

Dexamethasone and Salbutamol Stimulate Human Lung Fibroblast Proliferation

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Background: Asthma is characterized by bronchial hyperreactivity and airway remodeling. Subepithelial fibrosis, a feature of remodeling, is accompanied by activation of fibroblasts to myofibroblasts, with excessive proliferation and increased collagen, extracellular matrix protein, and profibrogenic cytokine production. Mast cells are important in the development of asthma and its fibrotic changes.

Objective: In this study, we aimed to investigate the direct effect of the drugs most frequently used in asthma, that is, glucocorticosteroids (dexamethasone) and shortacting β_2 -agonists (salbutamol), on human lung fibroblast proliferation when unstimulated or activated by mast cells or eotaxin.

Methods: Subconfluent human fetal lung or bronchial fibroblasts were incubated with different concentrations of the drugs (24 h) 6 activators, and [³H]-Thymidine was added (24 h) to measure their proliferation. IL-6 production in the supernatants of confluent monolayers cultured in the presence of the drugs or forskolin (24 h) was analyzed by enzyme-linked immunosorbent assay.

Results: Both drugs alone and in the presence of the activators enhanced fibroblast proliferation in a seemingly synergistic way for both fetal and bronchial fibroblasts. Dexamethasone was found to decrease IL-6 production, while salbutamol increased it.

Conclusions: These observations if corroborated by in vivo data may possibly account for the deleterious effect of long-term therapy with β_2 -bronchodilators and inhaled glucocorticosteroids on the natural history of asthma.

Key Words: fibroblasts, lung, proliferation, dexamethasone, salbutamol, IL-6

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Asthma is a chronic inflammatory disease characterized by bronchial hyperreactivity and airway remodeling.

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Remodeling features are damage and shedding of airway epithelium, increased number of goblet cells, mucous gland hypertrophy, increased fibroblast/myofibroblast numbers, and increased airway smooth muscle mass and neovascularity.^{1–3} Fibroblasts are thought to play a major role in the development of the structural changes in the airways by altering their phenotype from a quiescent cell into α -actin containing myofibroblasts that display enhanced proliferation, increased collagen and other extracellular matrix protein production, expression of adhesion molecules, and production of profibrotic and proinflammatory cytokines/chemokines and growth factors.^{1–4}

The early asthmatic response is typically initiated by activation of tissue-resident mast cells. The subsequent infiltration and activation of several additional inflammatory cells and notably that of the eosinophils account for the late phase of an asthma attack. Persistence of airway inflammation may explain the chronic disease. We and others have shown that mast cells and eosinophils are pivotal cells not only in initiating and maintaining airway inflammation in asthma but also in activation of fibroblasts to become myofibroblasts.^{5,6} The profibrotic effect of mast cells is due to several mediators, including histamine, tryptase, IL-6, nerve growth factor, eotaxin, TGF- β , TNF- α , and others.^{7–10}

Treatment of asthma usually includes short-acting β_2 -agonists for the relief of bronchoconstriction and, if needed, inhaled glucocorticosteroids to treat airway inflammation. Glucocorticosteroids have generally been considered to have antifibrotic activity based on data from in vitro studies carried out on fibroblasts from different anatomical origins. However, results obtained with lung fibroblasts have been inconclusive or contradictory because some have found that these drugs enhance fibroblast proliferation^{11–14} and some have shown that they inhibit proliferation and production of profibrotic cytokines.^{12,15} Moreover, although very effective in reducing airway inflammation, the efficacy of glucocorticosteroids in attenuating airway fibrosis is still controversial.^{16–18} Few studies have investigated the effect of short-acting β_2 -agonists on fibroblast proliferation and also with contrasting results.^{19,20} In this study, we were interested to examine the effect of salbutamol as a paradigm of short-acting β_2 -agonists and dexamethasone and as a paradigm of glucocorticosteroids and their combination on human fetal lung and adult bronchial fibroblast proliferation by themselves and in the presence of mast cells or eotaxin as stimulators.

MATERIALS AND METHODS

Fibroblasts

The human fetal lung fibroblast line MRC-5 (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum (FCS) (DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin—all from Biological Industries, Beit Haemek, Israel) at 37°C, 5% CO₂, and used for assays between the second and seventh passages. Human bronchial fibroblasts were obtained from endobronchial biopsy specimens of normal mucosa from 3 subjects (age, 54–64 yr; 1 female and 2 male subjects not receiving any medications) undergoing fiberoptic bronchoscopy (performed according to

2% FCS in 3 to 4 wells for each treatment. Treatment with TGF-β (10 ng/mL) (R&D) was used as a positive control. After an additional incubation for 24 hours, 1 μL of [³H] Thymidine (2 or 5 mCi/well) (DuPont NEN, Boston, MA) was added to each well, and the cells were incubated for an additional 24 hours. At the end of the incubation, cells were washed with cold phosphate buffered saline, fixed with methanol, and precipitated with 5% trichloroacetic acid (Merck, Darmstadt, Germany). The cells were lysed with 0.1% NaOH, transferred to scintillation vials, and counted in a β-counter (LKB 1211 Rackbeta; Pharmacia, Uppsala, Sweden). The results were obtained as counts per minute per well and are presented as the percentage of change (increase or decrease) in the fibroblast proliferation after the treatment compared with the cells that were incubated with medium alone:

$$100 \times \frac{\text{Value post incubation with sonicates or drugs or mast cells or drugs and mast cells}}{\text{Value post incubation with medium alone}}$$

published guidelines) for assessment of contralateral localized lung cancer, as previously described.⁸ Informed consent was obtained. Bronchial fibroblasts were expanded and cultured in DMEM/10% FCS and passaged weekly by Trypsin-EDTA (Biological Industries) treatment. They were used for assays between the second and fourth passages.

Mast Cells and Mast Cell Sonicates

The human mast cell leukemia cell line HMC-1 (donated by Dr J. Butterfield, Mayo Clinic, Rochester, MN) was used as a source of mast cells.²¹ HMC-1 cells were cultured in Iscove's medium (Biological Industries) supplemented with 10% FCS containing Fe [2], 50 U/mL penicillin, 0.05 mg/mL streptomycin, and 160 mg/mL (1.2 mM) monothioglycerol. Cells were passaged twice a week.

HMC-1 cells were centrifuged (120g, 5 min) and resuspended in 2 to 5 mL in DMEM/2% FCS (0.75–1:75 × 10⁵/mL) and bath sonicated (2 min, 0°C; Heat Systems/Ultrasonic 380W, duty cycle 5 s; output power, 50%). Sonicates were then microcentrifuged for 5 minutes at 4°C, 120g (Hettich Ultracentrifuge; Hettich Zentrifugen, Tuttlingen, Germany) to remove cell debris. The debris-free sonicate supernatant ("sonicate") was collected into aliquots and stored at –80°C.

Fibroblast Proliferation Assay

Fibroblast proliferation was assessed using the [³H]-Thymidine incorporation assay. Fibroblasts were plated in 96-well plates (3–4 × 10³ cells per well in 200 μL DMEM/10% FCS). After incubation for 24 hours, the wells were rinsed twice with DMEM/2% FCS, and dexamethasone (Sigma Chemicals, St Louis, MO) (stock solution 10⁻² M in ethanol) or salbutamol (stock solution 10⁻⁴ M in phosphate buffered saline; Sigma), at different concentrations or mast cell sonicates (15 × 10³), or eotaxin/CCL11 (500 ng/mL) (R&D, Minneapolis, MN) was added in 200 μL DMEM/

IL-6 Release

To evaluate the presence of IL-6 released in the culture medium, an ELISA kit (PeproTech, Rocky Hill, NJ) with sensitivity of 32 to 2000 pg/mL was used. For these experiments, fibroblasts were cultured in 24-well plates (3 × 10³ cells per well in 200 μL DMEM/10% FCS) until confluent. At this point, the wells were rinsed twice with DMEM/5% FCS and dexamethasone, salbutamol, or forskolin (stock solution 5 mg/mL in ethanol; Sigma) at different concentrations was added in 200 μL DMEM/5% FCS. After an 18-hour incubation, supernatants were collected and kept frozen at –80°C until assayed.

Statistical Analysis

Each experiment was performed in at least triplicates for 3 or 4 times. The results are presented as the mean ± standard error of the mean and were analyzed by the unpaired Student *t* test or analysis of variance test as appropriate for each case. *P* values of <0.05 were considered significant.

RESULTS

Dexamethasone and Salbutamol Increase Fetal Lung Fibroblast Proliferation Alone and When Stimulated by Mast Cells

Fetal lung fibroblasts were incubated for 48 hours with different concentrations of dexamethasone or salbutamol, and fibroblast proliferation was monitored by [³H]-Thymidine incorporation (Fig. 1). As shown in Figure 1A, the addition of dexamethasone caused upregulation in fibroblast proliferation in the range of concentration between 10⁻⁸ and 10⁻⁴ M in a bell-shaped manner. The maximal effect was achieved with 10⁻⁶ M (increase in proliferation, 350 ± 55%) (*P* < 0.0001) and was higher than that obtained with the addition of TGF-β. Addition of salbutamol (Fig. 1B) also induced increased fibroblast proliferation, although to a lesser extent than dexamethasone. This increase was maximal at 10⁻⁹ M (187 ±

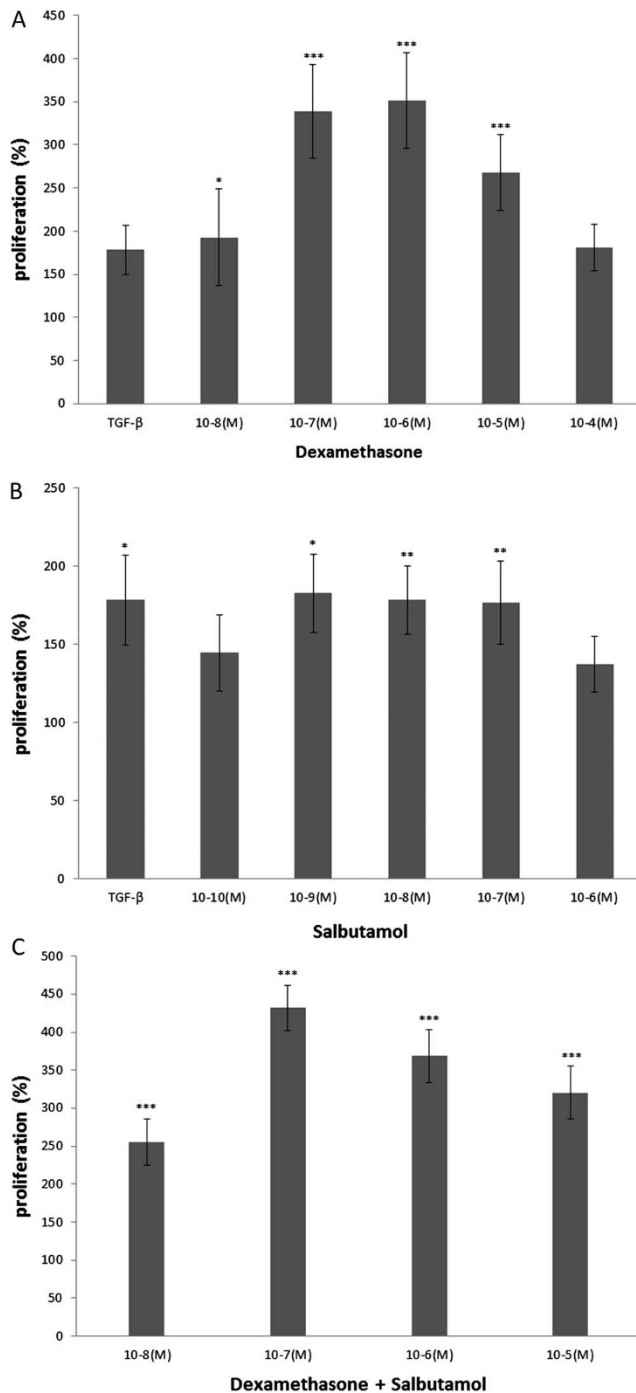


FIGURE 1. The influence of dexamethasone and salbutamol on fetal lung fibroblast proliferation. Fibroblasts were incubated for 48 hours with medium alone or with either dexamethasone (10^{-4} – 10^{-8} M) (A) or salbutamol (10^{-6} – 10^{-10} M) (B) or dexamethasone and salbutamol (10^{-5} – 10^{-8} M) (C). TGF- β (10 ng/mL) was used as a positive control. Proliferation was assessed by [3 H]-Thymidine incorporation, as detailed in Materials and Methods. Data are the mean \pm standard error of the mean of 4 experiments performed in quadruplicates. In medium alone, counts per minute of [3 H]-Thymidine incorporation were 474 ± 5 . * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$.

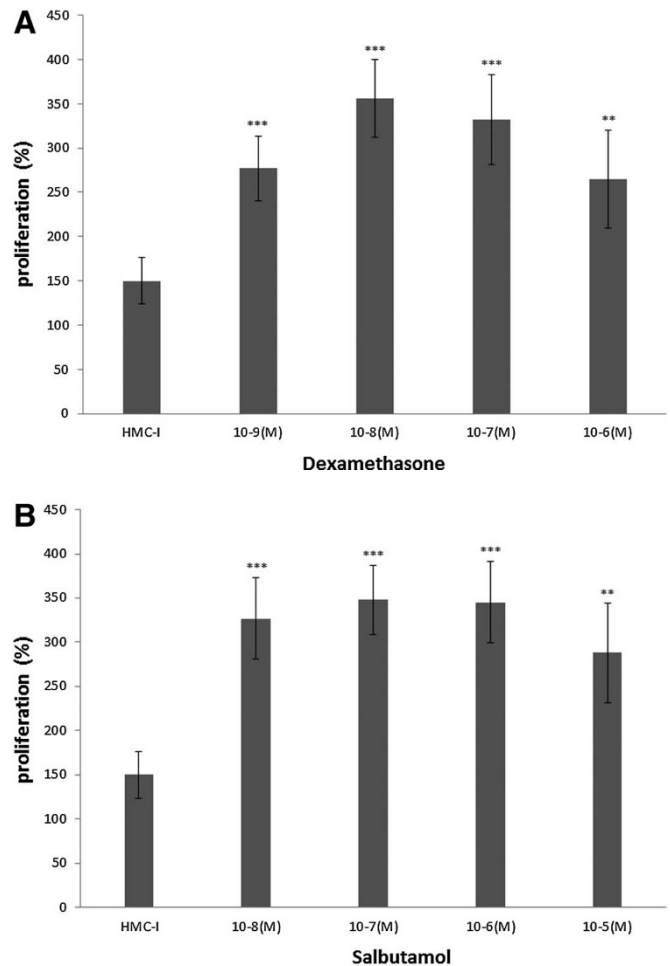


FIGURE 2. The influence of dexamethasone and salbutamol on mast cell-induced fetal lung fibroblast proliferation. Fibroblasts were preincubated for 30 minutes with either dexamethasone (10^{-6} – 10^{-9} M) (A) or salbutamol (10^{-5} – 10^{-8} M) (B). Mast cell (HMC-1) sonicates (15×10^3 cells per well) were then added, and cultures were incubated for further 48 hours. In the control, the fibroblasts were incubated in DMEM 2% alone or with mast cell sonicates alone. Proliferation was evaluated as described in the legend to Figure 1. Data are the mean \pm standard error of the mean of 5 experiments performed in triplicates. In medium alone, counts per minute of [3 H]-Thymidine incorporation were 219 ± 3 . ** $P < 0.01$; *** $P < 0.0001$.

31% at 10^{-8} M) and was similar at 10^{-8} and 10^{-7} M. When dexamethasone and salbutamol were added together at the same concentration (10^{-5} – 10^{-8} M), the 2 drugs significantly increased fibroblast proliferation in a synergistic fashion ($P < 0.0001$), with the maximal increase of $432 \pm 30\%$ at 10^{-7} M (Fig. 1C).

Next, we assessed the effect of the drugs on mast cell-induced fibroblast proliferation. Fetal lung fibroblasts were preincubated for 30 minutes with either dexamethasone or salbutamol, and then, mast cell sonicate (15×10^3 cells per well) was added for further 48 hours. As depicted in Figure 2, addition of mast cell sonicates increased fibroblast proliferation

by $150 \pm 26\%$ ($P < 0.05$). Preincubation of dexamethasone at different concentrations further enhanced this effect (Fig. 2A). The maximal increase in proliferation was observed with 10^{-8} M dexamethasone ($349 \pm 43\%$) ($P < 0.0001$). A similar result was obtained when the fibroblasts were incubated with different concentrations of salbutamol and mast cell sonicates, that is, salbutamol significantly increased fibroblast proliferation over the effect of the mast cell sonicates alone. The maximal increase in proliferation was observed with 10^{-7} M salbutamol and was $348 \pm 39\%$ more than the control ($P < 0.0001$) (Fig. 2B). In experiments performed with mast cell sonicate concentrations higher than 15×10^3 cells per well (ie, with 25×10^3 and 35×10^3 cells per well), still a significant increase in fibroblast proliferation ($P < 0.0001$) was observed with both dexamethasone (10^{-6} – 10^{-9} M) and salbutamol (10^{-5} – 10^{-8} M) preincubation.

In Table 1, the results obtained with 10^{-8} M dexamethasone and 10^{-7} M salbutamol are depicted. Dexamethasone at 10^{-8} M enhanced significantly by $387 \pm 41\%$ the fibroblast proliferation when preincubated with fibroblasts, thereafter receiving as a trigger 35×10^3 mast cell sonicate per well with no significant increase, however, from lower mast cell numbers. On the other hand, salbutamol increased in a dose-response fashion the fibroblast proliferation with increasing numbers of mast cell sonicates to achieve a maximum of $566 \pm 76\%$ enhancement with 35×10^3 mast cell per well at a concentration of 10^{-7} M.

Dexamethasone and Salbutamol Increase Human Bronchial Fibroblast Proliferation Alone and When Stimulated by Either Mast Cells or Eotaxin

When dexamethasone and salbutamol were added to human bronchial fibroblast subconfluent monolayers (Figs. 3A, B), both drugs caused an increase in fibroblast proliferation ($P < 0.05$). Dexamethasone displayed the maximal increase at 10^{-8} M ($174 \pm 17\%$) and salbutamol at 10^{-6} M ($145 \pm 16\%$). Addition of mast cell sonicates (25×10^3 cells per well) alike increased fibroblast proliferation as expected. Proliferation was further increased when cultures were preincubated with either dexamethasone or salbutamol (Fig. 4A). Cultures preincubated with dexamethasone

TABLE 1. Influence of Mast Cell Numbers on Dexamethasone and Salbutamol Increase in Fibroblast Proliferation

Mast Cell per Well	Increase in Fibroblast Proliferation (%)	
	Dexamethasone	Salbutamol
15×10^3	$349 \pm 37^*$	$348 \pm 39^*$
25×10^3	$352 \pm 36^*$	$405 \pm 58^*$
35×10^3	$387 \pm 41^*$	$566 \pm 76^*$

Fetal lung fibroblasts were preincubated with either dexamethasone (10^{-8} M) or salbutamol (10^{-7} M) and then incubated with different concentrations of mast cell sonicate. Fibroblast proliferation was evaluated and expressed as % increase over the value obtained with mast cell alone (as described in Materials and Methods). Data are the mean \pm standard error of the mean of 5 experiments performed in triplicates.

* $P < 0.0001$.

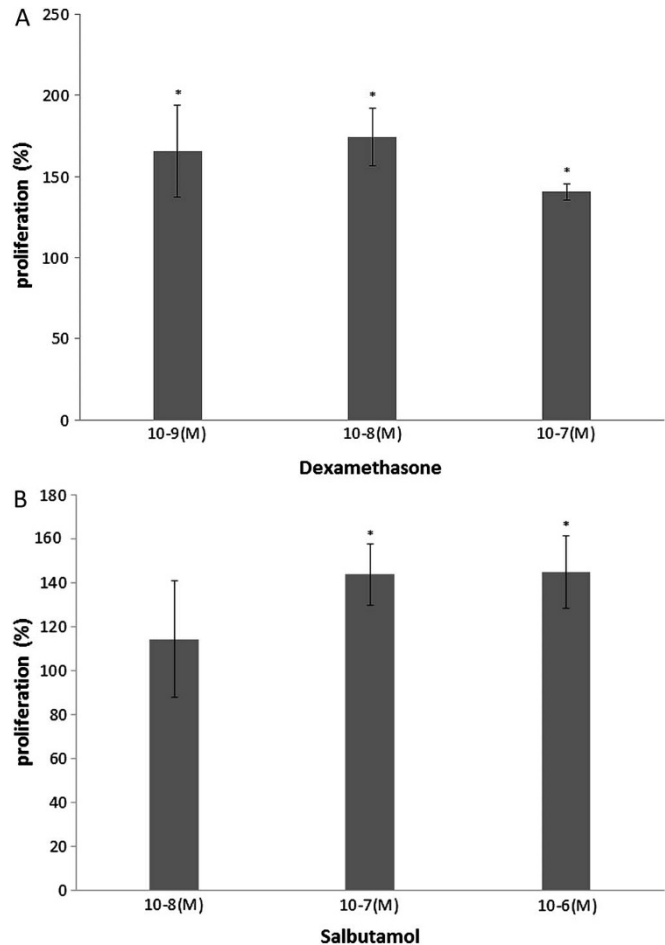


FIGURE 3. The influence of dexamethasone and salbutamol on bronchial fibroblast proliferation. Fibroblasts were incubated for 48 hours with medium alone or with dexamethasone (10^{-7} – 10^{-9} M) (A) or with salbutamol (10^{-6} – 10^{-8} M) (B). Data are the mean \pm standard error of the mean of 2 experiments performed in quadruplicates. In medium alone, counts per minute of [3 H]-Thymidine incorporation were 270 ± 2 . * $P < 0.05$.

displayed a significant increase in fibroblast proliferation compared with control and cultures incubated with sonicates alone. The maximal increase was obtained with 10^{-7} M dexamethasone and was $795 \pm 90\%$ ($P < 0.0001$). Salbutamol also increased the proliferative effect of mast cell sonicates (Fig. 4B). Interestingly, in this case, the influence of salbutamol was stronger than that of dexamethasone at the same concentration, and the maximal effect was $1069 \pm 96\%$ at 10^{-7} M.

To check whether salbutamol enhances fibroblast proliferation when they are exposed to a defined profibrogenic compound, eotaxin was added to cultures preincubated with the drug for 48 hours. Eotaxin by itself increased fibroblast proliferation by $146 \pm 9\%$. When together with salbutamol (10^{-5} – 10^{-8} M), a further significant increase was detected, that is, with a maximum of $261 \pm 18\%$ ($P < 0.0001$) reached with salbutamol concentration of 10^{-6} M (Fig. 5).

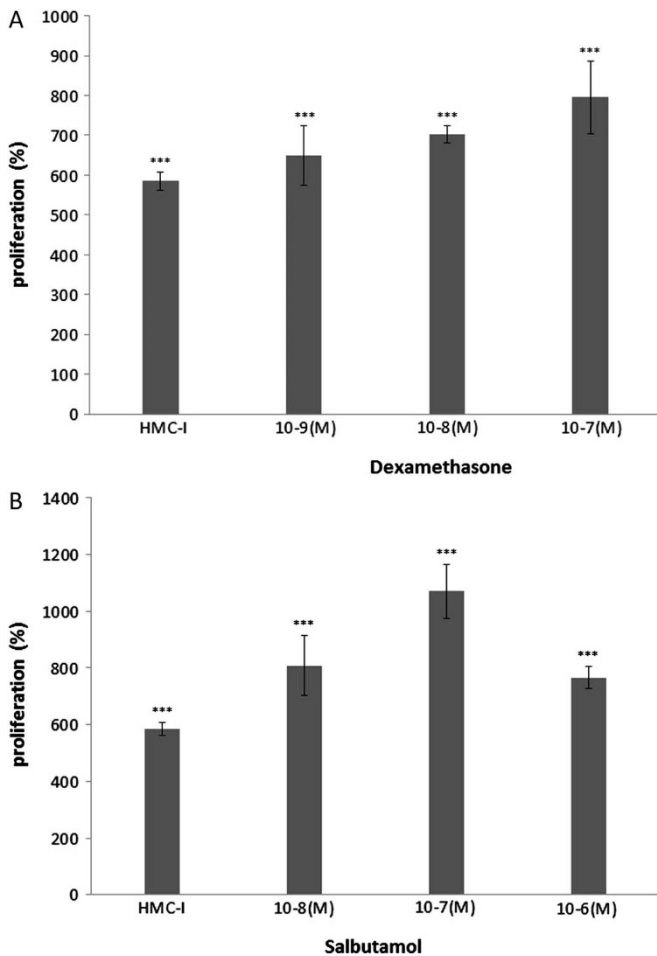


FIGURE 4. The influence of dexamethasone and salbutamol on mast cell-induced bronchial fibroblast proliferation. Fibroblasts were preincubated for 30 minutes with either dexamethasone (10^{-7} – 10^{-9} M) (A) or salbutamol (10^{-6} – 10^{-8} M) (B). Mast cell (HMC-1) sonicates (15×10^3 cells per well) were then added, and cultures were incubated for further 48 hours. In the control, the fibroblasts were incubated in medium alone or with mast cell sonicates alone. Data are the mean \pm standard error of the mean of 2 experiments performed in quadruplicates. In medium alone, counts per minute of [3 H]-Thymidine incorporation were 270 ± 2 . $***P < 0.0001$.

Dexamethasone and Salbutamol Influence Differently IL-6 Production From Fetal Lung Fibroblasts

As shown in Figure 6A, dexamethasone decreased in a dose-dependent fashion IL-6 production by confluent fetal lung fibroblasts (148% at 10^{-5} M; $P < 0.005$). Salbutamol in contrast enhanced this proinflammatory cytokine production also significantly with the maximal effect detected at 10^{-5} M of an increase of 129% ($P < 0.01$) (Fig. 6B).

Interestingly, when the 2 drugs were added together at 10^{-5} and 10^{-6} M, the inhibiting effect of dexamethasone was slightly decreased by the addition of salbutamol (not shown). In a series of experiments (Table 2), salbutamol and forskolin

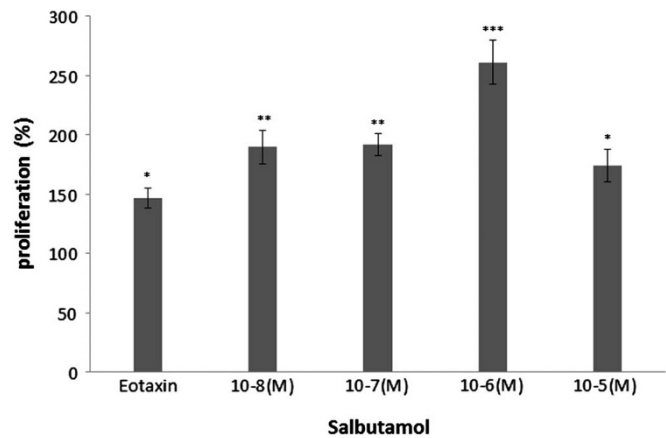


FIGURE 5. The influence of salbutamol on eotaxin-induced bronchial fibroblast proliferation. Fibroblasts were preincubated with salbutamol (10^{-5} – 10^{-8} M) for 30 minutes. Eotaxin (500 ng/mL) was then added to the wells for an additional 48 hours. In the control, the fibroblasts were incubated with medium alone or with eotaxin alone. In medium alone, counts per minute of [3 H]-Thymidine incorporation were 330 ± 5 . Data are the mean \pm standard error of the mean of 5 experiments performed in triplicates. $*P < 0.05$; $**P < 0.01$; $***P < 0.0001$.

were compared for their effects on IL-6 production from fetal lung fibroblasts. Similar increases were detected in IL-6 production relative to medium incubated wells. In the presence of salbutamol at 10^{-7} and 10^{-6} M, the increase in IL-6 found in the culture medium was $138 \pm 6\%*$ and $144 \pm 4\%*$, respectively, and with forskolin at 10^{-6} and 10^{-5} M, it was $149 \pm 4\%*$ and $151 \pm 4\%*$, respectively ($n = 3$, $*P < 0.05$).

DISCUSSION

It is well accepted that there is an increase in the number of fibroblasts in the airways of patients with asthma that correlates with thickness of lamina reticularis and disease severity. Moreover, fibroblast activation and differentiation to myofibroblasts are also evident.¹⁻⁴

In the present study, we aimed to investigate the in vitro effect of glucocorticosteroids and short-acting β_2 -agonists widely used as first-line antiasthmatic drugs on human lung fibroblast proliferation and IL-6 production. We specifically choose to evaluate fibroblast proliferation because this is the first hallmark of fibrosis taking place. IL-6 was selected among a plethora of proinflammatory profibrotic cytokines produced by the fibroblast²² that mainly influences the inflammatory response.^{23,24}

We found that dexamethasone and salbutamol alone and in combination increase both human fetal lung and human bronchial fibroblast proliferation. Moreover, we demonstrate for the first time that when the fibroblasts are preincubated with either drug and then with an activator such as mast cell sonicate or eotaxin, fibroblast proliferation is further increased. In contrast, the production of the proinflammatory and profibrotic cytokine IL-6 by confluent fibroblast monolayers was found to be decreased by dexamethasone, while it was increased in the presence of

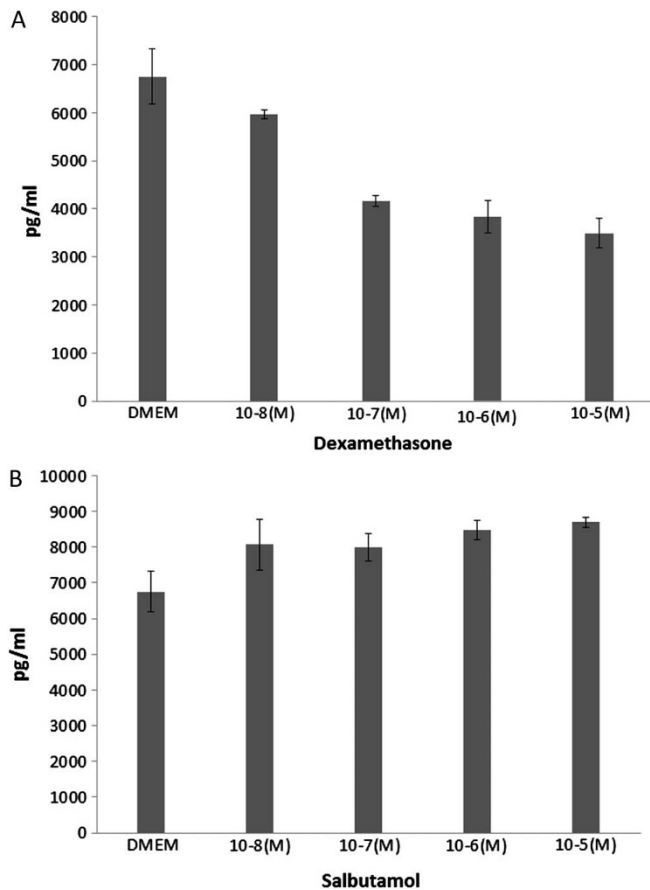


FIGURE 6. The influence of dexamethasone and salbutamol on IL-6 production by fetal lung fibroblasts. Fibroblasts were incubated for 18 hours with medium alone or with medium with either dexamethasone (10^{-5} – 10^{-8} M) (A) or salbutamol (10^{-5} – 10^{-8} M) (B). IL-6 production was evaluated in the culture supernatants, as described in Materials and Methods. Data are the mean \pm standard error of the mean of 2 experiments performed in quadruplicates.

salbutamol. When the 2 drugs were added together, dexamethasone was still able to slightly decrease IL-6 production.

We have previously shown that mast cells enhance proliferation of human lung fibroblasts, an effect also seen with histamine and to a lesser extent with tryptase.⁷ In a more recent study, we have also defined the profibrotic properties of eotaxin/CCL11,⁸ a central mediator of asthma that can also be produced by mast cells.²⁵

In that study, we did not find significant differences between the human fetal lung fibroblast line and human bronchial fibroblasts regarding their proliferative responses to eotaxin/CCL11. This was explained by the fact that they were found to express similar levels of surface CCR3. Similarly, in the present study, we did not observe significant differences in the response of the fetal lung and the bronchial fibroblasts toward the proliferative effects of either the drugs or the stimulants. This is an interesting observation because fibroblasts from cell lines and primary cultures might behave differently. The fact that we did not detect major differences between these 2 sources of fibroblasts might be due to the fact that primary cells did not need to be cultured and subcultured for extensive time and when used were not senescent.

Many studies have been carried out to investigate the effects of glucocorticosteroids on several sources of fibroblasts such as human fetal lines, or primary human lung, asthmatics endobronchial biopsies, human nasal polyps derived or mouse 3T3 and rat lung and even adipose tissue derived providing sometimes similar and sometimes contrasting results. We opted for the most commonly used source of human lung fibroblasts, the cell line MRC-5 and primary human bronchial fibroblasts.

Regarding our data, it is interesting to note that generally in the absence of the mast cell sonicate, dexamethasone effect was always greater than that of salbutamol. In this work, we showed for the first time that salbutamol caused a further increase in fibroblast proliferation in a dose-dependent fashion in fibroblasts treated with eotaxin. This proliferative event is probably carried out by the interaction of eotaxin with its receptor and by salbutamol with β_2 -receptors.

Glucocorticosteroids in general and specifically dexamethasone are known to decrease gene expression of proinflammatory cytokines. Several studies have found that dexamethasone decreases cytokine secretion by fibroblasts, among them IL-6.²⁶ The results of our study correlate with these findings. But also this decreasing effect is not universally detected and depends on the exposure duration and the cytokine under study.²⁷ Studies that tested the effect of β_2 -agonists on IL-6 secretion on different cell types found that these drugs enhanced IL-6 secretion.²⁸ As far as we know, the effect of β_2 -agonists on IL-6 secretion from lung fibroblasts has not yet been studied. We found that salbutamol increased slightly IL-6 production. Cyclic adenosine monophosphate (cAMP) is known as an IL-6 secretion enhancer (airway smooth muscle cells, 3T3 fibroblasts, and gingival

TABLE 2. Influence of Dexamethasone, Salbutamol, and Forskolin on IL-6 Release From Fetal Lung Fibroblasts

	Dexamethasone			Salbutamol			Forskolin	
	10 ⁻⁷ M	10 ⁻⁶ M	10 ⁻⁵ M	10 ⁻⁷ M	10 ⁻⁶ M	10 ⁻⁵ M	10 ⁻⁶ M	10 ⁻⁵ M
Change in IL-6 production	↓72 \pm 1%*	↓79 \pm 4%*	↓60 \pm 5%†	↑138 \pm 6%*	↑144 \pm 4%*	↑150 \pm 1%†	↑149 \pm 4%*	↑151 \pm 4%*

Fibroblasts were incubated with either medium alone (control) or with the indicated concentrations of the compounds, and the change in the IL-6 production was calculated relative to the control, as detailed in Materials and Methods. In medium alone, IL-6 production was 6700 \pm 450 pg/mL. The data are the mean \pm standard error of the mean of 2 experiments performed in triplicates.

* $P < 0.05$.

† $P < 0.01$.

fibroblasts), even though it has been reported that lung fibroblast cell line had an inverse relationship between the amount of cAMP and the amount of IL-6 secreted from the cell.²⁹

In our study, salbutamol and forskolin that increase cAMP production, both increased IL-6 secretion from the fibroblasts. These findings strengthen the assumption that the mechanism through which salbutamol affects the increase in IL-6 secretion is cAMP dependent. Interestingly, when dexamethasone and salbutamol were added together, the decrease in IL-6 production was smaller than the one obtained in the presence of dexamethasone alone.

Many mediators, including growth factors, IL-6, and other cytokines, are involved in fibroblast proliferation regulation. In a previous study, we found that when added to fetal lung fibroblasts, IL-6 increased their proliferation up to 140% (data not published). Therefore, we might speculate that salbutamol can influence lung fibroblast proliferation by causing IL-6 production. The effect of β_2 -agonists in general and specifically of salbutamol on fibroblast proliferation has almost not been studied. Agarwal and Glasel described the effect of treatment with opioid and β -adrenergic agonists on cell proliferation.³⁰ They found that although both receptors increase cAMP production, opioid agonists lowered the level of proliferation, while a β_2 -agonist (procaterol) increased cell proliferation. Silvestri et al²⁰ found that salmeterol decreased fetal lung line fibroblast proliferation induced by bFGF. The source of the cells is indeed similar to the cells in our study, but salmeterol is a long-acting β_2 -agonist as opposed to salbutamol.²⁰

Another important difference is that the study by Silvestri et al,²⁰ and other studies, tested the drug's effect on proliferation of cells that were incubated with a growth factor (in this case basic fibroblast growth factor). It is difficult to determine whether the drug's effect is independent of the tested growth factor and whether this can be compared with our research in which salbutamol's effect on fibroblasts was assessed either without additional treatment or in the presence of mast cells or eotaxin.

It is difficult to speculate how the dexamethasone carries out its profibrogenic response. Several mechanisms have been suggested regarding the fashion in which the glucocorticosteroids enhance fibroblast proliferation. For example, they can either enhance or decrease the activity of different growth factors (among them, insulin growth factor-1 and fibroblast growth factor) and their receptors (ie, platelet derived growth factor¹¹) or their production (ie, connective tissue growth factor³¹). The time of drug addition to the cultures can make a difference. When it is added after the cells reached confluence, it causes enhancement of proliferation, while drug administration during the exponential growth phase usually causes a decrease.³² In our protocol, we added dexamethasone to subconfluent monolayer and still it had a profibrogenic effect.

It is interesting to point out that the literature on the effect of drug combination on fibroblast proliferation presents a complex picture. Many researches describe an additive and a synergistic effect of glucocorticosteroids and β_2 -agonists. On the other hand, other studies have found classes of the two drugs to have antagonistic effects. Peters et al³³ reported that β_2 -agonists, among them salbutamol, have the ability to

lower the steroid effect on gene transcription by inhibiting the binding to the glucocorticosteroid responsive element on the DNA. On the other hand, salbutamol and salmeterol have been described as having the ability to activate the receptor by a mechanism related to cAMP increase.³⁴

Our findings obtained in vitro show that whatever the underlying mechanism(s) is, the drugs have a direct fibrogenic effect and a synergistic effect with central players, such as mast cell-derived mediators in the asthmatic process. If these in vitro observations are found to be true also in the in vivo complexity of the asthmatic patient with airway remodeling and fixed airway obstruction, it is then possible to speculate that this may not only be part of the natural course of the disease but a complication of the accepted drug treatment and a product of the drugs' interactions with the inflammation mediators. Therefore, if the profibrogenic effect of glucocorticosteroids and β_2 -agonists is reconfirmed in vivo, the combination of the remodeling and the drugs' effect can be dangerous. In conclusion, more in vivo studies are warranted to clarify what the risks and benefits are of monotherapy versus combination of inhaled glucocorticosteroids and short- and long-acting β_2 -agonists on airway remodeling in asthma.

REFERENCES

- Davies DE, Wicks J, Powell RM, Puddicombe SM, Holgate ST, et al. Airway remodeling in asthma: new insights. *J Allergy Clin Immunol.* 2003;111:215–225.
- Homer RJ, Elias JA. Airway remodeling in asthma: therapeutic implications of mechanisms. *Physiology (Bethesda).* 2005;20:28–35.
- Al-Muhsen S, Johnson JR, Hamid GL. Remodeling in asthma. *J Allergy Clin Immunol.* 2011;128:451–462; quiz 463–464.
- Lewis CC, Chu HW, Westcott JY, Tucker A, Langmack EL, et al. Airway fibroblasts exhibit a synthetic phenotype in severe asthma. *J Allergy Clin Immunol.* 2005;115:534–540.
- Okayama Y, Ra C, Saito H. Role of mast cells in airway remodeling. *Curr Opin Immunol.* 2007;19:687–693.
- Minshall EM, Leung DY, Martin RJ, Song YL, Cameron L, et al. Eosinophil-associated TGF-beta1 mRNA expression and airway fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol.* 1997;17:326–333.
- Garbuzenko E, Berkman N, Puxeddu I, Kramer M, Nagler A, Levi-Schaffer F. Mast cells induce activation of human lung fibroblasts in vitro. *Exp Lung Res.* 2004;30:705–721.
- Puxeddu I, Bader R, Piliponsky AM, Reich R, Levi-Schaffer F, Berkman N. The CC chemokine eotaxin/CCL11 has a selective profibrogenic effect on human lung fibroblasts. *J Allergy Clin Immunol.* 2006;117:103–110.
- Micera A, Vigneti E, Pickholtz D, Reich R, Pappo O, et al. Nerve growth factor displays stimulatory effects on human skin and lung fibroblasts demonstrating a direct role for this factor in tissue repair. *Proc Natl Acad Sci U S A.* 2001;98:6162–6167.
- Sommerhoff CP. Mast cell tryptases and airway remodeling. *Am J Respir Crit Care Med.* 2001;164:S52–S58.
- Warshamana GS, Martinez S, Lasky JA, Corti M, Brody AR. Dexamethasone activates expression of the PDGF-alpha receptor and induces lung fibroblast proliferation. *Am J Physiol.* 1998;274:L499–L507.
- Wen FQ, Kohyama T, Skold CM, Zhu YK, Liu X, et al. Glucocorticoids modulate TGF-beta production by human fetal lung fibroblasts. *Inflammation.* 2003;27:9–19.
- Da Silva CA, Kassel O, Lebouquin R, Lacroix EJ, Frossard N. Paradoxical early glucocorticoid induction of stem cell factor (SCF) expression in inflammatory conditions. *Br J Pharmacol.* 2004;141:75–84.
- Fouty B, Moss T, Solodushko V, Kraft M. Dexamethasone can stimulate G1-S phase transition in human airway fibroblasts in asthma. *Eur Respir J.* 2006;27:1160–1167.

15. Sabatini F, Silvestri M, Sale R, Serpero L, Giuliani M, et al. Concentration-dependent effects of mometasone furoate and dexamethasone on foetal lung fibroblast functions involved in airway inflammation and remodeling. *Pulm Pharmacol Ther.* 2003;16:287–297.
16. Olivieri D, Chetta A, Del Donno M. Effect of short term treatment with low-dose inhaled fluticasone propionate on airway inflammation and remodeling in mild asthma: a placebo controlled study. *Am J Respir Crit Care Med.* 1997;155:1864–1871.
17. Sont JK, Willems LN, Bel EH, van Krieken JH, Vandenbroucke JP, et al. Clinical control and histopathological outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. *Am J Respir Crit Care Med.* 1999;159:1043–1051.
18. Lundgren R, Soderberg M, Horstedt P, Stenling R. Morphological studies of bronchial biopsies from asthmatics before and after ten years of treatment with inhaled steroids. *Eur Respir J.* 1988;1:883–889.
19. Descalzi D, Folli C, Nicolini G, Riccio AM, Gamalero C, Scordamaglia F, Canonica GW. Anti-proliferative and anti-remodelling effect of beclomethasone dipropionate, formoterol and salbutamol alone or in combination in primary human bronchial fibroblasts. *Allergy.* 2008;63:432–437.
20. Silvestri M, Fregonese L, Sabatini F, Dasic G, Rossi GA. Fluticasone and salmeterol downregulate in vitro, fibroblast proliferation and ICAM-1 or H-CAM expression. *Eur Respir J.* 2001;18:139–145.
21. Butterfield JH, Weiler DA. In vitro sensitivity of immature human mast cells to chemotherapeutic agents. *Int Arch Allergy Appl Immunol.* 1989;89:297–300.
22. Berkman N, Levi-Schaffer F. Profibrotic and angiogenic factors in asthma. In: Izuhara K, Holgate ST, Wills-Karp M, eds. *Inflammation and Allergy Drug Design.* 1st ed. Oxford, UK: Wiley-Blackwell Publishing Ltd; 2011:237–252.
23. Chalaris A, Garbers C, Rabe B, Rose-John S, Scheller J. The soluble interleukin 6 receptor: generation and role in inflammation and cancer. *Eur J Cell Biol.* 2011;90:484–494.
24. Rose-John S, Waetzig GH, Scheller J, Grotzinger J, Seeger D. The IL-6/sIL-6R complex as a novel target for therapeutic approaches. *Expert Opin Ther Targets.* 2007;11:613–624.
25. Hogaboam C, Kunkel SL, Strieter RM, Taub DD, Lincoln P, Standiford TY, Lukacs NK. Novel role of transmembrane SCF for mast cell activation and eotaxin production in mast cell-fibroblast interactions. *J Immunol.* 1998;160:6166–6171.
26. Pujols L, Fuentes-Prado M, Fernández-Bertolín L, Alobid I, Roca-Ferrer J, Mullol J, Picado C. Lower sensitivity of nasal polyp fibroblasts to glucocorticoid anti-proliferative effects. *Respir Med.* 2011;105:218–225.
27. Suzuki T, Arakawa H, Mizuno T, Muramatsu K, Tadaki H, et al. Differential regulation of eotaxin expression by dexamethasone in normal human lung fibroblasts. *Am J Respir Cell Mol Biol.* 2008;38:707–714.
28. Futamura K, Orihara K, Hashimoto N, Morita H, Fukuda S, et al. Beta2-adrenoceptor agonists enhance cytokine-induced release of thymic stromal lymphopoietin by lung tissue cells. *Int Arch Allergy Immunol.* 2010;152:353–361.
29. Zitnik RH, Khiting NL, Elias JA. Glucocorticoid inhibition of interleukin-1 induced interleukin-6 production by human lung fibroblasts: evidence for transcriptional and post-transcriptional regulatory mechanisms. *Am J Respir Cell Mol Biol.* 1994;10:643–650.
30. Agarwal D, Glasel JA. Differential effects of opioid and adrenergic agonists on proliferation in a cultured cell line. *Cell Prolif.* 1999;32:215–229.
31. Dammeier J, Beer HD, Brauchle M, Werner S. Dexamethasone is a novel potent inducer of connective tissue growth factor expression. Implications for glucocorticoid therapy. *J Biol Chem.* 1998;273:18185–18190.
32. Yang JQ, Rüdiger JJ, Hughes JM, Goulet S, Gencay-Cornelson MM, et al. Cell density and serum exposure modify the function of the glucocorticoid receptor C/EBP complex. *Am J Respir Cell Mol Biol.* 2008;38:414–422.
33. Peters MJ, Adcock IM, Brown CR, Barnes PG. Beta adrenoceptor agonists interfere with glucocorticoid receptor DNA binding in rat lung. *Eur J Pharmacol.* 1995;289:275–281.
34. Eickelberg O, Roth M, Lörx R, Bruce V, Rüdiger J, Johnson M, Block LH. Ligand-independent activation of the glucocorticoid receptor by beta2-adrenergic receptor agonists in primary human lung fibroblasts and vascular smooth muscle cells. *J Biol Chem.* 1999;274:1005–1010.