Fluticasone and salmeterol downregulate in vitro, fibroblast proliferation and ICAM-1 or H-CAM expression

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ABSTRACT: β2-adrenoreceptor agonists have pharmacological properties that may suggest an inhibitory effect on various aspects of the inflammatory and repair processes that characterize asthma.

Since fibroblasts express β2-adrenoreceptors, the effects of different concentrations (0.1 – 100 nM) of fluticasone propionate (FP), salmeterol (S) and their combination (FP+S) on lung fibroblast proliferation and adhesion molecule expression were evaluated.

Stimulation of human foetal lung fibroblasts with a fibrogenic cytokine, basic fibroblast growth factor (bFGF), resulted in a [methyl-3H] thymidine ([3H]TdR) uptake, four-fold higher than that of control cultures (p < 0.0001) and was significantly inhibited by S, at all the concentrations tested (0.1 – 100 nM; p < 0.05). No changes in bFGF-induced cell proliferation were observed in the presence of FP (0.1 – 100 nM; p > 0.05, all comparisons). In addition, the association FP+S did not improve the inhibitory activity of S alone (p > 0.05, each comparison). An upregulation of intercellular adhesion molecule-1 (ICAM-1) expression was induced by tumour necrosis factor-α (TNF-α) (p < 0.0004), but not by interleukin-4 (IL-4) (p > 0.05), while none of the two cytokines were able to increase hyaluronic-cellular adhesion molecule (H-CAM) expression by lung fibroblasts (p > 0.05). A significant downregulation of ICAM-1 or H-CAM expression was demonstrated in the presence of FP or S, at all concentrations tested (0.1 – 100 nM; p < 0.01, each comparison). Interestingly, S (10 nM and 100 nM) was able to enhance the inhibitory activity of FP on ICAM-1 expression (p < 0.01), but not on H-CAM expression (p > 0.1).

These results show that in human foetal lung fibroblasts, fluticasone propionate and salmeterol are effective in modulating in vitro, different lung fibroblast biological functions that are likely to be involved in airway remodelling.


Bronchial asthma is a chronic inflammatory disorder of the airways associated with reversible airway obstruction and bronchial hyperreactivity to a variety of stimuli [1]. Although each of these components is recognized as an important part of the asthmatic phenotype, the primary underlying abnormality is thought to be the chronic airway inflammation that gives rise to reversible obstruction, hyperresponsiveness and tissue remodelling [2].

The pathological findings of asthma include not only infiltration of mononuclear cells, mast cells and eosinophils in the airway tissues, but also sub-basement membrane fibrosis [3]. Indeed, even in mild asthma, the inflammatory cells recruited in the airways release products that are able to mediate changes in airway patency and responsiveness [2], to damage bronchial epithelium and to stimulate myofibroblast proliferation and collagen depositing below the epithelial basement membrane [4]. As a consequence, two distinct processes can be observed: 1) regeneration ad integrum, leaving no residual trace of the previous injury; or 2) replacement by connective tissue, through the depositing of increased amounts of collagen (especially types III and V), polysaccharides (such as hyaluronic acid) and fibronectin by activated interstitial fibroblasts [2, 5, 6].

Besides being modulated by a variety of cytokines and mediators released by airway inflammatory and parenchymal cells, fibroblasts may also interact directly with inflammatory cells, as demonstrated by the frequent observation of membrane apposition of eosinophils and myofibroblasts in the bronchial mucosa of asthmatic patients [4, 6 – 8]. This close contact that is probably due to adhesion molecule expression on the interacting cell surface, may allow the stimulation of inflammatory cells by both direct cell-to-cell interaction and high cytokine concentrations [7].

Fibroblasts express a variety of surface molecules that include intercellular adhesion molecule-1 (ICAM-1, CD54) and hyaluronic-cellular adhesion molecule (H-CAM, CD44), which belongs to a family of transmembrane glycoproteins [7, 8]. During inflammation or experimental exposure to cytokines, adhesion
molecule expression is upregulated on both airway parenchymal cells and polymorphonuclear leukocytes and appears to be deeply involved in leukocyte transmigration and activation [9]. Indeed, eosinophils have shown to adhere to fibroblasts and to be activated through the binding of ICAM-1 to leukocyte integrin leukocyte function-associated antigen-1 (LFA-1, CD11a/CD18), which is expressed on the eosinophil surface [8, 10].

As a receptor for glycans hyaluronate, as well as for collagen and fibronectin [11], the CD44 transmembrane glycoprotein appears to be involved in regulation of cell locomotion and modulation of cell-to-cell and cell-to-matrix interactions that occur during fibrogenesis [8, 12].

Since asthma is characterized by airway inflammation and reversible bronchial obstruction, it is treated with anti-inflammatory drugs (i.e. corticosteroids) and bronchodilators, such as β2-adrenoceptor agonists [1]. The therapeutic activity of corticosteroids results, at least in part, from inhibition of inflammatory mediator synthesis, release by parenchymal and inflammatory cells, and prevention of some of the histological changes that characterize airway remodelling in asthma, including subendothelial fibrosis [1, 13]. Similarly, it has been recently shown that β2-adrenoceptor agonists have in vivo and in vitro pharmacological properties suggesting an inhibitory effect on various aspects of the inflammatory response related to asthma [14].

With this background, a study was designed to evaluate in vitro, the activity of an inhaled glucocorticoid (fluticasone propionate (FP)) and of a long-acting β2-adrenoceptor agonist (salmeterol xinafoate (S)) on fibroblast proliferation and adhesion molecule expression.

**Material and methods**

**Fibroblast culture**

GM 06114, a human foetal lung fibroblast cell line, was used in all the experiments performed. The GM 06114 cell line was selected because, in preliminary sets of experiments, it reacted to cytokine stimulation in a similar manner to human lung fibroblast primary cultures [7, 15]. Fibroblasts were cultured until confluence, then collected and spread into 96-well plates (7,000 cells well−1) or into 24-well plates (60,000 cells well−1) for the evaluation of adhesion molecule expression. The cells were incubated with Dulbecco’s Modified Eagle Medium (DMEM) (Euroclon Ltd, Paignton, Devon, UK) supplemented with 20% foetal calf serum (FCS; Euroclon Ltd) and penicillin/streptomycin (5,000 IU·mL −1) (ICN Biomedicals srl, Costa Mesa, CA, USA) for 24 h at 37 °C in 5% carbon dioxide (CO2). The medium was then removed and the cells resuspended in serum-free DMEM and incubated for an additional 48 h. The medium was then removed and replaced with fresh serum-free DMEM, in the presence of different concentrations of different stimuli: basic fibroblast growth factor (bFGF, 0.5–100 ng·mL −1) (SIGMA, Saint Louis, Missouri, USA), tumour necrosis factor-α (TNF-α, 0.5–20 ng·mL −1) (Euroclone Ltd) or interleukin-4 (IL-4, 0.5–20 ng·mL −1) (PeproTech EC Ltd, London, England). Unstimulated cells were used as negative control. After 24 h-incubation, cell proliferation and molecule adhesion expression were evaluated as described later.

**Cell proliferation assay**

Deoxyribonucleic acid (DNA) synthesis was evaluated as incorporation of tritiated thymidine ([methyl-3H] thymidine ([3H]TdR)) (Amerham International, Little Chalfont, Buckinghamshire, England). In preliminary experiments, cells were stimulated for 24 h with bFGF, TNF-α, or IL-4 at concentrations ranging 0.5–20 ng·mL −1, in order to determine which stimulus was most effective in inducing fibroblast proliferation. In the second set of experiments, the cells were stimulated for 24 h with bFGF at a final concentration of 5 ng·mL −1, plus FP or S, or plus the combination of the two drugs at concentrations ranging 0.1 nM–100 nM. Unstimulated fibroblasts were used as negative control, while cells stimulated with bFGF alone were used as positive control. [3H]TdR (0.1 microCi·well−1) was then added and, 18 h later, the cells were frozen and kept at -20°C for ≥24 h. After thawing, cells were collected on filter paper discs with an automatic cell harvester, the filters dissolved in 3 mL of scintillation fluid (ICN Biomedicals srl) in polypropylene vials and [3H]TdR incorporation was measured using a liquid scintillation counter [16]. Incorporation was expressed as count·min−1 (cpm). Each experiment was carried out in triplicate. Cell viability was evaluated by Trypan blue dye exclusion test (Euroclone Ltd) after 24-h stimulation [16].

**Evaluation of adhesion molecule expression by human foetal lung fibroblast**

Cells grown into 24-well plates and stimulated with TNF-α or IL-4 were trypsinized, collected, washed twice in DMEM, resuspended in DMEM plus 2% FCS and 0.1% sodium azide in NaCl 0.9%, and stained with monoclonal antibodies to evaluate adhesion molecule expression. Cell viability was evaluated by Trypan blue dye exclusion test. To evaluate the expression of ICAM-1 and H-CAM, 100 µL·well−1 of the cell suspensions were placed into round-bottomed microtiter 96-well plates. After incubation for 30 min at 4°C with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (MAB), antihuman (ah)-ICAM-1 (CD54) (Biosource International Inc., Camarillo, CA, USA) or antihuman (ah)-H-CAM (CD44) (Caltag Laboratories, Burlingame, CA, USA), the cells were washed twice and then fixed with 0.5% paraformaldehyde and analysed by immunofluorescence flow cytometry (Becton Dickinson Immunocytometry Systems; Mountain View, CA, USA). To compare the fluorescence intensities of different samples from the same
experiments, identical settings of the logarithmic amplifier were used and listmode files were analysed with CELLQuest software (Becton Dickinson). After conversion to linear fluorescence intensity units to obtain a linear function of fluorescence intensity over a wide range, the average background linear fluorescence obtained with the control antibody, the antihuman (ah)-CD3 FITC (Becton Dickinson), was subtracted from the average fluorescence intensity of the specifically stained cells. The possible changes in cell size in the different conditions were controlled by forward light scatter signal of the flow cytometer, proportional to cell size and algebraically adjusting to increases in cell surface area so that intensity in relative linear fluorescence units could be obtained. This adjustment permits the correlation of the average fluorescence intensity with the cell surface density of the stained antigens. All experiments were performed in triplicate. The intensity of fluorescence was expressed as mean fluorescence channel (mfc) [17].

To evaluate the effect of FP and/or S on adhesion molecule expression, fibroblasts were stimulated for 24 h with TNF-\(\alpha\) or IL-4 in the presence of different concentrations of FP, S or the association of the two drugs (0.1 – 100 nM). Evaluation of ICAM-1 and H-CAM was performed as described above. Cell viability was evaluated by Trypan blue dye exclusion test.

Data and statistical analysis

Mann-Whitney U-test or paired t-test were used to compare data, when appropriate. Data parametrically distributed are presented as arithmetic mean ± SEM. A p-value < 0.05 was set to indicate the level of statistically significant differences.

Results

Fibroblast proliferation and adhesion molecule expression following stimulation with basic fibroblast growth factor, tumour necrosis factor-\(\alpha\) or interleukin-4

Among the stimuli tested, bFGF was highly effective in inducing a dose-dependent lung fibroblast proliferation, statistically significant at all the concentrations tested (p < 0.01, each comparison with control cultures; fig. 1). In contrast, no significant increase in lung fibroblast proliferation was observed when the cells were stimulated with IL-4 or TNF-\(\alpha\) (p > 0.05, each comparison; fig. 1).

Unstimulated fibroblasts grown in DMEM medium showed negligible basal expression of ICAM-1 but elevated levels of H-CAM (fig. 2). TNF-\(\alpha\) (5 ng/mL) was able to induce an upregulation of ICAM-1 expression by lung fibroblasts (control: 6.88 ± 0.44 mfc; TNF-\(\alpha\) 5 ng/mL: 21.47 ± 2.44 mfc; p = 0.0004) (fig. 2). This upregulation was not seen with IL-4 (fig. 2).

Neither TNF-\(\alpha\) nor IL-4 were able to significantly increase the expression of H-CAM, probably because of its high basal expression (control: 129.71 ± 5.60 mfc) (fig. 2).

Effect of fluticasone propionate and/or salmeterol on fibroblast proliferation

At all the concentrations tested, FP had no significant effect on bFGF-induced fibroblast proliferation (fig. 3). Conversely, at the same concentrations, S was highly effective in inhibiting fibroblast proliferation (p < 0.01, each comparison with bFGF-stimulated cultures; fig. 3). While at the three highest concentrations tested, the inhibitory activity of S alone appeared to be higher compared to S plus FP, the levels of [\(^{3}H\)]TdR in the presence of S alone or of S + FP were not statistically different (p > 0.05, each comparison; fig. 3).

Effects of fluticasone propionate and/or salmeterol on adhesion molecule expression

When evaluating changes in adhesion molecule expression induced by FP and S, it was found that both drugs were able to significantly inhibit ICAM-1 and H-CAM expression on human lung fibroblast surface. Exposure to FP induced a dose-dependent downregulation of TNF-\(\alpha\)-stimulated ICAM-1 expression, significant at all the concentrations tested (p < 0.001, each comparison with TNF-\(\alpha\)-stimulated cultures; fig. 4). A significant downregulation of TNF-\(\alpha\)-stimulated ICAM-1 expression was also induced by S at all the concentrations tested (p < 0.01, each comparison with TNF-\(\alpha\)-stimulated cultures). The inhibitory effect of S was lower than that observed in the presence of FP at the three highest concentrations tested (p < 0.01; fig. 4). Comparison between the effect of FP alone and of FP + S showed that S at the two highest concentrations (10 and 100 nM), added to
FP at the same concentrations, enhanced the inhibition induced by FP alone on ICAM-1 expression (FP 10 nM 6.97 ± 1.00 mfc, FP 20 nM 4.53 ± 0.77 mfc, p ~ 0.0058; FP 100 nM 5.19 ± 0.84 mfc, FP 200 nM 4.63 ± 1.37 mfc, p ~ 0.008).

Since neither TNF-α nor IL-4 modified H-CAM expression, after the preliminary experiments the effect of the two drugs on this adhesion molecule was evaluated on unstimulated fibroblasts. Each concentration of FP or S alone significantly inhibited H-CAM expression (fig. 4). Particularly, FP tended to be stronger than S in inhibiting basal H-CAM expression, but these differences did not reach statistical significance (p ~ 0.1). In addition, S did not significantly modify the inhibitory activity of FP (p ~ 0.1; fig. 4). None of these effects induced by FP, S, or the combination of the two drugs, was associated with a statistically significant modification of cell viability (p ~ 0.1, each comparison; data not shown).

**Discussion**

Evaluating *in vitro*, a human foetal lung fibroblast cell line, it has been shown that long-acting β₂-agonist S is highly effective in inhibiting fibroblast proliferation and ICAM-1 and CD44 surface molecule expression. While no changes in cell proliferation were observed in the presence of FP, this drug was more effective than S in downregulating ICAM-1 and CD44 expression. Finally, associated S significantly...
INHIBITION OF FIBROBLAST ACTIVATION

The most surprising finding of the present study was that S was more effective than FP in inhibiting bFGF-induced fibroblast proliferation. β2-adrenoceptor agonists interact with specific cell membrane receptors, which activate signal transduction mechanisms leading to cellular responses that may be different in different cell types [14]. Indeed, in addition to inhibiting or reversing the contractile response of bronchial smooth muscles, these compounds modulate a variety of inflammatory and immuno-effector cell functions, including T-lymphocyte proliferation and mononuclear cell surface receptor expression [14, 16, 30]. Using a human foetal lung fibroblast cell line, this study has shown that S was highly effective in downregulating fibroblast proliferation and in reducing the expression of adhesion molecules that are likely to be involved in airway remodelling. In addition, at the two highest concentrations tested, S significantly increased the inhibitory activity of FP alone on ICAM-1 expression. These findings are in agreement with the results of a recent report, showing inhibition of ICAM-1 expression by human lung fibroblast primary cultures in the presence of budesonide and formoterol [31]. Indeed, β2-adrenergic receptor agonists may also activate glucocorticoid receptors in primary human lung fibroblasts [32].

Although more effective than S in reducing surface molecule expression, FP did not have a direct inhibitory activity on bFGF-induced fibroblast proliferation. Glucocorticosteroids influence not only protein translation, but also post-transcriptional processing of protein synthesis [33]. The complexity of the mechanisms involved in glucocorticoid activities may explain why cellular responses to these drugs are not only highly dependent on cell types, but also on cell functions [33–35].

Clinical studies demonstrate that in asthma, low-dose inhaled corticosteroids are not only able to...

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**Fig. 4.** – Effects of different concentrations of fluticasone propionate (●), salmeterol (○) or the association of the two drugs (△) on tumour necrosis factor-α (TNF-α) induced intracellular adhesion molecule (ICAM)-1 (CD54) expression (a) and on constitutive hyaluronic cellular adhesion molecules (H-CAM, CD44) expression (b) on surface of a human foetal lung fibroblast cell line. The different concentrations (0.1–100 nM) of the drugs are plotted against the mean fluorescence channel (mfc). Values expressed as mean ± SEM. □: unstimulated cells. *: p < 0.05; **: p < 0.01; ***: p < 0.001 versus control cell cultures grown in the absence of drugs.
downregulate the intensity of bronchial inflammation, but also to decrease subepithelial layer thickness [6, 14]. Indeed, in vivo corticosteroids may be effective in controlling tissue remodelling by inhibiting several aspects of the inflammatory process, such as regulation of the transcription of genes related to the production of cytokines able to activate leukocytes and lung parenchymal cells [5, 6, 31 – 33].

In summary, these data support at biological level the hypothesis that the combination of the two types of drugs, long-acting β2-adrenoceptor agonists and inhaled steroids, not only allows a better clinical control of asthma, but may also help to prevent long-term deterioration of lung function [1]. Although the concentrations of drugs used in vitro in this paper are similar to those found in peripheral lung tissue after 50 μg inhaled dose of salmeterol [35], the clinical importance of the experimental observations reported here deserves further investigation.

References

26. Damsky CH, Werb Z. Signal transduction by integrin receptors for extracellular matrix: cooperative process-