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Asthma is characterized by an irreversible subepithelial fibrosis with the appearance of myofibroblasts, which can be now considered important early participants in inflammatory responses as well as potential targets for anti-inflammatory drugs. In this study, we show that fluticasone propionate (FP), a powerful inhaled corticosteroid (ICS), displays novel anti-inflammatory effects on human lung fibroblasts during their myofibroblastic differentiation. Indeed, FP inhibits in lung myofibroblasts, at a very early stage of differentiation, the activation of Janus kinase/STAT pathways induced by IL-13 (tyrosine kinase 2, STAT1, STAT3, STAT6, mitogen-activated protein kinase). Contrarily, in mildly or fully differentiated myofibroblastic cultures, FP still displays a potential anti-inflammatory activity even if it only inhibits tyrosine kinase 2 phosphorylation. Moreover, FP inhibits constitutive and TGF-β-induced expression of α-smooth muscle actin, the main marker of myofibroblastic differentiation, both in very early and in mild differentiated myofibroblasts. Finally, FP displays an additional powerful anti-inflammatory effect, decreasing nuclear translocation of NF-κB independent of the degree of myofibroblastic differentiation. These data 1) suggest that myofibroblasts are priority targets for ICS, which is able to revert them to a normal phenotype even if they appear to be already engaged in their differentiation, and 2) may help to explain why asthma is improved by an early ICS treatment, whereas advanced asthma is more resistant to these drugs.


Fibroblast is a generic term for a population of cells present in all tissues that have previously been considered to exert a passive and relatively unselective role in the architectural support for other specialized cell types. At present, the existence of a phenotypic and functional heterogeneity within fibroblast populations is accepted, which may exert significant influence on the behavior of their associated tissue cell types.

Fibroblast heterogeneity may also occur even among the same tissue, as a consequence of an activation/differentiation process. The differentiated cells, called myofibroblasts, transiently (normal tissue repair) or permanently (pathological stromal reactions) express contractile proteins, such as α-smooth muscle actin (α-SMA). This cytoskeletal element is the most important marker of myofibroblastic differentiation because it causes a shift from a migratory/proliferative phenotype to a contractile phenotype (1) that has important consequences, for example, in lung remodeling process. When cultured in vitro, lung fibroblasts should be considered as permanently activated cells. Indeed, even resting postconfluent fibroblasts maintain phenotypic characteristics that are specific in vivo to activated myofibroblasts (1). Therefore, it is likely that cultured normal fibroblasts represent the initial step(s) of myofibroblastic differentiation before the induction of α-SMA.

Recently published data suggest that lung fibroblasts constitute a mixture of cellular subsets expressing different functional markers and probably have capacity to exert different immune functions (2–4). We have recently shown that Th2 cytokines, IL-4 and IL-13, specifically induce a proinflammatory-like signal transduction in cultured human lung myofibroblasts, leading to the expression of adhesion molecules and to the production of factors pivotal in the development of the inflammatory cascade (5–7).

These data strongly suggest that lung fibroblasts, representing ~30% of the resident lung cells, when activated play a major role in the remodeling of the lung microenvironment, acting not only as target cells, but also as effector proinflammatory cells (8). To date, airway fibroblasts were not considered as potential targets in asthma therapy, even if extensive studies have been developed to identify the direct inhibitory actions that corticosteroids display on inflammatory and structural cells involved in pulmonary and airway diseases (9).

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FP, fluticasone propionate; GAM, goat anti-mouse; GR, glucocorticosteroid receptor; ICS, inhaled corticosteroid; JAK, Janus kinase; TYK2, tyrosine kinase 2.
Fluticasone propionate (FP) is a highly lipophilic inhaled corticosteroid (ICS) that possesses the highest receptor affinity among corticosteroids, implying fast association and slow dissociation from the receptor protein. This explains the higher FP potency and its faster clinical response onset when compared with other corticosteroids. The receptor affinity of FP and the rank orders of affinities of other corticosteroids are well correlated with the induction of anti-inflammatory responses at the cellular level as well as with the clinical efficacy of these compounds. Thus, because glucocorticosteroid responses are primarily receptor mediated, the magnitude of their clinical effects and efficacy can be explained by the physicochemical properties of the corticosteroids that determine the glucocorticosteroid receptor (GR) interactions.

It was already demonstrated, in asthma and rhinitis studies, that regular topical FP therapy has a profound effect on an inflammatory cascade underlying the clinical disease expression. Glucocorticosteroids, such as FP, act intracellularly by binding to GR to form GR-glucocorticosteroid complex, which then modulates gene expression. The GR may regulate gene expression by complexing promoter regions of certain genes. The GR may regulate gene expression through a preformed GR-glucocorticosteroid complex, which then modulates gene expression (via the GR-glucocorticosteroid complex, which then modifies expression of certain genes. The GR may regulate gene expression directly by binding to the DNA or indirectly by interacting with other transcription factors. This may occur via various mechanisms, of which the interaction with the transcription factors, AP-1 and NFκB, has been best characterized.

In this study, we have analyzed the in vitro effects of FP, an ICS commonly used in asthma therapy, on behavior of human lung fibroblasts at different stages of myofibroblastic differentiation.

Materials and Methods

Cell culture

Twenty primary cultures from human airways of normal donors (embryonic fetal and adult) or from patients with Th2 lesions were established in primary culture by enzymatic digestion of the tissues. Phenotypic analysis of the fibroblastic cultures identified three types of cells: 1) cultures expressing less than 5% of α-SMA-positive cells (essentially from normal donors), 2) cultures expressing between 25 and 40% of α-SMA-positive cells (from pathological samples), and 3) fully differentiated cultures expressing 100% α-SMA-positive cells (from pathological samples). All these myofibroblastic primary cultures expressed the fibroblastic marker AS03 (5, 6).

For this study, we have chosen four fibroblastic lung primary cultures that represent the aforementioned different groups of myofibroblastic differentiation.

The origin and characteristics of cultured fetal lung fibroblasts (ICIG7) representing the very early step of myofibroblastic differentiation (5% α-SMA-positive cells) as well as of adult primary lung fully differentiated myofibroblasts (FPA, 100% α-SMA-positive cells), derived from a stromal Th2 reaction, have been previously described (11). Primary cultures BRONCO5 and BRONCO3 were obtained from lung tissue neighboring a neoplastic lesion and were adapted to in vitro culture after enzymatic digestion. These cells can be classified as mildly differentiated myofibroblasts (30% of α-SMA-positive cells). Cells were cultured in DMEM medium (Eurobio, Les Ulis, France) supplemented with 10% FCS (ATGC Biotechnologie, Noisy-le-Grand, France), 2 mM glutamine, and 1% antibiotic (Life Technologies, Cergy Pontoise, France) supplemented with 10% FCS (ATGC Biotechnologie, Noisy-le-Grand, France), 2 mM glutamine, and 1% antibiotic (Life Technologies, Cergy Pontoise, France), and incubated at 37°C and 5% CO2 conditions.

Cytokines and Abs

Human rIL-4 and rIL-13 were a generous gift from Schering-Plough (Darwin, France) and Sanofi Recherche (Labège Innopole, France), respectively. The anti-phospho-STAT1α (P-STAT1) and anti-phospho-STAT6 (P-STAT6) Abs were obtained from New England Biolabs (Beverly, MA); the anti-STAT3 Ab, the anti-p65 NFκB subunit, and the streptavidin FITC were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-STAT1, anti-phospho-tyrosine kinase 2 (Tyr2) (P-TYK2), anti-TYK2, and antiaactive mitogen-activated protein kinase (MAPK) were obtained from Transduction Laboratories (Lexington, KY) and Promega (Madison, WI). Antiphosphoserine I-Asp-specific polyclonal IgG were obtained from Calbiochem (San Diego, CA). The peroxidase-conjugated anti-rabbit IgG and the mAb Abs were obtained from Upstate Biotechnology (Lake Placid, NY). Biotinylated goat anti-rabbit IgG and peroxidase-conjugated goat anti-mouse (GAM) IgG were obtained from Immunotech (Marseille, France).

Immunoblotting and immunoprecipitation

Immunoblotting and immunoprecipitation were performed as described previously (12). Briefly, confluent cell cultures were serum starved overnight before each experiment to reduce the basal phosphorylation. Cells were washed and stimulated by various concentrations of IL-4 or IL-13 at 37°C for 15 min. Sister cultures of each condition were pretreated with 5 × 10−6 M FP at 37°C for 1 h, to investigate the FP effects on the signal transduction induced by the Th2 cytokines. Cells were then resuspended in lysis buffer containing 0.5% or 1% Nonidet P-40. Proteins were incubated with different Abs and immune complexes, then captured with protein G-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) overnight at 4°C. Complexes were washed, boiled, solubilized with Laemmli buffer, and separated on 7.5% or 12% SDS-polyacrylamide gels before transfer onto polyvinylidene difluoride membranes (DuPont/NECN, Boston, MA). Membranes were probed with Abs, revealed with peroxidase-conjugated Abs, and then visualized by ECL (Amersham, Les Ulis, France).

Detection of p65 NFκB nuclear translocation and analysis of α-SMA expression by confocal microscopy

For the detection of p65 NFκB nuclear translocation, normal fibroblasts, and myofibroblasts that had been cultured for 2 days were preincubated in serum-free medium either containing or lacking 10−6 M FP for 1 h at 37°C, and then stimulated for a further 15 min with or without TNF-α (10 ng/ml).

To determine α-SMA expression, 1-day cultured normal fibroblasts and myofibroblasts were incubated in the absence or presence of 10−6 M FP and/or TGF-β (5 ng/ml) for 72 h at 37°C.

Subsequently, cells from both experiments were washed and permeabilized with Ortho PermeaFix (Ortho Diagnostic Systems, Raritan, NJ) for 45 min, and indirect immunofluorescence was performed with rabbit Abs recognizing the p65 subunit of the NFκB complex or with mAb 1A4 that recognizes human α-SMA (DAKO, Glostrup, Denmark). Samples were then incubated with Alexa Fluor488 GARa (p65) or with Alexa Fluor594 GAM (α-SMA; Molecular Probes, Leiden, The Netherlands). To determine the nuclear localization of the p65 subunit of the NFκB complex, nuclei were washed by incubating the cells for 3 min at room temperature in the presence of propidium iodide (red nuclear staining). After staining, cells were washed and centrifuged in a Cytospin 3 (Shandon, Pittsburgh, PA) and analyzed by laser scanning confocal microscopy on a Leica TCS NT/SP Interactive Laser Cytometer equipped with confocal optics (Leica Microsystems, Wetzlar or RFA).

RT-PCR analysis of α-SMA expression

Oligonucleotide primers. Primers used in PCR and the annealing conditions for α-SMA were upper, 5′-GTC CAC CGC AAA TGA TCC TAA-3′, and lower, 5′-AAA CAT TAA CGA GTC AGT CAA-3′, amplification product 141 bp (58°C, 30 cycles); and for GAPDH, upper, 5′-GGT GAA CGG CAG CAT AGT CAA-3′, and lower, 5′-GAG GGA TCT CCC TCC TGG AAG A-3′, amplification product 240 bp (60°C, 20 cycles).

mRNA isolation and RT-PCR analysis. Total RNA was isolated from human lung fibroblasts by using TRizol reagent (Life Technologies), according to the manufacturer’s specifications. cDNA was synthesized from total RNA using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Life Technologies), according to the manufacturer’s instructions. In each PCR, 1 μCi/tube [α-32P]dCTP was included to label the PCR product. Aliquots of the RT-PCR were separated by PAGE. The gel was dried, and the results were visualized by autoradiography, as described (7).

For semiquantitative RT-PCR, amplified products of α-SMA were quantified by densitometric scanning of specific bands on negatives of GPDH transcripts in a PhosphorImager (Molecular Dynamics, Bondoufle, France), Water was used as a negative control, and some samples were amplified without reverse transcription to check for the presence of DNA contaminants.

Nuclear protein extraction and EMSA

ICIG7 cells treated with TNF-α and fluticasone, as described in Confocal microscopy, were extracted following a previously described protocol (13) Analysis of NF-κB DNA-binding activities in these extracts was performed by EMSA, as reported previously (14). The quantification of retarded oligonucleotides in the band corresponding to NF-κB was monitored by PhosphorImager quantification using a Storm apparatus. The supershift EMSA was performed by incubating the protein extracts with 1 μl of RelA-, RelB-, c-Rel-, and p50-specific antisera from Santa Cruz Biotechnology for 1 h prior to the addition of the radiolabeled oligonucleotide.
Results

Effects of FP on Janus kinase (JAK)-STAT signaling induced by IL-4 and IL-13 lung fibroblasts and myofibroblasts

IL-4 and IL-13 specifically act on cultured human lung myofibroblasts, activating the JAK-STAT signaling with an inflammatory-like pattern (5). We analyzed the ability of FP to interfere with these signal transduction pathways.

We investigated the effects of FP treatment on low passaged (p13) and older (p24) human lung fibroblasts at a very early stage of myofibroblastic differentiation (ICIG7) signal transduction, activated by IL-4 and IL-13 (Fig. 1). Upper panels are Western blots illustrating the phosphorylated proteins, while lower panels show the expression of the native proteins. Overall, our data show that Th2 cytokines induce the phosphorylation of TYK2, STAT-1, STAT-3, STAT-6, and MAPK, and that FP preferentially inhibits the action of IL-13 in young, but not in older cultures.

FIGURE 1. Tyrosine phosphorylation of TYK2, STAT1, STAT3, STAT6, ERK1, and ERK2 in response to IL-4, IL-13, and FP in normal lung fibroblasts. ICIG7 myofibroblasts, representing cells at an early step of myofibroblastic differentiation, were analyzed at different population-doubling levels (left panels, PDL13; right panels, PDL24). The cultures were preincubated (lanes 4–6) or not (lanes 1–3) with 10−8 M FP for 1 h at 37°C. Subsequently, the cultures were stimulated (lanes 2, 3, 5, and 6) or not (lanes 1 and 4) with 100 ng/ml IL-4 (lanes 2 and 5) and IL-13 (lanes 3 and 6) for 15 min at 37°C. Cell extracts were analyzed by Western blotting (upper blots) using Ab recognizing specifically the phosphorylated forms of TYK2 (A), STAT1 (B), STAT3 (C), STAT6 (D), ERK1, and ERK2 (E). Membranes were then reprobed with Ab recognizing the native proteins (lower blots). These pictures are representative of three independent experiments.
We also analyzed the activation of MAPK (extracellular signal-related kinase (ERK) 1 and ERK2) that have been described as possible targets for IL-4 and IL-13 (15, 16). In ICIG7 cells, ERK1 and ERK2 are constitutively phosphorylated, and both tested Th2 cytokines up-regulated their level of phosphorylation. FP decreased both constitutive and cytokine-induced phosphorylation of ERK1 and ERK2 (Fig. 1E).

When the same signaling pathways were studied in older ICIG7 cultures (right panels), we found the same pattern of basal or IL-4/IL-13-induced activation of JAK/STAT or MAPK. The exception was a less efficient phosphorylation of TYK2 and the lack of expression of STAT3β. The major difference was the loss of the inhibitory effect displayed by FP, which just inhibited the constitutive phosphorylation of TYK2 and the Th2-induced phosphorylation of MAPK.

Analysis of lower panels (Fig. 1, A–E) shows that in each sample the native proteins were homogeneously expressed. In some instances, reblotting the membrane with anti-tubulin mAbs confirmed the homogenous loading of the lysates in each group (data not shown).

In Fig. 2, we analyzed the above signal transduction pathways in the mildly differentiated human lung fibroblasts BRONCO5 (right panels) and in fully differentiated FPA myofibroblasts (left panels) at the same passage level (p13) tested in ICIG7 cultures. Upper panels are Western blots illustrating the phosphorylated proteins, while the lower panels concern the expression of the native proteins. These cells behaved similarly to older ICIG7 cells: Th2 cytokines activate most of the different pathways, but FP is less efficient, decreasing only TYK2 phosphorylation.

Indeed, we detected the constitutive phosphorylation of TYK2 (Fig. 2A) and of MAPK (Fig. 2E) as well as the stimulatory effects of IL-4 and IL-13. The only difference was that the two Th2 cytokines did not modify the basal level of phosphorylation of STAT3 in BRONCO5 and FPA cultures (Fig. 2D). Moreover, the
two myofibroblastic cell strains did not express STAT3. Concerning STAT1 (Fig. 2B) and STAT6 (Fig. 2C), we also confirmed that in BRONCO5 and FPA myofibroblasts, IL-4 and IL-13 induce the phosphorylation of these STAT proteins. A major difference in these myofibroblastic cultures was that FP did not interfere with these signal transduction pathways, with the single exception of TYK2. Indeed, FP partially inhibited the IL-13-induced phosphorylation of TYK2.

Because glucocorticosteroids inhibited the activation of the transcription factor NF-kB (10), in Fig. 2F we analyzed the extent of phosphorylation of the NF-kB inhibitor I-kB, to understand whether FP was active on this step of the NF-kB activation pathway.

In these experiments, we used an anti-I-kB mAb that recognizes both the phosphorylated protein (upper band) and the native one (lower band). I-kB was already phosphorylated in basal culture conditions, and treatment with Th2 cytokines and/or FP did not modify the intensity of phosphorylation of I-kB (upper band) nor induced the degradation of the native protein (lower band).

**FIGURE 3.** Cytoplasmic expression and nuclear translocation of NFκB p65 subunit in response to TNF-α and FP in lung myofibroblasts at different stages of differentiation. ICIG7 (A), BRONCO5 (B), and FPA lung myofibroblasts (C) were analyzed in basal culture conditions (panel 1), after stimulation for 15 min with 10 ng/ml TNF-α (panel 2) or with TNF-α and 10^−8 M FP (panel 3). The cells were permeabilized, labeled with anti-NFκB p65 subunit Abs, followed by incubation with Alexa Fluor 488 GARa and propidium iodide. These cells were analyzed with a laser confocal microscopy Leica (same rescale for all pictures). Propidium iodide stains nuclei in red, NFκB p65 subunit was identified by the green staining, and NFκB nuclear translocation showed nuclear yellow staining. As negative controls, cells were stained with propidium iodide and Alexa Fluor 488 GARa (D). In each panel, we analyzed a similar number of cells. These pictures are representative of three independent experiments.

**Effects of FP on NFκB activation in human lung fibroblasts and myofibroblasts**

**Confocal microscopy.** Subsequently, we tried to confirm the constitutive activation of the transcription factor NFκB in early (ICIG7), mild (BRONCO5), and fully differentiated (FPA) human lung myofibroblasts. By confocal microscopy, we analyzed the localization of p65 subunit (RelA) of the NFκB complex (green staining) inside the nuclei (red staining).

Fig. 3 represents different overlay pictures; cells in each sample displayed a cytoplasmic green staining that demonstrated the presence of the native NFκB p65 subunit. In contrast, the nuclear yellow staining identifies the cells expressing RelA at nuclear level. The three different lung myofibroblastic cultures (ICIG7, BRONCO5, and FPA) were analyzed 48 h after seeding during the proliferative phase. The percentage of cells presenting a nuclear localization for NFκB p65 subunit was ~10% of ICIG7 cultures (Fig. 3A1), but it increased up to 30–40% in BRONCO5 and FPA myofibroblastic cultures (Fig. 3, B1 and C1).

Treatment with TNF-α strongly increased the percentage of
cells expressing an activated NFκB in the three myofibroblastic lung cultures (Fig. 3, A2, B2, and C2), and FP efficiently counteracted this induction, blocking almost totally NFκB activation in all samples (Fig. 3, A3, B3, and C3). Fig. 3D represents the control samples treated with a nonrelated isotype-matched mAb and propidium iodide. In these cultures, nuclei were stained in red, whereas no background green staining was detected.

**Gel shift assay.** To confirm the inhibitory effect of fluticasone on basal and TNF-α-induced NF-κB, we performed gel retardation analysis of nuclear extracts from myofibroblastic cultures representing an early (Fig. 4A) and an intermediate stage (Fig. 4B) of differentiated cells, using as a probe an oligonucleotide encoding the κB sequence. As a control, we used an extract from a T cell line that had been activated for 3 h with PMA plus ionomycin. The results show that both cell types contained a constitutive κB-binding activity, which comigrated with the T cell NF-κB dimer that has been previously identified as RelA-p50 (17). TNF-α treatment of cells increased the binding of RelA-p50, whereas pretreatment with FP decreased it both in control and TNF-α-treated cells. The efficiency of the inhibition was quantified by Phosphor-Imager, and demonstrated that addition of FP decreased the basal NF-κB DNA binding by 15% in both cell strains and the TNF-α-induced NF-κB DNA binding by ~30% and 70% in ICIG7 and BRONCO cultures, respectively. These findings confirmed that FP efficiently inhibits TNF-α-induced activation of the prototypical RelA-p50 NF-κB dimer both in early and mildly differentiated lung myofibroblastic cells with a more powerful effect on the latter cells. The fact that the basal NF-κB activity was less reduced by FP might be due to a distinct mechanism by which NF-κB activity is generated in the absence of TNF-α. Gel retardation assays could not be performed on fully differentiated myofibroblasts owing to the too limited proliferative potential of these terminally differentiated cells.

The identity of the dimer was further confirmed by supershift experiments using Abs specific for several Rel/NF-κB proteins (Fig. 4C). Indeed, a supershift of the dimer was observed using an Ab directed against RelA, and the use of an Ab against the p50 subunit inhibited the binding.

**Effects of FP on α-SMA expression in human lung fibroblasts and myofibroblasts**

**Confocal microscopy.** In these experiments, we explored the capacity of FP to interfere with the myofibroblastic differentiation process, analyzing the effects of the drug on the constitutive or TGF-β-induced expression of α-SMA (cytoplasmic red staining). Indeed, recent data suggest that expression of α-SMA decreases in glomeruli from patients with IgA nephropathy treated with corticosteroids (18).

Constitutive expression of α-SMA was low (5% positive cells) in ICIG7 human lung myofibroblasts at an early stage of differentiation (Fig. 5A1), intermediate (up to 25–30% positive cells) in BRONCO5 mildly differentiated lung fibroblasts (Fig. 5B1), and complete (100% positive cells) in FPA fully differentiated lung myofibroblastic cultures (Fig. 5C1).

FP totally inhibited α-SMA expression in ICIG7 cells (Fig. 5A2), whereas the inhibition was only partial in BRONCO cells (Fig. 5B2) and absent in FPA cells (Fig. 5C2).

Treatment with TGF-β strongly increased the percentage of α-SMA-positive cells, both in ICIG7 (Fig. 5A3) and BRONCO5 cultures (Fig. 5B3), but not in FPA cultures (Fig. 5C3). Finally, FP completely inhibited α-SMA expression in both early (Fig. 5A4) and mildly differentiated myofibroblastic cultures (Fig. 5B4), treated with TGF-β, but was ineffective on totally differentiated lung myofibroblasts (Fig. 5C4).

**RT-PCR analysis.** To confirm the results from confocal microscopy on α-SMA modulation by FP and TGF-β, RT-PCR analysis was conducted using sets of primers described in Materials and Methods. Transfer to an adhesion surface displaying an increased mechanical tension may induce a rapid and stable myofibroblastic differentiation of normal fibroblasts (19). We adapted this technique to ICIG7 cells generating, within 4 days, fully differentiated ICIG7 myofibroblasts. Thus, in RT-PCR assays, we used ICIG7 cultures before and after their differentiation and in the mildly differentiated lung myofibroblastic cultures BRONCO5 and BRONCO3.

As shown in Fig. 6, the specific transcript (141 bp) for α-SMA, barely detectable in control ICIG7 cultures (Fig. 6A), was constitutively expressed in fully differentiated ICIG7 myofibroblasts (Fig. 6B) and in the mildly differentiated BRONCO5 (Fig. 6C) or BRONCO3 (Fig. 6D) cultures. TGF-β increased α-SMA expression in all the samples. Treatment with FP efficiently inhibited the constitutive α-SMA expression in all the cultures, with the exception of BRONCO3 cells, whereas TGF-β-induced expression of α-SMA was almost totally inhibited in all the samples. The transcript (240 bp) for the housekeeping gene GAPDH (used as internal standard) was expressed with the same intensity in all the samples.

**Discussion**

In this in vitro study, we show two new anti-inflammatory effects of an ICS on human lung resident cells. Indeed, FP inhibits, in human lung myofibroblasts, IL-13-induced JAK-STAT signaling, and basal or TGF-β-induced expression of the marker of myofibroblastic differentiation α-SMA. Moreover, FP efficiently inhibits basal and TNF-α-induced nuclear translocation of the p65 subunit of the transcription factor NFκB independently on the level of myofibroblastic differentiation. The latter result could be very important, because NFκB plays a major role, triggering several genes involved in the activation of the inflammatory process (10).
The inhibitory action of FP on the JAK/STAT pathway induced by IL-4 and IL-13 in lung fibroblasts is a new acquisition, even if recent studies report the existence of a cross-talk between the JAK-STAT and the glucocorticoid-nuclear receptor pathways. However, it must be stated that these studies report a synergistic effect among GR, STAT3, and STAT5, respectively (20, 21). Another recent study reports that overnight treatment with dexamethasone inhibits IL-2 activation of STAT5 in primary T cells. This inhibition is associated with a decreased expression of the IL-2Rα chain that also affects IL-4, IL-7, and IL-15 signaling (22).

On the contrary, our results show that a short exposure to FP inhibits the phosphorylation of STAT1, STAT3, STAT3β, and STAT6 induced by Th2 cytokines, with no effects on the IL-4/IL-13R expression. Moreover, lung fibroblasts at a very early stage of differentiation do not express the γ-chain, and IL-4/IL-13 signaling is mediated by a type II receptor (IL-4Rα/IL-13Rα1), whereas α-SMA-positive lung myofibroblasts constitutively express the γ chain (5–7), but once more its expression is not decreased by FP.

The inhibition seems to be more specific on the action of IL-13, and is also extended to the phosphorylation of TYK2 (the fourth member of the JAK family) and of the MAPK members, ERK1 and ERK2. These inhibitory effects seem to be more effective when myofibroblasts are treated early both in their life span and differentiation pathway; however, the loss of FP action on the JAK-STAT signaling in older lung myofibroblasts is not due to the acquisition of a generalized resistance to corticosteroids, because in these cells this drug is still able to inhibit NFκB. The therapeutic potential of corticosteroids may be very important, acting at different levels and cutting at a very early stage a cascade of inflammatory signals. In contrast, the residual effect observed in older early and in mild differentiated myofibroblasts (BRONCO5) on the phosphorylation of TYK2 may still have discrete anti-inflammatory effects (23, 24).

Th2 cytokines activate in vivo and in vitro lung fibroblasts, inducing matrix deposition, production of proinflammatory mediators, and expression of α-SMA (4–8, 25). Similar results on matrix deposition have also been reported in skin fibroblasts (26), even if the signal transduction activated by IL-4 and IL-13 in these cells (27) is much more restricted than that activated in lung fibroblasts (5). Thus, it is likely that activation of JAK2/TYK2/STAT6 pathways may be sufficient to induce matrix deposition, and the broader spectrum of signaling activated by Th2 cytokines in lung fibroblasts could account for a larger panel of activation of inflammatory mediators.

**FIGURE 5.** Detection of α-SMA in response to TGF-β and FP in lung myofibroblasts at different stages of differentiation. ICIG7 normal lung fibroblasts (A), BRONCO5 (B), and FPA lung myofibroblasts (C) were analyzed in basal culture conditions (panel 1), after stimulation for 72 h with 10^-8 M FP (panel 2), with 5 ng/ml TGF-β (panel 3), and with TGF-β and FP (panel 4). The cells were permeabilized, labeled with anti-α-SMA Abs, followed by incubation with Alexa Fluor GAM 594 GARa. These cells were analyzed with a laser confocal microscopy (same rescale for all pictures). As negative controls, cells were stained with Alexa Fluor 594 GAM (D). In each panel, we analyzed a similar number of cells. These pictures are representative of three independent experiments.
For instance, the different STAT members and MAPK control the activation of several genes involved in apoptosis, cell proliferation, and differentiation, and therefore in the inflammatory process. This happens by direct binding to specific sequences present in the promoter region of this gene or by formation of functional complex with other transcription factors, amplifying their competence for gene activation. The fact that FP inhibits the first step of their activation (phosphorylation) process potentially guarantees a very large spectrum on gene suppression. Our data also suggest that interplay between Th2 cytokines and FP on STATs activation may be much more complex. Indeed, ICI7G young cultures constitutively express STAT3β, a splice variant of STAT3 that behaves as a dominant-negative regulator of transcription. This is a very intriguing finding, because this competitor of STAT3 could theoretically interfere on the synergism between STAT3 and GR (28). Moreover, its phosphorylation is up-regulated by IL-13 and by the combined treatment with IL-4 and FP, whereas the corticosteroid totally inhibits STAT3β phosphorylation induced by IL-13. This different behavior is most likely due to the fact that IL-4 and IL-13, although sharing common receptors, may induce different signaling in the same type of cells (29). The fact that the two Th2 cytokines display an opposite control on the phosphorylation of STAT3β suggests potential differences in the pattern of gene activation controlled by STAT3 in the presence of FP.

The transcription factor NFκB is in the promoter region of several genes such as adhesion molecules, inflammatory cytokines, chemokine inflammatory enzymes, and receptors (30); thus, it exerts a dominant role in the induction and stabilization of the inflammatory process.

Therefore, inhibition of its activation by FP both in normal and irreversibly differentiated lung myofibroblasts could constitute one of the main anti-inflammatory effects in advanced asthma, considering that it has been recently reported that in patients with mild asthma, FP does not inhibit NFκB-DNA binding in bronchoalveolar lavage macrophages, and in airway epithelium of bronchial biopsies (26). Our results also confirm the preferential targeting of NFκB by FP and the lack of IκBα involvement (10). The inhibition of α-SMA expression by FP in early and mild differentiated lung myofibroblasts is also a quite original observation that may have very important consequences in the early intervention of asthma treatment. Indeed, the expression of this contractile protein causes on fibroblasts a shift from the migratory to the contractile phenotype, with important consequences on the lung function (1). A recent study showed that in patients with IgA nephropathy, in vivo corticosteroid treatment reduces the number of α-SMA-positive cells (18). However, the authors of this study (18) cannot determine whether this is a direct inhibitory effect of the corticosteroid, and recognize that such a direct effect has never been reported. Thus, our data represent the first direct evidence of the capacity for a corticosteroid to inhibit α-SMA constitutive and TGF-β-induced expression. Interestingly, the α-SMA expression is totally suppressed when FP is associated with TGF-β, even if this cytokine is the most powerful inducer of this contractile protein (31).

This approach allows a better definition of the early stages of myofibroblastic differentiation. Indeed, we distinguish, within BRONCO5 cultures, at least three different myofibroblastic subsets. The first one does not express α-SMA, but can be induced by TGF-β and is sensitive to FP. The second one constitutively expresses α-SMA and is sensitive to FP, while the third expresses α-SMA and is sensitive to FP only in the presence of TGF-β. It is possible that in mild differentiated myofibroblastic cultures, TGF-β also reactivates cells constitutively expressing this marker, which become sensitive to the inhibitory effects of FP. Interestingly, ICI7G cultures, undergoing a rapid and stable myofibroblastic differentiation by changing the adhesion substratum, behave as the third subset identified in BRONCO5 cells. This result strengthens the existence of a step in the myofibroblastic differentiation process characterized by a constitutive expression of α-SMA and by the sensitivity to inducing and inhibitory agents.

This mechanism could be relevant in vivo. Indeed, an early event during the induction of allergic airway inflammation is the production of the chemokine monocyte chemoattractant protein-1 that polarizes the Th2 cells, but also acts on lung fibroblasts, inducing collagen deposition and secretion of TGF-β (4). Thus, it is possible that an early treatment with FP may synergize with the TGF-β produced by fibroblasts, inhibiting the induction of α-SMA and the myofibroblastic differentiation.

Our in vitro data are in agreement with clinical studies that show that asthma could be improved by an early intervention with ICS, preventing the development of irreversible airway obstruction that occurs over time if the asthma is undertreated (32–35). This suggests that part of the therapeutic potential exhibited by ICS in vivo could be due to a direct effect on lung structural cells such as myofibroblasts.

Moreover, the fact that FP inhibition of α-SMA expression also occurs in cells already expressing this contractile protein suggests the possibility of an in vivo reversibility of the fibrosis by an early corticosteroid treatment, as suggested in recent in vivo studies on a different type of inflammatory/fibrotic disease (18).

In conclusion, we propose that FP may exert at least in part of its anti-inflammatory activity through a direct action on lung myofibroblasts. FP, in concentrations that probably occur in airway lining fluid during inhalation therapy (36), rapidly inhibits JAK-STAT signal transduction in lung myofibroblasts induced by IL-4 and IL-13. FP is efficacious not only on mild differentiated lung myofibroblasts by decreasing the α-SMA expression, but also acts on terminally committed myofibroblasts, decreasing the nuclear translocation of NFκB.
References


