AP-1 overexpression impairs corticosteroid inhibition of collagen production by fibroblasts isolated from asthmatic subjects

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 Jacques E, Semlali A, Boulet LP, Chakir J. AP-1 overexpression impairs corticosteroid inhibition of collagen production by fibroblasts isolated from asthmatic subjects. Am J Physiol Lung Cell Mol Physiol 299: L281–L287, 2010. First published June 11, 2010; doi:10.1152/ajplung.00360.2009.—Asthma is characterized by airway remodeling associated with an increase in the deposition of ECM proteins such as type I collagen. These components are mainly produced by fibroblasts. Inhaled corticosteroids are considered the cornerstone of asthma therapy. Despite substantial evidence as to the anti-inflammatory action of corticosteroids, their effect on controlling ECM protein deposition in the airways is not completely understood. This study determined the effect of dexamethasone (Dex) on collagen production by bronchial fibroblasts derived from asthmatic and healthy subjects. Expression of procollagen mRNA in fibroblasts from asthmatics and normal controls was determined by quantitative PCR. Regulation of the procollagen-α1 promoter was evaluated by transient transfections. Transforming growth factor-β (TGF-β) protein expression was determined by ELISA. Protein expression of glucocorticoid receptor (GR) and interaction with activator protein-1 (AP-1), a collagen regulatory transcription factor, was assessed by Western blots, communoprecipitations, and EMSA. AP-1 overexpression was performed by transient transfection using c-Fos/c-Jun expression plasmids. Dex significantly downregulated procollagen production and promoter activity in normal fibroblasts but had no effect on asthmatic fibroblasts. AP-1 and GR interaction increased after Dex stimulation in asthmatic fibroblasts. AP-1 overexpression in control fibroblasts abrogated collagen gene response to Dex. These results show that Dex failed to reduce collagen production in fibroblasts from asthmatic subjects. This impaired response may be related to AP-1 overexpression in these cells.

ASThma is defined as an inflammatory disease associated with physiological abnormalities attributed to airway remodeling (9). Deposition of interstitial collagen underneath the basement membrane is a characteristic feature of asthma. This seems to be due to the activation of mesenchymal cells (16, 24). Corticosteroids (CS) are considered the cornerstone of asthma therapy. Despite substantial evidence as to the anti-inflammatory effect of CS, their effect on controlling airway remodeling is not completely understood. Many studies have failed to address the effect of CS on established indices of remodeling, particularly collagen deposition. High doses of inhaled fluticasone for 8 wk reduced inflammation but not subepithelial collagen deposition (8). Moreover, oral CS did not decrease transforming growth factor-β (TGF-β) and types I and III collagens in moderate to severe asthma (11). Four-week treatment with inhaled budesonide failed to reduce basal membrane thickening in mild atopic asthmatics (23).

CS act by binding and activating the cytosolic glucocorticoid receptor (GR) (2). The activated GR undergoes nuclear translocation and interacts in either a cis- or trans-repressive manner to gene expression (5). The GR gene produces at least eight functional GR NH2-terminal isoforms via translational mechanisms (30). One of these isoforms, GR-β, is distinct from the classic ligand-activated GR, GR-α. GR-β is unable to bind CS and represses CS-sensitive genes. It is located in the nucleus of cells independent of hormone treatment. GR-β has an intact DNA-binding domain and can also inhibit GR-α activity by binding to GR-α as a heterodimer (4, 21, 37). GR-β is upregulated in glucocorticoid-insensitive asthmatics (20, 29).

The major anti-inflammatory effects of CS are thought to be due to repression of inflammatory and immune genes. However, protein-protein interactions between activated GR and transcription factors, such as NF-κB and activator protein-1 (AP-1), may also play an important role (10, 32, 34). GR dimerization-deficient mutant mice in which GR is unable to dimerize, and therefore bind to DNA, survive to adulthood, in contrast to GR knockout animals (36). In these animals, dexamethasone (Dex) was able to inhibit AP-1- and NF-κB-driven gene transcription, but the ability to facilitate DNA binding-mediated effects such as cortisol suppression and thymocyte apoptosis was markedly attenuated.

AP-1 is one of the main transcription factor involved in the regulation of the procollagen-α1 promoter by inhibiting its activity (14, 26). As AP-1 is recruited by both Dex and TGF-β, it is possible that CS modulate AP-1 activity and/or interaction with target genes. In this study, we compared the effect of Dex on procollagen-α1 synthesis in bronchial fibroblasts derived from healthy (NBF) and asthmatic (ABF) subjects at baseline and after TGF-β stimulation. We also evaluated the role of AP-1 in regulating the response of these cells to Dex.

METHODS

Bronchial fibroblast cell culture. Primary fibroblasts were isolated from bronchial biopsies of asthmatic (n = 8) and healthy (n = 8) subjects. The asthmatic subjects fulfilling the American Thoracic Society criteria for asthma (1) were recruited from the Asthma Clinic at Hôpital Laval (Québec, Canada). They used only an inhaled β2-agonist on demand. None used inhaled or systemic CS. All asthmatic patients were atopic nonsmokers [mean age = 28 yr, forced expiratory volume in 1 s (FEV1) = 98.3 ± 5.0% predicted, concentration of β2-agonist that induces 20% decline in FEV1 (PC20) = 4.9 ± 5.5 mg/ml]. Healthy subjects (mean age = 29 yr, FEV1 = 89.7 ± 6.4% predicted, PC20 = 99.9 ± 48.1 mg/ml) were nonatopic nonsmoker with no history of asthma or other pulmonary or systemic diseases. Bronchial biopsies were obtained by bronchoscopy from asthmatic and healthy subjects as previously described (17). The study was approved by the Ethics Committee at the Institut Universitaire de

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Cardiologie et de Pneumologie de Québec, and written informed consent was obtained from all subjects before entry into the study. Isolated fibroblasts were characterized by immunofluorescence and flow cytometry using a mouse anti-vimentin and a mouse anti-human fibroblast antigen Ab-1 antibody (Calbiochem, San Diego, CA) that shows no cross-reactivity with epithelial cells, endothelial cells, smooth muscle cells, or other cell types. This identification confirmed the purity of bronchial fibroblast cell culture as described previously (17).

NBF or ABF were plated at 2 \( \times 10^5 \) cells/well in 12-well plates (Costar) in 10% FBS-supplemented DMEM (Invitrogen, Burlington, Ontario, Canada) and allowed to adhere overnight before stimulation. Fibroblasts were then washed with PBS and stimulated for 6–24 h with TGF-\( \beta \_1 \) (5 ng/ml; Calbiochem) or Dex (10\(-8\) to 10\(-6\) M; Sigma, St. Louis, MO) in 1% FBS-supplemented DMEM.

**Real-time PCR.** Total RNA was extracted from stimulated fibroblasts using the RNeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada) following the manufacturer’s protocol. Concentrations of total RNA were measured by a fluorescent dye with the RiboGreen Total RNA Reagent (Qiagen) for 3 h. Transfected cells were then washed with PBS and stimulated for 6 –24 h in 10% FBS-supplemented DMEM (Invitrogen, Burlington, Ontario, Canada). Procollagen-I/H9251 total cDNA using MMLV RT and First-Strand Buffer (Invitrogen). Amplification was carried out with the DNA Engine Opticon (MJ Research, Waltham, MA). Procollagen-I/H9252 fibroblasts were then washed with PBS and stimulated for 6–24 h with TGF-\( \beta \_1 \) (5 ng/ml; Calbiochem) or Dex (10\(-8\) to 10\(-6\) M; Sigma, St. Louis, MO) in 1% FBS-supplemented DMEM.

**IL-6 was amplified with**

**Research, Waltham, MA). Procollagen-I/H9253**

**GAPDH, 1 mM EDTA, and 1% Triton X-100). The lysates (300**

**ng of proteins in reducing sample buffer**

**were subjected to SDS-PAGE Western blots using anti-GR antibody.**

**EMSAs.** Probe labeling and EMSA gels were performed as we (7) previously described. Briefly, cells stimulated or not with Dex for 0–360 min were suspended in a low-salt buffer (20 mM HEPES, pH 7.9, 0.1 mM EDTA, and 0.5 mM PMSF) and left on ice for 15 min. Detergent (10% Nonidet P-40) was added to the pellet and mixed vigorously before centrifugation. Cell pellets were suspended in high-salt buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100). The lysates (300 \( \mu g \)) were immunoprecipitated using 2 \( \mu g \) of anti-API-1 antibody (Santa Cruz Biotechnology) at 4°C overnight. The immunoprecipitants were subjected to SDS-PAGE Western blots using anti-GR antibody.

Double-stranded DNA probes corresponding to AP-1 (Promega) were end-labeled with \([\gamma-32P]ATP\) (Mandel) and 5 units of T4 DNA kinase (Invitrogen) for 1 h. The labeled probes were extracted with phenol-chloroform and used immediately for protein-DNA binding reactions. Each binding reaction used 2.5 \( \mu g \) of nuclear proteins, 0.5 ng of labeled probe, 2 \( \mu g \) of poly(dIdC) (Amersham) 50 \( \mu g \) of BSA (Sigma), 10 mM HEPES, pH 7.9, 4% glycerol, 1% Ficol, 25 mM KCl, 1 mM DTT, 0.5 mM EDTA, and 25 mM NaCl. The mix was incubated at room temperature for 30 min. Competition experiments were performed in the presence of 100 M excess of unlabeled probe. Samples were resolved on 4% non-denaturing polyacrylamide gels and exposed to an X-ray film overnight for autoradiography.

**AP-1 overexpression.** Expression plasmids RSV-c-Jun and CMV-c-Fos were kind gifts from Dr. Luc Bélanger (Centre de Recherche de l’Hôtel-Dieu de Québec, Québec, Canada). The empty vehicle plasmid pCI-neo was from Promega. NBF were cotransfected with 1 \( \mu g \) of both c-Jun and c-Fos expression plasmids or 2 \( \mu g \) of vehicle plasmid as described above. Following transfections, cells were cultured for 48 h to ensure forming of new AP-1 complexes.

Statistical analysis. All experiments in this study were performed at least three times. We used SAS software package, and analysis was performed by a statistician. A posteriori Tukey-Kramer multiple comparisons test was performed. Means \( \pm \) SE of quantitative vari-
ables were used as representative measures. All reported \( P \) values were two-tailed and declared significant at 0.05 levels.

RESULTS

Effect on procollagen-\( \alpha_{1} \)I gene expression. Real-time PCR analysis on bronchial fibroblasts shows that Dex at different doses (10\(^{-8}\), 10\(^{-7}\), and 10\(^{-6}\) M) inhibited procollagen-\( \alpha_{1} \)I mRNA in NBF at all doses tested (0.56 \pm 0.17-fold induction of nonstimulated cells, \( P = 0.02 \) at 10\(^{-8}\) M Dex; 0.54 \pm 0.02-fold induction, \( P < 0.001 \) at 10\(^{-7}\) M Dex; and 0.72 \pm 0.08-fold induction, \( P < 0.001 \) at 10\(^{-6}\) M Dex) but not in ABF (0.78 \pm 0.17-fold induction of nonstimulated cells, \( P = 0.16 \) at 10\(^{-8}\) M Dex; 0.92 \pm 0.11-fold induction, \( P = 0.24 \) at 10\(^{-7}\) M Dex; and 0.86 \pm 0.10-fold induction, \( P = 0.14 \) at 10\(^{-6}\) M Dex). We selected 10\(^{-7}\) M Dex as an optimal dose for all further experiments. TGF-\( \beta_{1} \) induced procollagen-\( \alpha_{1} \)I mRNA expression in both NBF and ABF (1.40 \pm 0.18- and 1.65 \pm 0.15-fold induction, respectively; \( P = 0.32 \)). Dex at 10\(^{-7}\) M did not inhibit TGF-\( \beta_{1} \)-induced procollagen-\( \alpha_{1} \)I mRNA expression in both cell types (Fig. 1).

As a control, we evaluated the effect of Dex on IL-6 gene expression. We found that Dex was able to reduce IL-6 mRNA expression in both NBF and ABF (0.33 \pm 0.15- and 0.23 \pm 0.08-fold induction of IL-6 expression compared with unstimulated controls, \( P = 0.01 \) and 0.0002, respectively).

Effect on procollagen I protein production. We measured the NH\(_{2}\)-terminal propeptide of procollagen I in supernatants of bronchial fibroblasts stimulated or not with TGF-\( \beta_{1} \) and/or Dex for 24 h. There was no difference in baseline production of procollagen I between NBF (122.6 \pm 3.6 \mu g/l) and ABF (129.1 \pm 11.9 \mu g/l; \( P = 0.37 \)). We found that TGF-\( \beta_{1} \) increased procollagen protein levels in both control and asthmatic subjects (165.4 \pm 6.3 \mu g/l in NBF, \( P = 0.02 \); and 157.6 \pm 7.5 \mu g/l in ABF, \( P = 0.03 \)), whereas Dex downregulated procollagen in NBF (98.2 \pm 12.6 \mu g/l in Dex-stimulated fibroblasts; \( P = 0.05 \)) but not in ABF (130.3 \pm 5.6 \mu g/l in Dex-stimulated fibroblasts; \( P = 0.30 \); Fig. 2).

Effect on procollagen-\( \alpha_{1} \)I promoter activity. To evaluate whether Dex has a direct effect on procollagen-\( \alpha_{1} \)I promoter activity, we performed transient transfections of bronchial fibroblasts with the wild-type procollagen-\( \alpha_{1} \)I promoter \((-174/-42)\) positioned upstream of a luciferase reporter gene. Transfected fibroblasts were stimulated with either TGF-\( \beta_{1} \) or Dex. \( \beta \)-Galactosidase-transfected fibroblasts were used as a positive transfection control, and we have observed constant transfection efficiencies during the different experiments. Figure 3 shows that Dex significantly downregulated collagen promoter
activity in normal controls compared with asthmatics (62.9 ± 6.2%, P = 0.0005 vs. 102.0 ± 8.8% activity, respectively, P = 0.77). TGF-β upregulated promoter activity both in normal controls (161.3 ± 19.2%; P = 0.0015) and asthmatics (261.4 ± 25.1%; P = 0.0003). Dex was unable to reduce TGF-β-induced collagen promoter activity in both types of cells.

**TGF-β protein production.** As TGF-β is a strong inducer of collagen production, we tested the effect of Dex on TGF-β release in culture supernatants of ABF and NBF. There was no difference in baseline production of TGF-β by NBF and ABF subjects (1,660.1 ± 205.2 and 1,992.4 ± 514.1 pg/ml, respectively; P = 0.27). Treatment with Dex was able to reduce TGF-β protein level in the supernatants of NBF (82.4 ± 4.1%; P = 0.0002) but not in ABF subjects (98.4 ± 7.1%; P = 0.82). The difference between Dex-treated NBF and ABF was statistically significant (P = 0.05; Fig. 4).

**GR expression.** Bronchial fibroblasts expressed the GR as shown in Fig. 5A. Both NBF and ABF expressed multiple isoforms of the receptor including the α-isofrom at 95 kDa. ABF subjects significantly overexpressed the α-isofrom. Using a specific GR-α isoform antibody, we also detected a significant overexpression of this natural CS antagonist in asthmatic cells (Fig. 5B). Using semiquantitative analyses of both isoforms, we calculated the agonist-to-antagonist ratio of both NBF and ABF. GR-α-to-GR-β ratios were the same in NBF (1.00 ± 0.04 arbitrary units) and from ABF (1.03 ± 0.11 arbitrary units; P = 0.40). Stimulation with Dex downregulated GR expression at 24 h in both types of cells, whereas TGF-β had no effect on GR expression (data not shown).

**GR-AP-1 interaction.** To determine whether AP-1 bound the GR after Dex treatment, communoprecipitations using a specific AP-1 antibody were performed after 30 or 60 min of Dex treatment. AP-1 was found to be strongly bound with GR in asthmatic cells at baseline compared with normal cells (2.53 ± 0.19-fold induction; P = 0.01). GR-AP-1 interactions began to increase in cells from normal and asthmatic subjects starting from 30 min of Dex treatment. However, addition of Dex for 60 min showed a significantly higher AP-1-GR interaction in asthmatics compared with normal controls (3.59 ± 0.35- vs. 1.68 ± 0.40-fold induction, respectively; P = 0.02; Fig. 6A).

Strong interaction of AP-1 with GR in asthmatics may explain its inability to repress promoter activity. To evaluate...
whether Dex could modulate interactions between AP-1 and its DNA consensus target sequence, we performed EMSA experiments using nuclear extracts of Dex-stimulated fibroblasts. As shown in Fig. 6B, EMSA controls showed a specific band appearing when nuclear proteins and AP-1 labeled probes were added together (C2) compared with labeled probes only (C1). A competition experiment with unlabeled probe (C3) showed that the specific band is AP-1. Untreated ABF showed higher levels of AP-1 than cells from healthy controls. Treatment with Dex increased AP-1 interactions with DNA in normal cells but diminished specific interactions between AP-1 and its consensus DNA sequence in ABF subjects.

AP-1 overexpression in NBF. NBF were cotransfected with plasmids expressing AP-1 components, and effect of Dex on procollagen-\(\alpha_1\)I gene expression was evaluated. Overexpression of AP-1 in bronchial fibroblasts was validated by Western blots (Fig. 7A). Figure 7B shows that Dex significantly inhibited gene expression in cells transfected with an empty plasmid (0.41 ± 0.10-fold induction; \(P = 0.02\)). However, NBF that overexpress AP-1 did not respond to Dex treatment (1.12 ± 0.43-fold induction compared with unstimulated transfected cells; \(P = 0.43\)).

**DISCUSSION**

In this study, we found that procollagen-\(\alpha_1\)I gene is nonresponsive to glucocorticoid stimulation in ABF. The effect of glucocorticoid treatment on subepithelial fibrosis in asthma is yet unclear. A clinical study by Ward et al. (38) has shown that treatment with high doses of fluticasone for a year was able to reduce basal membrane thickness but failed to reduce collagen synthesis. Recently, several studies challenged the previous findings. Fluticasone uptake was not able to reduce basal membrane thickness after 8 wk of treatment (8). Glucocorticoids increased procollagen synthesis by intestinal and vascular smooth muscle cells (19, 28). Dex increased procollagen I synthesis by primary bronchial fibroblasts when stimulated with ET-1, PDGF, and TGF-\(\beta\) (15). Also, we (11) have recently shown that immunoreactivity of type I collagen was not reduced in the submucosa of moderate to severe asthmatics after a 2-wk course of oral CS. Goulet et al. (18) have shown that CS reduced total ECM and collagen deposition in primary human lung fibroblasts when cultured in noninflammatory conditions but increased ECM deposition and collagen expression when cultured in proinflammatory conditions.
TGF-β is a strong inducer of collagen expression and is increased in asthmatic airways (11, 33). We found that TGF-β induced procollagen mRNA expression, protein levels, and promoter activity from NBF and ABF but cannot be antagonized by Dex treatment. A GR response element is present in the TGF-β gene promoter and is thus susceptible to CS treatment (31, 35). We found that the expression of TGF-β was reduced by CS in normal cells but not in cells from asthmatics. However, TGF-β was only inhibited by 20% of the overall expression in controls and could not fully explain the nonresponse to Dex on the collagen promoter observed in asthmatic cells. Interestingly, Dex was unable to inhibit TGF-β-induced procollagen mRNA expression, protein levels, and collagen gene expression in controls and could not fully explain the nonresponse to Dex on the collagen promoter observed in asthmatic cells. When NBF were stimulated with Dex, AP-1 and GR binding increased. Thus, in ABF, overexpression of AP-1 and GR binding levels between AP-1 and DNA interferes with Dex signaling to the collagen promoter. It has also been shown that TGF-β can induce effective pleurodesis in rabbits and remain effective even in the presence of high doses of CS (27).

The nonresponse observed in ABF was observed on the collagen gene, but Dex was still able to inhibit IL-6 expression in these cells, demonstrating that ABF had the capacity to be affected by glucocorticoids. The mechanism for this nonresponse was not a defect in GR. We found an overexpression of the α- and β-isofoms of GR, suggesting that cells from asthmatics were primed to respond to CS treatment.

The effect of Dex on healthy cells was found to act directly on the collagen promoter. The promoter sequence for procollagen I does not contain any GR response element, suggesting that Dex modulates collagen synthesis through the action of other transcription factors. Adcock et al. reported an increase in the basal levels of AP-1 DNA binding in patients with resistance to CS (3) and a possible cross talk between proinflammatory mediators and glucocorticoids (2). Glucocorticoid hormones inhibit basal and induced transcription of collagenase by interfering with AP-1 through a mechanism not involving DNA binding (25). The collagen promoter contains at least one site for AP-1 and acts as an inhibitory transcription factor (26).

It is thus likely that Dex-mediated collagen regulation involves AP-1. We found that AP-1 is overexpressed in cells isolated from asthmatic subjects, suggesting a higher possibility of interactions with glucocorticoid-activated GR. Baseline collagen expression between NBF and ABF is the same, and it is thus possible that higher levels of AP-1 in asthmatic cells may contribute to counterbalance high TGF-β expression in asthmatic cells. When NBF were stimulated with Dex, AP-1 interacts with GR and binding levels between AP-1 and DNA increased, possibly inhibiting the collagen promoter and reducing gene and protein expression. However, Dex stimulation reduced AP-1-DNA binding in asthmatics while increasing AP-1-GR binding. Thus, in ABF, overexpression of AP-1 and its increased interaction with GR may explain the nonresponse to Dex. To validate this possibility, we overexpressed AP-1 in NBF. We found that these cells that overexpress AP-1 no longer responded to Dex-mediated collagen gene inhibition, thus mimicking the condition observed in ABF.

In conclusion, we showed that collagen production by ABF is not modulated by CS. The impaired response may be due to TGF-β downregulation and overexpression of AP-1. More mechanistic studies are necessary to fully understand the impact of CS on subepithelial fibrosis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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