

Original article

In vitro effects of flunisolide on MMP-9, TIMP-1, fibronectin, TGF- β 1 release and apoptosis in sputum cells freshly isolated from mild to moderate asthmatics

Background: Corticosteroids play an important role in inflammation and remodelling of airways and are considered an important therapeutic target in asthma. Inflammation in asthma is characterized by a dysregulation of eosinophil apoptosis and of markers of airways remodelling. We evaluated the ability of flunisolide to inhibit *in vitro* the release of metalloproteinases-9 (MMP-9), tissue inhibitor metalloproteinases-1 (TIMP-1), transforming growth factor (TGF- β) and fibronectin by sputum cells (SC) as well as to induce sputum eosinophil apoptosis.

Methods: The SC, isolated from induced sputum samples of 12 mild-to-moderate asthmatics, were cultured for 24 h in the presence or absence of flunisolide (1, 10 and 100 μ M). The release of mediators was assessed by enzyme-linked immunosorbent assay (ELISA) whereas apoptosis was studied by TUNEL technique.

Results: Flunisolide (10 μ M) significantly reduced MMP-9 and TIMP-1 ($P = 0.0011$ and $P < 0.0001$ respectively) and increased MMP-9/TIMP-1 molar ratio ($P = 0.004$). In addition, flunisolide decreased TGF- β and fibronectin release by SC ($P = 0.006$; and $P < 0.0001$ respectively) and increased eosinophil apoptosis ($P < 0.001$).

Conclusions: These results demonstrate that flunisolide may play an important role in the inhibition of airway inflammation and remodelling, by promoting the resolution of eosinophilic inflammation and by inhibiting the release of MMP-9, TIMP-1, TGF- β and fibronectin.

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Key words: airway remodelling; asthma; flunisolide; metalloproteinases; sputum cells.

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Accepted for publication 23 December 2003

Asthma is characterized by airway inflammation and remodelling (1). Increasing evidence show that airway remodelling is a multi-step process leading to structural and geometric alterations of the airways and ultimately to several potential consequences. Although several mechanisms can contribute to the changes of the airway architecture, great emphasis has been attributed to those involved with the control of extracellular matrix synthesis and deposition (1). Indeed, it is believed that an increased production of extracellular matrix (ECM) components together with their reduced degradation can lead to an abnormal deposition with a consequent increased thickness of the airway wall.

Thus, the ECM is a dynamic structure and an equilibrium between synthesis (2) and degradation of ECM components is required for the maintenance of its homeostasis. Although many proteases can cleave ECM molecules, Zn²⁺-matrix metalloproteinases (MMPs) and their inhibitors are likely the normal physiologically relevant mediators of ECM degradation (3, 4). At least three subclasses of MMPs have been identified: collagen-

ases, gelatinases and stromelysins. They are mainly synthesized by connective tissue cells, granulocytes and monocyte-macrophages (5). The major physiological inhibitors of MMPs are the broad spectrum serum inhibitor α 2-macroglobulin and a special class of tissue inhibitors of metalloproteinases (TIMPs) (3). The balance between MMPs and their inhibitors is critical in tissue repair and remodelling, and its homeostasis plays an important role in the breakdown and deposition of ECM in the airways wall.

The ECM synthesis and deposition is also modulated by several growth factors (6). Among them, transforming growth factor (TGF- β) has been implicated in airway remodelling and pulmonary fibrosis (7, 8). The TGF- β family comprises several isoforms of TGF- β , which can be generated by cells including macrophages, epithelial cells, fibroblasts and eosinophils (9–13) and is considered to be a major fibrogenic cytokine (6, 13). In a normal lung, the bronchial epithelium appears to be the main location for TGF- β (14). Virtually all cells have specific membrane receptors for TGF- β (15).

The effects of corticosteroids on the release of ECM components as well as on factors modulating ECM homeostasis still remain controversial. Indeed, while some study have failed to show an effect on ECM synthesis and deposition, other have demonstrated that steroids have the potential to affect key mechanisms involved in ECM remodelling.

In the present study, we assessed the ability of flunisolide to inhibit the release of MMP-9, TIMP-1, TGF β and of fibronectin and to increase the eosinophil apoptosis by sputum cells (SC) isolated from asthmatic subjects with mild-to-moderate asthma.

Materials and methods

Patients

The study was performed in 12 subjects with mild-to-moderate asthma diagnosed and classified according to the GINA guidelines (16) and as previously described in detail (17).

None of the subjects was current or previous smoker. Patients were excluded from the study if they had undergone a severe exacerbation of asthma requiring hospitalization during the month preceding the study. Inhaled corticosteroids or oral corticosteroids had been withdrawn for at least 2 months prior to the commencement of the study, while the use of nedocromil sodium or cromoglycate had been stopped for at least 2 weeks and theophylline in the previous 48 h. The study was approved by the appropriate Ethics Committee and the patients gave informed consent.

Sputum induction and processing

Sputum induction and processing were performed according to the methods of Hargreave et al. without modification (18). Differential cell counts were performed on cytocentrifuge preparations stained with May-Giemsa-Gruenwald. In all cases 200 nonsquamous were counted by two blind observers and results are expressed as percentage of total nonsquamous cells.

Treatment of sputum cells with flunisolide

The SC were resuspended at the concentration of 1×10^6 /ml in RPMI 1640, 10% heat-inactivated (56°C, 30 min) fetal calf serum (FCS), 1% penicillin-streptomycin solution and 1 mmol/l L-glutamine (RPMI buffer). The SC were cultured in the absence or presence of concentrations of flunisolide ranging from 1 to 100 μ M for 24 h at 37°C in a humidified 5% CO₂ according to previous *in vitro* studies (19). At the end of the incubation time the cell viability was >85%, as assessed by trypan blue exclusion and supernatants were harvested and stored at -80°C for further analysis (MMP-9, TIMP-1, TGF β and fibronectin). Furthermore, cells of each experimental condition were recovered and fixed on slides to perform the TUNEL analysis on stimulated induced SC.

Measurement of MMP-9 and TIMP-1

Determinations of the absolute values of MMP-9 and TIMP-1 in supernatants of SC-stimulated in the presence or absence of flunisolide were assessed using commercially available specific enzyme-linked immunosorbent assay kits (ELISA) (Biotrak, Amersham International plc, Little Chalfont, UK) according to manufacturer's

instruction as previously demonstrated (20). These assays measure the total amount of each respective protein (MMP-9 and TIMP-1), whether free or complexed to one another or to matrix. The limits of detection was 0.6 ng/ml for MMP-9 and 1.25 ng/ml for TIMP-1.

Measurement of active TGF- β 1 in supernatants

The TGF- β 1 was measured in the supernatant of SC-stimulated in the presence or absence of flunisolide by ELISA (Predicta TGF- β 1 Kit, Genzyme, Cambridge, MA). In order to measure biologically active TGF- β 1, the samples were acidified using HCl for 1 h (21). After acidification the samples were neutralized to pH 7.0–7.4 with NaOH and measurements of TGF- β 1 were performed immediately. The detection limit of the assay was 0.05 ng/ml.

Measurement of fibronectin in supernatants

Fibronectin was measured in the supernatant of SC-stimulated in the presence or absence of flunisolide according to the technique of Campbell et al. (21) using a highly specific indirect ELISA assay utilizing a sandwich method, on dishes coated with anti-FN antibodies. The antibodies and standards were obtained from Calbiochem (La Jolla, CA). The limit of detection was 50 pg/ml.

Apoptosis evaluation

Apoptosis was assessed on slides fixed in paraphormaldehyde solution [4% in phosphate-buffered saline (PBS), pH 7.4] for 20 min and then for 20 min in ethanol absolute (25°C), using the TUNEL technique by an *in situ* cell death detection kit (Roche, Indianapolis, IN), as previously described (22). A total of 400 cells per slide were counted by two independent observers (R.D., M.P.) with use of a light microscope (Leica, Wetzlar, Germany). Results were expressed as percentage of positive cells. The inter-investigator coefficient of agreement was excellent ($\kappa = 0.96$).

Statistical analysis

Results of induced SC were expressed as medians and 25–75th percentiles. Statistical analysis of the effects of supernatants of SC cultured in the presence or absence of flunisolide, was performed with ANOVA test with Bonferroni Dunn correction.

Results

Demographic and cellular characteristics of patients

The median age of the asthmatic patients was 34 years as median and 25–75th percentiles 29–46. Forced expiratory volume (FEV₁) was 94% as median and 25–75th percentiles 76.2–101.5%. All asthmatic patients were no smoker subjects and of them seven were male. Analysis of SC is represented in Table 1. Asthmatic patients showed an elevated percentage of eosinophils.

Effects of flunisolide on MMP-9 and TIMP-1 release by SC

At baseline MMP-9 and TIMP-1 released by cultured SC was 52 (28–66.5) and 200 (110–265) ng/ml respectively; MMP-9/TIMP-1 molar ratio was 0.2 (0.2–0.3). Flunisolide

Table 1. Cell analysis of sputum samples

	Asthma
Squamous cells (%)	27.6 (15.4–40.4)
Corrected cell counts (million cell/ml)	0.92 (0.65–1.45)
Differential cell counts (%)	
Macrophages	36.9 (27.4–52.8)
Neutrophils	41.1 (24.6–52.6)
Lymphocytes	0.5 (0.2–0.9)
Eosinophils	13.7 (6.7–24.5)
Epithelial cells	0.00 (0.00–1.8)

Results are expressed as median with 25–75 percentiles.

was able to decrease the release of both these mediators, and exerted an inhibitory effect which was greater at a concentrations of 10 μ M (MMP-9: $P = 0.0011$; TIMP-1: $P < 0.001$, ANOVA test) (Fig. 1, panel A and B). We also found that flunisolide was capable of significantly increasing the MMP-9/TIMP-1 molar ratio ($P < 0.004$, ANOVA test) (Fig. 1, panel C).

Effects of flunisolide on TGF- β 1 and fibronectin release by SC

At baseline TGF- β and fibronectin released by cultured SC was 378 (311.5–458) and 154 (120–180) ng/ml respectively. Flunisolide was able to decrease the release of both these mediators, and exerted an inhibitory effect which was greater at a concentrations of 10 μ M (TGF- β : $P = 0.006$; ANOVA test) (Fig. 2) (fibronectin: $P < 0.0001$; ANOVA test) (Fig. 3).

Effects of flunisolide on SC apoptosis

The total cell count of the cells was not significantly different in sputum samples obtained before and after treatment with flunisolide. The percentage of squamous cells, the number

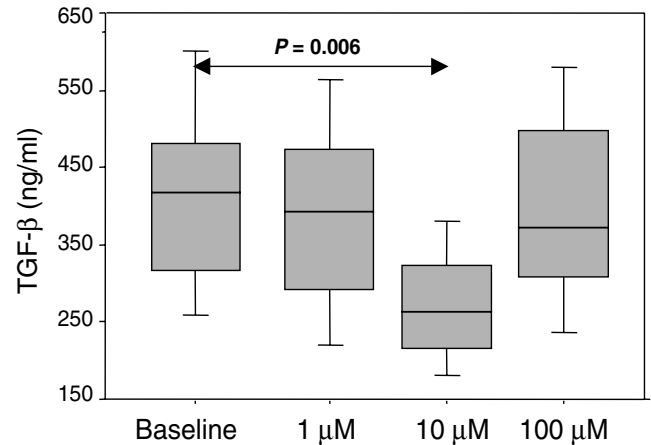


Figure 2. Effects of flunisolide (1, 10 and 100 μ M) on the release of transforming growth factor (TGF β), sputum cells. Statistical analysis by ANOVA test, shows a significant difference between baseline and flunisolide (10 μ M).

of macrophages, neutrophils, lymphocytes and epithelial cells was not statistically different after flunisolide treatment. By contrast, the number of apoptotic eosinophils was significantly reduced after incubation with flunisolide 10 μ M ($P < 0.0001$) (Fig. 4).

Discussion

Compelling evidences have demonstrated that asthma is an inflammatory disease associated with airway remodelling (23), and that this phenomenon may have several functional consequences. It is proposed that treatment or prevention of remodelling is a crucial element in asthma management.

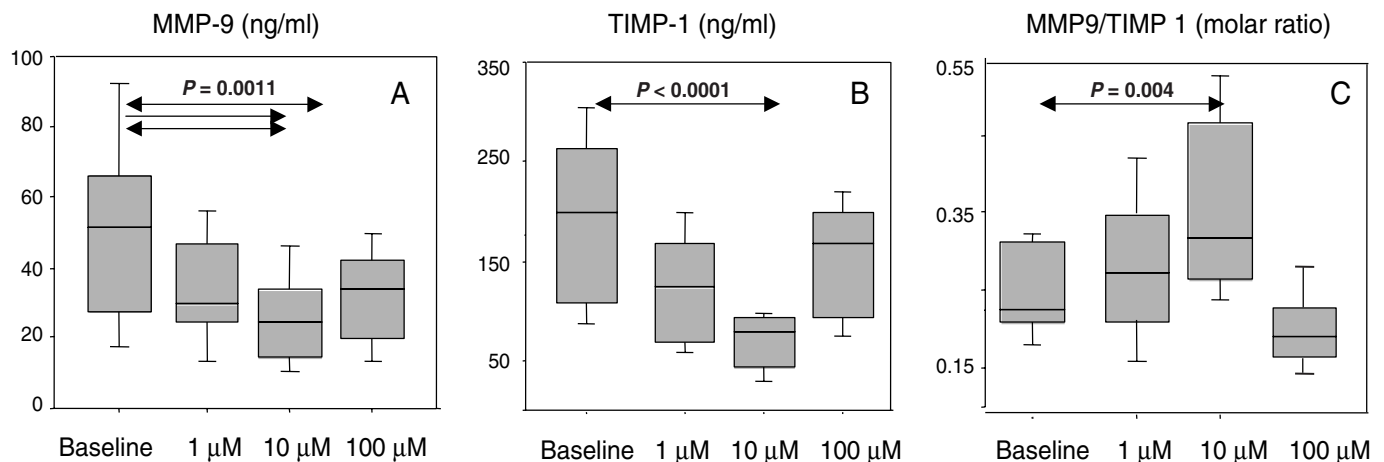


Figure 1. Effects of flunisolide (1, 10 and 100 μ M) on the release of metalloproteinases-9 (MMP-9, A), tissue inhibitor metalloproteinases-1 (TIMP-1, B) by sputum cells and on TIMP-1 to MMP9 molar ratio (C). Statistical analysis by ANOVA test, shows a significant difference between baseline and flunisolide (10 μ M).

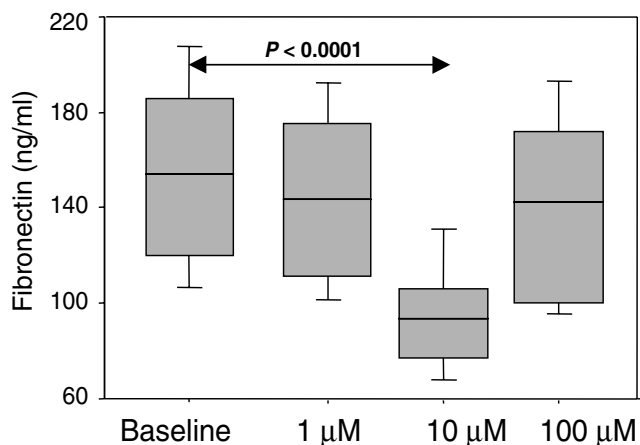


Figure 3. Effects of flunisolide (1, 10 and 100 µM) on the release of fibronectin. Statistical analysis by ANOVA test, shows a significant difference between baseline and flunisolide (10 µM).

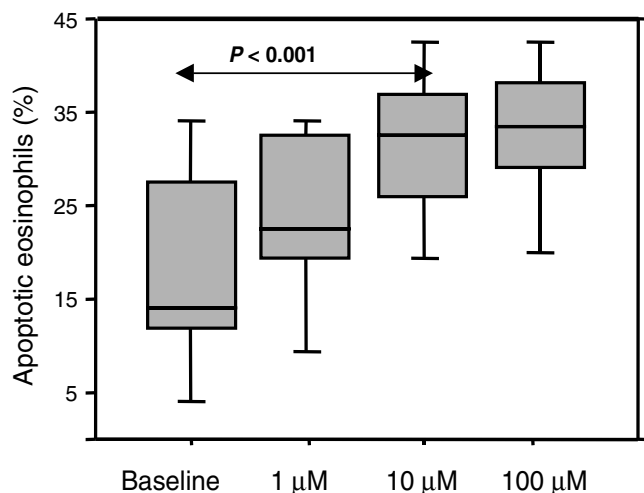


Figure 4. Effects of flunisolide (1, 10 and 100 µM) on the percentage of eosinophils apoptosis. Statistical analysis by ANOVA test, shows a significant difference between baseline and flunisolide (10 µM).

Currently available data indicate that the effects of steroids on airway remodelling are still controversial. The thickness of the reticular basement membrane is usually unchanged or only slightly reduced (24, 25). However, in one study, it has been shown that if the dose of inhaled corticosteroids is adapted on the degree of bronchial reactivity (BHR), a reduction in the reticular basement membrane pseudothickening can be achieved (26).

These morphological data correspond to the clinical observation that a prolonged treatment with inhaled corticosteroids improves both hypersensitivity and hyper-reactivity of the airways, thus suggesting an effect on components of remodelling (27–29).

This raises the question whether it would not be more beneficial to start inhaled corticosteroids at an early stage

of asthma to prevent the inflammatory cascade, which in turn induces structural remodelling, instead of reversing established lesions. This hypothesis has been tested in a rat model (30, 31) in which pretreatment with fluticasone propionate reduces airway inflammation and also partly prevents goblet cell hyperplasia, fibronectin deposition, thickening of the airway wall and the accompanying increase in BHR.

Mediators such as metalloproteinases (MMP-9 and TIMP-1), TGF-β and fibronectin are involved in airway remodelling. Previous studies by our group demonstrated that the release of these mediators were enhanced in sputum (20) and in bronchoalveolar lavage (BAL) fluids (32) of asthmatic subjects.

In the present study induced SC from mild-to-moderate asthmatics were cultured to assess their ability to spontaneously release high amounts of metalloproteinases, TGF-β and fibronectin and to respond to flunisolide. The results showed that flunisolide significantly reduced the release of MMP-9, TIMP-1, TGF-β and fibronectin as well as significantly increased MMP-9/TIMP-1 molar ratio and apoptosis of eosinophils recovered from induced sputum.

The concentration of flunisolide (10 µM) was chosen on the basis of the results of dose–response curve experiments as well as on previous evidences demonstrating that this dose was able to modulate cellular functions *in vitro* (19). Moreover, the dose used in our experiments is within the range of the dose of flunisolide commonly used in the therapy of asthma.

It should be noted that, in reality drugs or stimuli usually target the various cell types present in the airway such as inflammatory cells or structural cells and rarely just a single cell population, so we used airways from mild-to-moderate asthmatic patients to confirm the ability of flunisolide, in a concentration 10 µM, to influence the functional activation of airway inflammatory cells. The loose of the effect on mediator release and eosinophils apoptosis displayed by flunisolide 10 µM, obtained at a specific well-defined dose and not at doses either lower or higher than, may contribute to identified the dose effectively necessary to inhibit the airways immune cells activation in asthmatic patients treated with inhaled steroids.

It has been previously demonstrated that eosinophils, macrophages and neutrophils are able to release MMP-9, TIMP-1, TGF-β and fibronectin (5, 12, 32, 33). Our results showing that the concentration of these cytokines is reduced in SC culture supernatants after treatment with flunisolide together with the finding that eosinophils and macrophages are present in the induced sputum from our asthmatic patients strongly support the concept that these cells represent the major producers of these cytokines in our SC cultures and in the airways of the mild-to-moderate asthmatics.

We have shown that the treatment with flunisolide down-regulates the release of MMP-9 but also

down-regulates the release of its inhibitor TIMP-1. These results are consistent with previous observations that steroids may down-regulate the release of both MMP-9 and TIMP-1 (34, 35). Moreover, we have previously demonstrated that both these two molecules are less expressed in normal subjects when compared with asthmatic subjects but that the MMP-9/TIMP-1 ratio is decreased in asthmatics with respect to normal subjects (20). These concepts together with the finding that the treatment with flunisolide is able to increase the MMP-9/TIMP-1 ratio although in the presence of a reduction of the absolute release of both molecules strongly support the hypothesis that flunisolide is able to restore a condition, in terms of the ratio between the two molecules, present in normal subjects.

It is of interest that the flunisolide significantly inhibits the release of TGF- β . In fact, very few studies have assessed the effects of steroids on the levels of TGF- β released in the airways of asthmatic subjects. In general, TGF- β is known to play a crucial role in the pathogenesis of airway remodelling in asthma inducing formation of extracellular matrix and fibrosis. In addition, TGF β directly affects the level of TIMP-1, by increasing its release by several cell types (13). It is therefore likely that by inhibiting TGF- β , flunisolide not only reduced its effects on mesenchymal cells (fibroblasts and myofibroblasts) but also contributes to reduce the production of TIMP-1 by airway cells.

The fact that TGF- β has been demonstrated to abrogate the survival effects of IL-5 and haemopoietins on eosinophils thereby inducing their apoptosis (36, 37)

together with the fact that eosinophils of induces sputum of asthmatics express TGF- β (38) are consistent with our data showing both a reduced TGF- β release and an increased apoptosis in SC cultures after flunisolide treatment. In our system, the down-regulation of TGF- β release in the SC cultures induced by flunisolide could be related, at least in part, to an increased apoptosis of eosinophils, as well as of the other cell types present in the induced sputum samples, leading to the decrease of the number of the cells able to release TGF- β .

However, it is possible support the idea that flunisolide may prevent the airway remodelling in asthma further as showed by the inhibitory effect of flunisolide on fibronectin release. Fibronectin is indeed one of the ECM components that are overproduced in the airways of asthmatics and contributes to the increased thickness of the reticular layer of the basement membrane. Therefore, the reduced production of fibronectin resulting from the inhibitory effects of flunisolide may have profound beneficial consequences on airway remodelling.

In conclusion, this study provides new evidence about the effect of glucocorticosteroids on the release of key molecules involved in airway remodelling in asthma, and suggests that long-term clinical trials should be performed in asthmatics, both children and adults, in order to better appreciate their clinical relevance.

Acknowledgment

This study was supported by Valeas s.p.a Milan.

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