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Immunology Letters 112 (2007) 39–46
Immunology Letters
www.elsevier.com/locate/

Modulation of human lung fibroblast functions by ciclesonide: Evidence for its conversion into the active metabolite desisobutyryl-ciclesonide

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Received 23 May 2007; received in revised form 21 June 2007; accepted 26 June 2007
Available online 1 August 2007

Abstract

Background: Ciclesonide, an inhaled corticosteroid administered as inactive compound with almost no binding affinity for the glucocorticoid receptor, is clinically effective in asthma being converted by airway epithelial cells into its active metabolite desisobutyryl-(des)-ciclesonide.
Aim: To evaluate whether ciclesonide could directly modulate *in vitro* bronchial fibroblast functions being converted into des-ciclesonide by these pluripotent cells involved in the regulation of airway inflammation and remodelling.
Methods: Ciclesonide (0.09–9.0 μM) was added to a human adult lung fibroblast cell line (CCL-202), seeded in medium in the presence of the following cytokines and growth factors: (a) basic fibroblast growth factor (bFGF) for cell proliferation, measured by tritiated thymidine ([^{3}H]TdR) incorporation; (b) tumour necrosis factor (TNF)-α, to stimulate intercellular adhesion molecule (ICAM)-1 expression and monocyte chemoattractant protein-1 (MCP-1) and eotaxin release, evaluated by flow cytometry and ELISA, respectively; (c) transforming growth factor (TGF)-β_1, for induction of alpha smooth muscle actin (α-SMA) protein expression and modification of the organization of α-SMA stress fibres, evaluated by Western blot analysis and fluorescence microscopy.
Results: The presence of ciclesonide in cell cultures induced a significant downregulation of: (a) bFGF-induced fibroblast proliferation and TNF-α-induced ICAM-1 expression, at the 0.3–9.0 μM concentrations ($p < 0.05$); (b) TNF-α-induced MCP-1 release, at all the concentrations tested ($p < 0.05$); (c) TNF-α-induced eotaxin release, at the three highest concentrations (0.9–9.0 μM) ($p < 0.05$); (d) TGF-β_1-induced of α-SMA protein expression at the 0.3–3.0 μM concentrations, associated with a reduction in the organization of α-SMA stress fibres.
Conclusions: These data show at cellular level an effective anti-inflammatory activity of ciclesonide on human lung fibroblasts and support the hypothesis that also these cells, in addition to airway epithelial cells, may be involved in converting the parental compound into its active metabolite in the airways.

Keywords: Corticosteroid; Adhesion molecule; Chemokines; Myofibroblasts; Alpha smooth muscle actin

1. Introduction

Ciclesonide, the most recently developed inhaled corticosteroid for the treatment of asthma, is administered as a parent compound with almost no binding affinity for the glucocorticoid receptor [1–3]. Activation of ciclesonide occurs upon ester cleavage by endogenous esterases in the airways to form desisobutyryl-ciclesonide (des-ciclesonide) and this on-site activation results in a 100-fold greater relative glucocorticoid receptor binding affinity than ciclesonide [4,5]. Des-ciclesonide subsequently undergoes reversible esterification to fatty acids to form various fatty acid conjugates that may act as a storage pool for the slow release of des-ciclesonide in the lung [5–7]. Ciclesonide and fatty acid conjugates of des-ciclesonide are highly lipophilic [6–8], which may contribute to the high rate of absorption of ciclesonide and the subsequent retention of fatty acid esters in the lung.

Preliminary experiments suggest that human bronchial epithelial cells are highly effective in activating the drug, with detectable anti-inflammatory activity within 3 h, explaining the

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doi:10.1016/j.imlet.2007.06.010

high anti-inflammatory properties demonstrate in *in vivo* studies on patients with chronic inflammatory airway disorders, such as asthma [5]. Studies addressing whether other cellular components in the airway walls, in addition to bronchial epithelial cells, possess endogenous esterases to convert ciclesonide into its active metabolite are lacking. This could be a key issue, considering both the pharmacokinetic characteristics of the drug and the elevated numbers of cells involved in the pathogenesis of asthma [9]. Indeed, in the last decades it has become clear that, in addition to inflammatory cells, structural components of the airways, including fibroblasts, play a key role in modulating the inflammatory reaction and the repair processes that characterize the disease [9,10]. Indeed, fibroblasts have the capability to directly contribute to asthma pathogenesis, releasing a variety of chemokines and cytokines, expressing surface molecules, proliferating, producing extracellular matrix components and evolving into various cell types, including myofibroblasts [11,12].

It is not surprising, therefore, that because of the variety of pro-inflammatory and profibrotic functions displayed, airway fibroblasts should represent an important target for therapeutic interventions. Since these cells possess glucocorticosteroid receptors (GR) and the pharmacologic activity of these drugs may involve cell functions related both to inflammation and airway remodelling [13,14], it seemed interesting to test whether and to what extent ciclesonide could be activated into desciclesonide by lung fibroblasts to modulate their functions known to be effectively regulated by inhaled corticosteroids [13,14].

A study was therefore designed using a human adult lung fibroblast cell line to evaluate whether the presence of increasing concentrations of ciclesonide in cell culture medium could modulate *in vitro* a variety of fibroblast functions induced by specific cytokines and growth factors, i.e. cell proliferation, adhesion molecule expression, chemokine release and differentiation into alpha smooth muscle actin (α-SMA)-positive myofibroblasts.

2. Materials and methods

2.1. Fibroblast culture

Human adult lung fibroblast cell line (CCL-202), obtained from the American Type Culture Collection (Manassas, VA), was used in all the experiments performed. The cells were cultured in 75-cm^2 tissue culture flask with high-glucose medium Dulbecco's Modified Eagle medium supplemented with 10% FCS and penicillin/streptomycin (5000 IU/ml). The fibroblasts were passaged every ∼7 days after reaching confluence by dissociating monolayer with 1:1 0.05% trypsin and 0.1% ethylenediaminetetraacetate solution (EDTA). The medium was then removed and replaced with fresh serum-free DMEM containing, respectively: (a) basic fibroblast growth factor (bFGF) to evaluate cell proliferation; (b) tumour necrosis factor (TNF)-α to evaluate adhesion molecule expression and chemokine release; (c) transforming growth factor (TGF)-β_1 to measure α-SMA expression [14,15]. Fibroblasts used in the present study were between cell passages 5 and 15.

2.2. Evaluation of cell proliferation

Cells were stimulated for 24 h with bFGF (5.0 ng/ml), i.e. the lowest concentration found in preliminary experiments to be active in inducing CCL-202 fibroblast proliferation. Unstimulated fibroblasts were used as negative control. At the end of incubation period, DNA synthesis was measured as tritiated thymidine ([^{3}H]TdR) incorporation (Amersham International, Little Chalfont, Buckingham Shire, England), as previously described [14] and expressed as count per minute (cpm). Each experiment was carried out in triplicate. Cell viability was evaluated by Trypan blue dye exclusion test (Euroclone Ltd., Paignton, Devon, UK) after 24-h stimulation [14].

To evaluate the effect of ciclesonide on cell proliferation, fibroblasts were stimulated with bFGF in the presence of different concentrations (0.09–9.0 μM) of the drug. The vehicle used for corticosteroid dilution was DMSO and the final concentration of vehicle was <0.5%. At this concentration, DMSO did not affect cell viability, as assessed by Trypan blue dye exclusion.

2.3. Evaluation of adhesion molecule expression on human lung fibroblasts

To evaluate adhesion molecule expression, cells were grown into 24-well plate and stimulated for 24 h with TNF-α 5.0 ng/ml, i.e. the lowest concentration found, in preliminary experiments, to be active in stimulating CCL-202 fibroblast cell line. Then, the cells were trypsinized, collected, washed twice in DMEM and 100 μl per well of the cell suspension were placed into round-bottom microtiter 96-well plate (Costar Corporation, Cambridge, MA, USA). After incubation for 30 min at 4 °C with fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies (mAb) anti-human (ah)-ICAM-1 (CD54) (Caltag Laboratories, Burlingame, CA, USA), the cells were washed twice, fixed with 0.5% paraformaldehyde and then analyzed by immunofluorescence flow cytometry (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) [14].

To compare the fluorescence intensities of different samples from the same experiments, identical settings of the logarithmic amplifier were used and listmode files were analyzed with CELLQuest software (Becton Dickinson) as previously described [14]. All experiments were performed in triplicate. The intensity of fluorescence was expressed as mean fluorescence channel (mfc) [14]. To evaluate the effect of ciclesonide on adhesion molecule expression, fibroblasts were stimulated with TNF-α in the presence of different concentrations (0.09–9.0 μM) of the drug. Evaluation of ICAM-1 was performed as described above.

2.4. Chemokine release by human lung fibroblast

Monocyte chemoattractant protein-1 (MCP-1) and eotaxin concentrations in the cell supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (MCP-1: Amersham Biosciences and Eotaxin: Biosource International, Camarillo, CA, USA) [14].

To evaluate MCP-1 and eotaxin levels, cells (60,000 cells per well) were stimulated for 24 h with TNF-α (5.0 ng/ml). Unstimulated fibroblasts and cells cultured with the stimulus alone were used as negative or positive control, respectively. At the end of each incubation time, the culture supernatants were collected and kept frozen until being tested.

To evaluate the effect of ciclesonide on chemokine release, fibroblasts were stimulated with TNF-α in the presence of different concentrations (0.09–9.0 μM) of the drug. The measurement of MCP-1 and eotaxin was carried out according to the manufacturer's instructions [12] and expressed as pg/ml. Respectively the detection limits were 3.5 and 2.2 pg/ml for MCP-1 and eotaxin. The reproducibility of the assays for MCP-1 was as follows: intra-assay CV, 7.7%; inter-assay CV, 6.2%. The reproducibility of the assays for eotaxin was as follows: intra-assay CV, 4.9%; inter-assay CV, 5.8%.

2.5. Evaluation of α-SMA expression by human lung fibroblasts

Confluent human lung fibroblasts were growth-arrested by incubating in serum-free DMEM for 48 h before stimulating them with TGF-β_1 (Sigma–Aldrich Company, St. Louis, MO, USA) (10 ng/ml) for additional 48 h. To evaluate the inhibitory effect of ciclesonide, different concentrations of drug (0.3 or 3.0 μM) were added to human lung fibroblast cultures, at the beginning of the stimulation period. After 48 h incubation, myofibroblasts differentiation was evaluated as α-SMA expression by Western blotting for protein contents and by immunofluorescence analysis for cytoskeletal organization.

Western blot analysis was performed as described previously [16]. Briefly, human airway fibroblasts were washed with cold phosphate-buffered saline and resuspended in lysis buffer. Equal amounts of total protein [17] were loaded to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a PVDF membrane (Immobilon-P, Millipore, Bedford, MA). The membranes were blocked with 3% milk in Tris-buffered saline containing 0.05% Tween 20 and incubated with a mouse monoclonal IgG against the anti-human α-SMA monoclonal antibody (DakoCytomation, Gloslurp, Denmark) and the anti-human beta-actin (clone C4) (Boehringer Mannheim Inc., Mannheim, Germany). After incubation with the appropriate peroxidase-conjugated secondary antibody (1:1000, SuperSignal West Dura Extended Duration Substrate, Pierce Inc.), the bands were visualized by the enhanced chemiluminescence system (SuperSignal West Dura Extended Duration Substrate, Pierce Inc.) and relevant band intensities were quantified using a Versadoc Imaging System model 3000 (Bio-Rad Laboratories Inc., Hercules, CA).

Organization of α-smooth muscle actin was evaluated by immunofluorescence according to conventional indirect technique [15]. Briefly, fibroblasts were culture on glass coverslips (0.13×10^6 cells per glass) and stimulated for 48 h with TGF-β1 (10 ng/ml) in presence of ciclesonide (0.3 or 3.0 μM). Unstimulated fibroblasts were used as negative control while cells stimulated with TGF-β1 alone were used as positive control. After incubation, human airway fibroblasts were fixed in methanol for 5 min at −20 °C and then stained with mouse monoclonal antibodies against human α-smooth muscle actin (1:10; DakoCytomation, Gloslurp, Denmark) or with isotype matched control mouse IgG (Immunotech, Westbrook, ME, USA). FITC-conjugate goat anti-mouse IgG (heavy and light chain) (1:10. Immunotech, Beckman Coulter Company, Marseille, France) was used as secondary antibody. Slides were then cover-slipped using the Vectashield fluoromount (Vector Laboratories Inc., Burlingame, CA, USA) and stored at −20 °C until fluorescence analysis. The percentage of positive cells was determined on a total of 100 cells distributed in four different fields.

2.6. Evaluation of the concentrations of ciclesonide and des-ciclesonide in fibroblast culture supernatants

Culture supernatants from fibroblasts, cultured for 24 h in the presence of the highest concentration of ciclesonide (3.0 μM), with medium alone or with TNF-α, were analyzed for ciclesonide and des-ciclesonide concentrations by a high-performance liquid chromatography (HPLC) method using UV-detection [5]. Prior to HPLC, the samples were prepared by protein precipitation. The lower limit of quantification was 0.4 μM for both ciclesonide and des-ciclesonide.

2.7. Data and statistical analysis

Statistical analysis was performed using the statistical software package GraphPad Prism 3.02 (GraphPad Software Inc., San Diego, CA, USA). Mann–Whitney *U*-test or Student's *t*-test and Kruskal–Wallis or ANOVA were used to compare data, when appropriate. Data parametrically distributed are presented as arithmetic mean ± standard error of the mean. A probability value (p) lower than 0.05 was set to indicate the level of statistically significant difference.

3. Results

3.1. Inhibitory effects of ciclesonide on bFGF-induced fibroblast proliferation

At a concentration of 5 ng/ml, bFGF induced a significant upregulation of fibroblast DNA synthesis, measured as [^{3}H]TdR incorporation ($p < 0.01$), that was inhibited, in a concentration-dependent fashion, by ciclesonide ($p < 0.0001$) (Fig. 1A and B). The downregulating effect was statistically significant starting at the dose of 0.3 μM, while maximal inhibition was observed at the highest ciclesonide concentration (9.0 μM) and was 66.01 ± 2.49%. The inhibitory activity observed was not related to a toxic effect of the drug since fibroblast viability, determined by Trypan blue dye exclusion test, was ≥98% at the end of the incubation periods (not shown).

3.2. Inhibitory effects of ciclesonide on TNF-α-induced adhesion molecule expression and chemokine release

TNF-α, 5 ng/ml, was effective in enhancing ICAM-1 expression on fibroblasts after 24 h-incubation ($p < 0.01$) (Fig. 2A

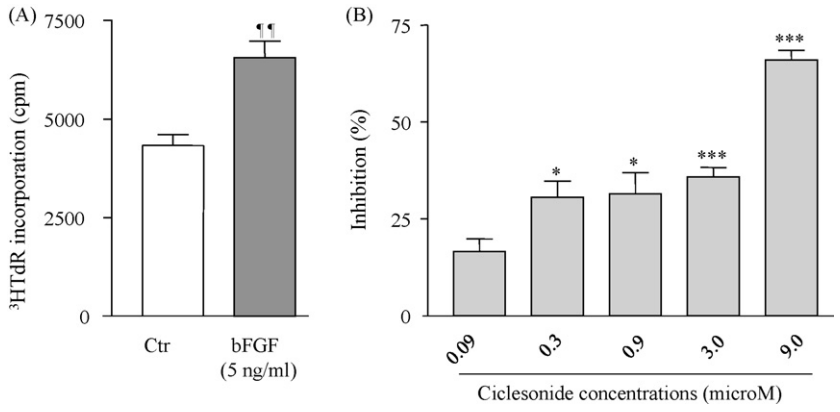

Fig. 1. Basic fibroblast growth factors (bFGF)-induced cell proliferation and effect of different concentrations of ciclesonide on the proliferative response of a human adult lung fibroblast cell line (CCL-202). (A) The effect of bFGF on cell proliferation, evaluated as tritiated thymidine ([^{3}H]TdR) incorporation and expressed as count per minute (cpm) is shown on the ordinate, whereas the different experimental conditions are reported on the abscissa. (B) The percentage of inhibition is shown on the ordinate whereas the different concentrations (0.09– μM) of the drug are reported on the abscissa. The data, representing the results of 12 experiments, are expressed as mean ± standard error of the mean. ¶¶$p < 0.01$, *vs.* unstimulated cells; *$p < 0.05$, ***$p < 0.001$, *vs.* cells grown in the absence of the drug.

and B) as illustrated by the representative flow-cytometry histograms of ICAM-1 expression shown on the top of the figure. When ciclesonide was added to the culture medium, the TNF-α-induced upregulation in ICAM-1 expression was inhibited in a dose-dependent fashion ($p < 0.0001$), statistically significant starting at 0.3 μM (Fig. 2C). Maximal inhibition was observed at the highest ciclesonide concentration (9 μM) and was 34.94 ± 5.36%.

Evaluating chemokine released by unstimulated fibroblasts, we found that, after 24-h incubation, the levels of MCP-1 were over fivefold higher than those of eotaxin (803.6 ± 215.9 and 157.7 ± 37.7 pg/ml, respectively; $p < 0.001$). Independently from the degree of the constitutive activity, TNF-α (5 ng/ml) induced a similar ∼2-fold increase of both chemokines released ($p < 0.05$, each comparison), significantly downregulated, in a dose-dependent fashion by ciclesonide (Figs. 3A and B and 4A and B). However, the efficiency of the inhibitory activity seemed to be different for the two chemokines, being statistically significant for MCP-1 release at all the concentrations tested, i.e., from 0.09 to 9.0 μM ($p < 0.05$,

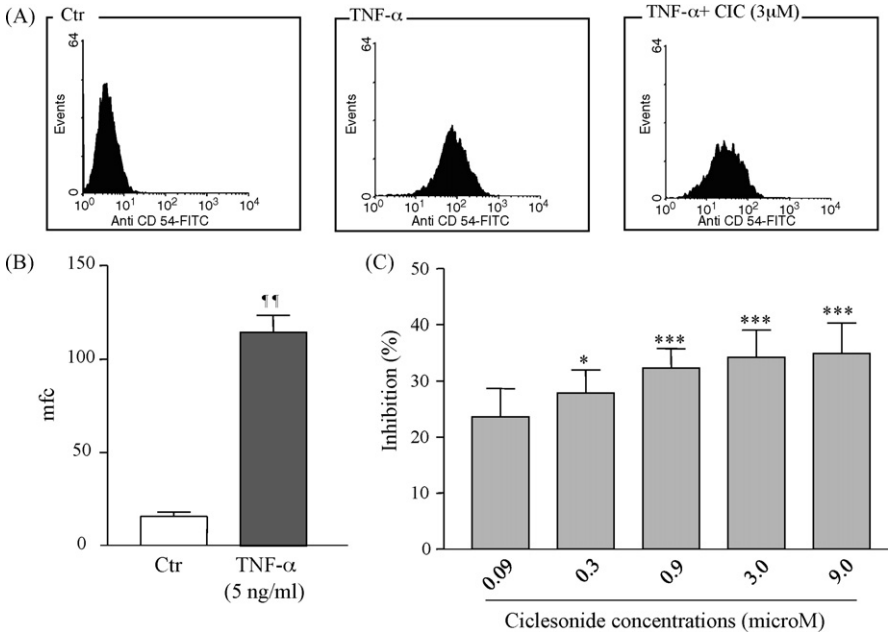

Fig. 2. Effect of tumour necrosis factor (TNF)-α on intercellular adhesion molecule (ICAM)-1 expression by a human adult lung fibroblast cell line (CCL-202) and effects of different concentrations of ciclesonide on the expression of this adhesion molecule. (A) A representative flow-cytometry histograms of ICAM-1 expression under basal conditions (Ctr), or after stimulation with TNF-α (TNF-α) or in the presence of ciclesonide at the highest concentration [TNF-α + CIC (3 μM)]: the fibroblast number is reported on the ordinate and the green fluorescence intensity given by anti-human CD54 FITC-conjugated monoclonal antibody on the abscissa as mean fluorescence channel (mfc). (B) The effect of TNF-α on ICAM-1 expression expressed as mean fluorescence channel (mfc) is shown on the ordinate, whereas the different experimental conditions are reported on the abscissa. (C) The percentage of inhibition is shown on the ordinate whereas the different concentrations (0.09–9 μM) of the drug are reported on the abscissa. The data are expressed as mean ± standard error of the mean. ¶¶$p < 0.01$, *vs.* unstimulated cells; *$p < 0.05$, ***$p < 0.001$, *vs.* cells grown in the absence of the drug.

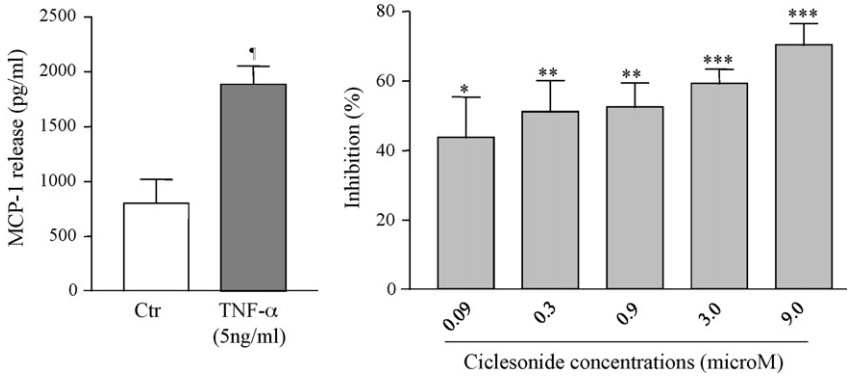

Fig. 3. Effect of tumour necrosis factor (TNF)-α on monocyte chemoattractant protein-1 (MCP-1) release by a human adult lung fibroblast cell line (CCL-202) and effects of different concentrations of ciclesonide on MCP-1 release. (A) The effect of TNF-α on MCP-1 release expressed as pg/ml is shown on the ordinate, whereas the different experimental conditions are reported on the abscissa. (B) The percentage of inhibition is shown on the ordinate whereas the different concentrations (0.09–9 μM) of the drug are reported on the abscissa. The data are expressed as mean ± standard error of the mean. ¶$p < 0.05$, *vs.* unstimulated cells; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, *vs.* cells grown in the absence of the drug.

each comparison) (Fig. 3B) while, on eotaxin release, the statistically significance was observed only at the highest three concentrations (0.9, 3.0 and 9.0 μM) (Fig. 4B). Maximal inhibition was observed at the highest ciclesonide concentration (9 μM), being 70.25 ± 6.32% for MCP-1 and 85.80 ± 3.59% for eotaxin release. The inhibitory activity on ICAM-1 expression and on MCP-1 and eotaxin release was not related to a toxic effect of the drug since fibroblast viability, determined by Trypan blue dye exclusion test, was always ≥98% at the end of the incubation period (not shown).

3.3. Effect of ciclesonide on myofibroblast differentiation

α-SMA expression at protein level, protein content and organization and number of α-SMA positive cells were evaluated. Unstimulated fibroblasts showed by Western blot analysis a weak endogenous expression of α-SMA protein, enhanced by the presence of TGF-β_1 (10 ng/ml) in the culture medium which was effectively inhibited by ciclesonide (0.3 or 3.0 μM) (Fig. 5A, top panel). Quantitative evaluation demonstrated that the amount of α-SMA protein in ciclesonide (0.3 or 3.0 μM) treated fibroblasts was also significantly lower than the constitutive expression, detected in untreated cells (Fig. 5A, bottom panel). However, as compared with control cultures, the number of α-SMA positive myofibroblasts was higher in cell cultures exposed to TGF-β_1 but also in those exposed to both ciclesonide concentrations.

Immunofluorescence analysis demonstrated the enhanced α-SMA actin expression by TGF-β_1 was due to an increase in the number of α-SMA positive cells as well as to polymerization of α-SMA filaments (Fig. 5B and C). In the presence of ciclesonide (0.3 and 0.3 μM), a decrease in the number of cells expressing α-SMA was observed, with a clear reduction in the organization of α-SMA stress fibres (Fig. 5D and E).

Taken together, these data suggest that the inhibitory effect of ciclesonide on TGF-β_1-induced myofibroblast differentiation is related to the ability of the drug to interfere on both α-SMA filaments synthesis and polymerization.

3.4. Evaluation of the concentrations of ciclesonide and des-ciclesonide in fibroblast culture supernatants

Evaluation of culture supernatants from fibroblasts grown for 24 h in the presence of ciclesonide 3 μM, demonstrated only the

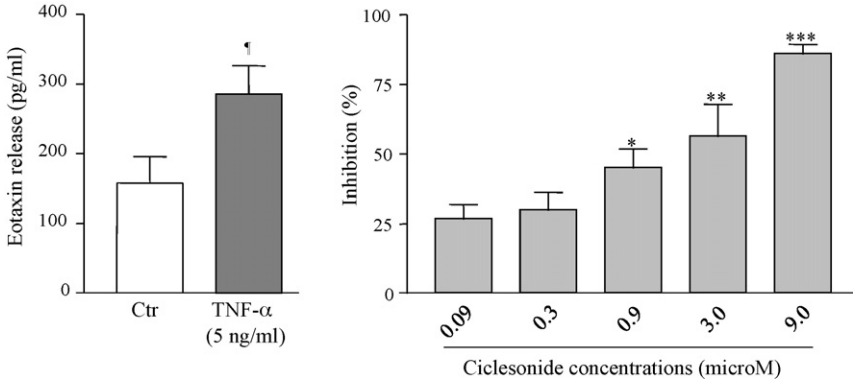

Fig. 4. Effect of tumour necrosis factor (TNF)-α on eotaxin release by a human adult lung fibroblast cell line (CCL-202) and effects of different concentrations of ciclesonide on eotaxin release. (A) The effect of TNF-α on eotaxin release expressed as pg/ml is shown on the ordinate, whereas the different experimental conditions are reported on the abscissa. (B) The percentage of inhibition is shown on the ordinate whereas the different concentrations (0.09–9 μM) of the drug are reported on the abscissa. The data are expressed as mean ± standard error of the mean. ¶$p < 0.05$, *vs.* unstimulated cells; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, *vs.* cells grown in the absence of the drug.

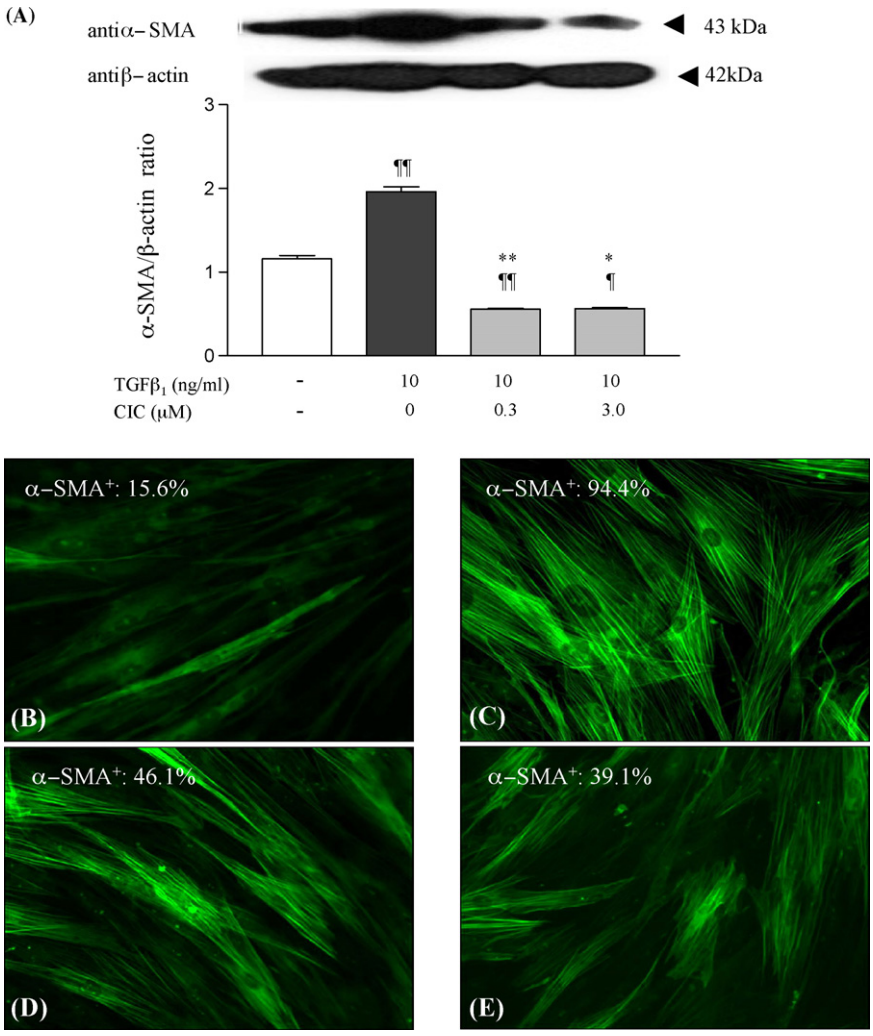

Fig. 5. Effect of different concentrations of ciclesonide (0.3–3.0 μM) on myofibroblasts differentiation in human lung fibroblasts (CCL-202) induced by transforming growth factor-β_1 (10 ng/ml). Panel (A) Western blot analysis of α-smooth muscle actin (α-SMA) expression in human lung fibroblasts. Whole-cell lysates (40 μg of total protein/lane) were subjected to 10% SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and probed with anti-α-SMA or anti-β-actin antibodies as described in Section 2. (Top panel) Representative blottings for α-SMA and β-actin (top panel) are shown from one of three experiments, which gave similar results. Arrows indicate the molecular size of the α-SMA (43-kDa) and β-actin (42-kDa) characteristic bands, respectively. (Bottom panel) After densitometric analysis, data were normalised to β-actin and plotted as mean ± standard error of the mean. ¶¶$p \leq 0.01$, *vs.* unstimulated cells; *$p \leq 0.05$, **$p \leq 0.01$, *vs.* cells grown in the absence of the drug. Panels (B–E) α-SMA organization in human lung fibroblasts (CCL-202). Fibroblasts were stimulated for 48 h with TGF-β1 (C) in presence or absence of ciclesonide (0.3 or 3.0 μM) (D, E, respectively). Unstimulated fibroblasts were used as negative control (B). After stimulation, cells were stained with anti-human α-smooth muscle actin and viewed under fluorescent microscope as described in Section 2. Original magnification 20×.

presence of detectable amounts of des-ciclesonide but not of the parental compound ciclesonide while, when incubated in culture medium without cells, ciclesonide appeared to be stable with no detectable amounts of des-ciclesonide.

4. Discussion

Evaluating an adult lung fibroblast cell line stimulated by mediators involved in the pathogenesis of asthma, we demonstrated that ciclesonide was able to downregulate a variety of functions involved in airway inflammation and remodelling. These included cell proliferation, adhesion molecule expression, chemokine release and differentiation into α-SMA-positive myofibroblasts. Since ciclesonide was added to cell cultures as an inactive compound, these results show that, in addition to airway epithelial cells, also lung fibroblasts may activate the parent compound in the pharmacodynamically active principle des-ciclesonide to exert anti-inflammatory functions on the same cells.

Ciclesonide, an inhaled corticosteroid developed for the treatment of asthma, possesses pharmacokinetic characteristics which are associated with high efficacy, favourable tolerability, and low risk for systemic side effects [1,8]. *In vitro* studies have demonstrated the conversion of ciclesonide to des-ciclesonide in animal and lung tissues [3,4], and specifically that human bronchial epithelial cells can activate the parent compound to exert anti-inflammatory activity within 3 h [5], with the subsequent fatty acid esterification of the active metabolite [6,7]. Because of the high rate of absorption of the drug with the possible subsequent retention in the lung structures [2], it was

important to evaluate whether, in addition to airway epithelial cells, other cellular constituents of the respiratory tissues, i.e. fibroblasts, could able to convert ciclesonide to its pharmacodynamically active principle with modulation of a variety of functions related to inflammation and remodelling.

The role of fibroblasts in the airways was thought to be exclusively the maintenance of bronchial and alveolar structure integrity [12]. However, recent evidences strongly suggest that these cells may also be integral components of the inflammatory responses that characterize chronic airway disorders, such as asthma [11,12,18]. Indeed fibroblasts, in response to cytokines and growth factors have the ability not only to secrete intercellular matrix components, but also release immunomodulatory products, express surface receptors important for cell adhesion and leukocyte activation, and differentiate into myofibroblasts, cells that may contribute to the enhancement of bronchial constriction through the expression of contractile proteins [11,12,18].

The CCL-202 cell line was selected for the studies here presented because, in preliminary experiments, it reacted to growth factors and cytokine stimulation similarly to human airway fibroblast primary cultures [19,20]. Indeed, CCL-202 fibroblast showed a significant proliferative response to bFGF, increased ICAM-1 expression and MCP-1 and eotaxin release when stimulated with TNF-α, and enhanced α-SMA expression in response to TGF-β_1.

The effects of glucocorticosteroids on airway fibroblast growth are still controversial [14,21,22]. Using adult fibroblasts, we clearly demonstrated that ciclesonide was able to significantly downregulate the bFGF-induced DNA synthesis, even if a strong inhibition was detected only at the highest drug concentrations (9.0 μM). The different results reported in the literature are probably due to the differences fibroblast characteristics (degree of differentiation, tissue of origin, health versus disease) rather than to the specific potency of the different molecules.

In agreement with previous reports [14,19,23,24], we found that the different fibroblast activities appear to be regulated by ciclesonide at different levels, ranging from 17 to 66% for cell proliferation, from 24 to 35% for ICAM-1 expression and respectively from 44 to 70% and 27 to 86% for MCP-1 and eotaxin release, while quantitative evaluation by Western blot showed that the amount of α-SMA protein in ciclesonide treated fibroblasts was even below the extent of the constitutive expression, detected in untreated cells.

These findings are consistent with the concept that the efficacy of these drugs may be not only cell-specific but also stimulus-specific [25,26]. In addition, when evaluating the effects of the drugs on all the fibroblast activities, the extended concentration-response curves were still incomplete, being nowhere near the “no effect levels”, also with the lowest concentrations of steroid used (0.09 μM).

The present study demonstrated that ciclesonide not only inhibited fibroblast differentiation into myofibroblast by downregulating α-SMA protein expression but also interfered with α-SMA polymerization. In addition to being a marker differentiation, α-SMA is thought to be functionally important for myofibroblast contraction, being expressed throughout the philogenetic tree in cells whose main function is contraction and exerting tension on cytoskeletal components, such as microtubules or intermediate filaments, organized as a tensegrity structure [27,28].

The demonstration that ciclesonide modulates α-SMA filaments synthesis and polymerization suggests that treatment with this drug could improve asthma symptoms not only through inhibition of classical inflammatory signals but also by acting on contractile properties of myofibroblasts.

Besides acting directly on GR-sensitive genes, the GR complex can indirectly alter gene transcription by activating transcription factors and the complexity of the mechanisms involved in glucocorticoid activities may explain why cellular responses are highly dependent not only on cell types but also on cell functions and on mechanisms involved in cell activation [27,29].

In our experimental system we did not evaluate at what extent ciclesonide can influence protein translation or post-transcriptional processing of protein synthesis. Nevertheless the data here presented show that fibroblasts are able to convert ciclesonide into the pharmacodynamically active principle and extend the profile of the anti-inflammatory activity of the drug to these cells. At biological level, these data support the hypothesis that ciclesonide, besides achieving a good clinical control of asthma, may also help in preventing the long-term deterioration in lung function described in this disease [30].

Although the concentrations of drugs used *in vitro* in this paper are similar to the concentrations obtained in airway tissue [2], the clinical relevance of the experimental observations here reported deserves further clinical investigations.

Acknowledgement

This work was supported by a grant from ALTANA Pharma S.p.A., Italy.

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