EFFECT OF CORTICOSTEROIDS AND LONG-ACTING β_2-AGONISTS IN A HUMAN CELL CULTURE BASED *IN VITRO* MODEL OF AIRWAY INFLAMMATION AND TISSUE REMODELING

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Stéphanie Goulet

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Auf Antrag von

Prof. Alex N. Eberle

Prof. Therese Resink

Prof. Kurt Ballmer-Hofer

Prof. Michael Roth

Prof. Michael Tamm.

Basel, den 19 September 2006

Prof. Dr. Hans-Jacob Wirz

Dekan der Philosophisch-Naturwissenschaftlichen Fakultät

To the ones I love
and miss…

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Stéphanie

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LIST OF ABBREVIATIONS

°C	Celsius
a.u.	arbitrary densitometry units
ASMC	airway smooth muscle cell
BALF	bronchoalveolar lavage fluid
bp	base pair
C/EBPα	CCAAT/enhancer binding protein alpha
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
cDNA	complementary deoxyribonucleic acid
cm	centimetre
CO_2	carbon dioxide
COPD	chronic obstructive pulmonary disease
cpm	counts per minute
CTGF	connective tissue growth factor
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
FCS	fetal calf serum
g	gram
GAGs	glycosaminoglycans
GM-CSF	granulocyte/macrophage-colony stimulating factor
GR	glucocorticoid receptor
GRE	glucocorticoid-response elements
h	hour
HAT	acetyl transferase
HDAC-2	histone deacetylase-2
Hsp90	heat shock protein 90
IgE	immunoglobulin E
IL	interleukin
LABA	long-acting beta$_2$-agonists
M	molar
mA	milliampere
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
MMPs	matrix metalloproteinases
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor-kappaB
ng	nanogram
nM	nanomolar
PCAF	p300/CBP activating factor
PCR	polymerase chain reaction

pg	picogram
RNA	ribonucleic acid
sec	second
SEM	standard error of the mean
TGF-β_1	transforming growth factor-beta$_1$
TIMPs	tissue inhibitor of matrix metalloproteinases
TNF-α	tumor necrosis factor-apha
U	unit
UV	ultraviolet
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
WHO	World Health Organization
µCi	microcurie
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar

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SUMMARY

Asthma and chronic obstructive pulmonary disease (COPD) are characterized by chronic airway inflammation and remodeling. In non-pathological conditions tissue homeostasis is maintained by the balance between extracellular matrix (ECM) synthesis and degradation. However, this equilibrium is altered in asthma and COPD leading to structural changes such as increased ECM deposition. Fibroblasts and airway smooth muscle cells (ASMC) are the main source of collagen and glycosaminoglycans (GAGs) and transforming growth factor-β_1 (TGF-β_1) is the most potent ECM stimulator *in vitro*. In turn, the ECM influences cell proliferation, migration, differentiation, and secretion of cytokines and growth factors. Thus, ECM deposition may perpetuate the inflammatory and remodeling processes, contributing to progression and severity of diseases. Inhaled corticosteroids and long acting β_2-agonists (LABA) are the current therapy for asthma and COPD but their effect on airway remodeling is not clear. Therefore, the purpose of this thesis was to investigate the effect of corticosteroids and LABA on airway inflammation and tissue remodeling using a human cell based *in vitro* model.

In the present thesis, primary human lung fibroblasts and ASMC were cultured under serum-free condition to reflect a non-inflammatory environment or with 5% fetal calf serum (FCS) and/or TGF-β_1 to mimic inflammation. This experimental design allowed us to assess the effect of corticosteroids and LABA on total ECM, collagen and GAGs deposition, cell proliferation, cytokines release, collagen and ECM mediators mRNA expression, and gelatinolytic activity under both non-inflammatory and inflammatory conditions.

Concerning the effect of corticosteroids and LABA on ECM deposition by fibroblasts, we showed that 5% FCS and TGF-β_1 increased total ECM and collagen deposition. Under serum-free condition corticosteroids reduced ECM deposition and the effect was partly mediated by the glucocorticoid receptor and collagen *de novo* synthesis. Interestingly, in the presence of 5% FCS corticosteroids had the opposite effect. LABA reduced total ECM and collagen deposition under both conditions. Combined drugs further decreased ECM deposition under serum-free condition whereas they counteracted each other in 5% FCS, independently of TGF-β_1. Furthermore, we showed that 5% FCS and TGF-β_1 increased GAGs secretion and deposition. Under serum-free condition corticosteroids inhibited GAGs secretion and

deposition whereas LABA alone had no effect but partly reversed the effect of corticosteroids. On the contrary, in 5% FCS corticosteroids and LABA increased GAGs deposition. Upon TGF-β_1 stimulation, similar effects were obtained except in 5% FCS, where both drugs decreased the TGF-β_1-induced GAGs secretion and deposition. Together, these findings indicate that the effect of corticosteroids but not of LABA, on ECM deposition by fibroblasts is altered by ongoing inflammation.

The effects of corticosteroids and LABA on GAGs, matrix metalloproteinases (MMPs) and total ECM/collagen deposition by ASMC from healthy, asthma and COPD patients were also investigated. First, no difference in GAGs secretion and deposition was observed between ASMC from healthy, asthma and COPD patients. Under serum-free condition corticosteroids inhibited GAGs secretion and deposition while LABA had no clear effect nor modulated the effect of corticosteroids. In 5% FCS corticosteroids decreased GAGs secretion to a greater extent than LABA and their combination resulted in the same effect as corticosteroids alone. None of the drugs had any effect on GAGs deposition. Second, we assessed the effect of the drugs on ASMC-derived MMPs. In ASMC only proMMP-2 gelatinolytic activity was detected, which was decreased in asthma and COPD patients compared to healthy controls. However, corticosteroids and/or LABA had no effect on proMMP-2 activity. Lastly, we demonstrated that corticosteroids and LABA generated similar effects on total ECM and collagen deposition by ASMC from healthy subjects as observed with fibroblasts.

In conclusion, our novel findings suggest that the action of corticosteroids and LABA on ECM deposition differs under non-inflammatory and inflammatory conditions in lung fibroblasts and ASMC. In the presence of acute inflammation with vessel leakage, increased ECM deposition may be regarded as an attempt of stromal cells to block further serum and cells infiltration into the tissue and this effect would be supported by corticosteroids. The addition of LABA would counteract the corticosteroid-induced ECM deposition and therefore their combination with corticosteroids may depend on the inflammatory status of the patient. Thus, when airway inflammation is resolved, combination therapy may beneficially reduce pathological tissue remodeling. Thus, our findings might have implications for the short- and long-term treatment strategies in regard to airway remodeling in asthma and COPD.

CHAPTER 1

INTRODUCTION

Asthma and chronic obstructive pulmonary disease (COPD) are among the world's most prevalent diseases. Both are characterized by chronic inflammation and structural alterations resulting in lung functional abnormalities, with partly reversible airflow limitation in asthma and irreversible airway obstruction in COPD. In the following, asthma and COPD will be defined and their epidemiology, risk factors and pathogenesis will be described. Then, the characteristics of airway remodeling will be outlined. Finally, the actual therapy for asthma and COPD and its effect on airway remodeling will be discussed.

1.1. ASTHMA

Definition

Asthma is a complex airway disorder resulting in airflow limitation that presents different clinical phenotypes in adults and children. Exacerbations of asthma (attacks or worsening of asthma symptoms and lung function) can be fatal in absence of effective treatment. The Global Initiative for Asthma defined asthma as:

> *"a chronic inflammatory disorder of the airways in which many cells and elements play a role. The chronic inflammation causes an associated increase in airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment*" [1].

Asthma is characterized by airway hyperresponsiveness, reversible airflow obstruction, chronic airway inflammation and tissue remodeling. Airway hyperresponsiveness is defined as an exaggerated bronchoconstriction in response to a wide variety of stimuli. Airflow limitation can be caused by several factors including acute bronchoconstriction, mucus plugging, smooth muscle hypertrophy and hyperplasia. All these manifestations may lead to symptoms of wheezing, coughing, mucus secretion, shortness of breath and chest tightness.

Epidemiology

Asthma is a major health problem throughout the world. It is estimated that as many as 300 million people of all ages and ethnic origins suffer from asthma. The World Health

Organization (WHO) and the National Institute of Health reported that mortality due to asthma has now reached over 180 000 cases annually and that its prevalence is rising by 50% every decade, especially among children [1-3]. Furthermore, asthma is a leading cause of hospitalization in young children in industrialized countries. Asthma severity and morbidity, which refers to the impact of the disease on a person's quality of life (medication, hospitalization, absence from work/school and disability) are also increasing [3]. Therefore, asthma is a substantial human and economic burden for the society [4].

Risk factors

Host factors

Asthma usually develops in childhood and genetic and environmental factors contribute to its progression. Individuals with a family history of atopy, which is defined as the production of abnormal amounts of immunoglobulin E (IgE) antibodies in response to common environmental allergens, have a higher risk of developing airway responsiveness and asthma. Indeed, atopy is causing more than 50% of all asthma cases [5, 6]. Gender, age and race may also contribute to prevalence of asthma. Over the last years, gene profiling from asthmatic tissues have provided evidences that genes related to apoptosis, arginine metabolism, tissue repair, proteases and pro-inflammatory cytokines may be involved in the pathogenesis of the disease [7-15] but no specific gene has been identified to be responsible for the disease.

Environmental factors

In addition, the likelihood that asthma will develop in predisposed individuals depends on environmental factors such as exposure to allergens (house dust mites, pets, cockroaches, grass pollen), cigarette smoke, occupational sensitizers (chemical irritants, fumes) and air pollution [16, 17]. In addition, cold air, physical exercise, respiratory tract infections and strong emotional stress can trigger asthma exacerbations [1].

Pathogenesis

Airway inflammation

Airway inflammation in asthma is a multicellular process that involves mast cells, T_H2 lymphocytes and eosinophilic infiltration and their mediator release (Fig. 1.1). The inflammatory changes can occur throughout the central and peripheral airways and often vary with the severity of the disease [18, 19].

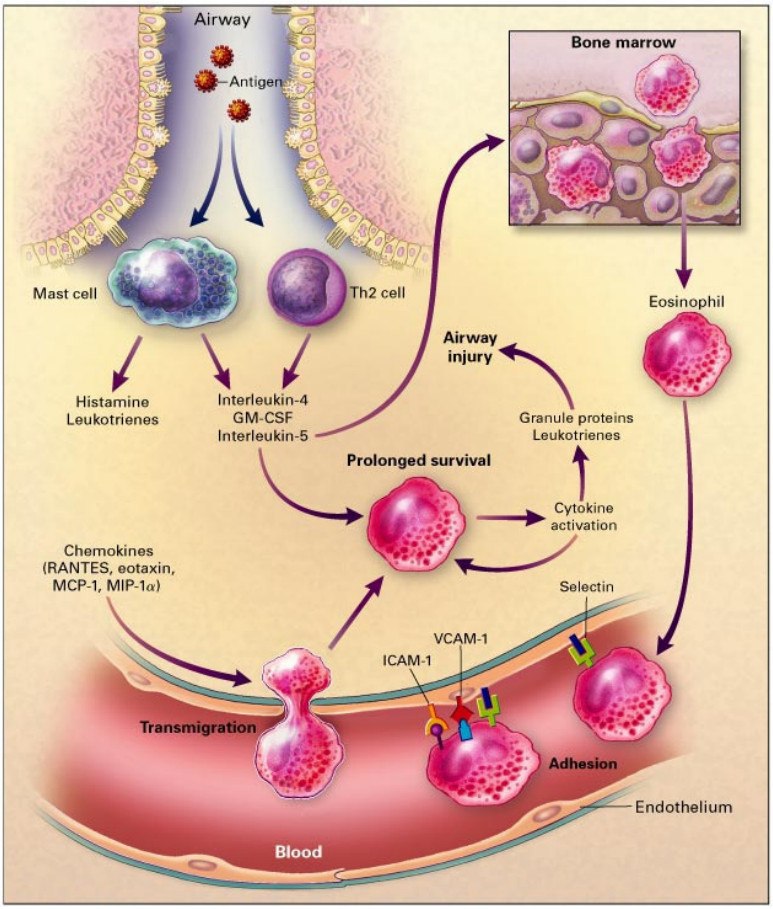

Figure 1.1. Asthmatic allergic inflammation following allergen exposure in sensitized individuals. Inhaled antigen activates mast cells and T_H2 cells in the airways and results in release of mediators (histamine, leukotrienes) along with cytokines (IL-4, IL-5, GM-CSF). These various compounds induce localized inflammatory cell influx and activation through upregulation of chemokines and adhesion molecules. Figure by Busse and Lemanske with permission from N Engl J Med [20].

Mast cell

Asthma is typically triggered by allergens and the classical inflammatory response takes place in two distinct phases: an early-phase reaction followed by a late-reaction [21]. The early-phase reaction of a sensitized individual initiates when IgE antibodies bind with high-affinity to their receptors FcεRI on mast cells, leading to their activation. Mast cells respond within minutes, they degranulate and release their preformed mediators such as histamine and tryptase and newly synthesized products such as prostaglandin D_2 and leukotriene C_4. These mediators induce constriction of the airways and may also alter vascular permeability. This early-phase reaction usually resolves within an hour. Four to six hours later, a prolonged late-phase reaction may develop as a result of cytokines and chemokines secreted by resident and recruited inflammatory cells. Mast cells further contribute to this chronic inflammation

through the release of pro-inflammatory cytokines including interleukin (IL)-4, IL-5, IL-13, tumor necrosis factor-α (TNF-α), and granulocyte/macrophage-colony stimulating factor (GM-CSF) which regulate the development of eosinophilic inflammation [21, 22].

T_H2 lymphocyte

As reviewed by Busse and Lemanske, the "T_H2 hypothesis" proposes that an imbalance in favor of T_H2 cells leads to allergic inflammation in asthma [20]. Indeed, the allergic inflammatory response in asthma is largely regulated by T_H2 cytokines including IL-4, IL-5 and IL-13 [23-25]. Briefly, IL-5 regulates differentiation, activation and survival of eosinophils and stimulates their release into the circulation [26, 27]. IL-4 controls T_H2 cell differentiation and expression of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells [28]. IL-13 shares many functions with IL-4 but it is also involved in airway hyperresponsiveness, airway fibrosis, epithelial damage and mucus production [29, 30]. Interestingly, genes for these cytokines cluster in a region constantly associated with asthma linkage studies [31]. In addition, transgenic mice overexpressing these cytokines exhibit airway hyperresponsiveness and pathologies similar to the one observed in asthma patients [32]. Furthermore, the transcription factor GATA-3, which is a key regulator of T_H2 response, is overexpressed in asthmatic airways [33, 34], whereas the transcription factor T-bet, which controls T_H1 differentiation, is undetectable [35, 36]. Thus, in asthmatics T_H2 cytokines increase the magnitude and/or prolong the inflammatory response.

Eosinophil

Despite ongoing controversy, evidences indicate a major role for eosinophils in the pathogenesis of asthma [37, 38]. In the bone marrow, IL-5 regulates the terminal differentiation of eosinophils, their activation and their release into the circulation. Eosinophils migrate to the inflamed area by rolling, through interactions with selectins and adhere to the endothelium via binding of integrins to adhesion molecules [39]. Under the influence of various cytokines and chemokines, eosinophils migrate through the extracellular matrix (ECM) of the airways and their survival is prolonged by IL-5 and GM-CSF [40, 41]. Activated eosinophils release pro-inflammatory cytokines such as IL-2, IL-4, IL-5, IL-13 and transforming growth factor-β_1 (TGF-β_1), chemokines (RANTES, eotaxin), LTC_4 and cytotoxic granule proteins. All these mediators orchestrate vascular permeability, mucus secretion, fibrosis, smooth muscle contraction, tissue damage and inflammation [42].

Neutrophil

Prominent neutrophil infiltration preferentially occurs in severe asthma, which cases are usually less sensitive to corticosteroids treatment. Indeed, neutrophil levels are elevated in sputum from exacerbated asthmatics, in bronchial washes from patients with status asthmaticus and in autopsy samples from patients who died of asthma [43-46].

Airway remodeling

Structural changes of the airway wall, collectively referred as airway remodeling, may result from chronic inflammation or chronic tissue injury. Briefly, tissue remodeling includes thickening of the airway wall, damages to the epithelium, airway smooth muscle cell (ASMC) hypertrophy/hyperplasia, angiogenesis and ECM deposition [47] and will be detailed later.

Treatment

There is no curative treatment for asthma. The basis of therapy consists of long-term suppression of airway inflammation with corticosteroids plus relief of airway constriction with bronchodilators such as long-acting β_2-agonists (LABA) [48]. Leukotriene receptor antagonists, which act to suppress inflammation and are also bronchodilators, have been successfully introduced for the treatment of asthma some years ago [49]. However, their efficacy is inferior to the combination of corticosteroids and LABA. Potential drug targets are under investigation for asthma, focusing on the inhibition of T_H2 cytokine activity [50, 51]. For example, a monoclonal antibody against IL-5 has been shown to reduce circulating eosinophils after allergen challenge in subjects with asthma but had no effect on bronchoconstriction [52]. These findings suggest that eosinophils might rather be a surrogate marker than a pathophysiologically important cell type in asthma. A recombinant human IL-4 receptor showed efficacy in preventing decline in lung function and symptoms in patients with moderate and persistent asthma [53]. A recombinant humanized monoclonal IgE antibody showed positive results by attenuating allergic responses and improving asthma symptoms and exacerbations, and is now approved for treatment of patients not properly controlled with corticosteroids and LABA [54, 55]. In summary, inhaled corticosteroids and LABA remain the cornerstone therapy for asthma.

1.2. CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Definition

COPD is a preventable disease characterized by slowly progressive development of airflow limitation that is poorly reversible by treatment. The Global Initiative for Chronic Obstructive Lung Diseases defined COPD as:

> "*a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases*" [56].

COPD is a complex chronic inflammatory state that encompasses 1) chronic bronchitis, defined by chronic cough, mucus secretion and obstruction of the small airways; 2) obstructive bronchiolitis, defined by small airway obstruction with inflammation and fibrosis; and at the end stage 3) emphysema, with enlargement of airspaces and loss of elasticity as a result of permanent destruction of the alveolar walls in the lung parenchyma [57]. Most COPD patients have a combination of these three pathological mechanisms assumed to be all induced by smoking but may differ in their proportion. The characteristic symptoms of COPD are cough, wheeze, sputum production, breathlessness upon physical efforts and frequent episodes of bronchitis or chest infections. COPD develops slowly over many years and is usually diagnosed when the disease is already advanced.

Epidemiology

COPD is not accurately diagnosed and treated, resulting in underestimation of the actual burden of the disease [58]. Based on data from the WHO in 2004, COPD is ranked as the 4th leading cause of death worldwide and as the 13th cause of morbidity. In addition, the worldwide prevalence of COPD in 2002 was estimated to be 11.6/1000 in men and 8.77/1000 in women [59]. By 2020, COPD is predicted to become the 3rd leading cause of death and the 5th commonest cause of morbidity [60-62]. COPD patients have to be treated for years, resulting in substantial economic and social burdens.

Risk factors

Host factors

To date, the only proven genetic risk factor for COPD is the severe hereditary deficiency of α_1-antitrypsin, a major circulating inhibitor of serine proteases protecting tissues against

degradation, which affects about 1 in 2000-5000 individuals and accounts for 1 to 3% of COPD cases [63]. Beside α_1-antitrypsin, genes involved in proteolysis/antiproteolysis, xenobiotic metabolism, antioxidation, mucociliary clearance and inflammation may potentially be involved in the pathogenesis of COPD [64-69]. Impaired lung growth in premature babies, diets deficient in antioxidants and gender may also contribute to the susceptibility of developing COPD [67, 70].

Environmental factors

COPD is likely caused by an interaction between genetic factors and environmental exposures. In industrialized countries, cigarette smoking is by far the most important risk factor for COPD, accounting for approximately 90% of all cases [71-73]. However, there is a great variability in lung function among smokers as only 10 to 20% develop clinically relevant symptoms of COPD. Other environmental risk factors for COPD include occupational dusts and chemicals (vapours, irritants, fumes), indoor/outdoor air pollution and bacterial/viral infections [56, 74].

Pathogenesis

COPD comprises pathological changes in the central and peripheral airways, lung parenchyma and pulmonary vasculature. These changes include progressive destruction and aberrant repair of the lung parenchyma and result from chronic airway inflammation, proteases/antiproteases imbalance and oxidative stress (Fig. 1.2).

Airway inflammation

Both COPD and asthma involve chronic airway inflammation but with distinct characteristics [75]. In COPD, pulmonary inflammation predominantly affects the peripheral airways and lung parenchyma and correlates with the severity of airflow limitation [76-81]. The ongoing inflammation is associated with increased number of macrophages, neutrophils and $CD8^+$ T lymphocytes, which all release multiple mediators such as pro-inflammatory cytokines, leukotrienes, chemokines, growth factors and proteases [82-87]. Oxidants derived from cigarette smoke and inflammatory cells activate macrophages to release pro-inflammatory mediators including TNF-α, IL-8 and LTB_4, mostly regulated by the transcription factor nuclear factor-κB (NF-κB) [88-90]. These mediators enhance neutrophil recruitment, orchestrate tissue destruction and chronic inflammation, and perpetuate bronchoconstriction, airway remodeling and mucus hypersecretion [79, 91, 92]. Interestingly, smoking cessation

does not resolve airway inflammation, suggesting that there might be mechanisms that maintain the chronicity of inflammation once it is established [93].

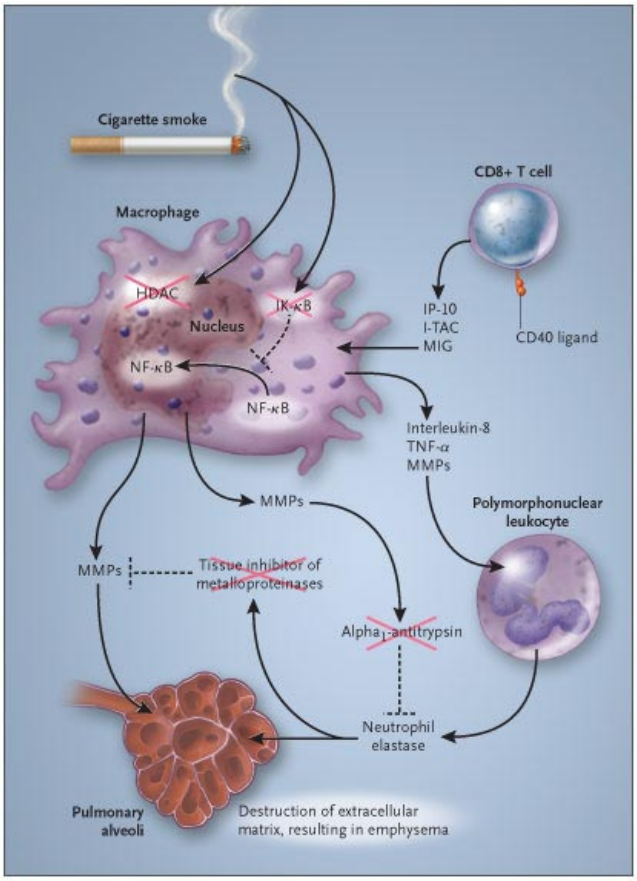

Figure 1.2. Complexity of the inflammatory, proteases/antiproteases and oxidative stress response in COPD. Reactive oxygen species resulting from cigarette smoke and inflammatory cells promote inactivation of histone deacetylase (HDAC), leading to transcription of NF-κB-mediated cytokines, neutrophil chemokines (TNF-α, IL-8) and proteases. These factors further recruit and activate neutrophils to the lung. Through their mediator release, $CD8^+$ T cells also activate macrophages. Besides digesting ECM, matrix metalloproteinases (MMPs) and neutrophil elastase degrade their inhibitors, the tissue inhibitor of metalloproteinases (TIMPs) and α_1-antitrypsin, enhancing their matrix-degrading capacities leading to emphysema. Figure by Shapiro with permission from N Engl J Med [92].

Proteases/antiproteases imbalance

In COPD, there is a loss of elasticity and destruction of the lung parenchyma, indicating that elastin and collagen are major targets of serine, cysteine proteases and MMPs [94, 95]. Neutrophils secrete the serine proteases neutrophil elastase and cathepsins, MMP-8 and MMP-9, all contributing to ECM destruction. Alveolar macrophages also secrete MMP-2, MMP-9, MMP-12 and cathepsins [86, 96-98]. The levels of these proteases are elevated in bronchoalveolar lavage fluid (BALF) and sputum of COPD patients [99-101]. Under

physiological tissue repair, proteases are in balance with their specific inhibitors, including α_1-antitrypsin and TIMPs, but this balance is altered in COPD [95, 102].

Oxidative stress

Each puff of cigarette smoke generates 10^{15} to 10^{17} free radicals. These reactive oxygen species and the one produced by inflammatory cells decrease the antiprotease activity and activate transcription of neutrophil chemokines [103-105]. These cellular damages may amplify the inflammatory response, mucus secretion, bronchoconstriction and exacerbations in COPD patients [57, 106, 107].

Treatment

At present, no therapies are effective at reversing progression of COPD but they increase physical capacity and reduce symptoms, number and severity of exacerbations. Smoking cessation at all stages of COPD is the most important therapeutic intervention to reduce progression of the disease [108]. Beside smoking cessation, COPD guidelines recommend the use of bronchodilators, anticholinergics or LABA, as the first line of treatment for symptomatic COPD patients [109-111]. In addition, high-dose of inhaled corticosteroids have been shown to reduce the frequency and/or severity of exacerbations in patients with severe COPD but were ineffective at reducing the progression of the disease [112-116]. The inflammation in COPD is largely resistant to the anti-inflammatory effects of corticosteroids [117-119]. This resistance seems to be caused by reduced activity of the nuclear enzyme HDAC-2 required for corticosteroids to switch off inflammatory genes [120-122]. Nevertheless, inhaled corticosteroids are recommended as an add-on therapy to anticholinergics and LABA in patients with severe COPD and frequent exacerbations, leading to improvement of lung function together with a marked reduction in symptoms [123-126].

1.3. AIRWAY REMODELING

Airway remodeling leads to changes in structures of the airways and connective tissue through different dynamic processes such as differentiation, migration and proliferation of structural cells. Although the exact relationship between airway remodeling and the pathogenesis of asthma and COPD remains unclear, remodeling definitely occurs in both diseases with differences in the structures, anatomic sites and cell types involved [127-130].

Airway remodeling in asthma

Characteristics

Airway remodeling in asthma seems to occur in childhood with pathological structural changes appearing even before the development of symptoms [131-134]. As shown in Fig. 1.3, epithelium damages, thickening of the reticular basement membrane, subepithelial fibrosis, ASMC hyperplasia and hypertrophy and increased vascularity are the major features of remodeling in asthma. Furthermore, these pathological changes have been associated with the duration and severity of the disease [135-140].

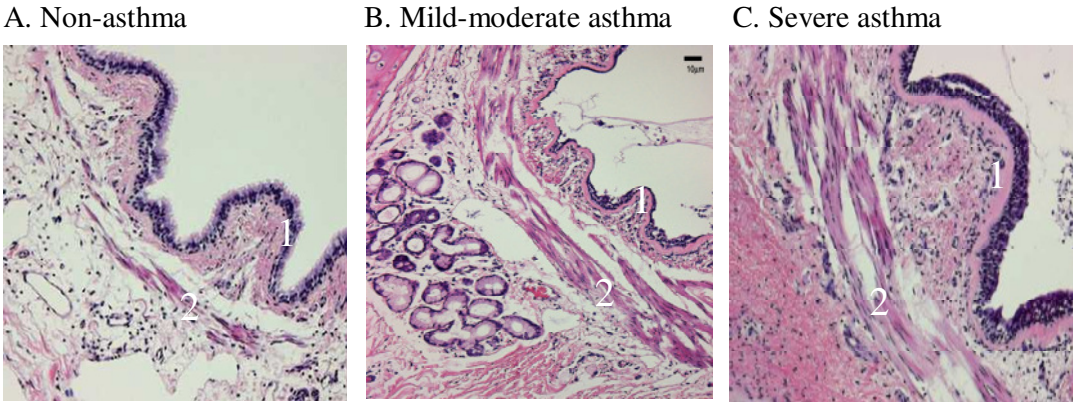

Figure 1.3. Structural changes in airways of asthma patients. Histology of airways of (A) an individual who died of causes unrelated to asthma, (B) a patient with mild-moderate asthma and (C) a patient with severe asthma who died of status asthmaticus. (1) A marked thickening of the basement membrane and (2) increased smooth muscle mass are apparent in the asthmatic airways. Figure by Borger *et al.* [141].

Epithelial damage

In asthma, the bronchial epithelium is highly abnormal with epithelial cell desquamation also termed "epithelial shedding", epithelial metaplasia, goblet cell hyperplasia and increased mucus secretion [142-144]. The number of goblet cells in asthma correlates with the expression and activation of mucins, thereby contributing to mucus hypersecretion, lumenal plugging and asthma severity [145-149].

Reticular basement membrane thickening

The "true" basement membrane consists of the lamina rara and lamina densa and is not altered in asthma patients. However, the lamina reticularis localized just below the basement membrane is 10 to 15 μm in depth, which is typically twice the thickness found in non-diseased bronchi [150]. Indeed, this subepithelial fibrosis results from increased deposition of collagen (types I, III, V), fibronectin, tenascin and glycosaminoglycans (GAGs) [151-156]

and correlates with the number of myofibroblasts below the reticular basement membrane [157, 158]. Furthermore, the increase in reticular basement membrane parallels the whole airway wall thickening in patients with asthma [159, 160].

ASMC hypertrophy and hyperplasia

Hyperplasia or hypertrophy of ASMC is another hallmark of the submucosa in asthma patients and plays a critical role in the development of airway narrowing [161-163]. *In vitro*, ASMC from asthma patients have a higher proliferation rate compared to non-asthmatics [164]. In addition, they produce more growth factors and ECM proteins, suggesting an autocrine regulation of their own proliferation [165-167]. This increased proliferation in asthmatic ASMC has been linked to a translational defect in the transcription factor CCAAT/enhancer binding protein α (C/EBPα), an inhibitor of ASMC proliferation [168].

Angiogenesis and increased vascularity

Increased airway vascularity in mild-to-severe asthmatics has been shown to contribute to airway wall thickening and oedema [169-172]. This elevated number of vessels has been associated with overexpression of vascular endothelial growth factor (VEGF) and angiogenesis [173-178]. Furthermore, overexpression of VEGF in the airway wall seems to correlate with basement membrane thickening in asthma patients [179].

Pathogenesis

The precise sequence of events during the development of airway remodeling in asthma remains poorly understood. However, inflammatory, epithelial and structural cells are actively involved via secretion of cytokines and growth factors. In turn, these mediators may affect the phenotype, proliferation and secretory response of these same cells. To date, three hypotheses have been suggested to trigger remodeling in asthma: chronic inflammation, defect in epithelial repair and epithelial/mesenchymal communication and mechanical stress.

Repeated episodes of allergic inflammation

The most accepted hypothesis on the origin of remodeling in asthma is that structural changes are secondary to an underlying chronic inflammation. Indeed, repeated episodes of acute allergic inflammation constantly activate mast cells, T_H2 lymphocytes and eosinophils, leading to increased secretion of mediators. *In vitro*, various pro-inflammatory cytokines and growth factors, including IL-4, IL-13, TNF-α and TGF-β_1, have been shown to increase collagen type I, III, tenascin and GAGs synthesis by lung fibroblasts [180-184]. Moreover,

the serine protease tryptase released by degranulating mast cells has been reported to stimulate fibroblasts and ASMC proliferation as well as type I collagen synthesis [185]. In addition, eosinophilic inflammation has been linked to thickening of the reticular basement membrane [186-188]. Therefore, chronic inflammation constantly activates inflammatory cells, which produce pro-inflammatory cytokines and growth factors, promoting mesenchymal cells differentiation and increased ECM synthesis.

Defective epithelial repair and epithelial-mesenchymal communication

Airway remodeling in asthma may also result from a defect in epithelial repair with reactivation of epithelial-mesenchymal trophic unit [189, 190]. This describes the interactions between the bronchial epithelium and the mesenchymal cells immediately underneath and is essential for lung branching and morphogenesis. Accordingly, repeated epithelial injuries can result in prolonged activation of the epithelial-mesenchymal trophic unit and may lead to tissue remodeling [191]. In asthma, the epithelial repair is altered and induces the secretion of epidermal growth factor, TGF-β_1 and fibroblast growth factor, which stimulate differentiation and proliferation of myofibroblasts, leading to increased ECM deposition [143, 192-195].

Mechanical stress

Airway obstruction produces constant folding of the airway wall. In response to such mechanical stress, epithelial cells adapt their phenotype and signal to nearby mesenchymal cells in order to activate tissue repair [196]. *In vitro*, mechanical stress stimulates the secretion of growth factors by epithelial cells and differentiation of fibroblasts and ASMC toward a pro-fibrotic profile [197-202].

Clinical consequences

Because increased airway wall thickening results in reduced airway calibre, airway remodeling is a significant contributor to airway narrowing, resulting in persistent airway obstruction, hyperresponsiveness and lung function decline [47, 203]. Accordingly, thickening of the reticular basement membrane seems to correlate with increased airflow limitation and airway hyperresponsiveness [204-207]. Remodeling features may also affect mechanical properties of the airway wall resulting in its stiffening [208, 209]. Furthermore, increased vascularity and mucus hypersecretion account for considerable swelling of the airway wall and obstruction of the lumen [149, 171].

Interestingly, depending on the localization and severity of the structural changes, these may protect against excessive tissue damages. Fundamentally, thickening of the basement membrane may prevent infiltration of inflammatory cells and proteins into the lung connective tissue. Indeed, airway wall thickening has been shown in some studies to reduce airway reactivity and constriction in asthma patients [150, 210-212]. Such protective effects may also be partly explained by the fact that remodeling makes the airway stiffer in order to resist dynamic compression during bronchoconstriction.

Airway remodeling in COPD

Compared to asthma, less is known about the remodeling process in COPD. The structural changes appear later in life in the pathogenesis of the disease, are closely associated with smoking and occur primarily to the peripheral airways and parenchyma (Fig. 1.4).

Structural alterations and functional consequences

Peripheral airways

In COPD, the bronchial mucosa is characterized by epithelial cell metaplasia, goblet cell hyperplasia, mucous gland enlargement, ASMC hypertrophy and infiltration of inflammatory cells into the tissue [78, 127, 130, 213-215]. Pulmonary vascular changes such as thickening of the vascular wall, angiogenesis and increased vascularity are also important features of remodeling in COPD [171, 216-218]. In contrast to asthma, the thickness of the reticular basement membrane is very variable and subjected to controversy in COPD [129]. These pathological changes lead to mucus hypersecretion, thickening of the airway wall, airway narrowing and airway obstruction that can induce airflow limitation in COPD [81, 219-221].

Lung parenchyma

In addition to collagen, elastin and proteoglycans confer the lung parenchyma its resilience necessary to undergo repetitive physiologic stress during breathing and are the major ECM components degraded in emphysema [222-224]. Emphysema describes the destruction of alveolar walls and parenchyma, histologically represented as holes in the connective tissue, which reduce the lung elastic recoil in COPD patients. Seeming controversial, airway and alveolar wall fibrosis with increased number of fibroblasts may also be present in parallel with parenchymal destruction. Interestingly, tissue repair with increased collagen and elastin deposition has also been reported in the alveolar walls of COPD patients [225, 226]. Furthermore, it was recently reported that collagen deposition even correlated with lung

destruction in human emphysema [227]. Together, emphysema and active tissue repair are major pathologic features of COPD and contribute to airflow obstruction and limitation responsible for severity of the disease [56].

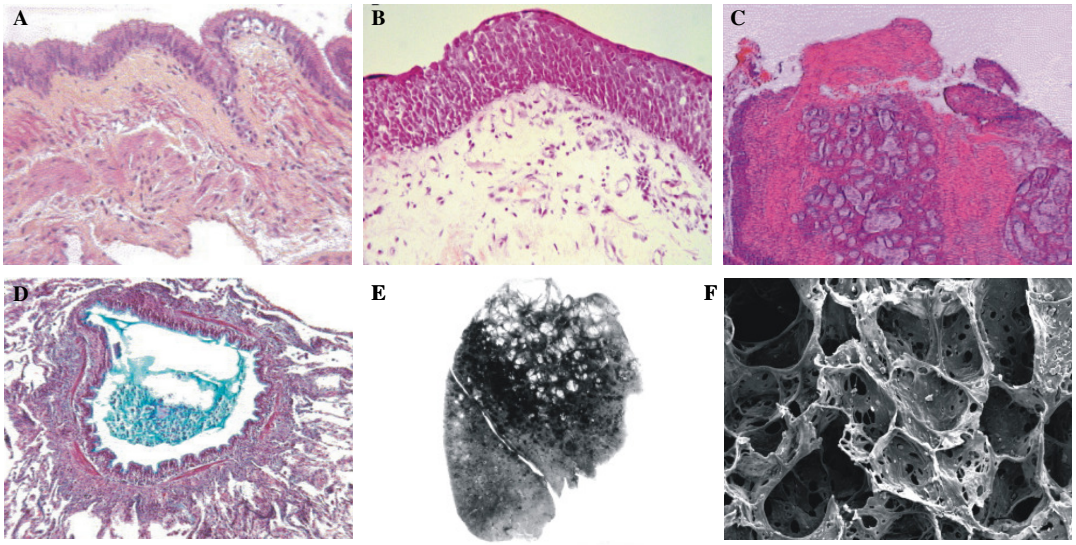

Figure 1.4. Structural alterations of the peripheral airways and parenchyma in smokers with COPD. (A) Presence of goblet cells in the epithelium, thickening of the airway wall with inflammatory cells infiltration and increase in smooth muscle mass. (B) Epithelial squamous metaplasia and basement membrane of normal thickness. (C) Mucous gland hyperplasia. (D) Inflamed small airway with thickened wall and lumen occlusion with inflammatory exudates of mucus and cells. (E) Gross appearance of emphysema. (F) Lung alveoli showing microscopic emphysema and subsequent enlargement of alveolar spaces. Figures by Jeffery [127], Hamid *et al.* [228] and Hogg [229].

Pathogenesis

In COPD, all these structural changes are mostly attributed to direct injury and inflammation from repeated exposures to cigarette smoke, environmental and/or occupational pollutants. In peripheral airways, the extent of inflammation is likely to be associated with aberrant repair process [127, 230]. The primary mechanism of lung parenchyma destruction is though to be an imbalance of endogenous proteases and antiproteases in the lung [95, 231]. Accordingly, increased expression and/or activity of proteases have been reported in BALF and lung tissue of patients with emphysema [90, 99, 101, 232-236]. Their role in the development of the disease was further highlighted by animal studies. For example, instillation of serine proteases in rodents induced infiltration of neutrophils in the lung tissue and structural changes resembling human emphysema [237-239]. Furthermore, disruption of the alveolar wall and airspace enlargement were reported in transgenic mice overexpressing MMP-1 and in TIMP-3 deficient mice [240, 241]. In addition, cigarette smoke-induced emphysema was prevented in

mice deficient for macrophage metalloelastase MMP-12 [96]. These studies show that a proteases/antiproteases imbalance in favour of proteolysis leads to lung tissue destruction. Thus, chronic inflammation and aberrant repair process seem to be the underlying cause to airway wall thickening and parenchymal destruction in COPD.

Airway remodeling in asthma and COPD: a summary

In summary, remodeling of the airway wall occurs in both asthma and COPD with differences in cellular infiltrates and structural changes as summarized in Table 1.1. Briefly, airway inflammation in asthma is characterized by mast cells, eosinophils and $CD4^+$ T lymphocytes whereas in COPD, macrophages, neutrophils and $CD8^+$ T lymphocytes are predominantly involved. Regarding the structural changes, epithelial shedding and reticular basement membrane thickening occur only in asthma, increased smooth muscle occur in large airways in asthma and in small airways in COPD, and emphysema is a feature of COPD only.

Table 1.1. Airway inflammation and remodeling in asthma and COPD. Adapted from [128-130].

Structural alterations	Asthma	COPD
Predominant anatomic localization	large airways	small airways and alveoli
Inflammatory cells	mast cells, eosinophils, $CD4^+$ T lymphocytes	macrophages, neutrophils $CD8^+$ T lymphocytes
Epithelial shedding	+ + +	-
Goblet cell hyperplasia	+ +	+ +
Squamaous cell metaplasia	+	+ + + +
Mucous gland hypertrophy	+ + +	+ + + +
Reticular basement membrane thickening	+ + +	-
Increased smooth muscle mass	+ + + (large airways)	+ + (small airways)
Airway wall thickening	+ +	+ + (small airways)
Airway wall fibrosis	+ (when severe)	+ +
Angiogenesis and increased vascularity	+ +	+
Emphysema	-	+ + +

1.4. ASTHMA AND COPD CURRENT THERAPY

Inhaled corticosteroids

Inhaled corticosteroids such as budesonide and fluticasone are the most effective anti-inflammatory agents currently available for the long-term treatment of asthma [1]. They reduce symptoms through inhibition of inflammatory cell infiltration in the bronchial mucosa and decrease of pro-inflammatory mediators [242]. Their predominant anti-inflammatory

effect is to "switch off" inflammatory genes through the interaction between the activated glucocorticoid receptors (GR) and pro-inflammatory transcriptions factors, such as NF-κB (Fig. 1.5) [243-250]. Corticosteroids diffuse across cell membranes and bind to GR in the cytoplasm. In an unbound state, GR remain in the cytosol and are complexed to several different proteins including the chaperones heat shock protein 90 (Hsp90). On ligand binding, the GR undergo conformational changes and dissociate from Hsp90, leading to nuclear translocation of the corticosteroid-GR complexes. Then, corticosteroid-GR block histone acetylation at the site of inflammatory gene transcription, either by direct binding to NF-κB-associated coactivators or by recruitment of HDAC-2, leading to suppression of activated inflammatory genes [251, 252].

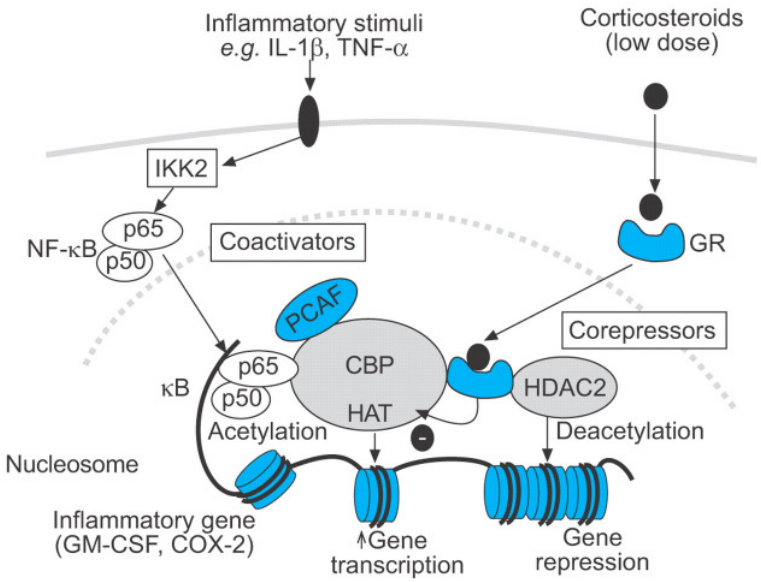

Figure 1.5. "Switch off" of activated inflammatory genes by corticosteroids. Many inflammatory genes are activated by stimuli (IL-1β and TNF-α), resulting in activation of NF-κB, which translocates to the nucleus. NF-κB binds to specific recognition sites in the promoter regions of responsive genes and subsequently recruits transcriptional coactivators, such as CBP and p300/CBP activating factor (PCAF), that have intrinsic acetyl transferase (HAT) activity, leading to increased transcription of inflammatory genes. Corticosteorids bind to GR in the cytosol. These complexes translocate to the nucleus and inhibit HAT by interacting with coactivators and by recruiting HDAC-2, leading to suppression of inflammatory genes. Figure by Barnes *et al.* [250].

Corticosteroids may also "switch on" anti-inflammatory gene expression (Fig. 1.6). Activated corticosteroids-GR complexes translocate to the nucleus, form dimers and interact with glucocorticoid-response elements (GRE) in the promoter region of corticosteroids-sensitive genes. GR-GRE interactions lead to recruitment of coactivators molecules, such as CREB-binding protein (CBP), resulting in histone acetylation, chromatin unwinding and transcription of genes encoding anti-inflammatory proteins [249, 253].

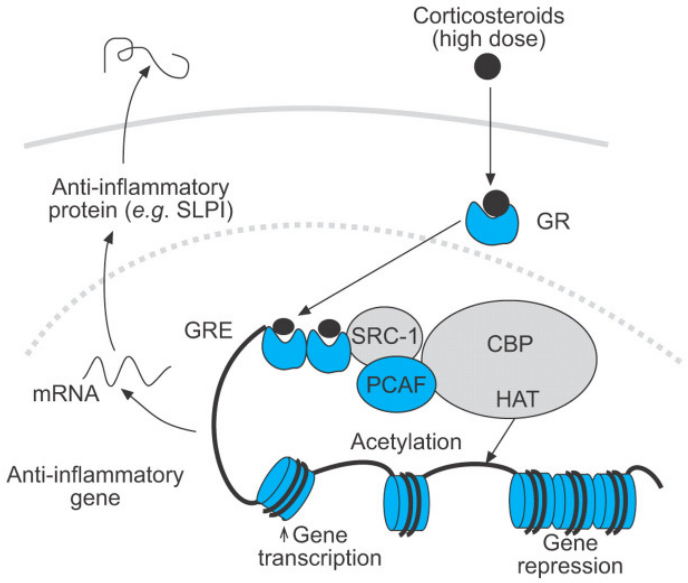

Figure 1.6. "Switch on" of anti-inflammatory gene expression by corticosteroids. Corticosteroids bind to cytoplasmic GR and translocate to the nucleus, where the corticosteroids-GR complexes bind to GRE in the promoter region of corticosteroids-sensitive genes and bind to coactivator molecules with HAT activity, causing acetylation and activation of genes encoding anti-inflammatory proteins. Figure by Barnes *et al.* [250].

Airway inflammation in COPD is corticosteroids-resistant. High doses of corticosteroids have no effect on the inflammatory cells, cytokines profiles or proteases/antiproteases imbalance in COPD patients [117-119]. This lack of response may be secondary to increased oxidative stress as a result of cigarette smoking, leading to reduced HDAC-2 activity [120, 122]. Recently, the bronchodilator theophylline has been shown to stimulate HDAC activity in COPD, consequently restoring the anti-inflammatory actions of corticosteroids [254].

Inhaled LABA

LABA such as formoterol and salmeterol are used as bronchodilators to bring rapid relief of airway bronchoconstriction. LABA exert their biological and therapeutic effects through the cell surface β_2-adrenergic receptors, which are members of the G-protein-coupled receptor family. Ligand-binding involves activation of the G_s-protein that in turn activates adenyl cyclase. This process leads to production of cyclic adenosine monophosphate (cAMP) and subsequent activation of protein kinase A, which then phosphorylates intracellular regulatory proteins involved in the control of muscle tone, such as myosin light chain kinase. Increased cAMP also results in inhibition of intracellular calcium and calcium release, leading to ASMC relaxation [255]. Besides, LABA exhibit non-bronchodilatory properties such as inhibition of pro-inflammatory mediators release and prevention of plasma leakage [256, 257].

Combination therapy

The combination of corticosteroids and LABA results in better outcomes than higher doses of corticosteroids in asthma [258-260] and COPD [124, 125, 261] with better symptoms control and an additive or synergistic reduction of airway inflammation. Although much of the benefits of the combination likely are through their specific mechanisms of action (anti-inflammatory versus bronchodilation), some of the additive effects may be due to the ability of LABA to enhance GR activation and nuclear translocation and to the ability of corticosteroids to increase β_2-adrenergic receptor synthesis [257, 262-266].

Effect on airway remodeling

Despite substantial evidence on the effect of inhaled corticosteroids on airway inflammation in asthma, data on remodeling are more limited and not clear [267]. While short-term corticosteroid treatment showed no significant change on the thickness of the reticular basement membrane [205, 268, 269] longer therapy resulted in a significant reduction mediated via decreased ECM deposition, growth factor and MMPs expression [154, 206, 270-275]. Inhaled corticosteroids have also been shown to reduce airway submucosa vascularity in asthma [170, 276, 277]. In contrast to the inhibitory effect of corticosteroids on cell proliferation of ASMC derived from healthy subjects [278], the absence of C/EBPα appeared to be responsible for the enhanced proliferation of ASMC from asthma patients and might further explain the failure of corticosteroids to inhibit their proliferation *in vitro* [168, 279]. All these structural changes result in an overall airway wall thickness, which seems to be reduced by short-term treatment with inhaled corticosteroids in asthma [138].

Less is known about the effect of LABA on airway remodeling. Briefly, LABA have been shown to reduce airway vascularity in asthma *in vivo* [280] and cell proliferation of healthy-derived ASMC [281]. Finally, combination therapy has been shown to inhibit ASMC proliferation to a greater extent than each drug alone through synchronized activation of GR and C/EBPα [282]. The effect of corticosteroids and/or LABA on structural alterations in COPD is not yet investigated. All together, these studies suggest a potential therapeutic role of the current asthma and COPD therapy in reversing the airway remodeling process.

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CHAPTER 2

OBJECTIVES

Prevalence of asthma and COPD is rapidly increasing worldwide. Both diseases result from chronic airway inflammation and tissue remodeling. Airway remodeling is characterized by various structural changes, including increased ECM deposition, more specifically of collagen and GAGs mainly synthesized by fibroblasts and ASMC. The basic therapy for asthma and COPD consists of corticosteroids and LABA, which are efficient at controlling symptoms, bronchoconstriction and inflammation but their effect at preventing or reversing lung tissue remodeling is unclear.

Therefore, the general aim of this thesis was to develop a human cell culture based *in vitro* model of airway inflammation and tissue remodeling in order to investigate the modulation of ECM deposition by lung fibroblasts and ASMC and their response to the actual asthma and COPD therapy.

The specific objectives of this thesis were:

- To develop an *in vitro* model mimicking airway inflammation and active tissue repair.
- To assess (*i*) the effect of corticosteroids and LABA, alone or in combination, on total ECM, collagen and GAGs deposition by primary human lung fibroblasts under non-inflammatory and inflammatory conditions and (*ii*) the mechanisms potentially involved in the drug effect.
- To investigate (*i*) the effect of corticosteroids and LABA on total ECM, collagen and GAGs deposition as well as gelatinolytic activity by primary human ASMC under non-inflammatory and inflammatory conditions and (*ii*) whether ASMC derived from asthma and COPD patients respond the same way as ASMC from healthy subjects.

CHAPTER 3

EFFECT OF CORTICOSTEROIDS AND LABA ON LUNG TISSUE REMODELING *IN VITRO* IN PRIMARY HUMAN LUNG FIBROBLASTS

Asthma and COPD are characterized by chronic airway inflammation and tissue remodeling, which includes increased deposition of ECM components, such as collagen and GAGs mainly produced by fibroblasts. In turn, these ECM proteins influence cell proliferation, migration, synthesis of pro-inflammatory mediators and growth factors, which may further sustain the inflammatory and remodeling processes. Thus, the ECM is a dynamic structure playing a key role in lung tissue structural alterations. Unfortunately, *in vivo* this pathological remodeling is not prevented or reversed by the current anti-inflammatory drugs and bronchodilators. A better understanding on the regulation of ECM components in lung fibroblasts by different mediators involved in lung diseases and by the mainstay therapies might provide new potential targets to prevent airway remodeling in patients. Therefore, using primary human lung fibroblasts we investigated the effect of corticosteroids and LABA in relation to tissue remodeling.

This chapter includes results of the following projects:

- Opposite effect of corticosteroids and LABA on serum- and TGF-β_1-induced ECM deposition by primary human lung fibroblasts. Manuscript accepted in the *Journal of Cellular Physiology*.
- Effect of corticosteroids and LABA on serum- and TGF-β_1-induced total GAGs secretion and deposition by primary human lung fibroblasts. Ongoing research project.

These results were presented as poster-discussion (2006) and oral presentation (2005) at the American Thoracic Society International Conference (San Diego, California, USA) and the abstracts were published in *Proceedings of the American Thoracic Society*.

3.1. OPPOSITE EFFECT OF CORTICOSTEROIDS AND LABA ON SERUM- AND TGF-β_1-INDUCED ECM DEPOSITION BY PRIMARY HUMAN LUNG FIBROBLASTS

Stéphanie Goulet[1], Michel P. Bihl[1], Franco Gambazzi[2], Michael Tamm[1], and Michael Roth[1,3*]

[1]Pulmonary Cell Research, Department of Research and [2]Department of Thoracic Surgery, University Hospital Basel, 4031 Basel, Switzerland; [3]The Woolcock Institute of Medical Research, University of Sydney, New South Wales 2050, Australia

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Abstract

Asthma and chronic obstructive pulmonary disease are characterized by chronic airway inflammation and major structural lung tissue changes including increased extracellular matrix (ECM) deposition. Inhaled corticosteroids and long-acting β_2-agonists (LABA) are the basic treatment for both diseases but their effect on airway remodeling remains unclear. In this study we investigated the effect of corticosteroids and LABA, alone or in combination, on total ECM and collagen deposition, gene expression, cell proliferation and IL-6, IL-8 and TGF-β_1 levels by primary human lung fibroblasts. In our model, fibroblasts in 0.3% albumin represented a non-inflammatory condition and stimulation with 5% FCS and/or TGF-β_1 mimicked an inflammatory environment with activation of tissue repair. 5% FCS increased total ECM, collagen deposition, cell proliferation and IL-6, IL-8 and TGF-β_1 levels. In 0.3% albumin corticosteroids reduced total ECM and collagen deposition, involving the glucocorticoid receptor (GR) and downregulation of collagen, Hsp47 and Fli1 mRNA expression. In 5% FCS corticosteroids increased ECM deposition, involving upregulation of COL4A1 and CTGF mRNA expression. LABA reduced total ECM and collagen deposition under all conditions partly via the β_2-adrenergic receptor. In combination, the drugs had an additive effect in the presence or absence of TGF-β_1 further decreasing ECM deposition in 0.3% albumin whereas counteracting each other in 5% FCS. These data suggest that the effect of corticosteroids but not of LABA, on ECM deposition by fibroblasts is altered by serum. These findings imply that as soon as airway inflammation is resolved, long-term treatment with combined drugs may beneficially reduce pathological tissue remodeling.

Introduction

Prevalence of asthma and chronic obstructive pulmonary disease (COPD) is rising worldwide and both diseases are characterized by chronic airway inflammation often leading to irreversible structural changes of the lung. The airway remodeling features include increased micro-vascularization, vessel leakage, smooth muscle cell hyperplasia and hypertrophy, thickening of the lamina reticularis and increased interstitial extracellular matrix (ECM) deposition [1-6]. It is still not clear whether the pathological ECM deposition results from chronic airway inflammation or is a pre-condition that contributes to the inflammatory process. Several studies reported that an increase of collagen contributed to airway hyperresponsiveness and correlated with subepithelial fibrosis and the number of fibroblasts

in the submucosa of asthma patients [7-11]. Therefore, ECM synthesis and degradation by fibroblasts play an essential role in lung tissue homeostasis and repair.

TGF-β_1 contributes to the daily and pathological ECM turnover in the lung and also modulates cell proliferation, differentiation, migration and apoptosis [12, 13]. TGF-β_1 expression is increased in the bronchoalveolar lavage fluid and in the airways of patients with asthma [14-16] and in the airway epithelium of patients with COPD [17, 18]. TGF-β_1 can function either as a pro- or anti-inflammatory cytokine on inflammatory cells and regulate ECM production mainly by fibroblasts located in the airway wall [19, 20]. In non-diseased human lung fibroblasts we previously reported that TGF-β_1 increased IL-6 secretion and stimulated the synthesis of ECM proteins, specifically of collagens and glycosaminoglycans [21-23]. Studies have shown a significant correlation between the number of TGF-β_1 positive cells, collagen synthesis and subepithelial fibrosis in asthma patients [15, 24, 25].

The current therapy for asthma and COPD consists of inhaled corticosteroids and long-acting β_2-agonists (LABA), applied alone or in combination. The anti-inflammatory actions of inhaled corticosteroids are well characterized but their effect on airway remodeling in asthma is controversially discussed [26-30] and not well-studied in COPD [31]. Even though the combination of inhaled corticosteroids and LABA resulted in better improvement of clinical outcome in patients [32], its influence on ECM is incompletely understood.

In this study, we used primary human lung fibroblasts to assess tissue remodeling, more specifically ECM deposition, in the early phase of lung inflammation and its modulation by corticosteroids and LABA. In this regard, an interesting report showed that the transcriptional program of serum-deprived primary human neonatal foreskin fibroblasts in response to 10% FCS was related to activation of wound healing processes, including cell migration, proliferation, inflammation and angiogenesis [33]. Another well-established model of wound healing and remodeling is the contraction of 3D collagen gels by fibroblasts and it was recently reported that both FCS and TGF-β_1 significantly augmented collagen gel contraction in a concentration-dependent manner [34]. Based on these findings, we assumed that fibroblasts under serum-free condition reflected a non-inflammatory environment, whereas stimulation with 5% FCS and/or TGF-β_1 mimicked an early stage of inflammation with vessel leakage, serum infiltration and activation of tissue repair. Under these two experimental conditions we investigated the effect of corticosteroids and LABA, alone or in combination,

on the release of pro-inflammatory markers, total ECM and collagen deposition, gene expression and cell proliferation.

Materials and Methods

Cell culture

Primary fibroblasts were established from non-diseased peripheral lung tissue samples obtained from patients undergoing lung resection following approval by the ethics committee (Faculty of Medicine, University Hospital Basel). Cells were grown in RPMI 1640 (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% FCS (Gibco BRL Invitrogen, Basel, Switzerland) and 1% MEM-vitamins (Cambrex Bio Science). Cells were treated with dexamethasone (Calbiochem, Lucerne, Switzerland), fluticasone, salmeterol (GlaxoSmithKline, London, UK), budesonide, formoterol (AstraZeneca, Lund, Sweden) and recombinant human TGF-β_1 (Sigma, Schnelldorf, Germany). Prior to any stimulation fibroblasts were serum-deprived for 24 h in 0.3% human albumin. Fibroblasts between passages 3 and 6 were used for all experiments.

IL-6, IL-8 and TGF-β_1 levels

Confluent serum-deprived fibroblasts were treated with corticosteroids and/or LABA (10^{-7} M) in 0.3% albumin or 5% FCS with or without TGF-β_1 (2.5 ng/ml) for 48 h. Conditioned cell culture media were collected and IL-6, IL-8 (Orgenium, Helsinki, Finland) and TGF-β_1 (R & D Systems, Minneapolis, MN, USA) levels were determined by ELISA according to the manufacturer's instructions.

Total ECM and collagen deposition

Total ECM and collagen deposition were assessed as previously described [21]. Into 24-well plates confluent serum-deprived fibroblasts were treated with corticosteroids and/or LABA (10^{-10} to 10^{-6} M) in 0.3% albumin or 5% FCS with or without TGF-β_1 (0.1 to 5 ng/ml) in the presence of 1 µCi/ml [^{3}H]-proline (Amersham, Little Chalfont, UK). To assess the signaling pathways, fibroblasts were pre-treated for 30 min with the glucocorticoid receptor (GR) antagonist RU486 (Sigma) or the β_2-adrenergic receptor antagonist propranolol (Calbiochem) at 10^{-6} M before adding a corticosteroid or a LABA (10^{-7} M). [^{3}H]-proline incorporation was measured by liquid scintillation counting 48 h after stimulation.

Total ECM and collagen deposition were calculated as follows: 1) cpm in solubilized ECM without collagenase was defined as "total ECM deposition", 2) (cpm in supernatant without collagenase x 100) / (cpm in supernatant without collagenase + cpm in solubilized ECM without collagenase) was defined as "percentage of background", and 3) cpm in supernatant with collagenase – (% background x cpm in supernatant with collagenase) was defined as "collagen deposition".

Reverse transcriptase-polymerase chain reaction

Confluent serum-deprived fibroblasts were treated with corticosteroids and/or LABA (10^{-7} M) in 0.3% albumin or 5% FCS for 48 h. Total RNA was isolated using RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) and reverse-transcribed using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). cDNA was amplified with the following primers (sense and antisense, respectively; MWG-Biotech, Ebersberg, Germany): β-actin (5'-GTACGTTGCTAT CCAGGCTGTGC-3', 5'-TCAGGCAGCTCGTAGCTCTTCTC-3', 335 bp), COL1A1 (5'-CC CCTGGCGCTGTCGGTCCTG-3', 5'-GGGCGCCAAGGTCTCCAGGAACA-3', 201 bp), COL1A2 (5'-CCCCCTGGCTTCGCTGGTGAGA-3', 5'-TTGCCAGGGTAACCGCGCTCT C-3', 343 bp), COL3A1 (5'-CCCTGCTGGTGCTCCCGGTCCT-3', 5'-CAGCGGCTCCAA CACCACCACAG -3', 397 bp), COL4A1 (5'-TGCCGGGCCTACTGGTCCAAGAG-3', 5'-GGGGCACGGTGGGATCTGAATG-3', 477 bp), COL5A1 (5'-CCCCCGGCCCTACTGGT GAACC-3', 5'-CGGGCGGGGTTCTGCTGCGT-3', 783 bp), CTGF (5'-GTGGAGTATGTA CCGACGGCC-3', 5'-ACAG GCAGGTCAGTGAGCACGC-3', 588 bp), Hsp47 (5'-GCGCA GCAGCAAGCAGCACTAC'-3, 5'-CGCGCCCGTAGATGTCCTGGTCA-3', 679 bp), and Fli1 (5'-CCGCGCCACCACCCTCTACA-3', 5'-GGCCCGGCTCAGCTTGTCGTAAT-3', 486 bp). PCR conditions were initial denaturation at 95°C for 3 min then denaturation at 95°C for 30 sec, primer annealing at 58°C for 30 sec and extension at 72°C for 1 min for 20-25 cycles, followed by a final extension at 72°C for 5 min. PCR reactions were size fractionated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide stain under UV light. The intensity of each band was analyzed by densitometry using NIH Image J software and the relative mRNA expression of target gene was normalized to the β-actin control.

Cell proliferation

Proliferation was measured by [^{3}H]-thymidine incorporation. Fibroblasts (5000 cells/well) were seeded into 96-well plates, grown until 60% confluence and serum-deprived for 24 h in 0.3% albumin. Cells were stimulated with 5% FCS and treated with corticosteroids and/or

LABA at 10^{-7} M in the presence of 2 μCi/ml [^{3}H]-thymidine (Amersham). Incorporated [^{3}H]-thymidine was determined by liquid scintillation counting 30 h after stimulation.

Statistical analysis

Data are expressed as means ± SEM. Student's *t*-test was performed and differences were considered statistically significant if $P < 0.05$ compared to control or as indicated.

Results

5% FCS and TGF-β_1 increased the release of IL-6 and IL-8

Confluent fibroblasts in 0.3% albumin secreted low levels of IL-6 (889 ± 514 pg/ml) and IL-8 (85 ± 43 pg/ml) within 48 h and stimulation with 5% FCS significantly increased cytokine levels by 4.5-fold ($P < 0.05$) and 19-fold ($P < 0.01$), respectively (Fig. 3.1A,B). TGF-β_1 increased IL-6 and IL-8 levels in 0.3% albumin but did not reach significance compared to control. In the presence of 5% FCS TGF-β_1 significantly augmented IL-6 (9819 ± 3299 pg/ml) and IL-8 (8255 ± 3373 pg/ml) levels compared to non-treated cells ($P < 0.05$; Fig. 3.1A,B). Corticosteroids (dexamethasone, fluticasone and budesonide at 10^{-7} M) significantly reduced both spontaneous and FCS-induced IL-6 and IL-8 release by approximately 75% ($P < 0.05$), two LABA (salmeterol and formoterol at 10^{-7} M) had no effect, and their combinations resulted in similar decreases as observed with the corticosteroids alone ($P < 0.05$; Fig. 3.1C,D). Similar results were obtained in the presence of TGF-β_1 (data not shown). The vehicle of the drugs, with a maximal final concentration of 0.001% DMSO, had no effect on IL-6 or IL-8 secretion by fibroblasts.

TGF-β_1 and 5% FCS additively increased total ECM and collagen deposition

In 0.3% albumin TGF-β_1 significantly increased total ECM and collagen deposition in a dose-dependent manner over a concentration range from 0.1 to 5 ng/ml ($P < 0.05$; Fig. 3.2A,B). Since 5% FCS initiated a pro-inflammatory response in primary human lung fibroblasts we investigated the effect of FCS on total ECM and collagen deposition. As depicted in Fig. 3.2C, 5% FCS alone significantly increased total ECM deposition by 3-fold compared to 0.3% albumin (6375 ± 464 versus 2125 ± 187 cpm; $P < 0.001$). When combined, 5% FCS and TGF-β_1 additively increased total ECM deposition compared to 5% FCS or TGF-β_1 alone ($P < 0.001$; Fig. 3.2C). Similar effects were observed on collagen deposition (Fig. 3.2D).

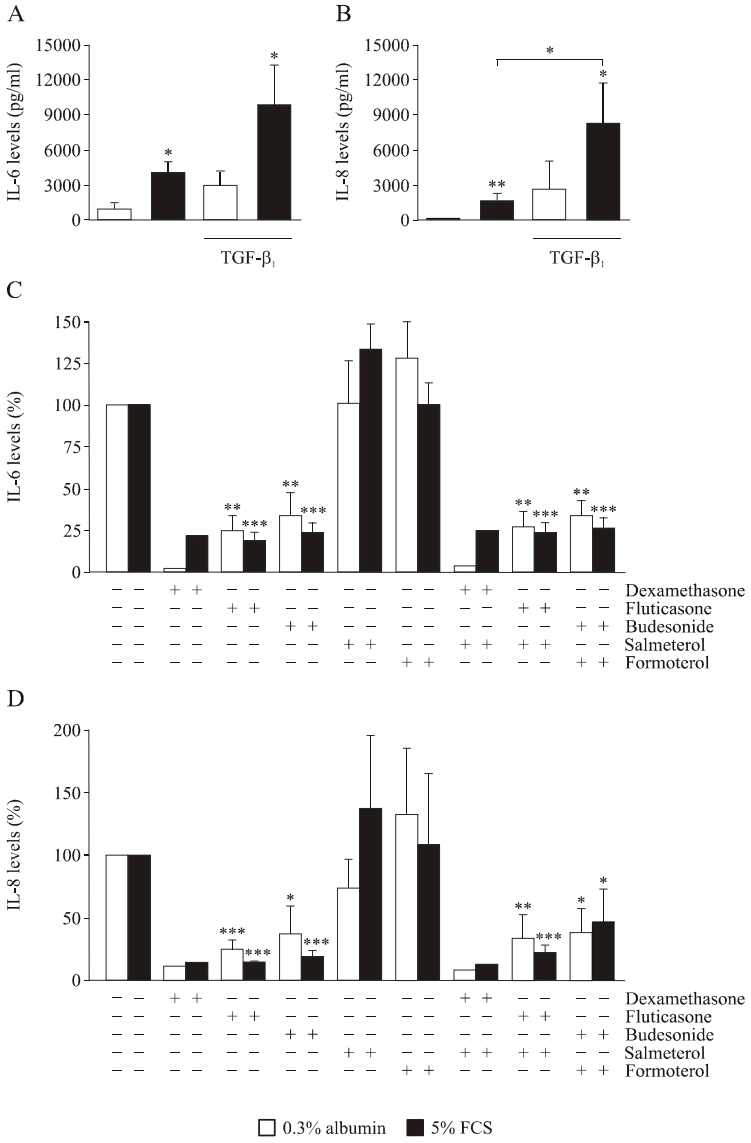

Figure 3.1. 5% FCS and TGF-β_1 increased IL-6 and IL-8 levels, which were decreased by corticosteroids but not LABA. Confluent serum-deprived fibroblasts were stimulated with 5% FCS and/or TGF-β_1 (2.5 ng/ml) for 48 h. Supernatants were collected and (A) IL-6 and (B) IL-8 levels were determined by ELISA (n = 4 cell lines). Cells were treated with corticosteroids and/or LABA (10^{-7} M) in 0.3% albumin or 5% FCS and (C) IL-6 and (D) IL-8 levels were measured (n = 3 cell lines, n = 1 for dexamethasone). Bars represent means ± SEM expressed as (A,B) pg/ml and as (C,D) % of control, where 0.3% albumin and 5% FCS were defined as 100%. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

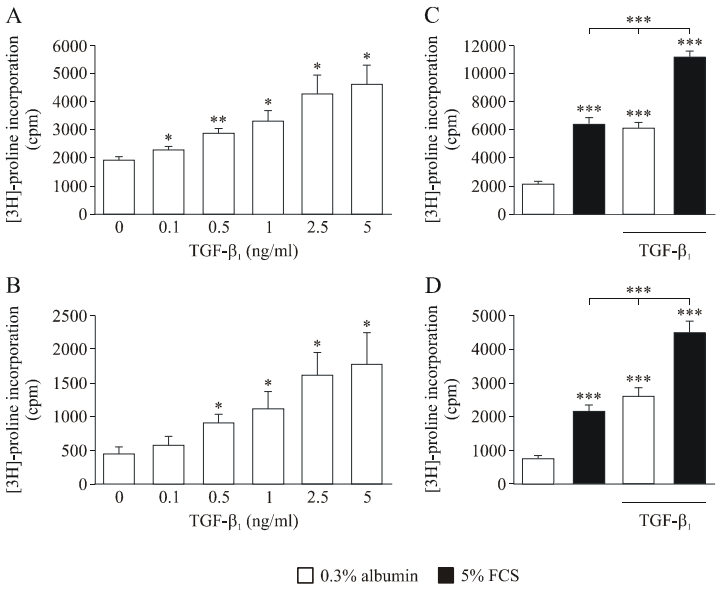

Figure 3.2. TGF-β_1 and 5% FCS increased total ECM and collagen deposition. Confluent serum-deprived fibroblasts were stimulated with TGF-β_1 (0.1 to 5 ng/ml) in 0.3% albumin for 48 h and (A) total ECM and (B) collagen deposition were determined by [^{3}H]-proline incorporation (n = 2 cell lines). Cells were stimulated with 5% FCS and/or 2.5 ng/ml TGF-β_1 and (C) total ECM and (D) collagen deposition were determined (n = 8 cell lines). Bars represent means ± SEM expressed as cpm. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Serum reversed the inhibitory effect of corticosteroids on total ECM and collagen deposition

We evaluated the effect of corticosteroids (on total ECM and collagen deposition at concentrations from 10^{-10} to 10^{-6} M in the presence or absence of 5% FCS (Fig. 3.3). The data of [^{3}H]-proline incorporation are expressed as percentage of change from control, where 100% was defined as total ECM and collagen deposition in 0.3% albumin or 5% FCS. In 0.3% albumin dexamethasone, fluticasone and budesonide decreased total ECM deposition in a dose-dependent manner with a significant maximal reduction of 32 ± 6%, 24 ± 5% and 34 ± 4%, respectively, at 10^{-6} M ($P < 0.001$; Fig. 3.3A-C). Interestingly, in 5% FCS the effect of corticosteroids on total ECM deposition was opposite to the effect observed in 0.3% albumin at therapeutically relevant concentrations. Dexamethasone further increased the FCS-induced total ECM deposition by 24 ± 5% at concentrations ≥ 10^{-8} M ($P < 0.01$; Fig. 3.3A). Fluticasone increased the FCS-induced total ECM deposition by 17% from concentrations ≥ 10^{-9} M but without a clear dose-dependent response ($P < 0.05$; Fig. 3.3B). Budesonide dose-dependently increased the FCS-induced total ECM deposition by 15 ± 7% at 10^{-9} M, and up to 30 ± 11% at 10^{-6} M ($P < 0.05$; Fig. 3.3C). Similarly to their effect on total ECM deposition,

corticosteroids decreased collagen deposition in 0.3% albumin, while increased it in 5% FCS (Fig. 3.3D-F).

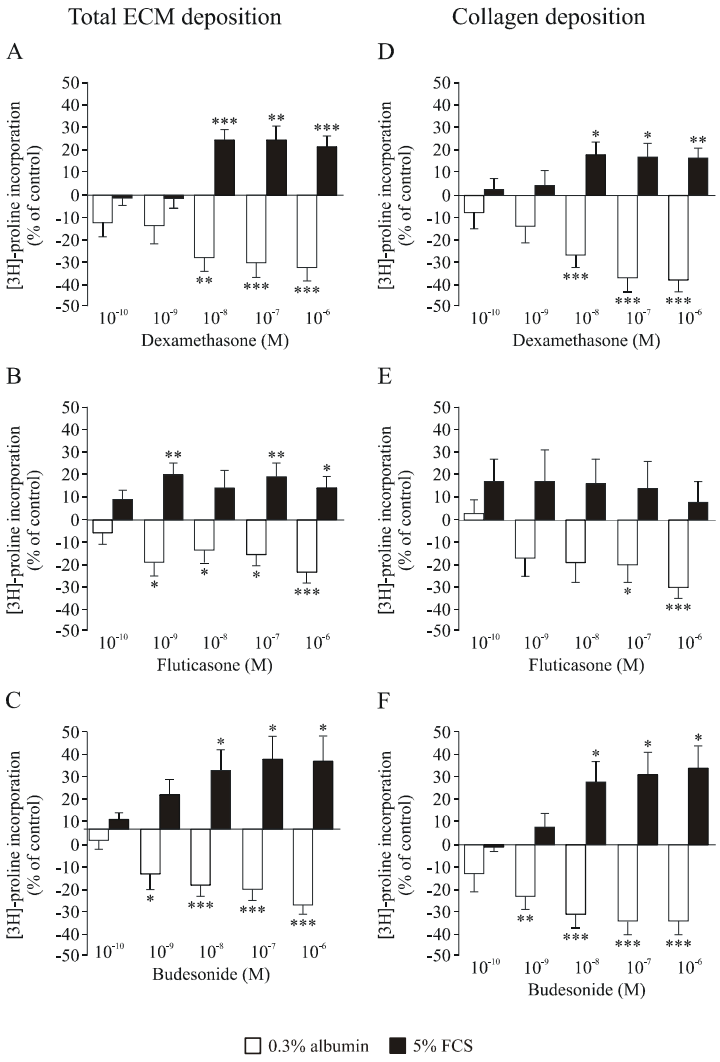

Figure 3.3. Opposite effects of corticosteroids on total ECM and collagen deposition depended on the presence of 5% FCS. Confluent serum-deprived fibroblasts were treated for 48 h with increasing concentrations (10^{-10} to 10^{-6} M) of (A,D) dexamethasone, (B,E) fluticasone or (C,F) budesonide in 0.3% albumin or 5% FCS and total ECM and collagen deposition, respectively, were determined by [^{3}H]-proline incorporation. Bars represent means ± SEM expressed as % of change from control, 0.3% albumin or 5% FCS. *P < 0.05, **P < 0.01 and ***P < 0.001 (n = 5 cell lines).

LABA reduced total ECM and collagen deposition

The effect of LABA on total ECM and collagen deposition in the presence or absence of serum is shown in Fig. 3.4. In both 0.3% albumin and 5% FCS salmeterol and formoterol (10^{-10} to 10^{-6} M) significantly decreased total ECM deposition (P < 0.05; Fig. 3.4A,B) and collagen deposition (P < 0.05; Fig. 3.4C,D) compared to the respective controls. However,

there was no clear dose-dependent response and both LABA reached their maximal effect at a clinically relevant concentration of 10^{-9} M. Thus, in contrast to corticosteroids the inhibitory effect of LABA on total ECM and collagen deposition was not affected by the presence of serum.

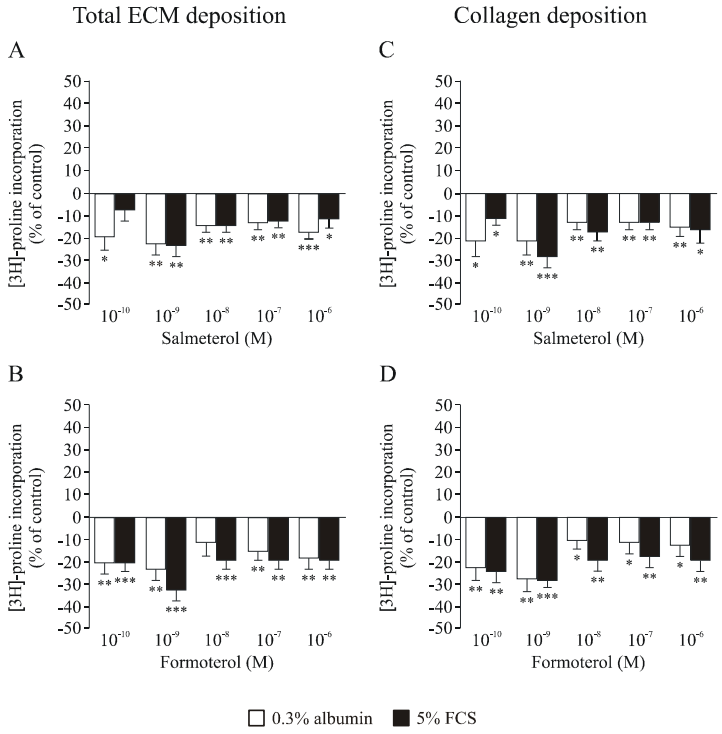

Figure 3.4. LABA decreased total ECM and collagen deposition in the presence or absence of serum. Confluent serum-deprived fibroblasts were treated for 48 h with increasing concentrations (10^{-10} to 10^{-6} M) of (A,C) salmeterol or (B,D) formoterol in 0.3% albumin or 5% FCS and total ECM and collagen deposition, respectively, were determined by [^{3}H]-proline incorporation. Bars represent means ± SEM expressed as % of change from control, 0.3% albumin or 5% FCS. *P < 0.05, **P < 0.01 and ***P < 0.001 (n = 5 cell lines).

Combination of corticosteroids and LABA showed an additive effect on total ECM and collagen deposition

Based on the above described results we assessed the effect of the combination of corticosteroids and LABA, all at 10^{-7} M, on total ECM and collagen deposition (Fig. 3.5). In 0.3% albumin all combinations significantly additively decreased total ECM deposition by 45 to 55% compared to the respective controls (P < 0.001) or to each drug alone (P < 0.05; Fig. 3.5A-C). Interestingly, in 5% FCS the addition of LABA reduced the corticosteroid-induced total ECM deposition close to control levels. Salmeterol counteracted the stimulatory effect of dexamethasone (17 ± 8 versus 48 ± 11%; P < 0.05; Fig. 3.5A) and fluticasone (30 ± 11 versus 68 ± 11%; P < 0.05; Fig. 3.5B) on total ECM deposition. Formoterol also reduced the

budesonide-induced total ECM deposition (8 ± 11 versus 59 ± 11%; $P < 0.01$; Fig. 3.5C). The three drug combinations showed similar effects on collagen deposition. In 0.3% albumin combined corticosteroids and LABA further decreased collagen deposition compared to each drug alone, while in 5% FCS LABA completely prevented the corticosteroid-induced collagen deposition (Fig. 3.5D-F).

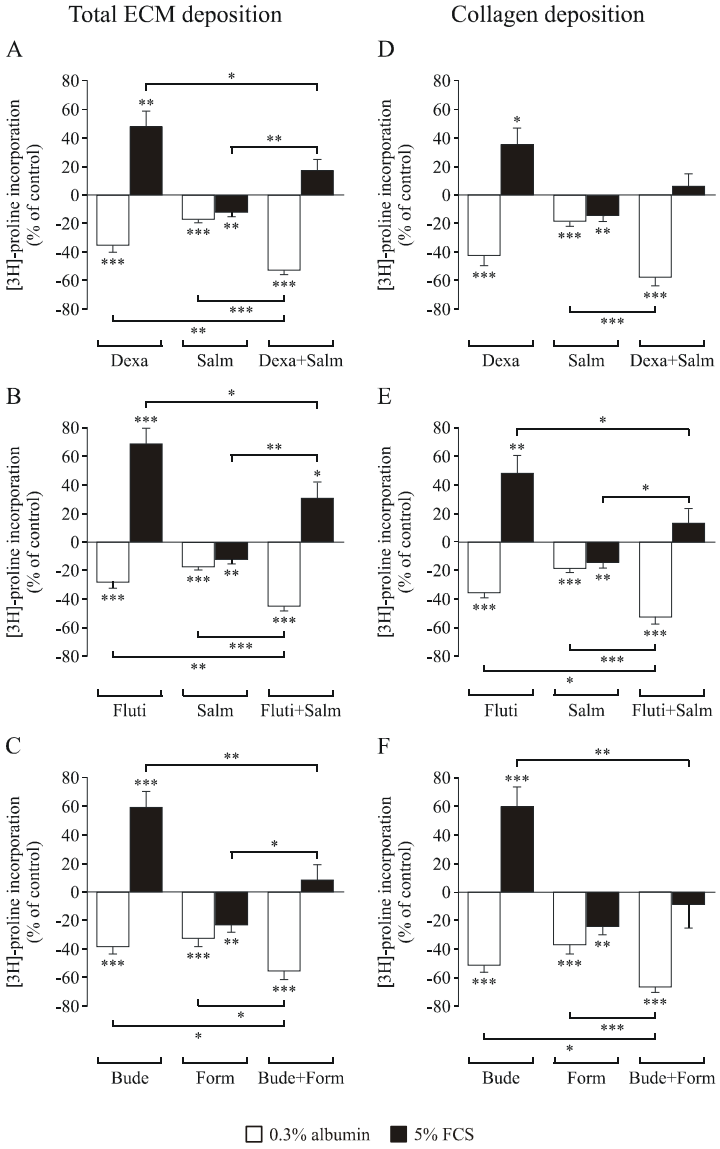

Figure 3.5. Combined corticosteroids and LABA had an additive effect on total ECM and collagen deposition. Confluent serum-deprived fibroblasts were treated with corticosteroids and LABA (10^{-7} M), alone or in combination, for 48 h in 0.3% albumin or 5% FCS and [^{3}H]-proline incorporation was determined. The following drug combinations were investigated: (A,D) dexamethasone and salmeterol (Dexa+Salm), (B,E) fluticasone and salmeterol (Fluti+Salm), and (C,F) budesonide and formoterol (Bude+Form) on total ECM and collagen deposition, respectively. Bars represent means ± SEM expressed as % of change from control, 0.3% albumin or 5% FCS. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (n = 5 cell lines).

The additive effect of corticosteroids and LABA on TGF-β_1-induced total ECM and collagen deposition

Regarding the relevance of TGF-β_1 for airway remodeling we investigated the effect of corticosteroids, LABA and their combination on TGF-β_1-induced total ECM and collagen deposition in the presence or absence of serum (Fig. 3.6). The data are expressed as percentage of change from control, where 100% was defined as total ECM and collagen deposition after TGF-β_1 (2.5 ng/ml) stimulation in 0.3% albumin or 5% FCS. In 0.3% albumin dexamethasone, fluticasone and budesonide significantly decreased the TGF-β_1-induced total ECM deposition by 29 ± 6% ($P < 0.01$), 29 ± 4% ($P < 0.001$) and 46 ± 5% ($P < 0.001$), respectively (Fig. 3.6A-C). The LABA, salmeterol and formoterol, significantly decreased the TGF-β_1-induced total ECM deposition by 25 ± 2% and 45 ± 5%, respectively ($P < 0.001$). Combined corticosteroids and LABA further decreased the TGF-β_1-induced total ECM deposition by a range from 51 ± 6% to 72 ± 4% compared to control ($P < 0.001$) or to each drug alone ($P < 0.05$; Fig. 3.6A-C).

In 5% FCS dexamethasone, fluticasone and budesonide further enhanced the stimulatory effect of combined FCS and TGF-β_1 on total ECM deposition by 29 ± 9% ($P < 0.05$), 36 ± 7% ($P < 0.001$) and 33 ± 4% ($P < 0.001$), respectively (Fig. 3.6A-C). In contrast, salmeterol and formoterol significantly decreased the stimulatory effect of 5% FCS and TGF-β_1 on total ECM deposition by 17 ± 3% and 40 ± 7%, respectively ($P < 0.001$). When combined, the drugs counterbalanced each other, the addition of LABA attenuating the stimulatory effect of corticosteroids on 5% FCS- and TGF-β_1-induced total ECM deposition close to control levels (Fig. 3.6A-C). Similar results were observed for collagen deposition (Fig. 3.6D-F).

Role of the GR and the β_2-adrenergic receptor in the corticosteroid- and LABA-mediated effect on total ECM and collagen deposition

We investigated the role of the GR and the β_2-adrenergic receptor in the effect of corticosteroids and LABA on total ECM and collagen deposition in the presence or absence of serum (Fig. 3.7). In 0.3% albumin the GR antagonist RU486 alone did not affect total ECM deposition but completely blocked the ECM decreasing effect of dexamethasone (104 ± 6 versus 81 ± 5%; $P < 0.01$) or budesonide (102 ± 6 versus 71 ± 5%; $P < 0.001$) but had no significant effect on fluticasone (Fig. 3.7A). In the presence of 5% FCS RU486 alone significantly increased total ECM deposition compared to control (123 ± 6%; $P < 0.01$), however, it reduced the corticosteroid-increased total ECM deposition with a significant difference only for dexamethasone (116 ± 5 versus 139 ± 7%; $P < 0.05$; Fig. 3.7B). In 0.3%

albumin and 5% FCS the β_2-adrenergic receptor antagonist propranolol alone did not affect total ECM deposition but partly altered the inhibitory action of formoterol and salmeterol (Fig. 3.7C,D). For collagen deposition, similar results were obtained (Fig. 3.7E-H).

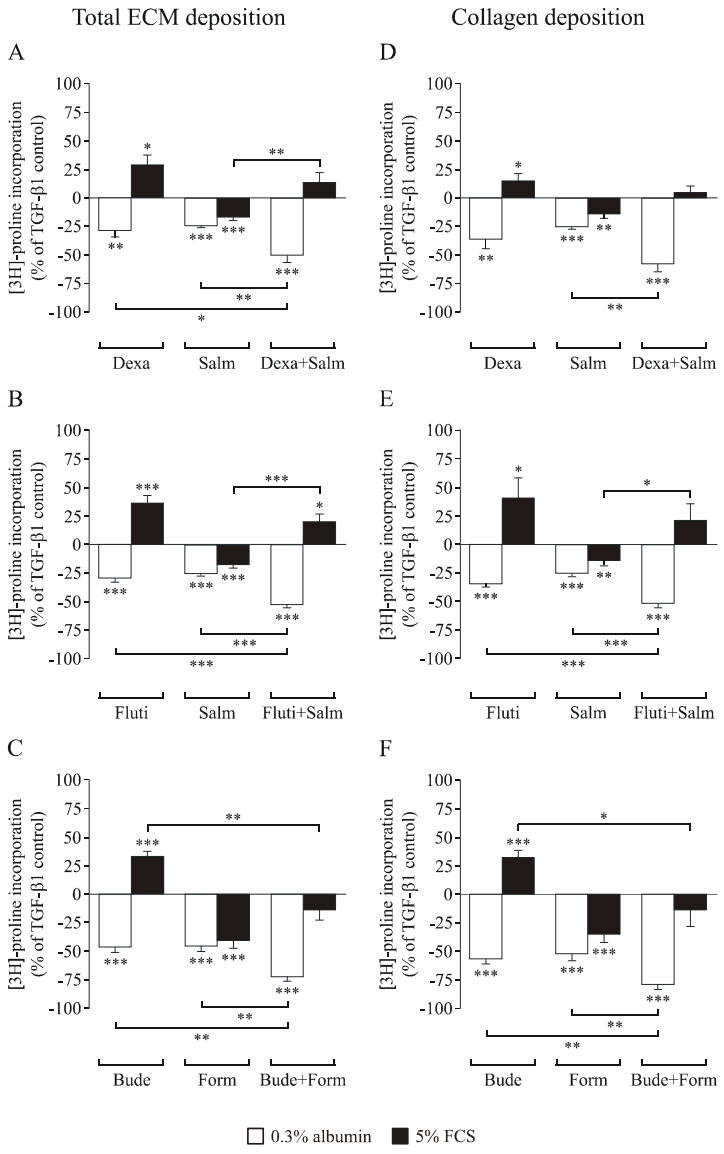

Figure 3.6. Additive effect of corticosteroids and LABA on TGF-β_1-induced total ECM and collagen deposition. Confluent serum-deprived fibroblasts were treated with corticosteroids and LABA (10^{-7} M), alone or in combination, for 48 h in the presence of 2.5 ng/ml TGF-β_1 in 0.3% albumin or 5% FCS and [^{3}H]-proline incorporation was determined. The drug combinations investigated were: (A,D) Dexa+Salm, (B,E) Fluti+Salm and (C,F) Bude+Form on TGF-β_1-induced total ECM and collagen deposition, respectively. Bars represent means ± SEM expressed as % of change from control, TGF-β_1 stimulation in 0.3% albumin or 5% FCS. *P < 0.05, **P < 0.01 and ***P < 0.001 (n = 5 cell lines).

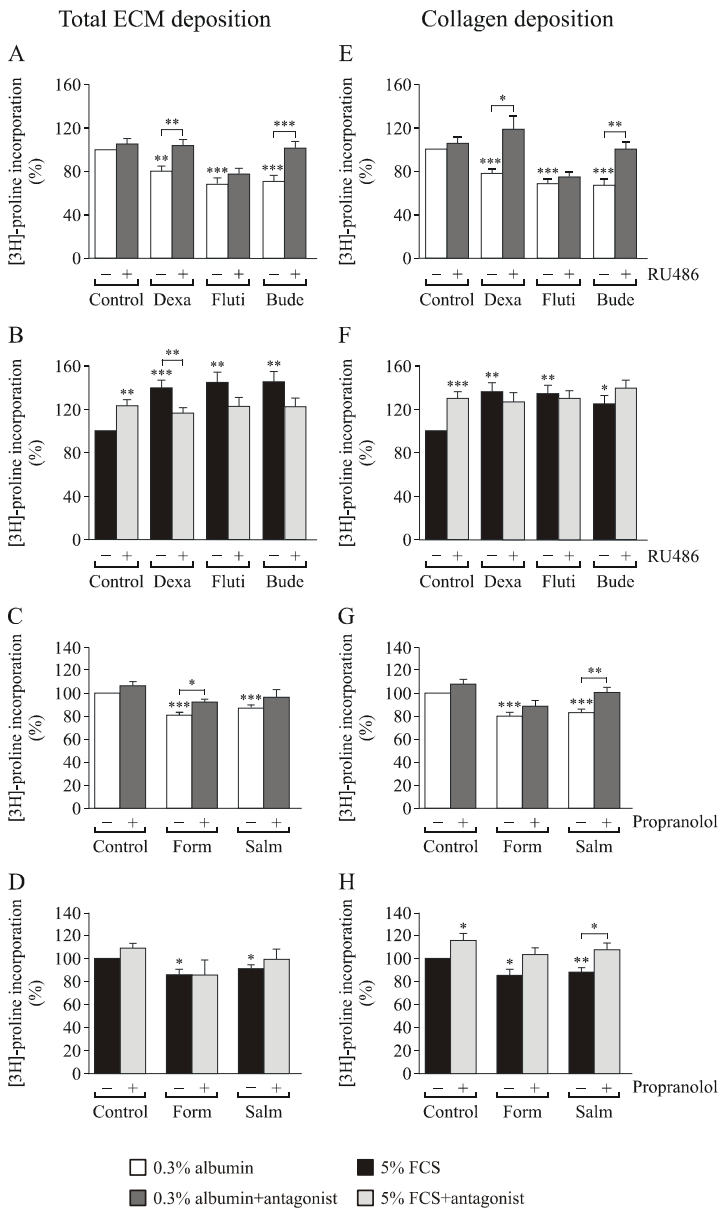

Figure 3.7. The GR and the β_2-adrenergic receptor are partly involved in the corticosteroid- and LABA-mediated effect on total ECM and collagen deposition. Confluent serum-deprived fibroblasts were pre-treated for 30 min with RU486 or propranolol (10^{-6} M) before adding corticosteroids or LABA (10^{-7} M) in 0.3% albumin or 5% FCS for 48 h. (A-D) Total ECM and (E-H) collagen deposition were determined by [^{3}H]-proline incorporation. Bars represent means ± SEM expressed as % of control, where 0.3% albumin and 5% FCS were defined as 100%. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (n = 3 cell lines).

Effect of FCS and corticosteroids on TGF-β_1 secretion

Next we assessed whether the effect of serum and corticosteroids on ECM deposition correlated with TGF-β_1 secretion. Therefore, we determined the level of active and total (active plus latent) TGF-β_1 in conditioned media of fibroblasts treated with fluticasone (10^{-7} M) in 0.3% albumin or 5% FCS for 48 h (Fig. 3.8). Neither 0.3% albumin nor 5% FCS

medium contained any active TGF-β_1, however following chemical activation 5% FCS medium contained 585 pg/ml of total TGF-β_1 corresponding to its latent form (data not shown). As shown in Fig. 3.8A, fibroblasts in 0.3% albumin secreted 227 ± 69 pg/ml active and 396 ± 94 pg/ml total TGF-β_1. Stimulation with 5% FCS significantly increased both active (464 ± 89 pg/ml) and total (1059 ± 202 pg/ml) TGF-β_1 levels compared to 0.3% albumin ($P < 0.01$). Fluticasone treatment did not significantly modify TGF-β_1 levels under both 0.3% albumin and 5% FCS conditions (Fig. 3.8A).

Modulation of collagen, CTGF, Hsp47 and Fli1 mRNA expression by corticosteroids and LABA

To further study the control mechanisms underlying the observed effect of cortico-steroids and LABA on total ECM and collagen deposition, we investigated the effect of budesonide and formoterol (10^{-7} M) on gene expression of different types of collagen and mediators involved in ECM synthesis (Fig. 3.8). Expression of the target genes was analyzed by densitometry analysis and normalized to β-actin and the relative gene expression from two cell lines is summarized in Table 3.1. As depicted in Fig. 3.8B by a representative PCR gel, budesonide downregulated COL1A1, COL1A2, COL3A1, COL5A1, heat shock protein 47 (Hsp47) and Fli1 mRNA expression in 0.3% albumin within 48 h but had no effect on COL4A1 and CTGF. Formoterol had no clear effect on gene expression in 0.3% albumin. When added to budesonide formoterol did not further modulate the expression of our target genes as observed with budesonide alone under serum-free condition.

Interestingly, we did not observe any upregulation in gene expression of collagens or ECM mediators when cells were stimulated with 5% FCS for 48 h. Furthermore, transcription of most collagen genes was not affected by budesonide, formoterol or their combination in the presence of 5% FCS. However, densitometry analysis revealed that budesonide alone or combined with formoterol in 5% FCS tended to increase COL4A1 compared to non-treated cells (Table 3.1). In addition budesonide upregulated CTGF, and downregulated Fli1 and to a smaller extent Hsp47 mRNA expression compared to control in 5% FCS. Formoterol decreased gene transcription of CTGF and had no effect on Hsp47 and Fli1 in 5% FCS. The addition of formoterol partly inhibited the effect of budesonide on CTGF mRNA expression but did not reach control level. In contrast, formoterol did not modulate the effect of budesonide on Hsp47 and Fli1 mRNA expression in the presence of 5% FCS (Fig. 3.8B).

Similar results were obtained with fluticasone and salmeterol under the same conditions (n = 2 cell lines; data not shown), suggesting a drug class effect on gene expression.

Table 3.1. Relative collagen and ECM mediators mRNA expression. Relative expression measured by densitometry. Data represent means ± SEM expressed as % of control, where the relative mRNA expression (after normalization with β-actin) in non-treated primary human lung fibroblasts in 0.3% albumin was defined as 100% (n = 2 cell lines, n =1 for Hsp47).

	Condition	Control	Budesonide	Formoterol	Budesonide + Formoterol
COL1A1	0.3% albumin	100	64 ± 22	100 ± 27	52 ± 25
	5% FCS	104 ± 26	92 ± 16	121 ± 4	88 ± 24
COL1A2	0.3% albumin	100	84 ± 10	103 ± 13	69 ± 7
	5% FCS	98 ± 15	82 ± 14	111 ± 13	80 ± 16
COL3A1	0.3% albumin	100	58 ± 9	128 ± 29	61 ± 28
	5% FCS	93 ± 23	83 ± 21	134 ± 18	95 ± 23
COL4A1	0.3% albumin	100	153 ± 6	100 ± 40	107 ± 17
	5% FCS	112 ± 44	194 ± 12	99 ± 13	181 ± 3
COL5A1	0.3% albumin	100	72 ± 25	101 ± 35	68 ± 30
	5% FCS	101 ± 32	86 ± 26	116 ± 12	84 ± 24
CTGF	0.3% albumin	100	150 ± 46	85 ± 13	104 ± 37
	5% FCS	73 ± 11	231 ± 22	52 ± 20	171 ± 47
Hsp47	0.3% albumin	100	30	76	27
	5% FCS	91	78	104	51
Fli1	0.3% albumin	100	57 ± 15	98 ± 9	48 ± 9
	5% FCS	102 ± 16	46 ± 15	103 ± 8	57 ± 26

Corticosteroids and LABA decreased fibroblast proliferation

We assessed the effect of corticosteroids and LABA (10^{-7} M) on cell proliferation in subconfluent fibroblasts by [^{3}H]-thymidine incorporation (Fig. 3.8). 5% FCS significantly induced fibroblast proliferation compared to 0.3% albumin (10 952 ± 1020 versus 4250 ± 796 cpm; $P < 0.01$; Fig. 3.8C), and this effect was significantly decreased by corticosteroids and LABA ($P < 0.01$) but did not reach significance for salmeterol (Fig. 3.8D). In combination, corticosteroids and LABA tended to have a stronger anti-proliferative effect compared to control ($P < 0.01$) but this additive effect was not significant when compared to each drug alone (Fig. 3.8D).

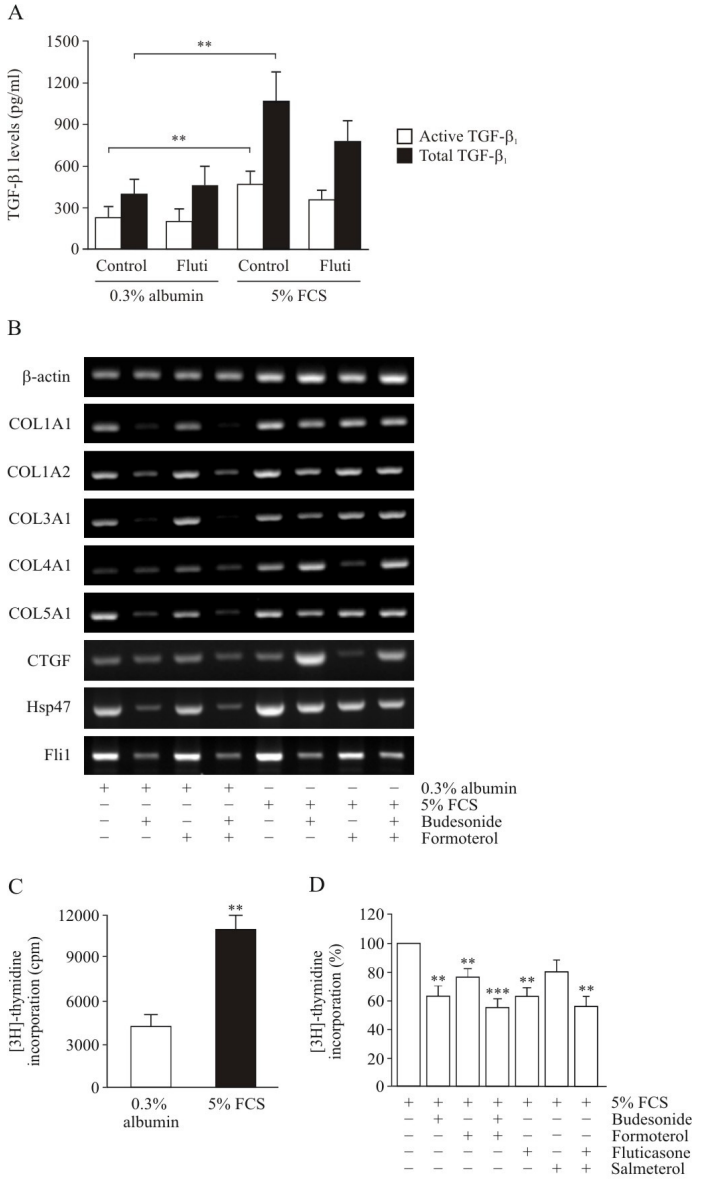

Figure 3.8. Possible mechanisms by which corticosteroids and LABA modify total ECM and collagen deposition. (A) Confluent serum-deprived fibroblasts were treated with fluticasone (10^{-7} M) for 48 h in 0.3% albumin or 5% FCS and active and total TGF-β_1 levels in the conditioned media were measured by ELISA. Bars represent means ± SEM expressed as active and total TGF-β_1 in pg/ml (n = 4 cell lines). (B) Confluent serum-deprived cells were treated with budesonide and/or formoterol (10^{-7} M) in 0.3% albumin and 5% FCS and total RNA was isolated after 48 h. The PCR data are representative of two cell lines. (C,D) Subconfluent serum-deprived fibroblasts were stimulated with 5% FCS, treated with corticosteroids and LABA (10^{-7} M), and cell proliferation was determined by [^{3}H]-thymidine incorporation 30 h after stimulation. Bars represent means ± SEM expressed as (C) cpm or as (D) % of control, where 5% FCS was defined as 100% (n = 6 cell lines). $^{**}P < 0.01$, $^{***}P < 0.001$.

Discussion

In this study we showed that serum significantly increased IL-6 and IL-8 levels as well as cell proliferation by primary human lung fibroblasts, and both pro-inflammatory cell responses

were inhibited by corticosteroids. FCS and TGF-β_1 increased total ECM and collagen deposition significantly and showed an additive effect when combined. In addition, stimulation with 5% FCS increased TGF-β_1 levels compared to serum-free condition. Under non-inflammatory condition corticosteroids inhibited total ECM and collagen deposition, involving the GR and downregulation of COL1A1, COL1A2, COL3A1, COL5A1, Hsp47 and Fli1 mRNA expression. In contrast, in the presence of serum corticosteroids significantly increased total ECM and collagen deposition, not involving the GR, TGF-β_1 secretion or collagen gene transcription. LABA did not alter IL-6 and IL-8 levels but significantly reduced total ECM and collagen deposition regardless of the presence of serum. LABA also decreased the FCS-induced fibroblast proliferation. The effect of LABA on ECM deposition did not involve the β_2-adrenergic receptor or collagen gene transcription. The combination of corticosteroids and LABA showed additive effects on total ECM and collagen deposition in the presence or absence of serum and was not affected by TGF-β_1 stimulation.

In the healthy lung, connective tissue fibroblasts are embedded in their specific local ECM and show low proliferation rate. We assumed that this condition can be mimicked by confluent layers of primary human lung fibroblasts under serum-free condition. It has been shown that serum starvation activates genes involved in the controlling mechanisms that maintain tissue homeostasis such as fat metabolism and negative cell-cycle control [33, 35]. Furthermore, Iyer *et al.* reported that under serum starvation gene expression of pro-inflammatory cytokines such as IL-6 and IL-8 were downregulated and then upregulated by stimulation with serum [33], which we also observed in confluent primary human lung fibroblasts. Therefore, we assumed that serum in cell culture condition not only stimulates proliferation of subconfluent cells but also mimics inflammation in confluent cells. Surprisingly, none of the two studies mentioned above observed any effect of serum starvation or FCS stimulation on gene expression of ECM compounds as reported by others [36].

Increased levels of IL-6 and IL-8 were reported in lung inflammation and we and others have shown earlier that lung fibroblasts are a source of both cytokines especially when stimulated with TGF-β_1 [23, 37, 38]. Based on our previous studies, we assessed the release of these two pro-inflammatory cytokines in response to serum and we showed that 5% FCS significantly increased IL-6 and IL-8 release by confluent fibroblasts compared to 0.3% albumin. In agreement with their anti-inflammatory properties [39], corticosteroids significantly

downregulated the spontaneous and FCS-induced release of both cytokines. However, the effect of LABA on pro-inflammatory mediators is controversially discussed and may depend on the cell type and stimulus [40-42]. Here, LABA alone did not affect IL-6 and IL-8 secretion by fibroblasts, and in combination, LABA did not further modulate the decrease of the corticosteroids under both serum-free and 5% FCS conditions.

Lung inflammation is associated with vessel leakage and consequently the surrounding tissue is exposed to non-physiological serum and growth factor levels which can stimulate ECM synthesis [25, 36]. Therefore, the effect of corticosteroids and LABA on total ECM and collagen deposition during lung inflammation is of interest. Accordingly in our *in vitro* model of inflammation and remodeling, TGF-β_1 and 5% FCS significantly increased total ECM and collagen deposition by confluent fibroblasts. We cannot exclude that the FCS-induced increase of ECM deposition was partly mediated via TGF-β_1. Indeed, increased levels of total TGF-β_1 were measured in the conditioned media of confluent fibroblasts stimulated with 5% FCS for 48 h.

Then we investigated the effect of corticosteroids on ECM deposition under both non-inflammatory and inflammatory conditions. In 0.3% albumin corticosteroids dose-dependently decreased total ECM and collagen deposition by fibroblasts, correlating with downregulation of COL1A1, COL1A2, COL3A1, and COL5A1 mRNA expression. It was previously reported that the decrease of collagen synthesis and mRNA by glucocorticoids is mediated via the GR [43]. In our model, the role of the GR in the corticosteroid-mediated effect on total ECM and collagen deposition in 0.3% albumin was partly confirmed as RU486 counteracted the inhibitory effect of dexamethasone and budesonide on ECM deposition but not of fluticasone.

To further elucidate the signalling mechanisms involved we assessed the effect of corticosteroids on CTGF, a well-described stimulus of ECM synthesis in mesenchymal cells [44, 45]. In 0.3% albumin CTGF mRNA expression was not affected by budesonide, suggesting that the inhibitory effect of corticosteroids on ECM deposition by fibroblasts under non-inflammatory condition is not mediated via CTGF. In 0.3% albumin budesonide downregulated mRNA expression of Hsp47, a chaperone protein critical for collagen maturation associated with type I procollagen expression [46, 47], indicating that corticosteroids may affect collagen conformation. We further investigated the effect of budesonide on Fli1, a member of the Ets transcription factors family that has been shown to

suppress collagen transcription in skin fibroblasts [48, 49]. This is the first study to report that primary human lung fibroblasts expressed Fli1 mRNA. However, Fli1 gene expression was downregulated by budesonide in 0.3% albumin and therefore suggests that Fli1 was not available to suppress collagen in lung fibroblasts under non-inflammatory condition. As a consequence, Fli1 can not mediate the ECM reducing effect of corticosteroids under serum-free condition.

Interestingly, the effect of corticosteroids on total ECM and collagen deposition by lung fibroblasts was different in the presence of serum, which we used as a model for inflammation and tissue repair. Accordingly, in our model 5% FCS significantly increased total ECM and collagen deposition by confluent fibroblasts and were further increased by corticosteroids. This corticosteroid effect only partly involved the GR and was not mediated by collagen gene expression. This assumption is further supported by the observation that RU486 alone significantly increased total ECM and collagen deposition in the presence of serum. RU486 acts as a competitor for glucocorticoids and occupies the GR binding site, still the complex migrates to the nucleus and recognises the respective glucocorticoid response element in gene promoters but does not affect transcriptional activity [50]. In addition to its function as a direct transcription factor the GR interacts with other gene control proteins in the cytosol and the effect of RU486 on this mechanism has not been studied in extension [51]. Thus, our data suggest that the increasing effect of corticosteroids on total ECM and collagen deposition may occur at the post-transcriptional level.

We further assessed possible signalling pathways by which corticosteroids could achieve their distinct serum-dependent effect on total ECM and collagen deposition. Our data indicate that corticosteroids do not affect TGF-β_1 production or activation by primary human lung fibroblasts, therefore the effect of corticosteroids on FCS-induced total ECM and collagen deposition did not involve TGF-β_1. Similar to the reported effect of dexamethasone on CTGF in different cell types [52, 53], budesonide increased CTGF mRNA expression in the presence of 5% FCS, which may subsequently stimulate collagen synthesis. In 5% FCS budesonide downregulated Hsp47 mRNA expression, implying that procollagen conformation may also be affected by corticosteroids under inflammatory condition. Furthermore, budesonide downregulated Fli1 mRNA expression in 5% FCS, suggesting that under this condition the corticosteroid-induced ECM deposition may result from the lack of Fli1. Indeed, in dermal

fibroblasts and scleroderma human skin Fli1 levels inversely correlated with collagen type I and contributed to uncontrolled ECM deposition [49].

Our *in vitro* findings may relate to clinical studies investigating the short- or long-term effect of inhaled corticosteroids on airway wall remodeling. The increased total ECM and collagen deposition by fibroblasts under inflammatory condition may explain why short-term corticosteroids treatment did not significantly change collagen expression in the bronchial mucosa of asthma patients [28, 29, 54]. We showed that only under serum-free condition corticosteroids reduced total ECM and collagen deposition, which may reflect the observations that long-term/high-dose treatment with inhaled corticosteroids significantly reduced airway remodeling [10, 27, 55]. Furthermore, our findings may provide the basis to understand why high-dose corticosteroids may cause skin atrophy in non-inflamed tissue as previously reported [56].

Surprisingly, the effect of LABA on ECM turnover has not been assessed extensively. In our study LABA significantly reduced total ECM and collagen deposition with similar efficacy regardless of the presence of serum. However, this inhibitory effect of formoterol and salmeterol was only partly mediated via the β_2-adrenergic receptor and could not be explained by collagen gene transcription. Interestingly, in 5% FCS formoterol downregulated CTGF mRNA expression and may account for a subsequent decreased collagen deposition. Formoterol did not change Hsp47 or Fli1 mRNA expression under both serum-free and 5% FCS conditions. Thus, under inflammatory condition LABA seem to reduce ECM deposition by downregulating CTGF and not affecting the expression of the negative control element Fli1. In regard to remodeling, Roberts *et al.* reported no change in the sub-basement membrane collagen deposition after six weeks of salmeterol treatment in patients with stable asthma [57]. In contrast, three months treatment with salmeterol reduced vascular remodeling in asthma patients [58]. In summary the effect of LABA on remodeling has to be further investigated as the effects might either depend on the duration of therapy or may be cell type specific.

While the combination of inhaled corticosteroids and LABA has a beneficial effect on asthma symptoms, its effect on airway remodeling is not clear. In our study combined corticosteroids and LABA had an additive effect on total ECM and collagen deposition under both 0.3% albumin and 5% FCS in the presence or absence of TGF-β_1. Most importantly, in 5% FCS LABA inhibited the corticosteroid-induced ECM deposition. Moreover, formoterol

counteracted the budesonide-induced upregulation of CTGF mRNA expression in 5% FCS, which may partly explain the effect of LABA on corticosteroid-induced collagen deposition in the presence of serum. However, under both serum-free and 5% FCS conditions the effect of combined budesonide and formoterol on collagen, Hsp47 and Fli1 gene expression was similar to budesonide alone. It was reported in children with asthma that six weeks treatment with budesonide plus formoterol did not further reduce circulating procollagen levels compared to budesonide [59]. In a murine allergen-induced model of airway remodeling the combination of fluticasone and salmeterol over a period of two weeks significantly increased fibronectin and collagen in the airway wall compared to placebo or fluticasone [60]. These observations imply that under chronic inflammation, adding LABA to corticosteroids does not further reduce the remodeling process in the lung. However, the effect of the combined drugs was only studied over a short period and with limited conclusions for dose-efficacy in both studies implying the necessity of further clinical, cellular and molecular biology studies.

In addition to total ECM and collagen deposition, cell proliferation is another important feature of tissue remodeling. In agreement with others [61], we observed a significant decrease of the 5% FCS-induced fibroblasts proliferation by corticosteroids and LABA, and have reported earlier that this effect was dose-dependent [62]. Thus, these findings make it unlikely that the corticosteroid-dependent increased total ECM and collagen deposition under inflammatory condition were directly linked to cell proliferation.

In conclusion, our data suggest that the effect of corticosteroids on total ECM and collagen deposition depends on the presence of serum but is not affected by TGF-β_1. We hypothesize that corticosteroids enhance ECM deposition in the initial phase of inflammation when serum infiltration is present. This increased ECM may be regarded as an attempt of fibroblasts to limit further infiltration of serum and inflammatory cells into the tissue and this effect would be further supported by corticosteroids. Our data indicate that the combination of corticosteroids and LABA whilst inflammation is present would counteract the corticosteroid-induced ECM deposition and therefore, be the treatment of choice instead of higher doses of corticosteroids. Then, as soon as inflammation is significantly diminished, both classes of drugs would contribute to a reduction of ECM in the lung but their concentrations should be carefully adjusted to avoid continuous matrix decrease and tissue loss as side effects. Thus, our findings might have implications for the treatment of patients with asthma and COPD in regard to lung tissue remodeling.

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3.2. EFFECT OF CORTICOSTEROIDS AND LABA ON FCS- AND TGF-β_1-INDUCED TOTAL GAGS SECRETION AND DEPOSITION BY PRIMARY HUMAN LUNG FIBROBLASTS

Background and Aims of the study

Within the lung ECM, collagen and elastin fibers are embedded in a hydrated gel, often called the "ground substance". Critical constituents of this matrix are GAGs, a family of highly charged polysaccharides that are ubiquitously distributed. There are different types of GAGs: heparin/heparan sulphate, chondroitin sulphate, dermatan sulphate, keratan sulphate and hyaluronic acid. Most GAGs, except hyaluronic acid, are usually covalently linked to a core protein with which they form proteoglycans. Both GAGs and proteoglycans are involved in many important biological functions including regulation of ECM water balance, cell adhesion, proliferation, migration and modulation of growth factor and cytokine activity [1], thus contributing to lung tissue stability by regulating its biomechanical and elastic properties [2, 3]. Each GAG can exist in different lengths of the polysaccharide chains and in turn, the length determines its function.

In the lungs of asthma and COPD patients, the structure and composition of the ECM are altered as a result of uncontrolled airway wall remodeling. This pathology is characterized by increased deposition of ECM constituents including GAGs and proteoglycans [4-9]. Recently, proteoglycan deposition has been observed in the airways basement membrane in allergen-challenged animal models of asthma [10, 11]. In chronically inflamed airway tissue, fibroblasts are assumed to represent the main activated cell type contributing to the excessive deposition of collagen and other ECM constituents [12, 13]. Accordingly, serum-stimulated [14] and mechanically-stimulated [15] fibroblasts from asthma patients have been shown to produce more proteoglycans than fibroblasts from healthy individuals.

TGF-β_1 is a potent stimulator of ECM synthesis in lung fibrotic processes [16, 17] and is increased in asthma and COPD [18-22]. Beside stimulating collagen production, TGF-β_1 regulates the synthesis of different GAGs and proteoglycans [23-25]. Previously, our group has demonstrated that the three isoforms of TGF-β stimulated synthesis and deposition of collagen and GAGs in primary human lung fibroblasts [26, 27].

To this day, inhaled corticosteroids and LABA are the most effective drugs to control the clinical symptoms associated with asthma and COPD but very little is known about their potential effects on GAGs and proteoglycans within the human lung. Therefore, the aims of this study were: (*i*) to investigate total GAGs secretion and deposition by primary human lung fibroblasts under non-inflammatory and inflammatory conditions; (*ii*) to assess the effect of corticosteroids and LABA on total GAGs secretion and deposition; and (*iii*) to compare whether the combination of both drugs would result in a stronger effect than each drug individually.

Experimental design

Primary human lung fibroblasts in 0.3% albumin were used to mimic a non-inflammatory environment and were stimulated with 5% FCS and/or TGF-β_1 to reproduce an inflammatory response with active tissue repair [28]. Confluent serum-deprived fibroblasts were stimulated with 5% FCS and/or TGF-β_1 (2.5 ng/ml) and treated with corticosteroids and/or LABA at 10^{-7} M for 48 h. Total GAGs secretion and deposition were determined by [^{3}H]-glucosamine incorporation in the cell culture media and in the cell layer with deposited ECM as detailed in the Materials and Methods section (Annex).

Results

FCS and TGF-β_1 increased total GAGs secretion and deposition

We first assessed total GAGs secretion and deposition by fibroblasts under non-inflammatory and inflammatory conditions. The [^{3}H]-glucosamine incorporation data for total GAGs secretion and deposition by fibroblasts in 0.3% albumin were defined as control and standardized to 100%. As depicted in Fig. 3.9A, 5% FCS and TGF-β_1 alone significantly increased GAGs secretion by 232 ± 29% and 191 ± 23%, respectively, compared to control ($p < 0.01$). Stimulation with 5% FCS combined with TGF-β_1 further increased GAGs secretion compared to non-stimulated cells in 0.3% albumin (254 ± 25%; $p < 0.01$) but the increase was not significant compared to each stimulus alone (Fig. 3.9A).

Similar results were obtained for total GAGs deposition. Stimulation with 5% FCS and TGF-β_1 significantly induced GAGs deposition to the same extent compared to 0.3% albumin (188 ± 18% and 193 ± 18%, respectively; $p < 0.01$). The combination of 5% FCS and TGF-β_1 further increased GAGs deposition compared to control (228 ± 29%; $p < 0.001$) but the

increase was not additive compared to 5% FCS or TGF-β_1 alone (Fig. 3.9B). Thus, 5% FCS and TGF-β_1 enhanced total GAGs secretion and deposition to a similar extent in fibroblasts.

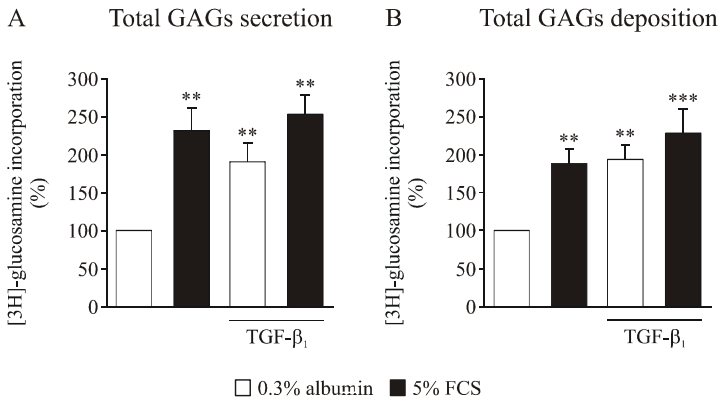

Figure 3.9. Effect of 5% FCS and TGF-β_1 on total GAGs secretion and deposition by primary human lung fibroblasts. Confluent serum-deprived fibroblasts were stimulated with 5% FCS and/or TGF-β_1 (2.5 ng/ml) for 48 h. (A) Total GAGs secretion and (B) deposition were determined by [^{3}H]-glucosamine incorporation. Bars represent means ± SEM expressed as percentage of control, where 0.3% albumin was defined as 100% (n = 5-7 cell lines). ** $p < 0.01$, *** $p < 0.001$.

Effect of corticosteroids and LABA on total GAGs secretion and deposition

We investigated the effect of corticosteroids (budesonide and fluticasone) and LABA (formoterol and salmeterol), alone or in combination, on total GAGs secretion and deposition by fibroblasts in the presence or absence of serum. The [^{3}H]-glucosamine incorporation data are expressed as percentage of change from control, corresponding to the standardized values described above for total GAGs secretion and deposition in 0.3% albumin and 5% FCS. Under serum-free condition budesonide significantly decreased GAGs secretion by 24 ± 5% compared to control ($p < 0.001$; Fig. 3.10A). The LABA formoterol alone had no effect but significantly counteracted the effect of budesonide in 0.3% albumin ($p < 0.01$). In the presence of 5% FCS budesonide and formoterol, alone or in combination, had no effect on GAGs secretion compared to control (Fig. 3.10A). As shown in Fig. 3.10B, fluticasone in 0.3% albumin significantly decreased GAGs secretion by 18 ± 5% compared to control ($p < 0.01$). Salmeterol alone had no effect on GAGs secretion while when combined with fluticasone, it partially reversed fluticasone's inhibitory effect (11 ± 5%; $p = 0.08$; Fig. 3.10B). Similarly to the other drugs, fluticasone, salmeterol and their combination had no clear effect on 5% FCS-induced total GAGs secretion by fibroblasts (Fig. 3.10B).

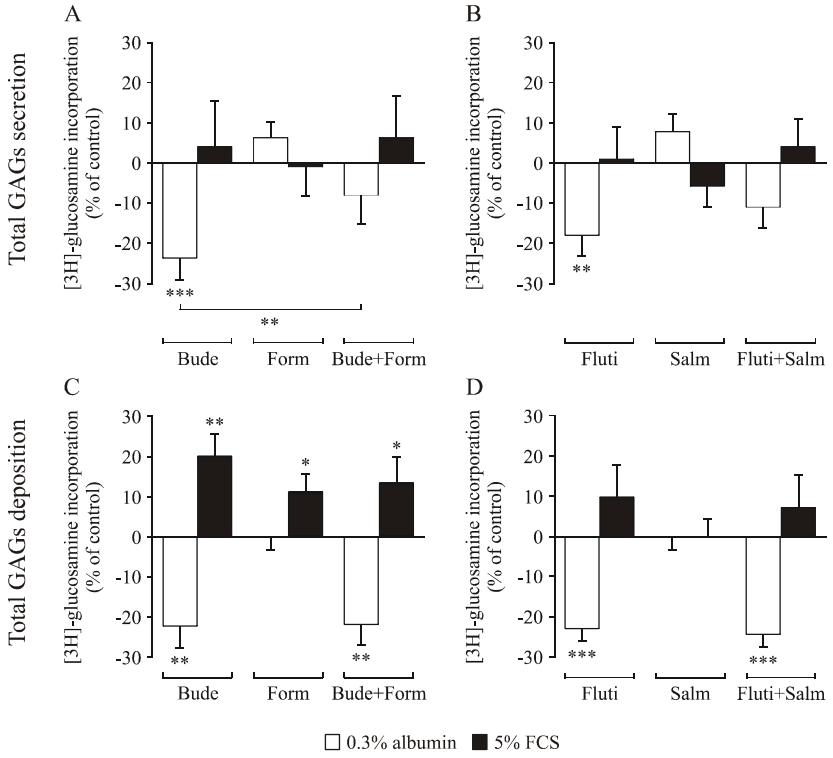

Figure 3.10. Effect of corticosteroids and LABA on total GAGs secretion and deposition in the presence or absence of serum. Confluent serum-deprived fibroblasts were treated with corticosteroids and LABA, alone or in combination (all at 10^{-7} M), in 0.3% albumin or 5% FCS for 48 h. The effect of (A,C) budesonide and/or formoterol (Bude/Form) and (B,D) fluticasone and/or salmeterol (Fluti/Salm) on total GAGs secretion and deposition were determined by [^{3}H]-glucosamine-incorporation. Bars represent means ± SEM expressed as percentage of change from control, where 0.3% albumin and 5% FCS were defined as 100% (n = 6-7 cell lines). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In regards to total GAGs deposition different effects of the drugs were observed. In 0.3% albumin budesonide significantly decreased GAGs deposition by 22 ± 5% compared to control ($p < 0.01$). Formoterol alone had no effect on GAGs deposition and did not alter the effect of budesonide (22 ± 5%; $p < 0.01$; Fig. 3.10C). On the other hand, in 5% FCS budesonide, formoterol and their combination significantly increased GAGs deposition by 20 ± 5% ($p < 0.01$), 11 ± 4% ($p < 0.05$) and 13 ± 6% ($p < 0.05$), respectively (Fig. 3.10C). Similarly, under serum-free condition fluticasone significantly decreased GAGs deposition by 23 ± 3% compared to control ($p < 0.001$) and salmeterol alone had no effect nor did it modulate the decreasing effect of fluticasone (25 ± 3%; $p < 0.001$; Fig. 3.10D). As described above for GAGs secretion, neither fluticasone nor salmeterol had any significant effect on 5% FCS-induced GAGs deposition (Fig. 3.10D). Therefore, these findings suggest that corticosteroids decrease total GAGs secretion and deposition under serum-free condition

whereas they have no clear opposite effect in the presence of 5% FCS. LABA alone have no effect and do not modulate the effect of corticosteroids under both conditions.

Effect of corticosteroids and LABA on TGF-β_1-induced total GAGs secretion and deposition

Since TGF-β_1 is a key factor in ECM synthesis we investigated the effect of corticosteroids, LABA and their combination on TGF-β_1-induced total GAGs secretion and deposition in the presence or absence of serum. The data are expressed as percentage of change from control, corresponding to total GAGs secretion and deposition after TGF-β_1 stimulation in 0.3% albumin and 5% FCS (data from Fig. 3.9) that were standardized to 100%. As shown in Fig. 3.11A, budesonide significantly decreased TGF-β_1-induced GAGs secretion by 31 ± 9% ($p < 0.05$) under serum-free condition. Formoterol alone had no effect but partially reduced the effect of budesonide, resulting in a non-significant decrease compared to control (24 ± 11%; $p = 0.054$). In 5% FCS budesonide, formoterol and their combination significantly decreased the TGF-β_1-induced GAGs secretion by 24 ± 4% ($p < 0.01$), 13 ± 4% ($p < 0.05$) and 24 ± 9% ($p < 0.05$), respectively (Fig. 3.11A). As observed previously in 0.3% albumin, fluticasone significantly decreased TGF-β_1-induced GAGs secretion by 32 ± 1% ($p < 0.001$) while salmeterol had no effect and only slightly attenuated the inhibitory effect of fluticasone (25 ± 2%; $p < 0.001$; Fig. 3.11B). In 5% FCS fluticasone and/or salmeterol had no significant effect on TGF-β_1-induced GAGs secretion (Fig. 3.11B).

Similar effects were obtained on TGF-β_1-induced total GAGs deposition. Under serum-free condition budesonide significantly decreased TGF-β_1-induced GAGs deposition by 39 ± 8% ($p < 0.01$) but formoterol had no effect and did not modulate the inhibitory effect of budesonide (40 ± 8%; $p < 0.01$; Fig 3.11C). Fluticasone, salmeterol and their combination significantly decreased TGF-β_1-induced GAGs deposition by 27 ± 3% ($p < 0.01$), 8 ± 3% ($p < 0.05$) and 33 ± 3% ($p < 0.001$) in 0.3% albumin (Fig. 3.11D). Interestingly, in the presence of 5% FCS all drugs slightly decreased the TGF-β_1-induced GAGs deposition but their effects were not significant compared to control (Fig. 3.11C, D). These data suggest that the effect of corticosteroids on total GAGs secretion and deposition in 5% FCS is reversed by further stimulation with TGF-β_1.

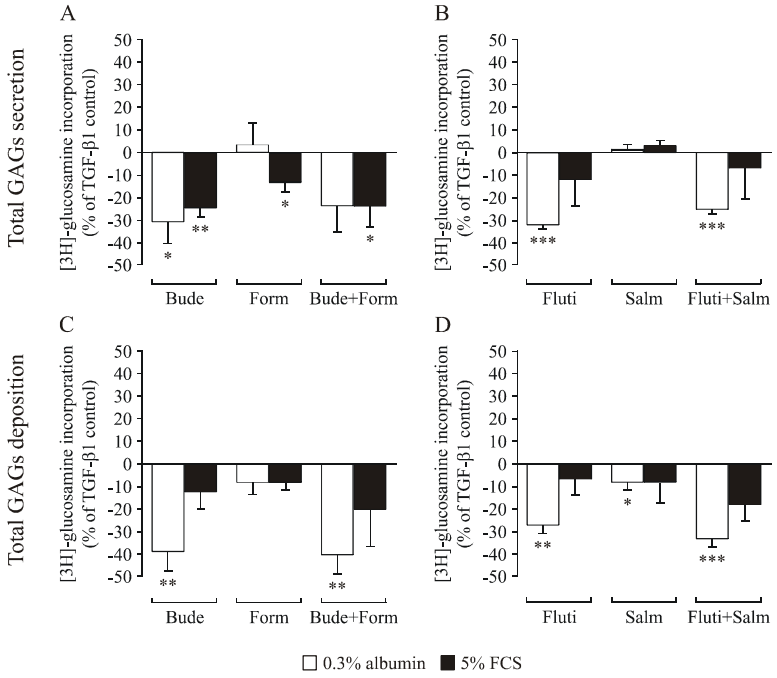

Figure 3.11. Effect of corticosteroids and LABA on TGF-β_1-induced total GAGs secretion and deposition. Confluent serum-deprived fibroblasts were stimulated with TGF-β_1 (2.5 ng/ml) and treated with corticosteroids and/or LABA (10^{-7} M) in 0.3% albumin or 5% FCS for 48 h. The effect of (A,C) Bude/Form and (B,D) Fluti/Salm on TGF-β_1-induced total GAGs secretion and deposition were determined by [^{3}H]-glucosamine-incorporation. Bars represent means ± SEM expressed as percentage of change from control, where TGF-β_1 stimulation in 0.3% albumin and 5% FCS were defined as 100% (n = 4 cell lines). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Discussion

In this study, we showed that serum and TGF-β_1 significantly increased total GAGs secretion and deposition by primary human lung fibroblasts. Under non-inflammatory condition corticosteroids inhibited GAGs secretion and deposition but LABA had no effect. However, LABA partly reversed the inhibitory effect of corticosteroids on GAGs secretion when the drugs were combined. In contrast, in 5% FCS corticosteroids and LABA had no significant effect on GAGs secretion but they increased GAGs deposition. Corticosteroids and/or LABA had similar effects on TGF-β_1-induced total GAGs secretion and deposition under serum-free condition but in 5% FCS corticosteroids and LABA surprisingly decreased the TGF-β_1-induced total GAGs secretion and deposition compared to control.

As we reported for total ECM and collagen deposition [28], here we observed that 5% FCS and TGF-β_1 increased total GAGs secretion and deposition. Accordingly, a study by Ueki *et al.* showed that FCS dose-dependently increased the secretion of hyaluronic acid in a human

embryonic lung-derived fibroblast cell line [29]. Similarly, stimulation with 10% FCS upregulated total proteoglycan production by human lung fibroblasts compared to low-serum condition [30]. During pathological tissue remodeling, levels of cytokines and growth factors are increased and can further stimulate ECM production. One of the most potent pro-fibrotic mediators, TGF-β_1, has been shown to increase the secretion and deposition of GAGs and proteoglycans by fibroblasts [23-25]. Thus, our data are in accordance with the well-described increased GAGs synthesis under inflammatory conditions and during tissue repair.

Concerning the effects of the current mainstay drugs used in the therapy of asthma and COPD, our data suggest that corticosteroids reduced total GAGs secretion and deposition under serum-free condition and that their inhibitory effect was blocked or partially reversed in the presence of serum. In contrast, LABA alone had no significant effect regardless of the presence of serum. Combined drugs had an additive effect under serum-free condition and had a stimulatory effect similar to the one observed with corticosteroids in 5% FCS. Only few studies investigated the effect of asthma drugs on the regulation of GAGs synthesis. Silvestri *et al*. showed that both fluticasone and salmeterol downregulated the expression of hyaluronic-cellular adhesion molecule (H-CAM, CD44) in a human fetal lung fibroblast cell line under serum-free condition, which may slow down fibroblast migration and tissue repair [31]. Different pro-inflammatory stimuli enhance the synthesis of individual GAGs in fibroblasts. In primary human lung fibroblasts, it was reported that fluticasone reduced both constitutive and IL-1β/TNF-α-induced hyaluronic acid synthesis, that salmeterol alone had no effect and that their combination resulted in a marked inhibition of hyaluronic acid synthesis via hyaluronan synthase-2 mRNA [32]. Similarly, Todorova *et al.* showed that budesonide reduced FCS-induced proteoglycan production by lung fibroblasts, formoterol had no effect and their combination had a stronger effect than budesonide alone, which occurred primarily at the post-transcriptional level and without affecting cell proliferation [30]. Furthermore, an interesting study showed that during allergen exposure budesonide treatment increased the mean density of the proteoglycans biglycan and versican in the lung tissue [33]. Thus, the *in vitro* effects of corticosteroids and LABA on GAGs and proteoglycans synthesis are still controversial and may depend on the cell culture conditions.

TGF-β_1 is involved in lung tissue remodeling and we are the first to report on the effects of corticosteroids and LABA on TGF-β_1-induced GAGs synthesis and deposition under non-inflammatory and inflammatory conditions. We showed that corticosteroids significantly

decreased TGF-β_1-induced total GAGs secretion and deposition and that LABA alone had no effect nor did they modulate the effect of corticosteroids under serum-free condition. In 5% FCS corticosteroids and LABA decreased TGF-β_1-induced GAGs secretion and deposition. Our findings are partly in accordance with data presented by Todorova *et al*. at the American Thoracic Society meeting showing that the combination of budesonide and formoterol under low-serum condition decreased total TGF-β_1-induced proteoglycan production in lung fibroblasts to a greater extent than each drug alone [34]. Furthermore, we demonstrated that in 5% FCS stimulation with TGF-β_1 reversed the effects of corticosteroids and LABA, suggesting a common signalling pathway underlying the drugs response to FCS and TGF-β_1.

In summary, this study showed that the inhibitory effect of corticosteroids on total GAGs secretion and deposition was reversed or blocked under inflammatory conditions. We demonstrated that LABA alone had no clear effect on GAGs secretion and deposition and that combined drugs produced the same effect as corticosteroids alone under both non-inflammatory and inflammatory conditions. In regard to clinical aspects, our data imply that once the airway inflammation is attenuated by corticosteroids further treatment with corticosteroids alone or combined with LABA may decrease GAGs synthesis by resident fibroblasts, thereby reducing ECM deposition. On the other hand, if the inflammatory status of the patient is not controlled, corticosteroids and LABA may not have any effect on airway remodeling or may even stimulate ECM deposition by fibroblasts. The characterization of individual GAGs and analysis of synthases gene expression are currently under investigation. These may provide insight into the mechanisms underlying the different effects of corticosteroids and LABA on GAGs synthesis and deposition under non-inflammatory and inflammatory conditions.

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CHAPTER 4

EFFECT OF CORTICOSTEROIDS AND LABA ON LUNG TISSUE REMODELING *IN VITRO* IN PRIMARY HUMAN ASMC

Asthma and COPD are characterized by chronic airway inflammation and remodeling. Lung tissue remodeling includes ASMC hyperplasia and hypertrophy, increased ECM deposition and proteases/antiproteases imbalance in the airways. ASMC have properties beyond constriction and relaxation, they also contribute to lung tissue ECM turnover and remodeling by secreting various ECM components, MMPs and TIMPs. The current therapies of asthma and COPD are inadequate in preventing or reversing tissue remodeling. Thus, a better understanding of the mechanisms regulating ECM synthesis and degradation will be helpful to design new therapeutic strategies targeting the remodeling process. Therefore, using primary human ASMC from healthy, asthma and COPD patients we investigated the effect of corticosteroids and LABA on tissue remodeling.

This chapter includes results of the following three ongoing research projects:

- Corticosteroids and LABA differently modulate the secretion and deposition of total GAGs by primary human ASMC: a comparison from healthy, asthma and COPD patients.
- Effect of corticosteroids and LABA on gelatinolytic activity of primary human ASMC from asthma and COPD patients compared to healthy controls.
- Effect of corticosteroids and LABA on total ECM and collagen deposition by primary human ASMC.

4.1. CORTICOSTEROIDS AND LABA DIFFERENTLY MODULATE TOTAL GAGS SECRETION AND DEPOSITION BY PRIMARY HUMAN ASMC: A COMPARISON FROM HEALTHY, ASTHMA AND COPD PATIENTS

Background and Aims of the study

Proteoglycans are macromolecules composed of a protein core and one or more covalently attached GAG chains. GAGs represent long unbranched polysaccharides consisting of a repeating disaccharide unit and are divided into heparin/heparan sulphate, chondroitin sulfate, dermatan sulphate, kermatan sulphate and hyaluronan. GAGs are involved in various important biological processes, including regulation of cell adhesion, differentiation, proliferation and migration, modulation of growth factor and cytokine activity and collagen fibrillogenesis [1]. Furthermore, GAGs have been shown to alter the viscoelastic and biomechanical properties of the lung connective tissue [2-4]. Therefore, alterations in GAGs content and distribution during lung development and pathological processes indicate their essential role in health and disease [5].

Chronic airway inflammation in asthma and COPD often leads to irreversible structural changes of the lung. This tissue remodeling is characterized by ASMC hyperplasia and hypertrophy, thickening of the reticular basement membrane and increased deposition of collagen, fibronectin, tenascin and proteoglycans in the airways [6-9]. Indeed, an increase in the deposition of proteoglycans has been reported in asthmatic airways [10-14] and in allergen-challenged animal models of asthma [15, 16]. A positive correlation has been shown between airway responsiveness and deposited proteoglycans in the airway wall [11] as well as proteoglycan production by human lung fibroblasts [17]. In addition to fibroblasts, it was reported that ASMC synthesize GAGs and proteoglycans [18-20] but little is known about their regulation during inflammation.

Inhaled corticosteroids and LABA are the cornerstone treatment for asthma and COPD, as they reduce symptoms and improve lung function. Whether these drugs reverse the structural alterations in the lung of asthma and COPD patients is still under active debate and needs further attention. After investigating the effect of corticosteroids and LABA on total GAGs secretion and deposition in primary human lung fibroblasts, we expanded our research to primary human ASMC and our collaboration with The Woolcock Institute (Sydney,

Australia) allowed us to further assess ASMC from asthma and COPD patients. Therefore, the aims of our study were: (*i*) to compare total GAGs secretion and deposition by ASMC from asthma and COPD patients with ASMC isolated from healthy individuals under non-inflammatory and inflammatory conditions; and (*ii*) to investigate the effect of corticosteroids and LABA, alone or in combination, on total GAGs secretion and deposition by ASMC from healthy, asthma and COPD patients.

Experimental design

Primary ASMC were established as described in the Material and Methods section (Annex). Sub-confluent ASMC (80%) were serum-deprived for 24 h then incubated in 0.1% FCS to reflect a non-inflammatory environment or stimulated with 5% FCS thus imitating an inflammatory response with active tissue repair [21]. In addition, cells were treated for 24 h with corticosteroids and/or LABA (10^{-7} M) either in 0.1% or 5% FCS. Total GAGs secretion and deposition were determined by [^{3}H]-glucosamine incorporation in the cell culture media and in the cell layer with deposited ECM (Annex).

Results

Total GAGs secretion and deposition by ASMC from healthy, asthma and COPD patients

We first determined total GAGs secretion and deposition by ASMC from healthy, asthma and COPD patients under non-inflammatory and inflammatory conditions. The [^{3}H]-glucosamine incorporation data are expressed in cpm. As shown in Fig. 4.1, there was no difference in GAGs secretion and deposition between ASMC from healthy, asthma and COPD patients under non-inflammatory condition. Serum stimulation increased GAGs secretion compared to serum-free condition and reached significance only in ASMC from asthma patients (Fig. 4.1A) but had no clear effect on GAGs deposition (Fig. 4.1B). Thus, there was no difference in total GAGs secretion and deposition by ASMC from asthma and COPD patients compared to ASMC from healthy controls and they similarly responded to serum stimulation.

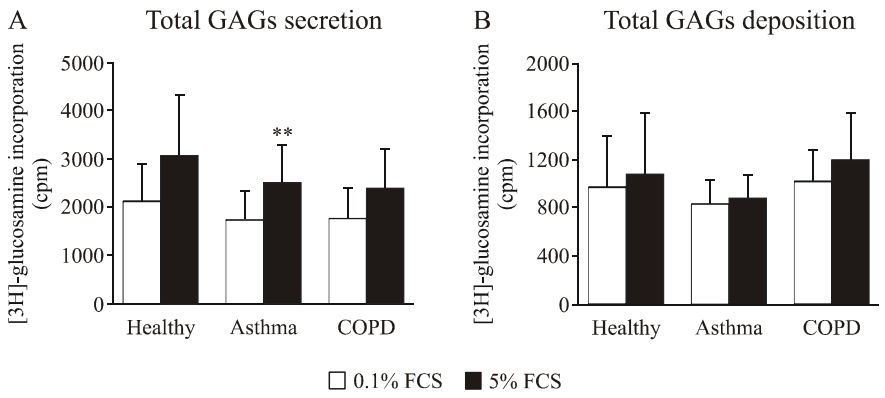

Figure 4.1. Total GAGs secretion and deposition by primary ASMC from healthy, asthma and COPD patients. Sub-confluent serum-deprived ASMC from healthy, asthma and COPD subjects were stimulated in 0.1% FCS or 5% FCS for 24 h. (A) Total GAGs secretion and (B) deposition were determined by [^{3}H]-glucosamine incorporation. Bars represent means ± SEM expressed in cpm (healthy, n = 5; asthma, n = 5; COPD, n = 2 cell lines). ** $p < 0.01$.

Effect of corticosteroids and LABA on total GAGs secretion

We then evaluated the effect of corticosteroids (budesonide and fluticasone) and/or LABA (formoterol and salmeterol) on total GAGs secretion after 24 h under non-inflammatory and inflammatory conditions. The [^{3}H]-glucosamine incorporation data are expressed as percentage of change from control, which correspond to the cpm values obtained in Fig. 4.1 without drugs treatment and standardized to 100%. As shown in Fig. 4.2A, under serum-free condition budesonide significantly decreased GAGs secretion by ASMC from healthy subjects (12 ± 3%; $p < 0.01$) while formoterol alone had no effect but partly reversed the effect of budesonide. In the presence of 5% FCS budesonide, formoterol and their combination had a tendency to decrease GAGs secretion compared to control (Fig. 4.2A). As depicted in Fig. 4.2B, fluticasone and/or salmeterol had similar effects than budesonide and/or formoterol on total GAGs secretion in ASMC from healthy individuals. In 0.1% FCS fluticasone significantly decreased GAGs secretion by 13 ± 2% ($p < 0.01$), salmeterol had no effect and the drugs combination resulted in the same decrease as observed with fluticasone alone (15 ± 3%; $p < 0.01$; Fig. 4.2B). Interestingly, these inhibitory effects were more important in the presence of 5% FCS. Indeed, fluticasone, salmeterol and their combination significantly reduced GAGs secretion by 26 ± 1% ($p < 0.001$), 14 ± 5% ($p < 0.05$) and 21 ± 8% ($p < 0.05$), respectively (Fig. 4.2B).

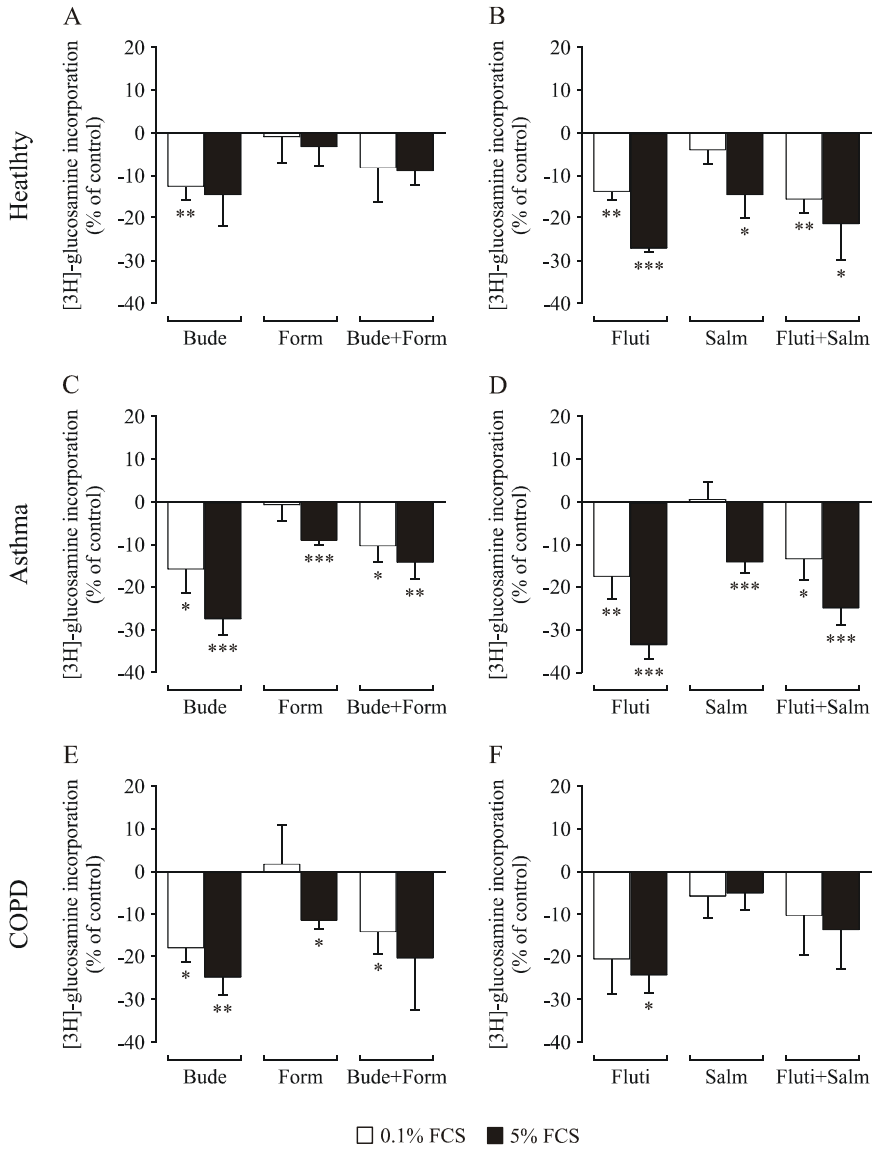

Figure 4.2. Effect of corticosteroids and/or LABA on total GAGs secretion by primary ASMC. Sub-confluent serum-deprived ASMC from healthy, asthma and COPD patients were treated with corticosteroids and/or LABA (10^{-7} M) in 0.1% or 5% FCS for 24 h and total GAGs secretion was determined by [^{3}H]-glucosamine incorporation. The following drug combinations were investigated: budesonide (Bude) and/or formoterol (Form) and fluticasone (Fluti) and/or salmeterol (Salm) in ASMC from (A, B) healthy, (C, D) asthma and (E, F) COPD patients, respectively. Bars represent means ± SEM expressed as percentage of change from control, which correspond to [^{3}H]-glucosamine incorporation of untreated cells in 0.1% and 5% FCS standardized to 100% (healthy, n = 5; asthma, n = 5; COPD, n = 2 cell lines). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In comparison to ASMC from healthy individuals, asthma patients-derived ASMC appeared to be more responsive to corticostereoids and/or LABA. Under serum-free condition budesonide significantly decreased GAGs deposition by 17 ± 6% ($p < 0.05$), formoterol alone had no effect and slightly modulate the inhibitory effect of budesonide (11 ± 4%; $p < 0.05$;

Fig. 4.2C). In 5% FCS GAGs secretion was significantly reduced by budesonide (30 ± 4%; $p < 0.001$), formoterol (10 ± 1%; $p < 0.001$) and their combination (15 ± 4%; $p < 0.01$; Fig. 4.2C). Similarly, in 0.1% FCS fluticasone significantly decreased GAGs secretion by 17 ± 5% ($p < 0.01$), salmeterol had no effect and did not modulate the effect of fluticasone (13 ± 5%; $p < 0.05$; Fig. 4.2D). In the presence of 5% FCS fluticasone, salmeterol and the drug combination significantly decreased total GAGs secretion by 33 ± 3%, 14 ± 2% and 24 ± 4%, respectively ($p < 0.001$; Fig. 4.2D), to a higher extent compared to the inhibitory effect observed in 0.1% FCS.

Similar effects were observed in ASMC from COPD patients. Under serum-free condition budesonide significantly decreased GAGs secretion by 18 ± 3% ($p < 0.05$), formoterol alone had no effect and did not reverse the effect to budesonide (14 ± 5%; $p < 0.05$; Fig. 4.2E). In 5% FCS budesonide and formoterol significantly reduced GAGs secretion by 25 ± 4% ($p < 0.01$) and 11 ± 2% ($p < 0.05$), respectively. Combined budesonide and formoterol also decreased GAGs secretion (20 ± 12%) but the effect did not reach significance due to a high variability between the two cell lines investigated (Fig. 4.2E). Similarly, under both serum-free and 5% FCS conditions fluticasone decreased GAGs secretion by 24% in average ($p < 0.05$ only in 5% FCS), salmeterol alone had no clear effect but slightly reversed the inhibitory effect of fluticasone (Fig. 4.2F). Thus, ASMC from asthma and COPD patients had similar responses to corticosteroids and LABA as healthy-derived ASMC under both non-inflammatory and inflammatory conditions.

Effect of corticosteroids and LABA on total GAGs deposition

We then assessed the effect of corticosteroids and/or LABA on total GAGs deposition by ASMC from healthy, asthma and COPD patients in the presence or absence of serum. In ASMC from healthy individuals under serum-free condition budesonide significantly decreased GAGs deposition by 14 ± 3% ($p < 0.01$), formoterol alone had no effect but further modulated the inhibitory effect of budesonide (29 ± 6%; $p < 0.01$; Fig. 4.3A). In 5% FCS neither budesonide nor formoterol had an effect on GAGs deposition but their combination had a significant decreasing effect (10 ± 3%; $p < 0.05$; Fig. 4.3A). Comparable results were obtained with fluticasone and salmeterol. Under serum-free condition fluticasone significantly inhibited GAGs deposition by 19 ± 3% ($p < 0.001$), salmeterol alone had no clear effect and a stronger reduction was observed by the combined drugs (28 ± 2%; $p < 0.001$; Fig. 4.3B). None of fluticasone and salmeterol, alone or in combination, had a significant effect on GAGs deposition in the presence of serum (Fig. 4.3B).

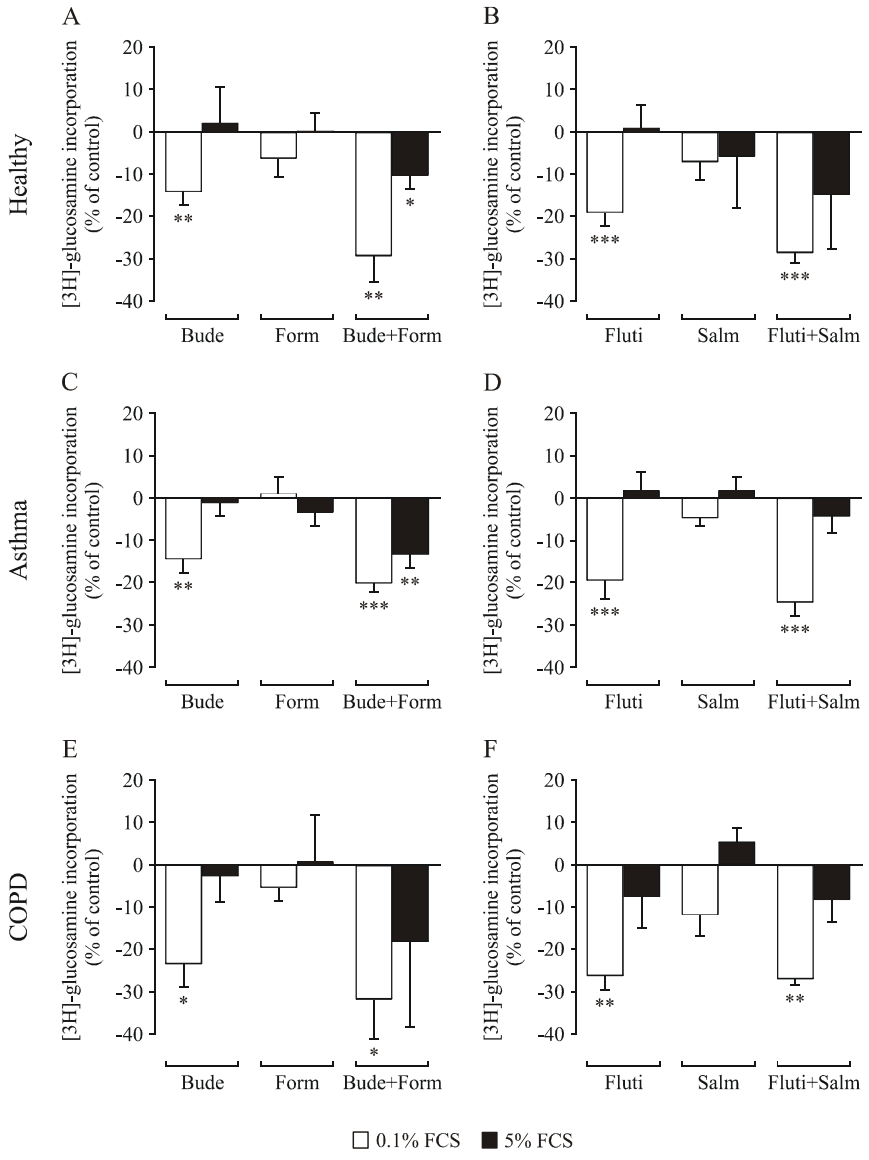

Figure 4.3. Effect of corticosteroids and/or LABA on total GAGs deposition by primary ASMC. Sub-confluent serum-deprived ASMC from healthy, asthma and COPD patients were treated with corticosteroids and/or LABA (10^{-7} M) in 0.1% or 5% FCS and total GAGs deposition was determined by [^{3}H]-glucosamine incorporation. The drug combinations investigated were budesonide (Bude) and/or formoterol (Form) and fluticasone (Fluti) and/or salmeterol (Salm) in ASMC from (A, B) healthy, (C, D) asthma and (E, F) COPD patients, respectively. Bars represent means ± SEM expressed as percentage of change from control, which correspond to [^{3}H]-glucosamine incorporation of untreated cells in 0.1% and 5% FCS standardized to 100% (healthy, n = 5; asthma, n = 5; COPD, n = 2 cell lines). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In ASMC from asthma patients similar results were observed. Under serum-free condition budesonide significantly decreased GAGs deposition by 14 ± 3% ($p < 0.01$), formoterol alone had no effect but significantly enhanced the inhibitory effect of budesonide (20 ± 2%; $p < 0.001$; Fig. 4.3C). In 5% FCS only the combination of budesonide and formoterol significantly decreased GAGs deposition (13 ± 3%; $p < 0.01$; Fig. 4.3C). Similarly, in 0.1%

FCS fluticasone significantly inhibited GAGs deposition (19 ± 4%; $p < 0.001$), salmeterol alone had no clear effect but slightly modulated the effect of fluticasone (24 ± 3%; $p < 0.001$; Fig. 4.3D). None of the treatments had any effect on GAGs deposition in 5% FCS-stimulated cells (Fig. 4.3D).

As depicted in Fig. 4.3E and F, in ASMC from COPD patients under serum-free condition budesonide and fluticasone significantly inhibited GAGs deposition by 23 ± 5% ($p < 0.05$) and 26 ± 3% ($p < 0.01$), respectively. Formoterol and salmeterol tended to decrease GAGs deposition but did not modulate the inhibitory effect of the corticosteroids. In contrast, no clear effect was obtained with any treatment in the presence of 5% FCS (Fig. 4.3E, F). Together, these findings showed that only corticosteroids under non-inflammatory conditions significantly decreased total GAGs deposition by ASMC from healthy, asthma and COPD patients.

Discussion

In this study our findings indicate no difference in the constitutive total GAGs secretion and deposition between ASMC from healthy, asthma and COPD patients and that serum stimulation had the tendency to increase total GAGs secretion. Under non-inflammatory condition corticosteroids significantly inhibited total GAGs secretion and deposition while LABA had no clear effect nor significantly modulated the effect of corticosteroids. Upon inflammatory stimulation corticosteroids significantly decreased GAGs secretion to a greater extent than LABA and their combination had a similar effect than corticosteroids alone. On the other hand, corticosteroids and/or LABA had no clear effect on total GAGs deposition in the presence of serum.

It is well documented that in the airways of asthma patients there is an increase in the number of ASMC [22] as well as an increase in the amount of total ECM surrounding ASMC in the connective tissue [23]. More profound examinations revealed an increase in hyaluronan and versican within and around the smooth muscle bundles, suggesting increased synthesis of specific GAGs and proteoglycans by ASMC from asthma patients [24]. ASMC can promote the formation of the interstitial ECM and ASMC derived from asthma or COPD patients may respond differently than cells from healthy individuals, both qualitatively and quantitatively [25, 26]. Interestingly, our findings showed that there were more GAGs secreted that deposited by ASMC from healthy, asthma and COPD patients but no difference in total

GAGs secretion and deposition between the three groups. We previously described that serum stimulation mimicked an inflammatory response *in vitro* and increased total ECM, collagen and GAGs deposition in primary non-diseased human lung fibroblasts [21, 27]. Here, we showed that serum stimulation slightly increased total GAGs secretion but not GAGs deposition and to a lower extent compared to fibroblasts as previously described.

Airway remodeling in chronic asthma and COPD may be viewed as a consequence of inflammation, resulting in release of cytokines and growth factors causing in turn, activation of fibroblasts and ASMC to produce more ECM macromolecules [19, 20, 28, 29]. Interestingly, Johnson *et al.* reported that passive sensitization of ASMC with serum from asthma patients significantly increased ECM production, including perlecan and chondroitin sulfate, but was not affect by beclomethasone treatment [18]. To our knowledge, this study is the only available on the effect of corticosteroids on GAGs synthesis by human ASMC. Our findings showed that corticosteroids significantly decreased total GAGs secretion by ASMC stimulated with 0.1% or 5% FCS. Interestingly, we demonstrated that corticosteroids decreased total GAGs deposition only under serum-free condition and had no effect in the presence of serum, which is in accordance with the study of Johnson *et al*. In addition, the effects of corticosteroids were not modulated by the combination with LABA under non-inflammatory and inflammatory conditions.

Preliminary data presented at the World Congress of Pharmacology held last July in Beijing, China, provide evidences that ASMC synthesized hyaluronic acid, heparin sulfate, dermatan sulfate and chondroitin sulfate [30]. There was no difference in the composition of GAGs between ASMC from healthy and asthma subjects. However, the levels of secreted hyaluronic acid were decreased by budesonide while they were increased by formoterol and to a greater extent by the drugs combination in ASMC from healthy and asthma patients. Thus, although total GAGs secretion and deposition by ASMC from healthy, asthma and COPD patients were similarly affected by corticosteroids and LABA, individual GAG may be differently regulated under non-inflammatory and inflammatory conditions.

In conclusion, our data indicate no difference in total GAGs secretion and deposition by non-treated ASMC from healthy, asthma and COPD patients in the presence or absence of serum. Corticosteroids decreased GAGs secretion under non-inflammatory and inflammatory conditions and were not modulated by LABA. Total GAGs deposition was decreased by corticosteroids under serum-free condition while not affected by the drugs in 5% FCS. These

findings imply that ASMC from healthy, asthma and COPD patients responded similarly to FCS, corticosteroids and/or LABA. The characterization of individual GAGs, the levels of hyaluronic acid in ASMC-conditioned media and the mRNA expression of GAGs synthases following the different treatments are in progress. These results will point out which GAGs are modulated by corticosteroids and/or LABA and which mechanisms may regulate their effects.

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4.2. EFFECT OF CORTICOSTEROIDS AND LABA ON GELATINOLYTIC ACTIVITY OF PRIMARY HUMAN ASMC FROM ASTHMA AND COPD PATIENTS COMPARED TO HEALTHY CONTROLS

Background and Aims of the study

In healthy individuals the physiologic turnover of total ECM in the airways has been estimated between 10 to 15% per day and results from the balance between the synthesis and degradation of ECM constituents controlled by MMPs and their inhbitors TIMPs [1]. Studies have suggested that in chronic inflammatory lung diseases, the proteases/antiproteases balance is altered. Indeed, enhanced MMPs expression has been reported in bronchial biopsies, serum, sputum and BALF of asthma and COPD patients [2-8]. In the lung MMPs are expressed by inflammatory and mesenchymal cells and play a key role in lung tissue homeostasis and remodeling [1, 9-12]. Although MMPs are being increasingly investigated in airway cell biology, little is known about the regulation of ASMC-derived MMPs in inflammatory lung diseases and the potential effect of corticosteroids and LABA. Therefore, the aims of this study were: (*i*) to compare MMPs secretion and activity kinetics by primary ASMC from healthy subjects to those of ASMC from asthma and COPD patients; and (*ii*) to determine the effect of corticosteroids and LABA, alone or in combination, on MMPs activity in ASMC obtained from healthy, asthma and COPD subjects.

Experimental design

Confluent serum-deprived ASMC were treated with the corticosteroid budesonide and/or the LABA formoterol (both at 10^{-8} M) in 0.1% FCS for 0.5, 1, 6 and 24 h. Conditioned media were collected, equalized for protein content and MMPs activity was determined by gelatin zymography (Annex).

Results

Gelatinolytic activity of ASMC from healthy, asthma and COPD patients

Zymograms of ASMC-conditioned media showed that growth-arrested cells under serum-free condition constitutively secreted the 72-kDa gelatinase proMMP-2 but not the 92-kDa gelatinase proMMP-9 (Fig. 4.4A). Other faint bands at higher molecular weight were also detectable and may correspond to proMMP-2 dimers or complexes of proMMP-2 with other

MMPs and/or TIMPs. No gelatinase activity was detected in 0.1% FCS medium alone (Fig. 4.4A). The gelatinolytic activity data are expressed as arbitrary densitometry units (a.u.). As depicted in Fig. 4.4B, proMMP-2 activity was significantly decreased in ASMC of asthma (8058 ± 115 a.u.; $p < 0.01$) and COPD patients (8190 ± 500 a.u.; $p < 0.05$) compared to ASMC of healthy individuals after 0.5 h incubation in 0.1% FCS (10 500 ± 307 a.u.). These values were defined as control and standardized to 100% for further analysis.

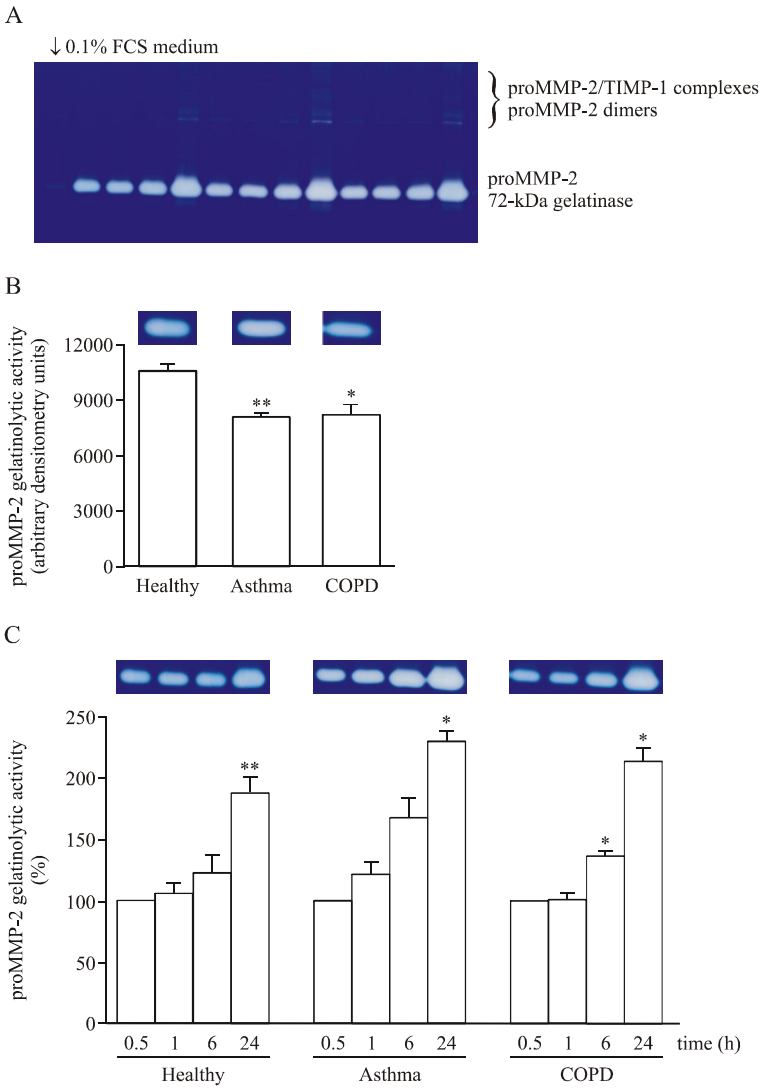

Figure 4.4. Gelatinolytic activity of ASMC from healthy, asthma and COPD patients. Confluent serum-deprived ASMC from healthy, asthma and COPD subjects were incubated in 0.1% FCS for 0.5, 1, 6 and 24 h and conditioned media were analyzed for MMPs activity by zymography. (A) Gelatinolytic activity in conditioned media of ASMC. (B) ProMMP-2 activity after 0.5 h incubation defined as control and standardized to 100%. (C) Time-kinetic of proMMP-2 production. Bars represent means ± SEM expressed as arbitrary densitometry units (B) and percentage (C). Representative zymograms are shown (healthy, n = 3; asthma, n = 2; COPD, n = 3 cell lines). * $p < 0.05$, ** $p < 0.01$.

We then investigated the kinetic of proMMP-2 gelatinolytic activity over 24 h and the following data are expressed as percentage. As displayed in Fig. 4.4C, proMMP-2 activity increased with time and a similar profile was observed in ASMC originating from healthy, asthma or COPD patients. In ASMC from healthy subjects proMMP-2 activity was significantly enhanced to 187 ± 12% after 24 h incubation in 0.1% FCS ($p < 0.01$). Compared to ASMC from healthy individuals, the autocrine production of proMMP-2 increased faster in the asthmatic cells, reaching 121 ± 9% after 1 h incubation and up to a significant increase of 229 ± 8% after 24 h compared to control ($p < 0.05$). In ASMC from COPD patients proMMP-2 activity was enhanced to 136 ± 3 and 213 ± 19% after 6 and 24 h incubation, respectively ($p < 0.05$; Fig. 4.4C). Thus, our data demonstrated that ASMC from asthma and COPD patients constitutively secreted less proMMP-2 than ASMC from healthy subjects but increased similarly with time.

Effect of corticosteroids and LABA on gelatinolytic activity of ASMC

We investigated the effect of budesonide and formoterol on the constitutive proMMP-2 secretion by ASMC. Budesonide and formoterol alone or in combination had no significant effect on proMMP-2 gelatinolytic activity of ASMC from healthy, asthma and COPD patients compared to non-treated cells after 0.5, 3 and 6 h incubation (Fig. 4.5). However, combined drugs tended to reduce proMMP-2 secretion by ASMC from COPD patients after 24 h incubation but this decrease failed to reached significance when compared to non-treated cells (169 ± 26 versus 213 ± 19%; $p = 0.068$; Fig. 4.5C). Thus, our findings showed no significant effect on proMMP-2 gelatinolytic activity by corticosteroids and LABA in ASMC from healthy, asthma and COPD patients.

Discussion

In this study, we investigated the effect of corticosteroids and LABA on gelatinolytic activity of ASMC originating from healthy, asthma and COPD patients. We showed that only proMMP-2 was constitutively secreted by ASMC and that proMMP-2 activity was significantly decreased in ASMC from asthma and COPD patients compared to healthy individuals but all similarly increased in a time-dependent manner. However, proMMP-2 secretion was not significantly affected by budesonide and formoterol, alone or in combination.

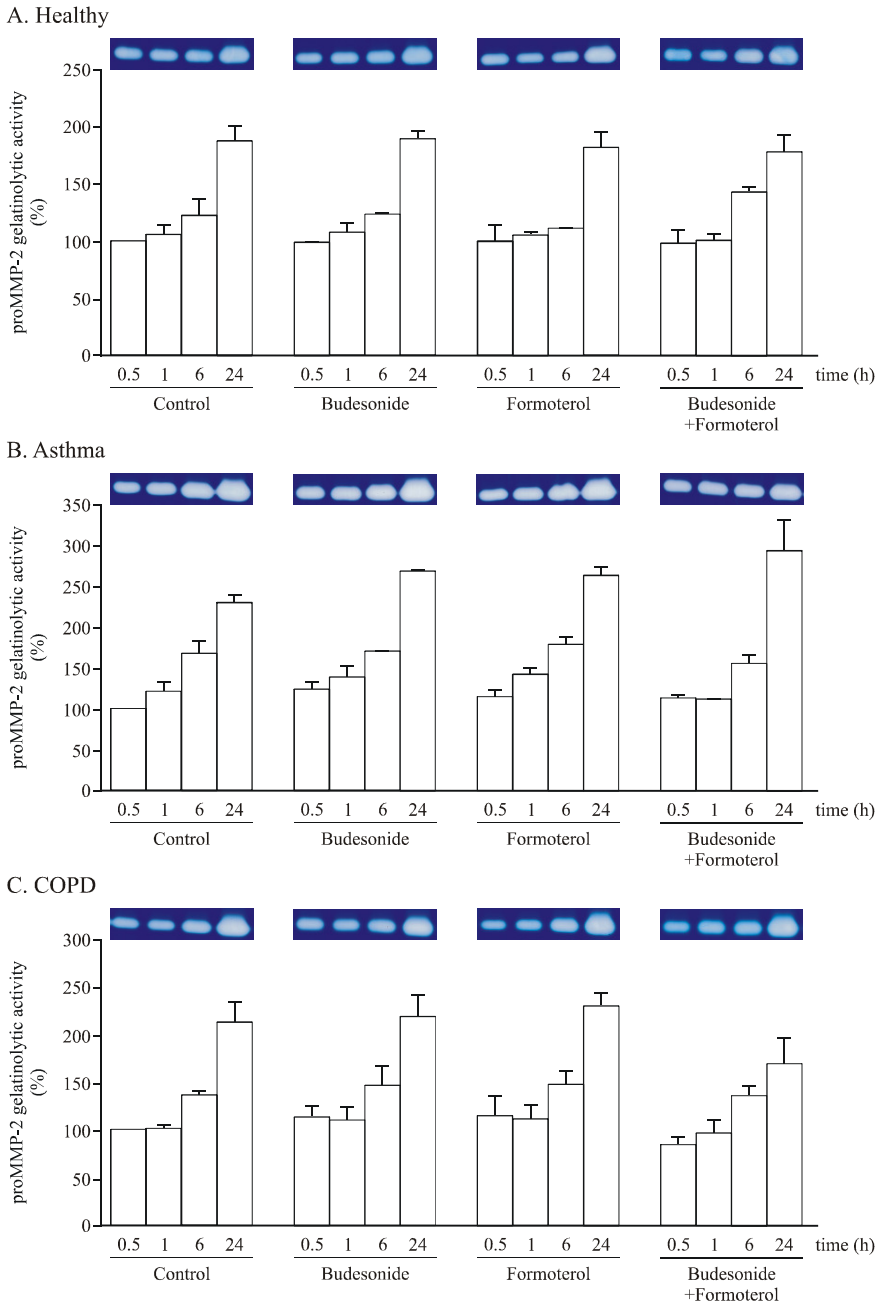

Figure 4.5. Effect of budesonide and formoterol on gelatinolytic activity. Confluent serum-deprived ASMC were treated with budesonide (bude) and/or formoterol (form) at 10^{-8} M in 0.1% FCS for 0.5, 1, 6 and 24 h. Gelatinolytic activity in conditioned media of ASMC from (A) healthy, (B) asthma and (C) COPD patients was determined by zymography. Bars represent means ± SEM expressed as percentage, with proMMP-2 activity of non-treated ASMC after 0.5 h incubation being strandardized to 100%. Representative zymograms are shown (healthy, n = 3; asthma, n = 2; COPD, n = 3 cell lines).

In the conditioned media of confluent ASMC under serum-free condition we detected gelatinolytic activity of proMMP-2 only. Previously published studies showed that ASMC constitutively released the 72-kDa gelatinase and further reported secretion of the 92-kDa gelatinase proMMP-9 when stimulated with pro-inflammatory mediators such as TNF-α [9,

13]. More recent studies reported that human ASMC express and secrete a spectrum of MMPs, including MMP-1, MMP-2, MMP-3, MMP-12 and MT1-MMP as well as their inhibitors TIMP-1 and TIMP-2 [14-18]. Furthermore, it was shown that upon stimulation with FCS or asthmatic serum MMPs secretion by ASMC from healthy individuals was upregulated [13, 15]. Therefore, it would be interesting to study modulation of different MMPs and TIMPs in primary human ASMC under both non-inflammatory and inflammatory conditions.

Compared to ASMC from healthy individuals, we observed a slight reduction in the constitutive gelatinolytic activity of proMMP-2 of ASMC from asthma and COPD patients. In addition, we showed that ASMC from healthy, asthma and COPD subjects had a similar kinetic profile of proMMP-2 secretion. Although the gelatinolytic activity of ASMC in lung diseases is not well described, other studies have reported increased MMP-1 and MMP-12 expression and secretion in asthmatic ASMC [18, 19]. Together with our observations, these findings suggest that ASMC-derived MMPs are differently regulated during pathological lung tissue remodeling. Furthermore, it has been reported that MMPs influence ASMC behaviors and functions, such as proliferation and migration [13, 17]. In addition to ECM degradation, MMPs can proteolytically process cytokines, chemokines and growth factors, suggesting that ASMC-derived MMPs may contribute to cell proliferation and ECM synthesis, thus potentiating the remodeling process [20, 21].

The role of the current asthma and COPD therapy in the regulation of MMPs production by mesenchymal cells is poorly understood. This study is the first one to address the effect of corticosteroids and LABA on gelatinolytic activity from primary human ASMC from healthy, asthma and COPD patients and we showed that both corticosteroids and LABA, alone or in combination, had no effect on proMMP-2 activity under serum-free condition. Previous studies mainly investigated the effect of corticosteroids on inflammatory cells-derived MMPs in serum, sputum and BALF of asthma patients. Hoshino *et al.* reported that 6 months of treatment with corticosteroids in asthmatics significantly reduced collagen deposition in the basement membrane via the downregulation of MMP-9 and upregulation of TIMP-1 expression [22]. Furthermore, the serum MMP-9:TIMP-1 ratio has been shown to correlate with corticosteroids response in severe asthma [5]. In regard of the effect of corticosteroids on MMPs in BALF from asthma patients data are controversially discussed. Mautino *et al.* reported a significant decreased levels of MMP-9 in BALF of corticosteroid-treated asthmatics close to levels measured in healthy subjects [3]. In contrast, no change in MMP-9

and TIMP-1 levels were reported in BALF and sputum of patients with mild asthma after budesonide treatment [23].

Our data indicate that ASMC do not contribute to MMP-9 levels in sputum or BALF in asthma patients. Based on data we previously presented at the European Respiratory Society meeting using an organoid model, we suggest that MMP-9 may originate from the epithelial cells, levels of which were decreased by corticosteroids [24]. An interesting study by Hakonarson *et al.* reported that dexamethasone repressed the IL-1β/TNF-α-induced gene expression of MMP-3, MMP-10 and MMP-12 in rabbit ASMC [25]. Accordingly, IL-1β-induced MMP-12 activity and expression was downregulated by dexamethasone in human ASMC [18]. Therefore, the effects of corticosteroids and LABA on MMPs expression, secretion and activity are MMP-specific and cell type-specific. In addition, the origin of various MMPs may depend on various factors, such as the source of samples, inflammatory status of the patient, severity of the disease, dose and duration of the treatment. Nevertheless, the role of MMP-2 and MMP-9 released by ASMC under inflammatory conditions needs further attention as both gelatinases were reported to be crucial for the infiltration of inflammatory cells and induction of airway hyperresponsiveness in a murine model of allergic asthma [26].

In conclusion, this study showed that the constitutive secretion of proMMP-2 over time was similar in ASMC from healthy, asthma and COPD subjects and was not affected by corticosteroids and/or LABA. Our findings suggest that corticosteroids have no effect on the constitutive gelatinolytic activity in mesenchymal cells. These findings may provide some explanations for the inefficacy of the current asthma and COPD therapy to reverse the remodeling process. Therefore, further investigations are needed to fully understand the effect of corticosteroids and/or LABA on ASMC-derived MMPs/TIMPs under both non-inflammatory and inflammatory conditions.

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4.3. EFFECT OF CORTICOSTEROIDS AND LABA ON TOTAL ECM AND COLLAGEN DEPOSITION BY PRIMARY HUMAN ASMC

Background and Aims of the study

ASMC play a key role in asthma specifically in the response to allergens and by controlling airflow. The airway wall of asthma patients is thicker compared to healthy individuals due in part to smooth muscle hyperplasia and hypertrophy and increased ECM deposition. In the area of the muscle bundles, ASMC express and release a variety of ECM molecules [1-3]. Various pro-inflammatory and pro-fibrotic mediators, including TGF-β_1, are increased in asthma and COPD patients, and further stimulate ECM secretion by ASMC, thereby potentiating the remodeling process. In turn, ECM components may influence the phenotype and function of ASMC, such as cell proliferation, survival, migration, mediator synthesis and plasticity [4-8]. Thus, the modulation of ASMC-derived ECM components may influence the overall lung tissue remodeling in both asthma and COPD.

Many clinical studies have reported that corticosteroids and LABA are ineffective in preventing or reversing airway remodeling in asthma and COPD patients. Although the complex interaction between the ECM and ASMC is being widely investigated, little is known about the effect of corticosteroids and LABA on ECM synthesis, deposition and regulation by ASMC *in vitro*. Therefore, the aims of this ongoing study are: (*i*) to assess the effect of corticosteroids and LABA on total ECM and collagen deposition by ASMC from healthy individuals under non-inflammatory and inflammatory conditions; (*ii*) to investigate whether total ECM and collagen deposition by ASMC is altered in asthma and COPD; and (*iii*) to compare the effect of corticosteroids and LABA, alone or in combination, on total ECM and collagen deposition by ASMC from asthma and COPD patients. Here we present preliminary data on ASMC from healthy subjects only.

Experimental design

Based on our cell culture model established in fibroblasts [9], confluent serum-deprived ASMC from healthy individuals under 0.3% albumin were assumed to reflect a non-inflammatory condition. To mimic an inflammatory environment ASMC were stimulated with 5% FCS and/or TGF-β_1 (2.5 ng/ml). In addition, ASMC were treated with

dexamethasone, budesonide, formoterol or salmeterol at concentrations ranging from 10^{-10} to 10^{-6} M in 0.3% albumin or 5% FCS for 48 h. Total ECM and collagen deposition were determined by [^{3}H]-proline incorporation (Annex). No statistical analysis was performed due to the low number of experiments.

Results

5% FCS and TGF-β_1 synergistically increased total ECM and collagen deposition

The [^{3}H]-proline incorporation data are expressed in cpm. As shown in Fig. 4.6A, 5% FCS increased total ECM deposition compared to 0.3% albumin (5256 ± 223 versus 1679 ± 618 cpm). TGF-β_1 tended to increase total ECM deposition compared to 0.3% albumin (2225 ± 412 versus 1679 ± 618 cpm). Stimulation with 5% FCS and TGF-β_1 synergistically enhanced total ECM deposition compared to 0.3% albumin and to each stimulus alone (10 800 ± 2571 cpm; Fig. 4.6A). Similar results were observed for collagen deposition. Stimulation with 5% FCS induced a 4-fold increased collagen deposition compared to 0.3% albumin (2745 ± 282 versus 746 ± 293 cpm; Fig. 4.6B). TGF-β_1 applied in serum-free condition slightly increased collagen deposition (1064 ± 167 cpm). When combined, 5% FCS and TGF-β_1 synergistically increased collagen deposition by 10-fold compared to 0.3% albumin (7309 ± 1554 cpm; Fig. 4.6B).

Effect of corticosteroids on total ECM and collagen deposition

We then investigated the dexamethasone and budesonide dose-response on total ECM and collagen deposition by ASMC. The results are expressed as percentage of change from control, corresponding to [^{3}H]-proline incorporation in 0.3% albumin and 5% FCS standardized to 100%. Dexamethasone had no clear effect on total ECM deposition in 0.3% albumin but further increased the FCS-induced total ECM deposition by approximately 60% at concentrations ≥ 10^{-8} M (Fig. 4.7A). Similar results were obtained with budesonide, having no clear effect on total ECM deposition in 0.3% albumin but dose-dependently increasing the FCS-induced total ECM deposition, reaching a 30% increase at concentrations ≥ 10^{-8} M (Fig. 4.7C).

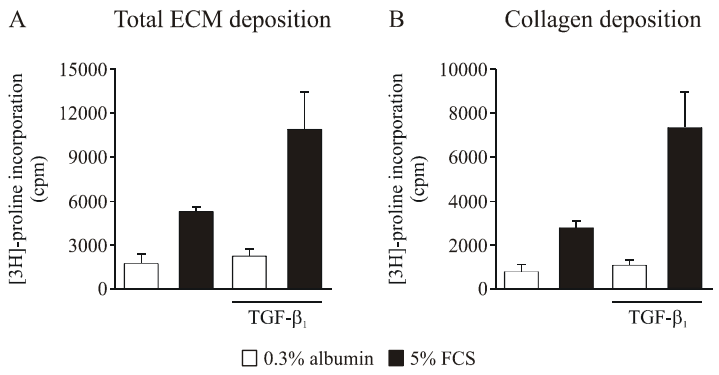

Figure 4.6. Effect of 5% FCS and TGF-β_1 on total ECM and collagen deposition by ASMC. Confluent serum-deprived ASMC from healthy subjects were stimulated with 0.3% albumin or 5% FCS and/or TGF-β_1 for 48 h. (A) Total ECM and (B) collagen deposition were determined by [^{3}H]-proline incorporation. Bars represent means ± SEM expressed in cpm (n = 2-3 cell lines).

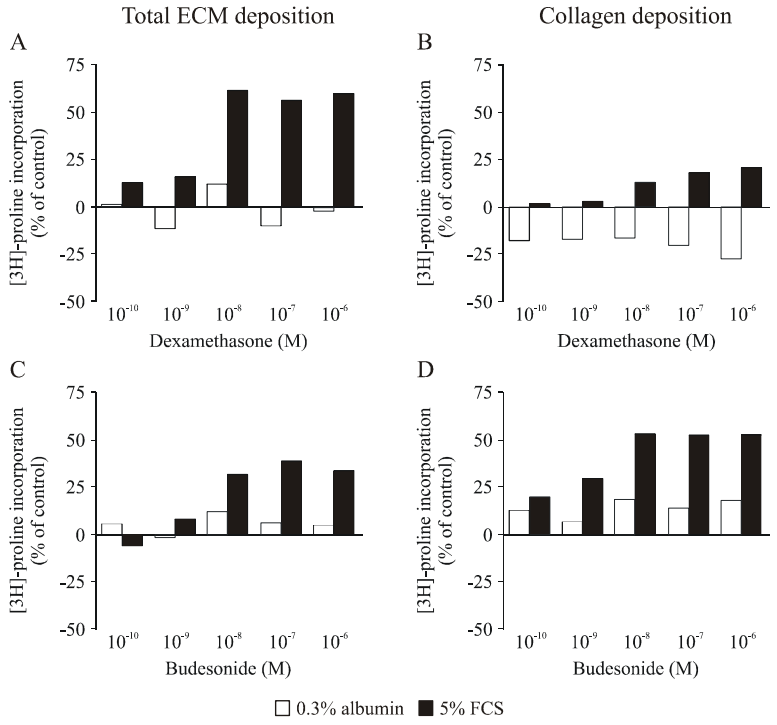

Figure 4.7. Effect of corticosteroids on total ECM and collagen deposition in the presence or absence of serum. Confluent serum-deprived ASMC were treated for 48 h with increasing concentrations (10^{-10} to 10^{-6} M) of (A, B) dexamethasone and (C, D) budesonide in 0.3% albumin or 5% FCS and total ECM and collagen deposition, respectively, were determined by [^{3}H]-proline incorporation. Bars represent means ± SEM expressed as percentage of change from control, where 0.3% albumin and 5% FCS were defined as 100% (n = 1 cell line).

In regard to collagen deposition, dexamethasone had opposite effects dependently on the presence or absence of serum. Dexamethasone dose-dependently decreased collagen deposition under serum-free condition and the reduction reached 28% at 10^{-6} M. In 5% FCS

dexamethasone had the opposite effect, increasing collagen deposition in a dose-dependent manner (Fig. 4.7B). Budesonide in 0.3% albumin tended to slightly increase collagen deposition and in 5% FCS it further increased the FCS-induced collagen deposition by 20% at 10^{-10} M up to 53% at concentrations ≥ 10^{-8} M (Fig. 4.7D).

LABA decreased total ECM and collagen deposition

Then we assessed the dose-response of two LABA on total ECM and collagen deposition under serum-free and 5% FCS conditions. Salmeterol (Fig. 4.8A,B) and formoterol (Fig. 4.8C,D) reduced total ECM and collagen deposition in both 0.3% albumin and 5% FCS. However, the decreasing effects which ranged from 18 to 40% compared to non-treated cells were not clearly dose-dependent.

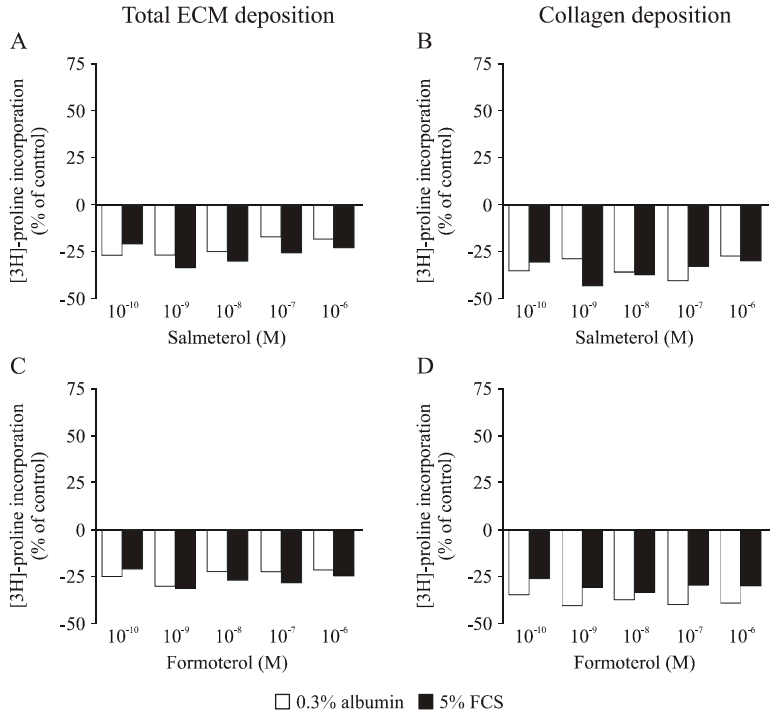

Figure 4.8. Effect of LABA on total ECM and collagen deposition in the presence or absence of serum. Confluent serum-deprived ASMC were treated for 48 h with (A, B) salmeterol and (C, D) formoterol at concentrations from 10^{-10} to 10^{-6} M in 0.3% albumin or 5% FCS and total ECM and collagen deposition, respectively, were determined by [^{3}H]-proline incorporation. Bars represent means ± SEM expressed as percentage of change from control, where 0.3% albumin and 5% FCS were defined as 100% (n = 1 cell line).

Discussion

These preliminary data showed that serum and TGF-β_1 synergistically increased total ECM and collagen deposition by primary ASMC from healthy subjects. Corticosteroids had no clear effect on total ECM and collagen deposition under serum-free condition and further increased the FCS-induced total ECM and collagen deposition in a dose-dependent manner. In contrast, LABA decreased total ECM and collagen deposition independently of the presence of serum.

ASMC hyperplasia and airway wall thickening are pathological features of airway remodeling in asthma and COPD. *In vitro*, ASMC express a wide variety of ECM macromolecules [3]. Here we showed that 5% FCS and TGF-β_1 increased total ECM and collagen deposition by ASMC. In primary human lung fibroblasts, we recently reported that 5% FCS and TGF-β_1 increased total ECM and collagen deposition to a similar extent and had an additive effect when combined [9]. Many studies have reported that TGF-β_1 stimulation upregulated expression of several ECM genes in ASMC [1, 10, 11]. Furthermore, it was recently shown that the TGF-β_1-mediated induction of fibronectin and collagen type I production by ASMC occurred in part by the autocrine release of CTGF [12].

Regarding the effect of the actual therapy for asthma and COPD, we showed that corticosteroids and LABA had similar effect on total ECM and collagen deposition by ASMC as described in fibroblasts [9]. In contrast to the significant inhibition on ECM deposition by corticosteroids in fibroblasts, no clear effect of corticosteroids under serum-free condition was obtained in ASMC. Interestingly, in both fibroblasts and ASMC corticosteroids further increased the FCS-induced total ECM and collagen deposition. LABA had a inhibitory effect under non-inflammatory and inflammatory conditions in both cell types.

Our collaborators previously showed increased ECM proteins deposition following sensitization of ASMC with asthmatic serum and no effect of corticosteroids [2]. In contrast, Hakonarson *et al.* showed in ASMC upregulation in the expression of ECM-related genes following stimulation with the pro-inflammatory cytokines IL-1β and TNF-α and that pre-treatment with dexamethasone repressed not only the upregulated inflammatory genes but also ECM genes [13]. Together with these studies, our findings suggest that ASMC from healthy individuals may respond differently to corticosteroids and/or LABA depending on the inflammatory stimuli. Our experiments need to be repeated for confirmation of the described

results and the effect of the drug combination should also be addressed. Further investigations comparing total ECM and collagen deposition by ASMC from healthy, asthma and COPD patients are needed. In addition, the effect of corticosteroids and LABA on ECM deposition by ASMC from asthma and COPD subjects under non-inflammatory and inflammatory conditions will be addressed as well as the signaling pathways involved in the drug effects.

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CHAPTER 5

KEY FINDINGS, CONCLUSION AND PERSPECTIVES

5.1. KEY FINDINGS

In the present thesis, we have shown that the effect of corticosteroids and LABA regarding ECM deposition depends on the inflammatory environment surrounding mesenchymal cells. Using different experimental approaches we were able to answer our three original objectives.

- This thesis provides the first description of an *in vitro* model mimicking inflammatory and remodeling conditions. Indeed, we have demonstrated that serum stimulation induced IL-6, IL-8 and TGF-β_1 release as well as cell proliferation in primary human lung fibroblasts and that these two inflammatory responses were inhibited by corticosteroids. In addition, FCS and/or TGF-β_1 increased total ECM, collagen and GAGs deposition, reflecting active tissue repair.

- Using our model, we showed that the inhibitory effect of corticosteroids on total ECM, collagen and GAGs deposition was reversed in 5% FCS. In contrast, LABA decreased total ECM and collagen deposition independently of the presence of serum but had no effect on GAGs deposition. Interestingly, combined corticosteroids and LABA showed an additive effect by decreasing total ECM and collagen deposition under serum-free condition and counterbalanced each other in FCS. However, the combined treatment had the same effect on GAGs secretion and deposition as corticosteroids alone.

  In addition, we showed that the inhibitory effect of corticosteroids on total ECM and collagen deposition under non-inflammatory condition was partly mediated via the GR and collagen gene expression, whereas the corticosteroid-induced ECM deposition in the presence of serum was not mediated via the GR but was associated with upregulation of CTGF mRNA expression. The effect of LABA was partly mediated via the β_2-adrenergic receptor and did not involve gene expression.

- We expanded our research to ASMC originating from healthy, asthma and COPD patients. We showed no difference in the constitutive GAGs secretion and deposition

between ASMC from healthy, asthma and COPD subjects and in their response to FCS, corticosteroids and/or LABA. Under serum-free condition corticosteroids but not LABA, decreased GAGs secretion and deposition. Surprisingly, in 5% FCS corticosteroids decreased GAGs secretion and had no effect on GAGs deposition. Furthermore, we showed that corticosteroids and LABA had similar effect on total ECM and collagen deposition as observed in fibroblasts. Finally, we demonstrated that the drug effects on ECM deposition were not modulated by MMPs.

5.2. CONCLUSION

In conclusion, our data suggest that the effect of corticosteroids on ECM deposition by fibroblasts is generally altered by serum, while this is not the case for LABA. Our *in vitro* model demonstrates new effects of the corticosteroids and LABA, alone or in combination, on ECM deposition by mesenchymal cells. Our findings indicate that in order to block the progression or to reverse airway remodeling in asthma and COPD patients, the actual therapy should be adjusted to the inflammatory status of the patients and the concentrations well controlled to avoid the occurrence of important side effects.

5.3. PERSPECTIVES

Our data raise the question whether it would not be more beneficial to start corticosteroids treatment at an early stage of asthma to prevent inflammation and consequently airway remodeling instead of trying to reverse established lesions. Addressing theses issues would provide more information about the role of structural cells and the importance of inflammation in the onset and progression of tissue remodeling and might elucidate which elements of the remodeling process can be prevented. This is indeed a hypothesis that could be tested using our *in vitro* model by pre-treating cells with corticosteroids before serum stimulation and measurement ECM deposition.

Furthermore, ECM deposition during airway inflammation is regulated by altered cell phenotype and response to various cytokines and growth factors. In this regard, signals in the inflamed airways of asthma and COPD patients could modulate ECM production by fibroblasts and ASMC and influence their response to therapy. Therefore, it would be of great interest to assess ECM deposition by fibroblasts from asthma and COPD patients and to further investigate their response to corticosteroids and LABA. An alternative would be to

investigate the influence of serum or BALF from asthma and COPD patients on ECM synthesis by healthy fibroblasts.

Finally, DNA microarray represents another interesting approach, which might elucidate potential mediators and mechanisms involved in the opposite effect of corticosteroids on ECM deposition by lung fibroblasts under non-inflammatory and inflammatory conditions. Obviously, the extrapolation of such experimental findings to the clinical management of asthma and COPD requires caution. However, they may provide insights into the mechanisms modulating lung tissue remodeling and may lead to new drug targets or help to optimize current therapy of asthma and COPD.

ANNEX

MATERIALS AND METHODS

Primary human lung cell cultures

Ethical committee

The primary human lung cell cultures were established from macroscopically normal parts of peripheral lung tissue obtained from patients undergoing partial lung resection as part of a lung cancer therapy. This protocol was approved by the Ethical committee of the Faculty of Medicine, University Hospital Basel (#M75/97). Tissues were collected by Dr. Franco Gambazzi from the Department of Thoracic Surgery. In addition, primary ASMC from healthy, asthma and COPD patients were provided by our collaborators from The Woolcock Institute of Medical Research (University of Sydney, Australia). Ethical permissions for these studies were obtained from the University of Sydney and the Central Sydney Area Health Service, and all patients gave written and informed consent.

Primary human lung fibroblasts

Primary human lung fibroblast cultures are well established in our laboratory and their phenotype was previously characterized and described by our group [1, 2]. Briefly, fibroblasts were established from sterile lung tissue samples stored at 4°C in sterile PBS (Cambrex Bio Science, Verviers, Belgium). Tissue samples were cut into small pieces of 1-2 mm^3 and 16-20 pieces were placed into 75 cm^2 flasks pre-wetted with 5 ml of RPMI 1640 (2.1 mM glutamine and 25 mM Hepes; Cambrex) supplemented with 10% FCS (heat inactivated for 15 min at 65°C; Gibco BRL Invitrogen, Basel, Switzerland) and 1% MEM-Essential minimum vitamins (Cambrex). Cultures were performed without antibiotics and were kept at 37°C in a humidified incubator containing 5% CO_2. Epithelial-like cells started to grow after few days followed by spreading of spindle shape-like fibroblasts from the tissue pieces. Medium was changed twice a week until fibroblasts reached confluence around the tissue pieces. Then, cells were washed three times with PBS and trypsinized [0.125% trypsin (Biochrom AG, Berlin, Germany) and 0.01% EDTA in PBS] for 3 min at 37°C. For the first passage, cells were re-suspended in 10% FCS medium in the same flask to avoid any cell loss and medium was changed the following day. The next passages were performed according to a 1:3 ratio. Fibroblasts displayed a typical spindle-shaped morphology, stained positive for fibronectin

and laminin but were negative for von Willebrand factor, Factor VIII, cytokeratin, and smooth muscle actin. Fibroblasts between passages 3 and 6 were used for all experiments.

Primary human ASMC

ASMC were mainly obtained from our collaborators at the Woolcock Institute (Sydney, Australia) and were established as previously described by Carlin *et al.* [3]. Briefly, non-asthmatic ASMC were obtained from bronchi of patients undergoing resection for either lung transplantation or carcinoma. Asthmatic ASMC were obtained from deep endobronchial biopsies performed by flexible bronchoscopy. Large bronchi of 5-15 mm internal diameter were dissected from the surrounding parenchyma. With the aid of a binocular microscope, the epithelial layer was removed, thus exposing the underlying smooth muscles. These smooth muscle bundles were dissected from the bronchi and placed into 25 cm^2 tissue flasks containing DMEM medium supplemented with 10% FCS, 20 U/l penicillin, 20 µg/ml streptomycin, and 2.5 µg/ml amphotericin B (GIBCO BRL, Life Technologies, Sydney). ASMC grew to confluence within approximately 3 to 4 weeks and were passaged into 75 cm^2 flasks. Cells were sent to our laboratory and then cultured in RPMI medium supplemented with 5% FCS and 1% MEM-vitamins until confluence. ASMC were also obtained from the University of Basel as described above and were grown in antibiotic-free medium. All experiments were performed with cells between passages 4 and 9. As described by Johnson *et al.*, ASMC appeared spindle shaped with central and prominent nucleoli, and pure populations were confirmed by positive staining for α-smooth muscle actin and calponin [4].

Cell stimulation and drug treatment

Serum starvation

Prior to stimulation and treatment, cells were growth-arrested by serum starvation for 24 h in low serum RPMI medium supplemented with 0.1% FCS or 0.3% human albumin. Medium was exchanged after 12 and 24 h to avoid auto-stimulation of the cells.

Cell stimulation

Stimulation at different time points with either 5% FCS or recombinant human TGF-β_1 (0.1 to 5 ng/ml; Sigma, Schnelldorf, Germany) was used to mimic an inflammatory environment.

Corticosteroids and LABA

The effect of corticosteroids and LABA on pro-inflammatory cytokine release, ECM deposition, MMPs activity, gene expression and cell proliferation was investigated in our cell

cultures. Three corticosteroids (dexamethasone [Calbiochem, Lucerne, Switzerland], fluticasone [GlaxoSmithKline, London, UK] and budesonide [AstraZeneca, Lund, Sweden]) and two LABA (salmeterol [GlaxoSmithKline] and formoterol [AstraZeneca]) were tested at concentrations ranging from 10^{-6} to 10^{-10} M for 24 or 48 h under both non-inflammatory and inflammatory conditions. Drugs were dissolved in DMSO, which final concentration (< 0.01% DMSO in the culture medium) had no effect on all measured parameters.

GR and β_2-adrenergic receptor antagonists

We assessed whether the effects of corticosteroids and LABA on total ECM and collagen deposition were mediated via their receptor. Fibroblasts were pre-treated for 30 min with the GR antagonist RU486 (Sigma) or the β_2-adrenergic receptor antagonist propranolol (Calbiochem), both at 10^{-6} M, before the addition of a corticosteroid or LABA (10^{-7} M).

Cellular biology

IL-6, IL-8 and TGF-β_1 ELISA

Conditioned cell culture media of confluent fibroblasts were collected after 48 h of stimulation and levels of IL-6, IL-8 (Orgenium, Helsinki, Finland), and total TGF-β_1 (R & D Systems, Minneapolis, MN, USA) were determined by ELISA according to the manufacturer's instructions.

Total proteins concentration

Total proteins concentration in conditioned media was measured using the Bradford protein assay (Bio-Rad Laboratories GmbH, Munich, Germany). The standard curve was established using bovine serum albumin (0-500 ng/ml) and the absorbance was measured at 595 nm with a microplate reader (SpectraMax 190, Molecular Devices Corporation, Sunnyvale, USA).

Gelatin zymography

Conditioned media were collected from ASMC after stimulation and/or treatment before assessing gelatinolytic activity by zymography according to the method previously detailed [6, 7]. Each sample (2 µg of proteins) were separated on a 10% polyacrylamide SDS gels containing 1 mg/ml porcine skin gelatin (Sigma) for 3 h at 50 mA constant current. After electrophoresis, gels were washed 2 x 30 min in 2.5% Triton-X to remove the SDS in order to allow the enzymes to recover their enzymatic activity. Gels were rinsed in water and then incubated in the enzyme activation buffer [50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 2 µM $ZnCl_2$, and 0.02% Brij-35] for 18 h at 37°C. During this incubation, the activated MMPs in

the gels digested the gelatine substrate. The following day, gels were stained for 3 h with 0.1% Coomassie Brilliant Blue (R-250, Bio-Rad) in 25% methanol and 10% acetic acid. Gels were then destained (25% methanol, 10% acetic acid) until the detection of transparent bands and dried overnight between two sheets of cellophane. Enzymatic activity was detected as colorless bands against a blue background and compared to pre-stained molecular weight marker (Bio-Rad). Quantification was performed by densitometry using the NIH Image J software and data expressed as arbitrary units (a.u.).

Total ECM and collagen deposition

Total ECM and collagen deposition were assessed by [^{3}H]-proline incorporation as previously described by our group [8, 9]. This well-established assay measures the incorporation of [^{3}H]-labeled proline into protein. The percentage of proline specifically incorporated into collagen is determined after digestion with collagenase. In our studies, primary human lung fibroblasts and ASMC were seeded into 24-well plates, grown until confluence, serum-deprived for 24 h in 0.3% human albumin and stimulated and/or treated in the presence of 1 μCi/ml [^{3}H]-proline (Amersham, Little Chalfont, UK) and 10 μg/ml L-ascorbic acid for 36 or 48 h. After removing the conditioned media, cells were lysed with 25 mM NH_4OH for 10 min at room temperature. The remaining deposited ECM was fixed in 70% ethanol (2 x 15 min) and washed 3 times with a buffer consisting of 50 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$ and 1 mM L-proline. The resulting deposited ECM was then incubated with 300 μl of collagenase buffer [50 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$ and 2.5 mM N-ethylmaleimide] with or without 120 U/ml of highly purified bacterial collagenase (type VII, *Clostridium histolyticum*; Sigma) for 4 h at 37°C. The supernatants were then carefully removed and collected into scintillation vials. The residual ECM was solubilized by an overnight incubation in 0.3 M NaOH containing 1% SDS. The levels of [^{3}H]-proline present in the supernatants and in the solubilized ECM was determined by liquid scintillation counting by a β-counter and expressed as counts per minute (cpm). All experiments were performed in duplicate.

Modification of deposited total ECM and collagen was calculated as follows: 1) cpm in solubilized ECM without collagenase was defined as "total ECM deposition"; 2) (cpm in supernatant without collagenase x 100) / (cpm in supernatant without collagenase + cpm in solubilized ECM without collagenase) was defined as percentage of background; and 3) cpm in supernatant with collagenase – (% background x cpm in supernatant with collagenase) was defined as "collagen deposition".

Total GAGs secretion and deposition

Total GAGs secretion and deposition were determined by using [^{3}H]-glucosamine incorporation (1 μCi/ml; Amersham) as previously described [2]. In order to measure total GAGs secretion, 500 μl of conditioned media were added to 2 ml of 95% ethanol containing 2.5% (w/v) sodium acetate and incubated overnight at 4°C in order to allow protein precipitation. The following day samples were centrifuged at 3000 x *g* for 30 min at 4°C and the pellets were dissolved by 0.2 mg/ml pronase (pre-incubated for 30 min at 40°C to eliminate glycosidase activity, *Streptomyces griseus*; Calbiochem) in 500 μl of pronase buffer consisting of 100 mM Tris-HCl (pH 7.5) and 1 mM $CaCl_2$. Samples were incubated at 40°C for 1.5 h prior to an overnight protein precipitation at 4°C as above. Samples were centrifuged and the pellets were dissolved in 250 μl of 0.5 M NaOH and transferred into scintillation vials. [^{3}H]-glucosamine was determined by liquid scintillation by a β-counter and expressed as cpm.

Total GAGs were also measured in the cell layer together with the deposited ECM. Cells were washed twice with ice-cold PBS and lysed with 200 μl RIPA buffer [1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate (pH 7.2)]. Proteins were first precipitated overnight at 4°C (800 μl of 95% ethanol containing 2.5% sodium acetate), centrifuged at 16 000 x *g* for 15 min and then the remaining pellets were dissolved by 0.5 mg/ml pronase in 200 μl of pronase buffer for 1.5 h at 40°C. GAGs were precipitated overnight at 4°C, centrifuged, resuspended in 250 μl of 0.5 M NaOH and [^{3}H]-glucosamine was determined by liquid scintillation.

Cell proliferation

Fibroblast proliferation was measured by [^{3}H]-thymidine incorporation in newly synthesized DNA. Fibroblasts were seeded at a density of 5000 cells/well into 96-well plates, grown until 60-70% confluence. Serum-deprived cells were stimulated with 5% FCS and treatments were applied in the presence of 2 μCi/ml [^{3}H]-thymidine (Amersham). After 30 h of stimulation, cells were washed twice with PBS and lyzed (100 μl of 1 M NaOH/well). Cells were harvested onto filter plates using the Filtermate Unifilter-96 Harvester (PerkinElmer Life Sciences, Boston, USA). After drying the filter plates, 20 μl of scintillation liquid/well were added and incorporated [^{3}H]-thymidine was determined in a β-counter (TopCount microplate scintillation counter; PerkinElmer Life Sciences). Data were expressed as cpm and all experiments were performed in sixtuplicate.

Molecular biology

RNA extraction and reverse transcription

Cells were plated in 100 x 20 mm dishes, grown until confluence and serum-deprived for 24 h prior to stimulation and/or drug treatment. After incubation, cells were washed twice with ice-cold PBS, scrapped and cell pellets were collected by centrifugation at 900 x *g* for 10 min. Total RNA was isolated using RNeasy Mini kit (Qiagen, Basel, Switzerland) and RNA concentration and purity were measured by spectrophotometry (NanoDrop Technologies Inc., Wilmington, DE, USA). The A_{260}/A_{280} ratio was used as an indicator of purity with optimal values ranging between 1.8 and 2.0 RNA samples were stored at -70°C until analysis. First strand cDNA was synthesized from 1 µg of total RNA using M-MLV Reverse Transcriptase according to the manufacturer's instructions (Promega, Madison, WI, USA).

Polymerase chain reaction

cDNA was amplified using *Taq* DNA polymerase (Promega). PCR conditions consisted on 1) an initial denaturation at 95°C for 2 min, 2) denaturation at 95°C for 30 sec, primer annealing at 58-60°C for 30 sec, and extension at 72°C for 1 min for 20-40 cycles, and 3) a final extension at 72°C for 5 min. PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide stain under UV light. The intensity of each band was analyzed by densitometry using the NIH Image J software and the relative mRNA expression of target gene was normalized to β-actin. All primers were purchased from MWG-Biotech AG (Ebersberg, Germany).

Statistical analysis

For statistical analysis, Student's *t*-test was performed using the free statistic software package R and $p < 0.05$ was considered significant.

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CURRICULUM VITÆ – STEPHANIE GOULET

Mittlere Strasse 33/79
4056 Basel, Switzerland
Phone: + 41 61 265 23 37
Fax: + 41 61 265 23 50
stephanie.goulet@stud.unibas.ch

From October 2006
193, rue Principale
Sainte-Justine, Québec, Canada
Phone: 418 383-3989
steph_goulet@hotmail.com

EDUCATION

Ph.D. in Biomedical Sciences **2002-2006**
Pulmonary Cell Research Laboratory, Department of Research
University Hospital Basel
Thesis title "Effects of corticosteroids and long-acting β_2-agonists in a human cell culture based *in vitro* model of airway inflammation and tissue remodeling"

Master in Sciences, Experimental Medicine **1999-2002**
Institut universitaire de cardiologie et de pneumologie
Hôpital Laval, Université Laval, Sainte-Foy, Québec
Master title "Role of matrix metalloproteinases and their inhibitors in a chronic murine model of allergic asthma"

Bachelor degree, Medical Biology **1996-1999**
Université du Québec à Trois-Rivières (UQTR), Trois-Rivières, Québec

College degree, Natural and Health Sciences **1994-1996**
Champlain-St.Lawrence College, Sainte-Foy, Québec

EXPERIENCES

Post-graduate courses, International Respiratory Meetings **2002-2006**
American Thoracic Society International Conference
European Respiratory Society Annual Congress

Key issues in Drug discovery and Development **2005**
Graduate course Pharmacentre Basel – ETH Zurich
Collaboration Novartis-Roche, Basel

Novartis Institute for Biomedical Research **2000, 2001**
Novartis Pharmaceuticals Corporation, East Hanover, New Jersey
Collaboration and training

University of Alberta, Edmonton, Alberta **1999**
Glaxo-Heritage Asthma Research Laboratory
Undergraduate training on the "Role of the cofactor tetrahydrobiopterin in nitric oxide production in rat peritoneal mast cell"

TECHNICAL SKILLS

Cellular and molecular biology

- Primary cell cultures from human lung tissue and biopsies : fibroblasts, airway smooth muscle cells, vascular smooth muscle cells, and epithelial cells; lung cancer cell lines
- Pharmacologic application *in vitro*
- Cell proliferation by [^{3}H]-thymidine incorporation

- Total, cytosolic and nuclear proteins extraction, Western blotting
- RNA extraction, RT-PCR, Northern blotting
- DNA extraction, purification and cloning
- Gene silencing (antisense)
- Quantification of extracellular matrix components deposition by [^{3}H]-proline and [^{3}H]-glucosamine incorporation
- Proteoglycan purification
- Enzymatic activity by zymography and reverse zymography
- ELISA and other colorimetric assays

Animal research

- Optimization of a chronic murine model of allergic asthma
- Intraperitoneal injection, gavage feeding, intranasal instillation, intratracheal canulation, bronchoalveolar lavage, and total/differential cell counts in mice
- Intraperitoneal injection, peritoneal lavage, and mast cells isolation in rats

OTHERS

Leadership and organizational skills

- Laboratory organization: orders, common cell cultures, biosecurity levels 1 and 2, radioactivity
- Writing of protocols, scientific manuscripts, grant applications
- In charge of Macherey-Nagel AG products distribution, Department of Research, Basel
- Supervision of undergraduate students
- Programm representative to socio-cultural activities in UQTR (1997-1999)

Languages: french and english, german (beginner level)

PUBLICATIONS AND MANUSCRIPTS IN PREPARATION

Goulet S, Bihl MP, Gambazzi F, Roth M, Tamm M. Opposite effect of corticosteroids and long-acting β2-agonists on TGF-β1-induced extracellular matrix deposition by primary human lung fibroblasts. Manuscript accepted to *Journal of Cellular Physiology*.

Bihl MP, Laule-Kilian K, Bubendorf L, Rutherford RM, Baty F, Kehren J, Eryüksel E, Staedtler F, Yang JQ, **Goulet S**, Gilmartin JJ, Tamm M, Brutsche MH. Progressive pulmonary sarcoidosis - a fibroproliferative process potentially triggered by EGR-1 and IL-6. *Sarcoidosis, vasculitis, and diffuse lung diseases* 2006;23(1):38-50.

Yang JQ, Rüdiger JJ, **Goulet S**, Gencay MC, Tamm M, Roth M. Cell density and inflammation affect compartmental distribution, activation and function of the glucocorticoid receptor in human lung fibroblasts. Manuscript submitted.

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Gosselin M, **Goulet S**, Wu-Wong JR, Wessale JL, Opgenorth TJ, Boulet LP, Battistini B. Effects of a selective ET(A)-receptor antagonist, atrasentan (ABT-627), in murine models of allergic asthma: demonstration of mouse strain specificity. *Clinical Science (Lond)* 2002;103 Suppl 48:367S-370S.

INTERNATIONAL COMMUNICATIONS

* This list includes oral and poster presentations done in international conferences during my Ph.D. studies. Please note that other presentations were done during my master studies and to local and national meetings. Detailed list is available.

XVth World Conference of Pharmacology, 2006

- 2006, Beijing (China), Abstract published in *Acta Pharmacologica Sinica* Suppl 1:2006.

  Karakiulakis G, Papakonstantinou E, **Goulet S**, Klangas I, Tamm M, Roth M. Glycosaminoglycan synthesis by airway smooth muscle cells is differentially modulated after treatment with β_2-agonists and corticosteroids.

American Thoracic Society International Conference

- 2006, San Diego (California, USA), Abstracts published *Proceedings of the American Thoracic Society* 3:2006.

  Goulet S, Papakonstantinou E, Bihl MP, Gambazzi F, Roth M, Tamm M. Effect of fluticasone and salmeterol on extracellular, collagen and glycosaminoglycan deposition in primary human lung fibroblasts.

  Borger P, **Goulet S**, Tamm M, Roth M. Steroids and β_2-agonists differentially modulate the expression of C/EBPα and calreticulin in airway smooth muscle cells.

  Hostettler KE, **Goulet S**, Roth M, Burgess JK, Black JL, Tamm M, Borger P. Primary human airway epithelial cells inhibit fibroblast proliferation through phosphorylation of p27.

- 2005, San Diego (California), Abstracts published *Proceedings of the American Thoracic Society* 2:2005.

  Goulet S, Tamm M, Roth M, Bihl MP. Effects of steroids and β_2-mimetics on TGF-β_1- and serum-induced extracellular matrix deposition in primary human lung fibroblasts.

  Yang JQ, Rüdiger JJ, **Goulet S**, Tamm M, Roth M. Glucocorticoid receptor traffic is different in confluent and subconfluent primary human lung fibroblasts.

- 2004, Orlando (Florida, USA), Abstracts published *American Journal of Respiratory and Critical Care Medicine*, 169(7):2004.

  Goulet S, Bihl MP, Roth M, Tamm M. Effects of steroids and β_2-mimetics on matrix metalloproteinases activity in an *ex vivo-in vitro* model of the human lung.

  Marcotte M, **Goulet S**, Israël-Assayag E, Cormier Y, Battistini B. Modulation of the MMP/TIMP system in patients with extrinsic allergic alveolitis (EAA): evidence of MMP/TIMP modulation in the BALF.

  Bihl MP, **Goulet S**, Rüdiger JJ, Roth M, Tamm M. IL-6 signaling and alternative splicing.

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  Goulet S, Bihl MP, Roth M, Tamm M. Gelatinolytic activity in the human lung: role of cell-cell interactions.

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- 2003, Vienna (Austria), Abstracts published *European Respiratory Journal*, 22 (Suppl 45):2003.

  Goulet S, Bihl MP, Roth M, Tamm M. Effects of steroids and β-mimetics on matrix metalloproteinases expression in human primary lung cells.

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