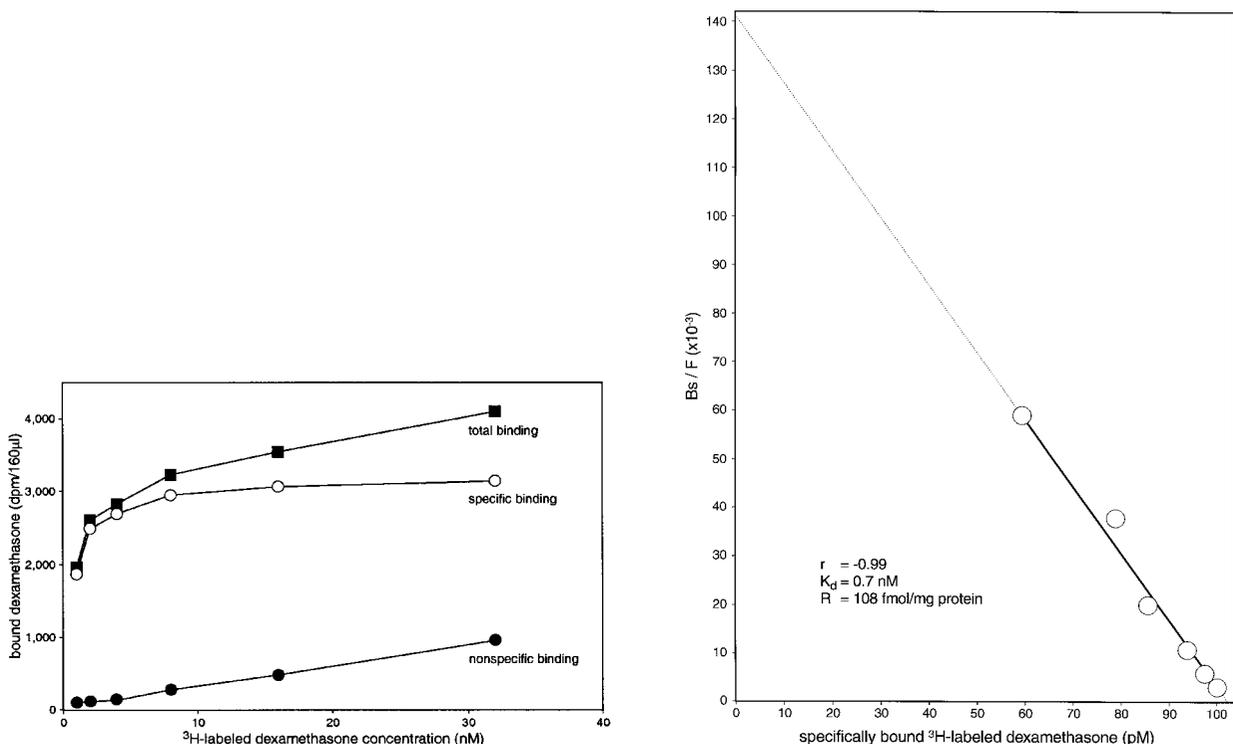


FIG. 2. (A) Binding curve of <sup>3</sup>H-labelled dexamethasone for cultured HBEC. The specific binding is represented by the difference between the total and nonspecific binding. The data points represent mean values of duplicate determinations. (B) Scatchard plot of the specific binding of <sup>3</sup>H-labelled dexamethasone to HBEC. On the ordinate is given the ratio of the number of specifically bound <sup>3</sup>H-labelled dexamethasone (Bs) to the number of free <sup>3</sup>H-labelled dexamethasone (F). One representative experiment out of 11 is shown. The  $K_d$  and the number of glucocorticoid binding sites per mg protein (R) were calculated using the negative inverse of the slope and the  $\times$  intercept, respectively.

recognized a prominent band at ~97 kDa in the HBEC and BEAS 2B preparations (Fig. 1). This estimated molecular mass is consistent with that reported previously for the human GR.<sup>20</sup> In the GR negative cell line COS-1, no immunoreactive protein was detectable.

After demonstrating the presence of GR protein, the number of GR and  $K_d$  values were studied in cultured HBEC from controls ( $n = 11$ ). Using established methods to identify GR, we could demonstrate specific binding of <sup>3</sup>H-labelled dexamethasone by these cells. A typical binding curve for cultured HBEC is shown in Fig. 2A. After Scatchard analysis of the data, the linear regression line obtained indicated a single class of GR (Fig. 2B).

### GR binding in HBEC COPD patients, smokers and controls

HBEC were isolated from bronchus tissue from eight COPD patients and 11 controls. The COPD and control group contained five and six smoking individuals, respectively. Cells were cultured for one passage. Thereafter, cytosols were prepared and the number of glucocorticoid binding sites and  $K_d$  were determined. Results are presented in Table 2 and Fig. 3. A significant difference was found between the  $K_d$  values in HBEC from smoking ( $K_d = 0.98 \pm 0.08$  nM) and nonsmoking controls ( $K_d = 0.76 \pm 0.10$  nM,  $P = 0.03$ ), but no significant difference was found between the mean number of binding sites ( $70.5 \pm$

**Table 2.** Glucocorticoid binding in HBEC from smoking and nonsmoking COPD patients and controls

|                     | Controls<br>Nonsmoking ( $n = 5$ ) | COPD patients<br>Smoking ( $n = 6$ ) | Nonsmoking ( $n = 3$ ) | Smoking ( $n = 5$ ) |
|---------------------|------------------------------------|--------------------------------------|------------------------|---------------------|
| R (fmol/mg protein) | $87.2 \pm 16.7$                    | $70.5 \pm 14.0$                      | $79.7 \pm 22.6$        | $68.4 \pm 12.8$     |
| $K_d$ (nM)          | $0.76 \pm 0.10$                    | $0.98 \pm 0.08^*$                    | $0.74 \pm 0.19$        | $0.71 \pm 0.15$     |

Data are shown as mean  $\pm$  SEM. R: number of glucocorticoid binding sites;  $K_d$ : dissociation constant. \*: significantly higher ( $P < 0.05$ ) than in nonsmoking controls.

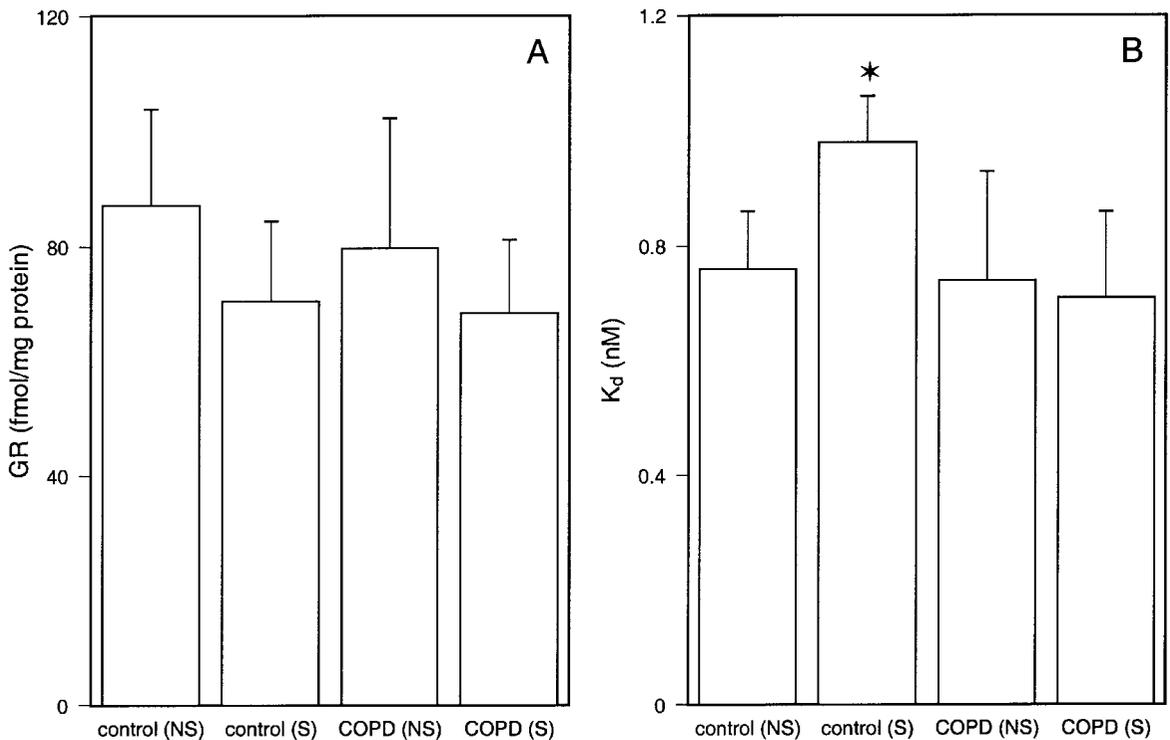


FIG. 3. Total number of GR (A) and  $K_d$  values (B) in cultures of HBEC from nonsmoking controls ( $n = 5$ ), smoking controls ( $n = 6$ ), nonsmoking COPD patients ( $n = 3$ ) and smoking COPD patients ( $n = 5$ ). Data are shown as mean  $\pm$  SEM. \*  $P < 0.05$  for smoking controls versus nonsmoking controls. sm: smoking.

14.0 and  $87.2 \pm 16.7$  fmol/mg protein, respectively,  $P = 0.3$ ). Furthermore, values measured in nonsmoking and smoking COPD patients ( $P = 0.6$  and  $P = 0.6$ , respectively) did not differ significantly. Nor was any significant difference observed in glucocorticoid binding sites or  $K_d$  between nonsmoking COPD patients and nonsmoking controls ( $P = 0.6$  and  $P = 0.6$ , respectively) or between smoking COPD patients and smoking controls ( $P = 0.9$  and  $P = 0.1$ , respectively).

## Discussion

In this study we identified and characterized specific GR in primary cultures of HBEC from COPD patients and controls. The presence of GR protein was demonstrated using Western blot analysis and  $^3\text{H}$ -dexamethasone binding studies. A significant decrease was found in the binding affinity of GR in HBEC from smoking controls compared with nonsmoking controls, but no significant difference was found between the mean number of specific glucocorticoid binding sites. Furthermore, values measured in nonsmoking and smoking COPD patients did not differ significantly. Nor was any difference observed between the mean number of binding sites and the  $K_d$  values in HBEC from nonsmoking COPD patients and the values measured in nonsmoking controls or between the values measured in smoking COPD patients and smoking controls.

HBEC are considered to play an important role in airway inflammation.<sup>1-5</sup> Inhaled glucocorticoids are used to suppress airway inflammation and, concomitantly, to improve clinical parameters. The finding that HBEC possess functional GR allows us to hypothesize that the clinical response to inhaled glucocorticoids, which mainly precipitate in the larger airways, results, at least partly, from the modulation of airway epithelial cell functions. From the  $K_d$  value of the GR in HBEC observed in our studies, we expect that effective *in vivo* glucocorticoid concentrations should be around 1 nM. In a recent study by Van den Bosch *et al.*<sup>21</sup> it was shown that at least 90 min after inhalation of 1.6 mg budesonide, lung tissue concentrations ranged from 2.1 to 8.9 nM. Therefore, we can assume that therapeutical inhalation of glucocorticoids results in such concentrations of glucocorticoids in lung tissue that interaction with GR in HBEC will occur.

In a previous study we found that the SV40/adenovirus transformed human bronchial epithelial cell line BEAS 2B contained a higher number of glucocorticoid binding sites compared with peripheral blood mononuclear cells (PBMC).<sup>8</sup> The number and quality of GR in target cells may determine the extent of glucocorticoid responsiveness.<sup>22</sup> HBEC were observed to contain less GR than the BEAS 2B cell line ( $87.4 \pm 16.5$  and  $370 \pm 14$  fmol/mg protein, respectively). This difference may be caused by the SV40/adenovirus transformation. HBEC contain a relatively high num-

ber of GR compared with PBMC, which contain approximately 30 fmol/mg protein (unpublished results). It would be interesting to investigate whether these differences in GR number result in differences in the functional response to glucocorticoids. As both epithelial cells and PBMC are able to produce IL-1 $\beta$ , the inhibition of IL-1 $\beta$  production by glucocorticoids may be a useful parameter to study this issue. Few data are available on the number of GR in other epithelial cells. A human epithelial duct cell line was found to contain 83–92 fmol GR/mg protein.<sup>23</sup>

Previously, we demonstrated that the inflammatory mediators IL-1 $\beta$  and LPS increased the GR number and  $K_d$  in BEAS 2B cells.<sup>8</sup> Similar results were found in other cell types.<sup>9–11</sup> These results suggest that inflammatory processes may indirectly influence the response of bronchial epithelium to glucocorticoid therapy via production of cytokines by infiltrating cells. It has been suggested that in COPD, bronchial inflammation is responsible for the development of airway hyperreactivity, and chronic airflow limitation.<sup>12</sup> Cigarette smoking causes an inflammatory reaction in the airways and can lead to the development of COPD.<sup>13</sup> We found a significantly decreased binding affinity of the GR in smoking controls when compared with values found in nonsmoking controls. These results indicate that the inflammation present in the airways of smokers may influence the binding affinity of GR receptors. The finding that *in vivo* inflammation found in smokers affects GR characteristics in HBEC is in accordance with our above mentioned findings *in vitro*. However, we did not find any difference in the binding affinity of the GR in smoking COPD patients when compared with values in nonsmoking COPD patients. A study by Linden *et al.*<sup>13</sup> showed that obstructed smoking COPD patients had significantly lower concentrations of inflammatory cells in the bronchial lavage fluid compared with nonobstructed smoking individuals. Therefore, it was suggested that obstructed and nonobstructed smokers may differ in the type of airway inflammation present. The presence of lower concentrations of inflammatory cells in obstructed smoking COPD patients in the study by Linden *et al.* may explain why in our study no differences in GR binding affinity were found between smoking and nonsmoking obstructed COPD patients. Furthermore, we did not find any difference in the binding affinity of the GR in COPD patients when compared with values found in controls. Different inflammatory mediators may be involved in COPD and in smoking and it remains to be established which mediators actually alter GR characteristics. The actual mechanisms by which inflammatory mediators, present in the airways of smoking controls can induce a decrease in GR binding affinity in HBEC remain to be established. Others have shown that altered expression of glucocorticoid-regulated

proteins appears to be mediated via interaction of the modulatory domain of the GR with transcriptional factors, such as AP-1.<sup>24–26</sup> Overexpression of AP-1 interferes with the function of the modulatory domain of the GR. Because cytokines can induce elevated levels of AP-1, it has been suggested that this may provide a plausible explanation for the decreased ligand binding affinity of nuclear GR for glucocorticoids found in T cells after incubation with IL-2 and IL-4.<sup>24–26</sup> Perhaps, a similar explanation could be given for the decreased GR binding affinity in HBEC induced by inflammatory mediators in the airways of smoking controls. Another explanation for decreased GR binding affinity could be a modulating effect of inflammatory mediators in the airways of smoking controls on the expression and phosphorylation of heat shock proteins, which are associated with the unliganded GR.<sup>27,28</sup> Bacterial products and cytokines can regulate the expression and phosphorylation of heat shock proteins and this may modulate glucocorticoid binding to the GR.<sup>27,28</sup> Further studies are necessary to clarify the exact mechanism of the effects of these inflammatory mediators found in the airways of smoking controls on GR.

We did not find any difference in the number of GR between COPD patients and controls, nor between smoking and nonsmoking controls. However, it cannot be excluded that differences, present between the groups *in vivo*, disappeared during isolation and culture of these cells. We tried to clarify this issue and tested cultures of HBEC at subsequent passages. Similar numbers of GR and  $K_d$  values were found in subsequent passages. Others have found that increased GR numbers and decreased GR binding affinity in T cells from steroid resistant asthmatic patients reverted to normal after 48 h in culture.<sup>29</sup> It would be interesting to study GR number and affinity directly on the bronchial tissue or directly after cell isolation. However, performance of a <sup>3</sup>H-dexamethasone binding assay, the most sensitive method to determine GR number and affinity, directly after cell isolation is not possible, because of the low cell number obtained. We are currently working on a method to quantify GR number in small cell samples by flow cytometry using several GR specific antibodies.

To our knowledge, no other studies have been published on GR number and  $K_d$  values in HBEC from smoking and nonsmoking COPD patients and controls. Until now, most investigations have concentrated on the analysis of GR in PBMC, comparing asthmatics with healthy individuals.<sup>30,31</sup> No differences in GR number or GR binding affinity were found between PBMC from asthmatics and controls.<sup>23,29</sup> Recently, Kam *et al.*<sup>10</sup> showed that PBMC from steroid resistant asthmatics had a significantly reduced GR binding affinity and an increased GR number when compared with normal subjects. Others have claimed that the clinical response to gluco-

corticoids can not be explained by abnormal GR number or affinity.<sup>32</sup> They suggested that the ability of GR to bind to their DNA binding sites (GRE) is impaired. Further studies are necessary to clarify whether the clinical response to inhaled glucocorticoids is related to the number and binding affinity of GR and which cells are involved in this response. As only a subgroup of COPD patients responds to inhaled glucocorticoids, it is of interest to look in resistant patients for correlations between the clinical response to glucocorticoids and the number of GR and their  $K_d$  in bronchial epithelial cells.<sup>33</sup>

In conclusion, we demonstrated that cultured HBEC possess a single class of specific GR and that the binding affinity of GR in HBEC from smoking controls was significantly decreased when compared with values found in nonsmoking controls. These results provide further evidence that the bronchial epithelium may be an actual target for glucocorticoid therapy. Furthermore, our findings are the first indication that altered GR characteristics are present in cultured HBEC from smokers. We hypothesize that this may result from the inflammation found in the airways from smokers.

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