

REVIEW

Mutual activation of pulmonary fibroblasts and eosinophils, and modulation by drugs in relation to asthma

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Introduction

Asthma is a disease characterized by inflammation, airway remodelling, variable airway obstruction and hyper-responsiveness. Usually, asthmatic patients are sensitized to one or more aeroallergens. A prominent cell involved in allergic inflammation is the eosinophil. In the past 15 years many studies have established the role of this cell in the pathogenesis of asthma. It is known that eosinophils of asthmatic patients are in an activated state and contribute to asthma symptoms by releasing their toxic contents in the tissue. Eosinophils can be activated in many different ways: by microbial products, platelet activating factor (PAF), immunoglobulins, complement factors, cytokines and by adhesion to other cells. However, it is still not completely understood which factors contribute to *in vivo* eosinophil activation in asthma.

The remodelling in the airways as seen in asthma is largely a result of altered fibroblast behaviour. These resident cells of the lungs, providing structure to the tissue, can also play an active role in inflammation. They are able to produce many different chemokines and cytokines upon stimulation. These products can, in turn, activate or attract inflammatory cells, e.g. eosinophils, to the lung.

The main goals of asthma treatment are inhibition of ongoing inflammation and bronchodilation. The most powerful anti-inflammatory agents are glucocorticoids, at present mostly applied by inhalation when asthma is stable. Bronchodilatory drugs such as β_2 -agonists are administered locally in the lungs by inhalation and provide relaxation of smooth muscle. Recent evidence indicates additional anti-inflammatory effects from long-acting β_2 -agonists as well. It is important to know how anti-asthma drugs interact with cells involved in the inflammatory process in the airways

and lung tissue, because this eventually may lead to clinical improvement of the patient.

In the following article we will review the available literature with respect to eosinophil and pulmonary fibroblast behaviour and their interaction in the ongoing inflammatory and remodelling processes of the airway and lung disease in asthma. Further we will discuss the influence of glucocorticoids and long-acting β_2 -agonists on eosinophil and fibroblast behaviour in relation to their beneficial clinical effects in asthma therapy.

Activation of pulmonary fibroblasts

The major role of fibroblasts is maintaining integrity, solidity and form of lung tissue. This is achieved by producing a vast variety of extracellular matrix (ECM) components, such as collagen, laminin, fibronectin, hyaluronic acid and proteoglycans. Recent investigations showed that the basement membrane in airways, forming a barrier between the external milieu of the lung lumen and epithelium on one side and the interstitium on the other side, is mainly created by deposition of ECM produced by fibroblasts [1]. In repair mechanisms after damage to airway epithelium, the production of ECM proteins is important, as well as proliferation of fibroblasts and epithelial cells. Fibroblasts are able to produce growth factors that affect fibroblasts themselves as well as adjacent tissue cells.

Very soon after the onset of asthma, a distinct increase in the basement membrane thickness can be seen histologically, which is thought to be caused by deposition of collagens [1]. Subepithelial fibrosis and an increase in the number of myofibroblasts have also been observed [2]. Already 24 h after allergen challenge, myofibroblast counts in biopsies of asthmatics are higher and these cells have a higher capacity of migration and contractility than before allergen challenge [3], suggestive of a role of myofibroblasts in asthma. Earlier reports have shown that stimulation

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with transforming growth factor (TGF) β , possibly released by activated eosinophils or epithelial cells, stimulates the conversion of normal fibroblasts into myofibroblasts [4]. Myofibroblasts are thought to be responsible for the increased ECM deposition [1] and increased contractility [5] in asthma. Thus, induction of myofibroblasts contributes to airway remodelling.

There are several studies indicating that eosinophils may affect fibroblasts, possibly contributing to the pathophysiology of asthma. Eosinophils adhere to fibroblasts by means of β_2 -integrin binding, and adhesion is increased after phorbol myristate acetate (PMA) stimulation of eosinophils, or interleukin-1 (IL-1)/tumour necrosis factor (TNF) stimulation of fibroblasts [6]. Conditioned media of guinea-pig peritoneal eosinophils increase human fetal lung fibroblast proliferation [6]. Furthermore, guinea-pig and human eosinophil extracts, as well as conditioned media from resting eosinophils induce an increased proliferation of human (dermal) fibroblasts *in vitro* [7]. Finally, sonicates of peripheral eosinophils derived from atopic individuals also increase proliferation of lung fibroblasts. This proliferative effect is thought to be partially mediated by TGF β and TNF α produced by eosinophils [8]. This was compatible with the observation that incubation with eosinophil cationic protein (ECP) increased the intracellular pool of proteoglycans of human lung fibroblasts [9]. However, proliferation appeared not to be affected. Cook and coworkers also reported that both intact and disrupted eosinophils derived from rat peritoneum inhibited proliferation of a human lung fibroblast cell line. PMA stimulation of eosinophils augmented the inhibitory effect; toxic effects of granule proteins were kept responsible for the decreased growth of fibroblasts in this study [10].

Further studies have elucidated that eosinophils are able to cleave ECM proteins by a metalloproteinase, disrupting the connective tissue in interstitial lung diseases [11]. This may also play a role in asthma. Eosinophils stimulated with IL-5 and PAF are able to degrade ECM by use of metalloproteinase-9 (MMP-9) *in vitro* [12]. Whereas an increase in collagen synthesis was found in dermal fibroblasts, collagen synthesis of lung fibroblasts was inhibited by stimulation with eosinophil sonicates [13], but lung fibroblast proliferation increased due to eosinophil-derived TGF β . *In vivo* data support the fibrotic effects of eosinophils on fibroblasts in *in vitro* studies. In asthmatic airways, activated eosinophils represent the major source of TGF β 1. TGF β 1 expression and fibrosis in airway wall tissue was larger in asthmatic patients than in normal controls and was inversely correlated with the level of FEV₁ (forced expiratory volume in one second) [14]. This suggests that eosinophils may be involved in fibrosis in asthma through TGF β production.

In another study, increased expression of MMP-9,

probably produced by eosinophils [15], was associated with collagen III, V and tenascin deposition in the basal membrane of the airway walls of asthmatic patients [16]. Furthermore, activation of eosinophils and fibroblasts was corroborated by the finding of increased amounts of ECP and hyaluronan, respectively, in bronchoalveolar lavage (BAL) from patients with asthma. Both ECP and hyaluronan levels correlated inversely with the level of FEV₁ and height of symptom score. A positive correlation between ECP and hyaluronan suggests a common activation of eosinophils and fibroblasts in asthma [17].

Another very important feature of fibroblasts, next to their contribution to fibrosis, is their ability to produce a large array of inflammatory cytokines and chemokines which are able to activate and attract inflammatory cells. Airway remodelling and inflammation in asthma is thought to take place primarily in the larger airways. However, infiltration of inflammatory cells in small airways [18,19] and lung parenchyma [20] has also been established recently. This indicates that inflammatory processes take place throughout the whole lung, and therefore parenchymal lung fibroblasts may also play a role in asthmatic inflammation. Upon stimulation with lipopolysaccharide (LPS), TNF α , IL-1 β or after tissue damage, parenchymal fibroblasts are able to secrete monocyte chemoattractant proteins (MCP-1,2,3), macrophage inflammatory proteins (MIP-1 α and β), RANTES (regulated on activation of normal T cells expressed and secreted) and eotaxin which are chemotactic to macrophages, monocytes, basophils, eosinophils and subsets of lymphocytes [21]. IL-8, IL-6, granulocyte-macrophage colony stimulating factor (GM-CSF) and stem cell factor (SCF) are cytokines produced by fibroblasts [22], and they are able to activate, amongst others, neutrophils, eosinophils and mast cells. The eosinophil degranulation product MBP was able to enhance IL-6 production of IL-1/TNF-stimulated lung fibroblasts, partly due to the cationic nature of the protein. However, in itself, MBP did not influence IL-6 or IL-11 production [23].

Fibroblasts are also able to interact with inflammatory cells directly via cell-cell contacts. They possess the capacity to express intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), by which they can bind to β -integrins on leucocytes [24,25]. In an *in vitro* model, we recently have shown that human lung fibroblasts increased both ICAM-1 and VCAM-1 expression after IL-1 β and TNF α stimulation. Interferon (IFN) γ specifically increased ICAM-1 expression while IL-4 only increased VCAM-1 expression [26]. The differential effects of IFN γ and IL-4 are in accordance with results of other studies. IFN γ increased ICAM-1 expression on skin fibroblasts, endothelium and bronchial epithelium [25,27,28], whereas IL-4 increased

VCAM-1 expression on endothelium, lung fibroblasts, skin fibroblasts and bronchial epithelium [27–29]. Little is known about the *in vivo* expression of adhesion molecules in the connective pulmonary tissue, yet the number of ICAM-1 and VCAM-1 positive blood vessels in bronchial biopsies is higher in asthmatic patients than in healthy controls [30]. Up-regulation of these adhesion molecules on endothelium and fibroblasts by cytokines may add to increased infiltration of inflammatory cells in the pulmonary tissue as seen in asthma via their binding to the corresponding adhesion molecules. Moreover, the selective increase of VCAM-1 on lung fibroblasts by the TH₂ cytokine IL-4 may contribute to selective eosinophil transmigration through the pulmonary tissue. Thus, lung fibroblasts may not only be involved in tissue remodelling, but may also add to inflammatory processes in asthma.

Activation of eosinophils

Eosinophil activation can be measured using many different parameters. They can be divided into the release of granule proteins, the production of arachidonic acid metabolites and superoxide, chemotactic mobility and reactivity to chemotactic stimuli, as well as modulation of surface receptors. Peripheral blood eosinophils derived from asthmatic patients release more of their granule products compared with control eosinophils. *In vivo*, granule proteins are found in increased quantity in sputum and BAL of asthmatic patients, indicating eosinophil activation [17,31]. In addition, there are also studies reporting clusters of free eosinophil granules (cfebs) in asthmatic airways [32]. In the past they were considered to be artefacts, but nowadays they are thought to reflect total disruption of eosinophils due to ultimate activation. Eosinophils from asthmatic patients are potent leukotriene producers after calcium-ionophore stimulation and mainly release eosinophil-specific LTC₄, a powerful bronchoconstrictor [33]. They produce superoxide anion upon stimulation with, for instance, PAF and fMLP, but also on more physiological stimulation after adhesion to VCAM-1 [34] and eotaxin incubation [35]. The capacity to produce superoxide is enhanced after endothelial transmigration [36] and this agrees with the fact that BAL eosinophils are more potent superoxide producers than blood eosinophils after stimulation [37].

There are also many membrane molecules indicative for the activation state of eosinophils. Eosinophils in BAL of asthmatic patients express ICAM-1, HLA-DR, CD4, CD44, CD69 and CD45RO spontaneously as well as after *in vitro* stimulation with cytokines [38]. Upon activation with, for example, PAF, fMLP, IL-3 and GM-CSF, the adhesion molecule CD11b, involved in adhesion and migration, is rapidly up-regulated [39]. CD11b expression on eosinophils

obtained from BAL is higher than the expression on blood eosinophils [40]. This can be caused a.o. by the process of transendothelial migration, resulting in an activated phenotype. The adhesion molecule L-selectin is constitutionally expressed on eosinophils and plays an important role in the initiation of adhesion to the vascular endothelium leading to transendothelial migration. Before firm adhesion takes place, it is shed from the surface. L-selectin shedding has been shown *in vivo* by a decreased L-selectin expression on BAL eosinophils compared with blood eosinophils [41] and is therefore also considered to be a characteristic of activation. *In vitro*, there are a number of activating agents that induce shedding such as PAF, fMLP and GM-CSF.

When eosinophils have crossed the endothelial barrier, they migrate through the interstitium before they can reach the epithelium and airway lumen. In the interstitium, fibroblasts and their ECM products constitute the connective tissue. There is limited knowledge of the implications of interactions between human eosinophils and human lung fibroblasts *in vivo*. Studies using cultures or separate mediators of these cells have led to more insight into mechanisms of interaction. The first studies to prove activating effects of fibroblasts on eosinophils were done using the 3T3 mouse fibroblast cell line. The group of Owen and Rothenberg discovered that 3T3 fibroblasts augmented eosinophil viability, hypodensity, superoxide and LTC₄ production in the presence of GM-CSF or IL-5 after co-culture [42–44], but direct cell–cell contacts were not necessary for the observed effects. These experiments were extended by Vancheri and coworkers, who showed an increase in eosinophil survival after co-culture with human fetal lung fibroblasts without addition of supplementary cytokines. They also showed that this effect was due to GM-CSF production by fibroblasts [45].

Further support came from a study showing that subepithelial myofibroblast cultures and their conditioned media also increased survival of eosinophils. This was due in part to delayed apoptosis of eosinophils by GM-CSF produced by fibroblasts. Blocking fibronectin in these fibroblast cultures did not affect eosinophil survival, suggesting that ECM proteins do not play a major role in maintaining eosinophil viability [46]. However, when eosinophils were cultured in wells coated with fibronectin, survival was prolonged and LTC₄ production increased by eosinophil adhesion to fibronectin through VLA-4 interaction and autocrine GM-CSF and IL-3 production [47,48]. Binding of soluble ICAM-1 also increased survival of eosinophils by an autocrine GM-CSF effect [49]. EPO release after 16 h of culture with human fetal lung fibroblasts was only significantly increased when both eosinophils and fibroblasts were pre-activated with C5a and TNF α , respectively. Both GM-CSF and adhesion molecules of fibroblasts were probably involved in release of

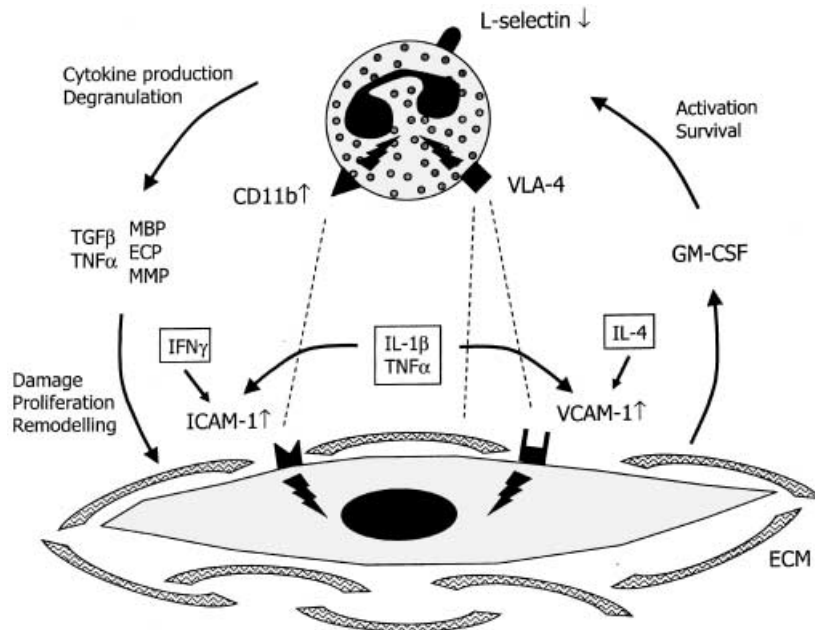


Fig. 1. Schematic representation of possible interactions between fibroblasts and eosinophils. Mutual activation can occur either by adhesion or production of soluble factors.

EPO [50]. Another study showed eosinophil activation after co-culture with lung fibroblasts, measured by an increased percentage of HLA-DR-positive eosinophils [51].

We have shown that human lung fibroblasts from normal individuals are able to activate isolated peripheral eosinophils from non-atopic individuals *in vitro* as demonstrated by increased CD11b expression and decreased L-selectin expression of eosinophils as well as an increased viability [52]. Activating effects on eosinophils were more pronounced when fibroblasts had been in contact with the proinflammatory cytokine IL-1 β . GM-CSF produced by fibroblasts appeared to be the most important factor in eosinophil activation. Only in the case of CD11b up-regulation, an influence of direct eosinophil-fibroblast contact could not be excluded. Eosinophil survival was partly dependent on GM-CSF production by fibroblasts, suggesting that additional factors are involved here. This indicates a different mechanism for enhancement of eosinophil survival by fibroblasts. In a comparable study of eosinophil survival by fibroblasts, GM-CSF was also found to be only partly responsible for increased viability [46]. In contrast to the findings with fibroblasts, GM-CSF produced by epithelial cells is totally responsible for the increased eosinophil viability [53].

The knowledge that pulmonary fibroblasts are able to activate eosinophils may have important implications for understanding the pathogenesis of asthma. Infiltration of eosinophils in the lungs of asthmatic patients leads to eosinophil-fibroblast contact and interaction (see Fig. 1), in addition to interactions with endothelium and epithelium. Fibroblasts thus may contribute to the activation of

eosinophils (mainly through the production of GM-CSF) as observed in pulmonary biopsies and BAL. Moreover, the finding that fibroblasts possibly act as important intermediate cells in the inflammatory response, places these cells in focus for modulation by therapeutic regimens currently used in asthma.

Influence of anti-asthmatic drugs

The treatment of asthma generally consists of inhaled glucocorticoids supplemented with inhaled β_2 -agonists. Glucocorticoids have a vast array of anti-inflammatory properties, resulting in improvement of asthma symptoms. They act on activation and infiltration of inflammatory cells as well as activation of resident tissue cells. Glucocorticoids in general have an inhibitory effect on eosinophil chemokine and cytokine release, survival, chemotaxis and membrane receptor expression. Recently, it has become clear that glucocorticoids also exert a large effect on activation of resident cells in the lung. They attenuate a.o. IL-6, IL-8 and GM-CSF production of fibroblasts [22], epithelium, endothelium and smooth muscle. Moreover, glucocorticoids inhibit the cytokine-induced increase in adhesion molecule expression (ICAM-1, VCAM-1 or ELAM-1) on epithelium, endothelium and fibroblasts. The inhaled glucocorticoid budesonide reduces the number of infiltrating eosinophils in sputum, BAL and bronchial tissue both before and after allergen challenge [54,55]. Levels of ECP in sputum also decrease significantly.

We have shown that budesonide also inhibits IL-1 β - (Fig. 2a), TNF α -, IFN γ - and IL-4-induced ICAM-1 and VCAM-1 up-regulation on human lung fibroblasts [56]. Moreover, a small reduction in basal ICAM-1 and VCAM-1

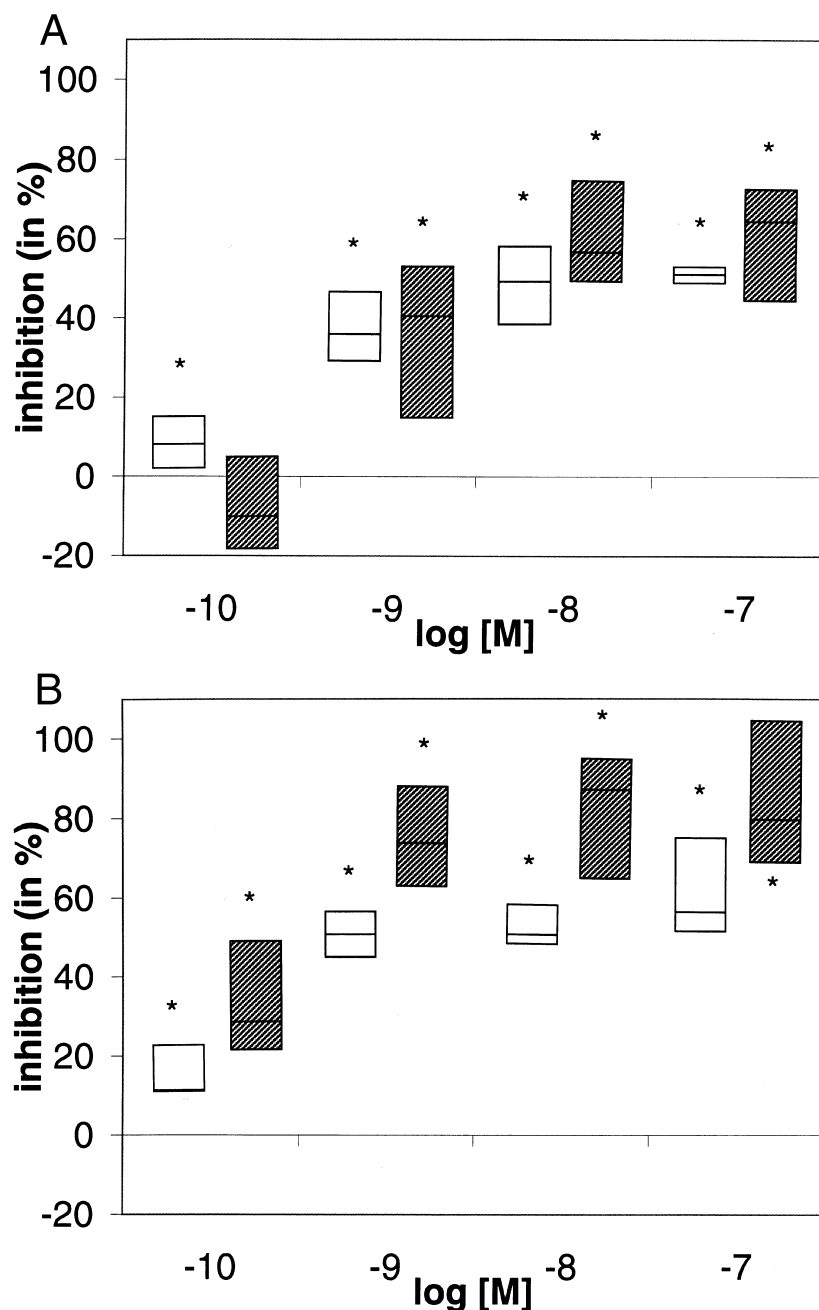


Fig. 2. Inhibition of ICAM-1 up-regulation (open bars) and VCAM-1 up-regulation (hatched bars) of human lung fibroblasts after IL-1 β (1 U/mL) stimulation by (a) budesonide and (b) formoterol in concentrations of 10^{-10} – 10^{-7} M. Results are presented as median percentages with interquartile range of inhibition. * $P < 0.05$ compared with expression after IL-1 β stimulation.

expression was found. Budesonide also inhibited the IL-1 β -induced production of IL-8 and GM-CSF (unpublished results). The beneficial effect of inhaled budesonide in asthma can therefore be due to the modulation of cytokine production and the expression of adhesion molecules on resident cells, e.g. fibroblasts in lung tissue, thus diminishing infiltration and activation of inflammatory cells. This is in accordance with clinical effects of budesonide, showing inhibition of eosinophil infiltration in the airway wall and eosinophil activation [57].

In our next study we investigated whether eosinophil activation (measured by modulation of CD11b and L-selectin expression) induced by fibroblast-conditioned medium (after IL-1 β stimulation) could be inhibited by budesonide. To this purpose lung fibroblasts were incubated with budesonide and conditioned medium was collected afterwards. The obtained conditioned media were incubated with eosinophils and their activation state determined. Eosinophils were also incubated directly with budesonide in the presence of conditioned medium of

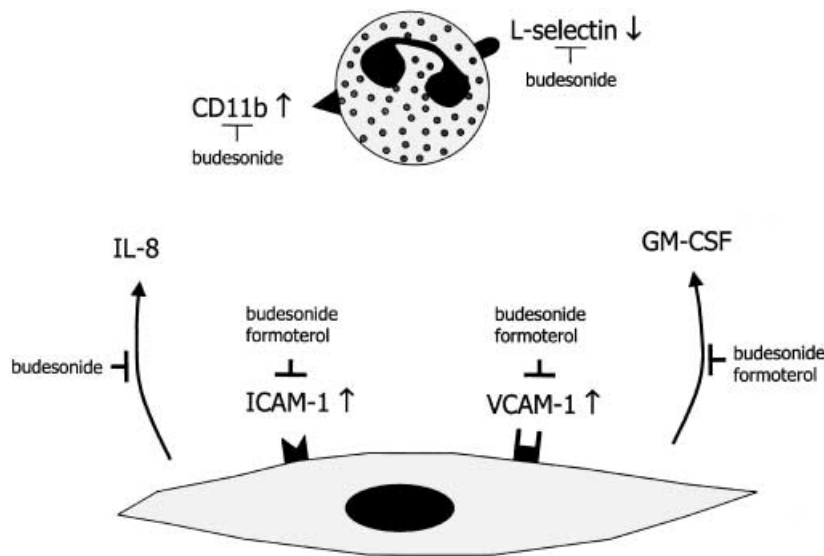


Fig. 3. Inhibitory effects of budesonide and formoterol on eosinophil and lung fibroblast activation.

fibroblasts unmodulated by drugs. These studies allowed us to discriminate between the effects of budesonide on fibroblasts and direct effects on eosinophils. We found that eosinophil activation by fibroblast-conditioned medium exposure was inhibited through modulation of fibroblasts by 10^{-9} M budesonide, probably through decreased GM-CSF production. In contrast, inhibition through direct modulation of eosinophils was less pronounced and observed only on eosinophils after longer incubation times (16 h) [58].

In vivo, resident lung tissue cells contribute to trans migratory and chemotactic processes and activation of inflammatory cells by production of cytokines and adhesion molecules. From the above mentioned studies, it seems that the anti-inflammatory effect of glucocorticoids is achieved mainly by suppression of resident lung tissue cell function and additionally by inhibition of infiltrating cell function.

Another group of drugs frequently used in asthma therapy are long-acting β_2 -agonists. Next to the beneficial effects such as prolonged bronchodilation, resulting in reduction of symptoms and increased lung function, there is increasing evidence that long-acting β_2 -agonists such as formoterol and salmeterol, may have some anti-inflammatory effects *in vivo* as well, although this idea has not yet been universally accepted. After formoterol treatment, guinea pigs show a decreased late asthmatic reaction (LAR) after challenge beside a decreased eosinophil influx and an inhibited respiratory burst [59,60]. Recently, evidence for a decreased early asthmatic reaction (EAR) and LAR as well as a decreased infiltration of mast cells and eosinophil into the airway wall tissue, but not in BAL or sputum, has been found in asthmatic patients after formoterol treatment [57,61], and comparable effects have been observed for salmeterol [62]. *In vitro* studies with

formoterol showed inhibition of granulocyte adhesion to vascular endothelium [63] and of PAF-induced chemotaxis and ECP release of eosinophils [64].

In contrast to an anti-inflammatory effect, it was recently found that β_2 -agonists, i.e. salmeterol, inhibit eosinophil apoptosis [65], and formoterol showed no direct inhibition of eosinophil activation (CD11b/L-selectin expression) [58]. However, we did find an anti-inflammatory effect on lung fibroblasts: formoterol inhibited the fibroblast-mediated eosinophil activation at 10^{-9} M after fibroblasts had been cultured with formoterol, probably through inhibition of fibroblast GM-CSF production [58]. IL-1 β -(Fig. 2b), TNF α -, IFN γ - and IL-4-induced ICAM-1 and VCAM-1 up-regulation on human lung fibroblasts were also inhibited by formoterol. Additionally, we found a small reduction in basal ICAM-1 and VCAM-1 expression [56].

The only report so far of β_2 -agonists inhibiting ICAM-1 expression comes from Oddera and colleagues, who described the inhibition of IFN γ -induced ICAM-1 expression on bronchial epithelial cells by the short-acting β_2 -agonist fenoterol [66]. Formoterol also inhibited IL-1 β -induced GM-CSF production, but not IL-8 production by lung fibroblasts (not published). This finding is partly in accordance with a recent study from Korn and colleagues, which showed that formoterol inhibits the TNF α -induced production of GM-CSF by bronchial epithelial cells, while IL-8 production is increased [67,68]. Therefore, although relaxation of smooth muscle cells in the bronchi is the major effect of formoterol treatment, anti-inflammatory effects on fibroblasts may be beneficial in asthma as well. Figure 3 shows an overview of the effects of budesonide and formoterol on eosinophils and lung fibroblasts.

It is very important to know how glucocorticoids and long-acting β_2 -agonists interact, as many asthmatic patients

regularly use these drugs simultaneously. One *in vitro* study showed blocking of glucocorticoid-induced inhibition of the respiratory burst and induction of apoptosis in eosinophils by β_2 -agonists [69]. However, there are many reports confirming that glucocorticoids and β_2 -agonists beneficially add to each other's effects. In clinical studies, the addition of salmeterol to beclomethasone dipropionate or fluticasone propionate provided better improvement in lung function and symptom control than the glucocorticoid alone [70,71]. The FACET (Formoterol and Corticosteroids Establishing Therapy) study showed an improvement of asthma symptoms, lung function and exacerbation rates when treatment with budesonide was combined with formoterol [72]. This may be a result of additive anti-inflammatory effects of long-acting β_2 -agonists in the lung. Synergistic inhibition of IL-8 release from airway smooth muscle cells has been reported for the combination of salmeterol and fluticasone propionate [73].

In recent work (not published), we have investigated the *in vitro* effect of the combination of formoterol and budesonide on ICAM-1, VCAM-1 up-regulation and GM-CSF, IL-8 production of human lung fibroblasts after IL-1 β stimulation. Formoterol exerted an additive effect on the inhibition of ICAM-1, VCAM-1 up-regulation and GM-CSF production by budesonide, but not on inhibition of IL-8 production. These results reflect the inhibitory capacity of formoterol alone. Consequently, there is no disadvantage at the level of human lung fibroblast activation in using these drugs simultaneously. On the contrary, beneficial effects as have been established in, for example, the FACET study [72] may partially have been achieved through additive inhibition of cellular infiltration, due to diminished adhesion molecule expression and GM-CSF production of lung fibroblasts by budesonide and formoterol.

Conclusion

The many ways by which pulmonary fibroblasts and eosinophils can interact, indicate an important place for these cells in the pathophysiology of asthma. Activated eosinophils can affect fibroblast function, leading to airway remodelling, by release of TGF β , MMP or granulation products. Pulmonary fibroblasts can activate eosinophils by GM-CSF and adhesion phenomena, thereby contributing to the inflammatory process in asthma.

Drugs used in asthma management are able to inhibit single and mutual activation of fibroblasts and eosinophils, i.e. glucocorticoids can inhibit both lung fibroblast and eosinophil activation. Budesonide inhibits cytokine-induced ICAM-1 and VCAM-1 expression, as well as GM-CSF and IL-8 production of fibroblasts. Modulation of eosinophil CD11b and L-selectin expression is also inhibited by budesonide. Surprisingly, long-acting β_2 -

agonists also may inhibit inflammatory responses of resident pulmonary cells, such as fibroblasts. It has been shown now that formoterol inhibits cytokine-induced ICAM-1 and VCAM-1 expression and GM-CSF but not IL-8 production. Formoterol also inhibits ECP release and chemotaxis of eosinophils, but not CD11b/L-selectin modulation. These inhibitory effects probably contribute to the clinical beneficial effects of these drugs in asthmatic patients. Furthermore, the combination of the β_2 -adrenergic agent formoterol and the glucocorticoid budesonide may show additional beneficial effects, while there are no indications that the combination is less effective.

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