GLUCOCORTICOID ACTIONS IN HUMAN BRONCHIAL EPITHELIAL CELLS

de werking van glucocorticoïden op humane bronchusepitheelcellen

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GLUCOCORTICOID ACTIONS IN HUMAN BRONCHIAL EPITHELIAL CELLS

DE WERKING VAN GLUCOCORTICOÏDEN OP HUMANE BRONCHUS-EPITHEELCELLEN

PROEFSCHRIFT

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"Labor improbus omnia vincit"
Vergilius

"De aanhouder wint"

Aan mijn ouders
Aan Theo

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1.1 Airway inflammatory diseases

Diseases characterized by airway inflammation affect a substantial proportion of the population. These diseases include asthma and chronic obstructive pulmonary disease (COPD). In the European Union, COPD and asthma, together with pneumonia, are the third most common cause of death (1). In the last decade much progress has been made towards the understanding of the mechanisms underlying airway inflammation. The production of cytokines and growth factors in response to irritants, infectious agents and inflammatory mediators plays an important role in the initiation, perpetuation and inhibition of acute and chronic airway inflammation. Today, several models exist to visualize the processes by which airway inflammation is perpetuated in diseases such as asthma and COPD. These processes include the persistence of the acute inflammatory response and neurogenic inflammation. Better understanding of these processes will provide us with new therapeutic approaches to the treatment of these common chronic diseases.

1.1.1 Asthma

Asthma is one of the most common chronic disorders in the Western world and affects about 10% of the population. Initially, the Greek term "ασθμα", used by Hippocrates (460-357 B.C.), was the name given to the disorder occurring in people with "difficult breathing". Today, "asthma" is still difficult to define, probably because of lack of understanding of the disease mechanism(s). In a recent international consensus report on diagnosis and treatment of asthma, the following current working definition of asthma was formed:

'Asthma is a chronic inflammatory disorder of the airways in which many cells play a role, including mast cells and eosinophils. In susceptible individuals this inflammation causes symptoms which are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment, and causes an associated increase in airway responsiveness to a variety of stimuli' (1).

The vast majority of asthmatic patients are categorized as having allergic (extrinsic or atopic) asthma. This disease often develops during childhood in association with a documented history of other allergic disorders (2). In these patients, allergen inhalation induces an IgE-mediated asthmatic reaction as described below. Non-allergic or intrinsic asthma occurs only in 10-20% of the patients. These patients do not have enhanced serum IgE levels and do not demonstrate any seasonal variation.

The initial event in allergic asthma is thought to be presentation of antigen by dendritic cells/monocytes/macrophages (known as antigen presenting cells) to $CD4^+$ helper T lymphocytes. Recognition of the presented antigen by these T lymphocytes and the generation of costimulatory signals by the antigen presenting cells results in the production of inflammatory mediators by both the helper T lymphocytes and the antigen presenting cells. The majority of these helper T lymphocytes belong to the subclass of T_h2 lymphocytes. This subset of T lymphocytes is able to produce a limited panel of cytokines including Interleukin (IL)-3, IL-4, IL-5, IL-10, IL-13 and granulocyte macrophage colony-stimulating factor (GM-CSF) (3). The netto effect of antigen presentation and the release of these cytokines is to promote the synthesis of specific IgE by B lymphocytes through the action of IL-4 on Ig isotype switching (4) and to enhance the differentiation, migration, and activation of eosinophils through the actions of GM-CSF, IL-3, and IL-5 (5,6). The IgE produced in asthmatic airways binds with high affinity to specific receptors (FcεRI) expressed on the surface of mast cells, basophils and eosinophils, and with lower affinity to a second type of specific receptors (FcεRII, CD23) on macrophages, eosinophils and bronchial epithelial cells.

Cross-linking of receptor-bound IgE with specific allergen results in the release of mediators of inflammation, including histamine, leukotrienes, platelet-activating factor (PAF) and various proteases which, through their direct effects on airway smooth muscle and the microvasculature, are responsible for the allergen-induced bronchial narrowing and wheezing (7). Once recruited from the circulation, eosinophils secrete cationic proteins such as major basic protein (MBP), eosinophil cationic protein (ECP) and reactive oxygen species into the airways (8-10). These products may cause epithelial damage (shedding) and can increase airway responsiveness (9-12).

It has been suggested that also epithelial cells play a role in airway inflammation. Interactions between airway epithelial cell products and effector inflammatory cells may directly contribute to the pathogenesis of airway inflammation in disorders such as asthma and COPD (Figure 1). Immunohistochemical studies performed on bronchial mucosal biopsy specimens have detected enhanced amounts of GM-CSF and monocyte chemoattractant protein-1 (MCP-1) in asthmatic patients compared with healthy controls (13,14). In addition, primary cultures of asthmatic bronchial epithelial cells secrete increased amounts of IL-6, IL-8, and GM-CSF (15). After stimulation, bronchial epithelial cells can also release chemotactic factors such as leukotriene B_4 (LTB_4) (16) and RANTES (regulated on activation, normal T cells, expressed and presumably secreted) (17). These airway epithelial cell mediators may then contribute to the recruitment, activation, differentiation and survival of eosinophils, lymphocytes, and macrophages in the airways of asthma and COPD patients.

Glucocorticoids have proven to be the most effective drugs in the treatment

of asthma (18). Chronic treatment with inhaled glucocorticoids reduces the number of inflammatory cells in the airways and more specifically, reduces the number of mast cells, eosinophils and lymphocytes (19). Additionally, glucocorticoid treatment is associated with repair of the airway epithelial structure (20). These treatment-induced morphological changes are associated with clinical improvement and a decrease of airway hyperresponsiveness (20).

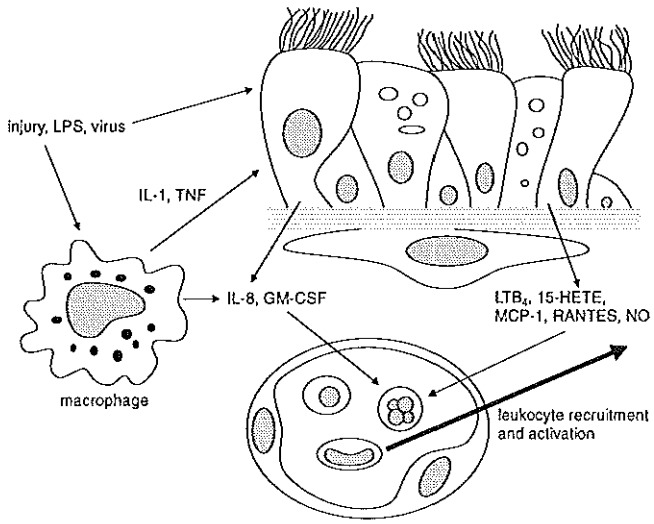

Figure 1. Cytokine interactions between bronchial epithelial cells and inflammatory cells. Inflammatory stimuli, *e.g.* LPS or viral infection, initiate release of mediators including LTB_4, 15-HETE, IL-8, GM-CSF, MCP-1, RANTES and NO from epithelial cells and IL-1 and TNF from macrophages, which further stimulate epithelial cells. The macrophages and epithelial cells augment the inflammatory response by releasing additional mediators that recruit and activate leukocytes. LPS: lipopolysaccharide; LTB_4: leukotriene B_4; 15-HETE: 15-hydroxyeicosatetraenoic acid; IL-8: interleukin-8; GM-CSF: granulocyte-macrophage colony-stimulating factor; MCP-1: monocyte chemoattractant protein-1; RANTES: regulated on activation, normal T cells, expressed and presumably secreted; NO: nitric oxide; IL-1: interleukin-1; TNF: tumor necrosis factor. Adapted from reference 30.

1.1.2 COPD

The European Respiratory Society (ERS) has taken the initiative of producing a consensus statement of COPD. The current working definition is as follows:

'Chronic obstructive pulmonary disease (COPD) is a disorder characterized by reduced maximum expiratory flow and slow forced emptying of the lung; features which do not change markedly over several months' (21).

The airflow limitation in COPD is generally progressive, may be accompanied by airway hyperreactivity and may be partial reversible. COPD includes two diseases: chronic bronchitis and emphysema.

Chronic bronchitis is defined as the presence of chronic productive cough for 3 months in each of two successive years in a patient in whom other causes of chronic cough have been excluded (22). *Emphysema* is defined as abnormal permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their wall and without obvious fibrosis (22). It has to be noted that the obstruction in many patients with COPD may include a significant reversible component and that some patients with asthma may go on to develop irreversible airflow obstruction indistinguishable from COPD.

The major characteristic of COPD is the presence of chronic airflow limitation that progresses slowly over a period of years. Inflammation, fibrosis, goblet cell metaplasia, and smooth muscle hypertrophy in terminal bronchioles are important causes of airflow obstruction; loss of alveolar attachement to bronchioles due to constructive changes in emphysema can also be of importance. The primary cause of COPD is without question exposure to tobacco smoke (23). Other factors that may play a role are occupational factors and alpha$_1$-antitrypsin deficiency (23). Lung function tests are used in the diagnosis of COPD as well as in the assessment of its severity, progression and prognosis. The presence of airflow limitation is recognized by a reduction in the ratio of forced expiratory volume in one second (FEV1) to vital capacity (VC). The main factor associated with rapid longitudinal decline in FEV1 and poor prognosis is smoking. Cessation of smoking reduces the rapid decline in FEV1. Therefore, active intervention to help patients stop smoking is the primary tool for adequate management of COPD.

Glucocorticoids are of great benefit in asthma, but their precise role in COPD has yet to be establised. Most studies suggest that 20 to 30% of patients with COPD improve if given oral steroid therapy (24).

1.2 Bronchial epithelium

1.2.1 Anatomy

The epithelial layer forms the interface between the respiratory system and the inspired air. It rests upon a connective tissue substratum consisting of a basement membrane, lamina propria and submucosa, which contains smooth muscle, glands and cartilage (Figure 2) (25,26). The bronchial epithelium is comprised of a pseudostratified ciliated epithelium, with ciliated cells occupying the majority of the luminal surface and basal cells in contact with much of the basement membrane (27). In addition, secretory cells, namely goblet and Clara cells, can be found in the bronchial epithelium. Each of the major cell types in the epithelium has distinctive histological characteristics.

Ciliated cells are terminally differentiated columnar epithelial cells which are thought to originate from basal or secretory cells (28). Mucociliary clearance is

the primary function of the ciliated cells. A cilium has a core of microtubules (axoneme) ensheathed in an extension of the plasma membrane. The axoneme of the cilium is made up of an outer ring of nine microtubule doublets surrounding a central pair. The microtubule assembly is anchored in the cell by a basal body. Side arms, extending from each microtubule doublet, composed of a protein called dynein that contains ATPase, play a major role in the bending movements of the cilia (29). The cilia in a given cell and its neighbours share a common orientation. By cell-cell interaction the beating of the cilia is maintained in effective, metachronous state (30).

Goblet cells make up 15-25% of the surface epithelium and contain large granules of varying electron density (31). The most important function of goblet cells is the production and secretion of airway mucus, in which inhaled particles, including viruses and bacteria can be trapped. Increased numbers of goblet cells have been found in patients with chronic bronchitis and after chronic exposure to cigarette smoke (32,33).

Clara cells contain membrane-bound, electron-dense secretory granules and produce the surfactant apoproteins A and B (34,35) and antileukoprotease (36). In addition they may participate in the clearance of noxious agents via the detoxification of inhaled substances (37).

Basal cells are flattened, pyrimidal-shaped cells with a small cytoplasmic/nuclear ratio (30). They anchor to the basement membrane by hemidesmosomes (38). In addition basal cells play a role in the attachment of the columnar cells to the basement membrane (38). The basal cell is considered as the stem cell of the bronchial epithelium (39).

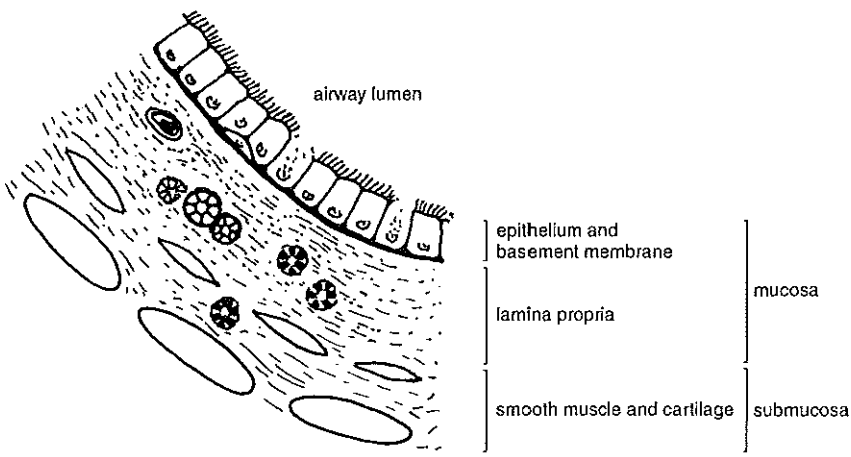

Figure 2. Schematic diagram of a bronchus.

1.2.2 Host defence

The bronchial epithelium has several functions, which act to protect the airspaces from airborn particles and preserve the normal respiratory function. These functions can be divided in three categories: 1) the integrity of the epithelium that contributes to the physical barrier function; 2) the secretion and ciliary function leading to effective mucociliary clearance; and 3) secretion of mediators, produced by the bronchial epithelial cells or by other cell types. Integration of all these functions is necessary to maintain a healthy epithelium.

Barrier function

The epithelial layer serves as a protective barrier to the underlying tissue. The epithelial integrity is maintained by adhesive mechanisms which are shown in Figure 3 (40). The desmosome (macula adherens) and the intermediate junction (zonula adherens) are the most effective adhesive mechanisms for maintaining cell-to-cell adhesion. The tight junctions (zonula occludens) provide a physical barrier function. The basal cells anchor to the basement membrane through hemidesmosomes.

The luminal cell membrane forms an impermeable barrier to macromolecules and infectious agents. Moreover, junctional complexes greatly limit the leakage of water and solutes into the airways. Epithelial cell products can be transported either to the luminal surface to interact with environmental agents or basally to affect neighbouring or distant cells. Exclusion of inhaled particulates and molecules from the basal surface protects the cells underlying the epithelium, which are sensitive to environmental agents. Exposure of cells of the airway mucosa, of mast cells and lymphocytes to environmental agents may result in deleterious effects upon airway function (41,42). Additionally, the bronchial epithelium provides an effective barrier against invasion by microbes (43). Attachment of microbes to the epithelium is believed to be the initiating event for infections of the lower respiratory tract. Injury of the airway epithelium by infection with viruses, particularly influenza virus, permits bacterial attachment, and is associated with greatly increased incidence of pneumonia (44,45).

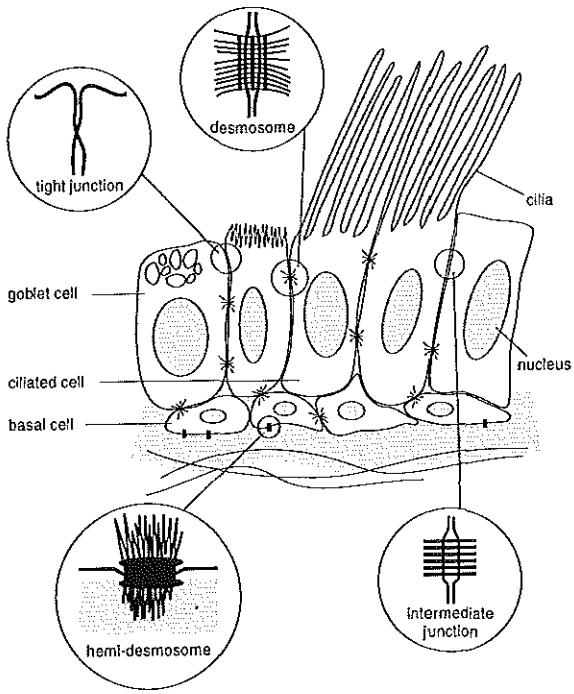

Figure 3. Schematic representation of the adhesion of the bronchial epithelium. Adapted from reference 40.

Mucociliary clearance

Inhaled particles, including bacteria and viruses, are cleared from the airways by trapping of the particle in mucus and clearance of the mucus by the co-ordinated beating of cilia. Surfactant, produced by Type II epithelial cells and Clara cells, assists in the clearance of particles by coughing and by changing the surface charge properties, making foreign particles "less sticky" and thus more easily cleared. The regulation of ciliary motility and mucociliary clearance is complex and not completely understood. However, it is clear that the cells and mediators of the immune system play an important role in altering and regulating mucociliary function.

Macrophages have the capacity to synthesize cytokines such as IL-1β and tumor necrosis factor-α (TNF-α), which appear to upregulate ciliary beat frequency. This upregulation has been related to the release of nitric oxide (NO) in a manner that requires induction of the inducible form of NO synthase (iNOS) and is blocked by glucocorticoids (46). In contrast, macrophages and activated polymorphonuclear cells can also produce cilia damaging proteases and oxidants, including hydrogen peroxide and superoxide (47,48). Activated

lymphocytes, which are a rich source of cytokines such as interferon (IFN)-γ, can upregulate ciliary motility as well by inducing iNOS in epithelial cells (49). Neural and hormonal mediators, such as β-agonists (50-52), bradykinin (53-55) and substance P (56,57) are known to upregulate ciliary beat frequency and affect NO synthesis. MBP, which is released from activated eosinophils, can impair ciliary motility (58). Histamine and leukotrienes, inflammatory mediators released from basophils, mast cells and platelets, have no effect on ciliary motility *in vitro*, but histamine has been shown to increase tracheal mucus velocity in human volunteers (59-61). Thus, the cells and mediators of the immune system can both impair and enhance mucociliary function. The complex regulation of these cells and their mediators is required to maintain this vital host defence function.

Epithelial secretion of mediators

In addition to the secretion of mucus, the bronchial epithelium secretes a number of mediators that provide protection against a wide variety of potentially injurious agents, including infection, reactive oxygen species and proteases. A number of substances with antibacterial activity, such as lysozyme and lactoferrin, are secreted by the epithelium (62-64). Other factors produced are the components of the complement system, which act as opsonins, allowing efficient phagocytosis by macrophages (65). The bronchial epithelium is constantly exposed to an oxidant-rich environment. Airway epithelial cells were found to have both intracellular and extracellular antioxidant activities. Three major intracellular antioxidant systems, the glutathione redox cycle, superoxide dismutase and catalase, are shared between airway epithelial cells and other lung cells (66). Pulmonary inflammatory cells are rich sources of proteases, which potentially contribute to the hypersecretion state which characterizes chronic airway inflammation (67). The bronchial epithelium is protected from the effects of these proteases by the presence of an excess of antiproteases.

The bronchial epithelium also secretes a large number of inflammatory mediators (including several arachidonic acid metabolites, cytokines and chemokines) and relaxant and contractile factors. The role of these mediators will be discussed further on.

1.2.3 Loss of function

Histopathological studies of asthmatic airways show epithelial damage and shedding, and infiltration of eosinophils and lymphocytes (9,68-70). In these studies an association between the eosinophils and the epithelial disruption has been found (9,69). The eosinophils are in their activated state and release cationic proteins such as MBP and ECP, and oxygen species which may directly damage airway epithelial cells (8-10). Large numbers of epithelial cells are also

found in asthmatic sputum during acute exacerbations and in bronchoalveolar lavage (BAL) fluid from asthmatics (71-73). Loss of airway epithelial cells may correlate with the degree of airway responsiveness in asthma (74). A significant negative correlation between epithelial cell number and bronchial hyperreactivity was seen in two bronchial biopsy studies, but not in others (71,75-77). Similarly, a significant correlation between epithelial cell numbers in BAL fluid and bronchial hyperreactivity has been demonstrated (71,73). However, in a recent study it was shown that the amount of abnormal epithelium was similar in fatal asthma, nonfatal asthma and nonasthma cases at post mortem (78). Furthermore, epithelial repair processes have received only little attention in asthma. Recently, it has become clear that the epithelial damage sites are dynamic structures where epithelial repair (dedifferentiation, migration and proliferation) is ongoing (79). How epithelial damage may participate in increased airway responsiveness is still not clear. Several mechanisms have been suggested:

Loss of a permeability barrier

Disruption of the permeability barrier between the external and the internal milieu will have several important consequences. Firstly, it will enable bacteria, viruses and toxic substances to directly penetrate the airway wall (40). Secondly, transport of mucus will be impaired due to loss of the cilial transport system (40). Thirdly, loss of bronchial epithelial cells may expose neuropeptide containing neurones to environmental stimuli. This leads to an axon reflex and liberation of neuropeptides (including substance P, neurokine A and B), which in turn can contract smooth muscle and can stimulate mast cells (80,81). Fourthly, permeability to antigen will be increased and antigens will be able to reach parenchymal inflammatory cells more readily (82).

Loss of metabolic activity

The bronchial epithelium normally expresses several enzymes, including neutral endopeptidase (NEP) and angiotensine-converting enzyme (ACE) which are involved in the metabolism and catabolism of active peptides (83). A number of peptides such as substance P and neurokinin A have potent contractile effects on airway smooth muscle (84). Loss of NEP has been suggested to contribute to increased airway reactivity in response to substance P (85,86). ACE may not be important in degrading neuropeptides since the enzyme inhibitor captopril does not affect tachykinin-induced responses in man (87).

Reduced epithelial-derived relaxing factors

There are now several studies in different species which show that the presence of bronchial epithelium modulates the contractile and relaxant effects of a number of drugs on the underlying smooth muscle (88-90). It is proposed

that the bronchial epithelium generates both relaxant and contractile factors that are lost when the epithelium is removed. Prostaglandin E_2 (PGE_2), for example, is a product of bronchial epithelial cells and has been suggested to mediate smooth muscle relaxation induced by airway epithelium (91-94). NO, has been suggested as an additional epithelial-derived smooth muscle relaxing factor and is also produced by bronchial epithelial cells (95). After the discovery of a vascular smooth muscle relaxing factor released by endothelial cells (endothelium derived relaxing factor, EDRF), many investigations were performed in order to identify a similar factor released by airway epithelium (epithelium derived relaxing factor, EpDRF). Until today, the existence and the putative role of EpDRF remains controversial.

1.2.4 Pro-inflammatory potential

Airway inflammation is associated with excessive production and activity of several mediators and cytokines released by inflammatory and resident cells in the airways. Now it is clear that the epithelium is not only an important target for the action of mediators and inflammation, but also an active participant in the inflammatory process. Bronchial epithelial cells are able to recruit inflammatory cells to the airways through the release of chemoattractants, to direct inflammatory cell migration across the epithelium through the expression of cell adhesion molecules, and to regulate the inflammatory activity of other cells through the release of mediators, like cytokines, chemokines, arachidonic acid metabolites and relaxant and contractile factors.

Recruitment of inflammatory cells

Chemotactic substances are released by airway epithelial cells *in vitro* in response to a number of stimuli. These stimuli include environmentally-derived factors such as cigarette smoke extract, endotoxin, organic dust extract, viruses, stimuli derived from cells intrinsic to the lungs including cytokines, substance P, neutrophil proteases, acetylcholine, and experimentally important stimuli such as calcium ionophore, phorbol myristate acetate, and opsonized zymosan (16,96-99). Within the first few hours after smoke or lipopolysaccharide (LPS) stimulation bronchial epithelial cells release arachidonic acid metabolites, such as LTB_4 and hydroxyeicosatetraenoic acids (HETE) with neutrophil, monocyte and eosinophil chemotactic activity (16,100-104). Subsequent to the initial response of the epithelial cells to a provocative stimulus, macrophage stimulation of the epithelial cells by TNF-α or IL-1β induces chemokine expression. Chemokines released by epithelial cells are thought to perpetuate the inflammatory response (30).

Chemokines are a group of chemotactic and pro-inflammatory cytokines. They represent a family of polypeptides with four conserved cysteine residues which can be subdivided into two large groups depending on whether or not

there is an intervening amino acid between the two cysteines, yielding the C-X-C and the C-C families. Chemokines of the C-X-C type include IL-8, neutrophil-activating peptide-2 (NAP-2), neutrophil-activating protein from epithelial cells (ENA-78), GROα,β and γ, macrophage inflammatory protein-2 (MIP-2) and platelet factor-4 (PF-4) and are predominantly chemotactic *in vitro* for neutrophils. Chemokines of the C-C type preferentially attract monocytes and include RANTES, MCP-1, macrophage inflammatory protein-1α and 1β (MIP-1α,β).

There is abundant evidence that bronchial epithelial cells can be induced to release IL-8, one of the most potent chemotactic factors for neutrophils (99,105,106). ENA-78, another member of the C-X-C branch has been identified in the conditioned medium of a human pulmonary epithelial cell line (A549) stimulated with TNF-α or IL-1β (107). Constitutive and stimulated expression of GR0α and GROγ, which attract neutrophils, has been found in short term cultured human bronchial epithelial cells (108). RANTES is a chemokine which is chemotactic for monocytes, eosinophils, basophils and T cells (109). RANTES has been identified as a product of cultures of bronchial epithelial cells (110,17) and A549 cells (111). Following stimulation with IL-1β, TNF-α or INF-γ, RANTES mRNA expression and protein production were increased (111,17). Both levels of RANTES mRNA and protein were reduced by IL-4 (17).

Expression of MCP-1, a chemoattractant for monocytes, basophils, eosinophils and subsets of lymphocytes, has been observed in airway epithelial cells (108). Furthermore, increased expression of MCP-1 has been found in bronchial tissue from asthmatic subjects (14).

Airway epithelial cells have also been shown to release PAF, a potent eosinophil chemoattractant (112,113) and MIP-2, a potent chemoattractant for neutrophils, after stimulation with TNF-α (114).

Cell-cell adhesion and interaction

A number of recent investigations have demonstrated the importance of intercellular adhesion molecule-1 (ICAM-1) expression by bronchial epithelial cells. ICAM-1 is the ligand for the CD11/CD18 leukocyte integrins expressed on the surface of neutrophils, monocytes, lymphocytes and eosinophils (115,116). During inflammation (or *in vitro* exposure to cytokines, such as TNF-α, IFN-γ or IL-1β) ICAM-1 expression on epithelial cells is enhanced and this will lead to the adhesion of leukocytes, followed by the maturation and activation of these cells in the epithelial compartment (117). Recent studies in primates provide strong evidence for the role of ICAM-1 in the adhesion of eosinophils and the maintenance of airway eosinophilia in association with asthma (118,119). Intravenous administration of anti-ICAM-1 antibodies attenuated both airway eosinophilia as well as bronchial hyperresponsiveness in these studies (118,119).

Bronchial epithelial cells are capable of expressing major histocompatibility complex (MHC) class II antigens (120,121). Expression of MHC class II antigens allows cells to present antigen to helper T cells. In normal airway epithelium the expression of MHC class II antigens (human leukocyte antigen (HLA)-DR, DQ and DP) is relatively low, but in cultured bronchial epithelial cells MHC class II expression can be stimulated by the addition of mast cell derived histamine or IFN-γ (122-124). Increased expresssion of HLA-DR has been reported on epithelial cells recovered from the lower respiratory tract of asthmatics compared with healthy individuals (120,121). Although the expression of HLA-DR is often associated with the presentation of antigen to lymphocytes, it has yet to be clearly demonstrated whether human bronchial epithelial cells are capable of this function.

Human bronchial epithelial cells express the $\alpha_{2\text{-}6}$ integrins, both *in vivo* and *in vitro* (125). These function as cell-surface receptors that bind to extracellular matrix components collagen, fibronectin and laminin and are thought to play an important role in: (1) the adherence of epithelial cells to the basement membrane and the underlying tissue; and (2) localization of leukocytes at sites of inflammation.

Modulation of inflammatory cell activity

The modulation of cellular responses by bronchial epithelial cells is thought to be an important aspect of the regulation of inflammatory responses in the lung (Table 1)(74). Epithelial cells may release several mediators and cytokines with pro- and anti-inflammatory effects and thus may produce both the critical pro-inflammatory or anti-inflammatory activities that control chronic airway inflammation (126).

Bronchial epithelial cells cultured from several species (including human) can generate numerous arachidonic acid mediators: LTB_4, PGE_2, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and 15-HETE. These mediators can be either bronchoconstrictive, pro-inflammatory (involvement in chemotaxis or vasodilation), or in some cases both. Agents such as irritant gases, which are thought to be one of the external triggers of asthma, are capable of inducing epithelial cells to release bronchoconstrictor and inflammatory mediators. For example, ozone and NO_2 augment PGE_2, $PGF_{2\alpha}$, LTB_4 and 15-HETE in human bronchial epithelium (127,128). Exposure to either of these irritant gases produces bronchial hyperreactivity. Thus, there may be a causative link between irritant gas exposure, release of mediators from the epithelium and airway hyperreactivity. In addition to eicosanoids, bronchial epithelial cells can also produce endothelin. Endothelin is a potent bronchoconstrictor and can promote airway smooth muscle proliferation. In asthma its expression is increased compared with controls (129). Another mediator released by bronchial epithelial cells is NO, which is a smooth muscle relaxing factor and has been demonstrated to modulate inflammatory cell chemotaxis (30,95).

Table 1 . Mediators expressed by the bronchial epithelium during inflammation

Mediators	Possible consequence	References
Lipid mediators:		
PGE_2, $PGF_{2\alpha}$, LTB_4, 15-HETE	Bronchospasm, mucus secretion, microvascular leakage, chemotaxis	16,91,101, 127,128
PGE_2, 15-HETE	Vasodilation, bronchospasm	16,91,127,128
Cytokines/chemokines:		
IL-1	Augment inflammation	135,136
IL-6	Augment/reduce inflammation	105,135,138
IL-8	Neutrophil recruitment	103,105,106
TNF-α	Augment inflammation	139
GM-CSF	Prolong eosinophil/neutrophil survival Increase eosinophil activation	15,131,132
MCP-1	Monocyte/eosinophil recruitment	108
RANTES	Eosinophil, monocyte, basophil, T cell recruitment	17,110,111
GROα	Neutrophil recruitment	108
GROγ	Neutrophil recruitment	108
PAF	Eosinophil recruitment	113
MIP-2	Neutrophil recruitment	114
TGF-β	Reduce inflammation	140,141
Other mediators:		
NO	Modulation chemotaxis, bronchodilation	30,95
Endothelin	Bronchospasm, smooth muscle proliferation	129

Airway epithelial cells and epithelial cell lines *in vitro* synthesize and secrete several pro-inflammatory cytokines. GM-CSF is produced by the bronchial epithelium under basal conditions (15,130-134). Its production can be enhanced by treatment with IL-1β (131,134). Bronchial epithelial cells from asthmatics produce more GM-CSF than controls (15,132). Epithelium derived GM-CSF may have an important role in inflammation *in vivo*, since, in cell culture, it prolongs neutrophil and eosinophil survival with concomitant cell activation (131-133). IL-1 production by bronchial epithelial cells was detectable after exposure to toluene diisocyanate (TDI) (135,136). Air exposure did not stimulate IL-1 production. It is therefore unlikely that it is released basally (136). IL-6 has been demonstrated in human bronchial epithelium either unstimulated, or after exposure to histamine, TDI, transforming growth factor-β (TGF-β), IL-1β or TNF-α (15,136-138). IL-6 and IL-8 production, along with GM-CSF, appears to be upregulated in bronchial epithelial cells from symptomatic asthmatics (15). The release of IL-8, GM-CSF and TNF-α is augmented by NO_2 treatment (139).

In addition to recruiting and stimulating inflammatory cells, epithelial cells may participate in the downregulation of inflammatory cells. TGF-β is present

in the epithelial lining fluid of the lung, and is present in the epithelium of injured lung (140,141). TGF-β has anti-inflammatory properties, such as the inhibition of IL-2-dependent proliferation of T cells and inhibition of cytokine production by macrophages (142,143). Other mediators with anti-inflammatory properties produced by bronchial epithelial cells include PGE_2 and IL-6. PGE_2 is capable of reducing the production of neutrophil chemoattractants by macrophages (144). IL-6 has been found to reduce inflammation in several models, including an *in vivo* model of pulmonary inflammation (145). In this model endotoxin and cytokines were injected into the trachea and IL-6 was observed to inhibit acute inflammation.

1.3 Glucocorticoids

Glucocorticoids are powerful agents in the treatment of several inflammatory diseases and are by far the most effective anti-inflammatory drugs used in asthma. The precise molecular interactions underlying their immunosuppressive effects are not yet clear (20). Recent studies have increased our understanding of the mechanisms of actions of glucocorticoids and this will lead to improved and more specific anti-inflammatory therapies in the future (146,147).

1.3.1 Glucocorticoid receptor

Glucocorticoids exert their effects by binding to a specific cytoplasmic receptor molecule. Glucocorticoid receptors (GR) are expressed almost in all cell types, although GR density may vary between different cell types (146). In recent years, a number of steroid receptors, including the GR, has been cloned, and their amino acid sequences have been determined (148-150). Certain areas of sequence homology were observed in these receptors and a family of receptor molecules was recognized to which not only the steroid receptors belonged, but also nuclear receptors for ligands like thyroid hormone and retinoic acid (151). GR from human and other species were found to consist of approximately 800 amino acid residues. Alternative splicing of the human GR pre-mRNA generated two highly homologous isoforms, termed human GRα (hGRα) and hGRβ (152,153). hGRβ differs from hGRα only in its COOH terminus with replacement of the last 50 amino acids of the latter with a unique 15 amino acid sequence. This difference renders hGRβ unable to bind glucocorticoids.

All members of the nuclear hormone receptor family share a characteristic three-domain structure, which was first described for the hGR (Figure 4) (154). The most conserved central domain contains many basic amino acids and a number of cysteines on strongly conserved positions. This domain is involved in direct binding of the receptor to DNA (155). This DNA-binding domain

contains two distinct loops of protein, each bound at their base via four cysteine residues to a single zinc ion, the so called "zinc clusters". The first, N-terminally located "zinc cluster" is believed to interact with the major groove of the DNA double helix (156). The second "zinc cluster" plays a role in the dimerization of two GR molecules (156,157). The DNA-binding domain is also known to have a transcription-activation function (148,158). The C-terminal steroid-binding domain is equal in size in all nuclear receptors studied, i.e. about 250 amino acids (151). It also contains the binding sites for the heat shock proteins (hsp) 90 (see below) (159,160). Removal of the steroid binding domain results in a constitutively active GR molecule, indicating that this part of the molecule acts as a repressor of the transcription-activation function. The size of the N-terminal domain is extremely variable (24-600 amino acids) in the nuclear receptors. The precise role of this third domain is still uncertain, but it is required in transcriptional activation (147). The region between the DNA binding and steroid binding domain has been termed the "hinge region", because of supposed flexibility in determining the three-dimensional structure (Figure 4). The hinge region contains sequences that are important for nuclear translocation and dimerization (159,160). The GR is phosphorylated (predominantly on serine residues at the N-terminal end), but the role of phosphorylation in steroid actions is not yet certain (161).

The expression of GR may be regulated by numerous factors either at transcriptional, translational or post-translational level (162). After glucocorticoid therapy, a downregulation of GR was observed, but the importance of this aspect in chronic glucocorticoid therapy is still unclear (163). Also *in vitro* a marked reduction of GR was found after exposure to glucocorticoids, although it is not certain whether this persists after prolonged treatment (164). Recent studies showed that the expression of antisense GR mRNA in transgenic mice resulted in lower levels of GR (165). Halving GR expression caused various abnormalities, including an increase in circulating cortisol and an increase in body fat (165).

Numerous conditions and agents in addition to glucocorticoids alter GR levels. Levels vary through the cell cycle, doubling in HeLa cells as they pass through late G_1 (166). Lymphocytes showed an increase in GR binding after stimulation with mitogens, which is probably a consequence of entry of cells into cycle (167,168). Inflammatory mediators may regulate both GR levels and affinity (169-172). Stimulation of normal T cells with IL-2 and IL-4 resulted in a significant increase in GR levels, but in a reduction of GR binding affinity for glucocorticoids (169). Glucocorticoid binding was increased by IL-1β, TNF-α and IL-6 in a B-cell line (CESS), in a monocyte-like tumor cell line (U937), and in a hepatoma cell line (HepG2) (170). IFN-γ has been shown to mediate increased GR expression in murine macrophages (171). However, until today it is not clear whether, under physiological conditions, there is an association between GR levels and the magnitude of the cellular response following administration of

glucocorticoids. A number of studies demonstrate a direct correlation between the concentration of GR in a cell and the cell's sensitivity to glucocorticoids (173,174). If cellular responsiveness is determined by receptor concentration, then the downregulation of receptor by its ligand may well be physiologically important.

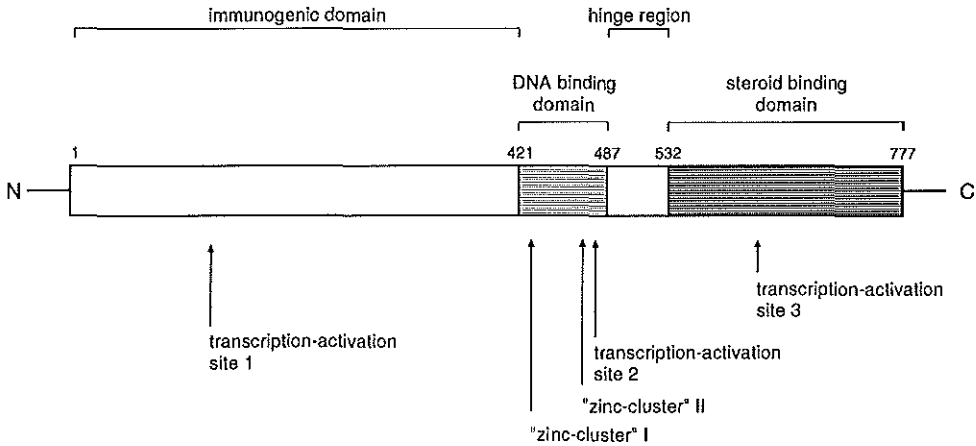

Figure 4. Linear alignment of the human glucocorticoid receptor. N and C represent the N-terminal and C-terminal end of the protein, respectively (154).

1.3.2 Mechanisms of gene transcription regulation

Glucocorticoids exert their effects on responsive cells by activating GR to regulate the transcription of certain target genes (175) (Figure 5). Due to their hydrophobic character, glucocorticoids can passively diffuse through the cell membrane. In the cytoplasm they bind to their receptors (148,176,177). The unliganded GR exists in the cytoplasm as a hetero-oligomer and interacts with two molecules of hsp 90 and with one molecule of hsp 70 (178,179). Hsp 56 presumably does not interact with the receptor itself, but interacts with and regulates the function of hsp 90 (152,180,181). Upon binding of glucocorticoids to their receptors, the receptor molecule undergoes some conformational changes, and becomes an activated complex, characterized by a high affinity for specific DNA sequences in the nucleus. This activation is accompanied by the dissociation of two types of heat shock proteins (hsp 90 and hsp 56) from the receptor (Figure 5). It is thought that the dissociation of hsp 70 takes place not in the cytoplasm, but at a later stage in the nucleus (152,182). Upon activation the receptor-glucocorticoid complex passes the nuclear membrane and enters the nucleus. Within the nucleus, the hormone-

activated GR can act in two ways, here referred to as type 1 and type 2 mechanism of action. The type 1 mechanism is characterized by the GR interacting with specific DNA sequences, whereas the type 2 mechanism involves interaction of the GR with other transcription factors in the absence of specific DNA binding.

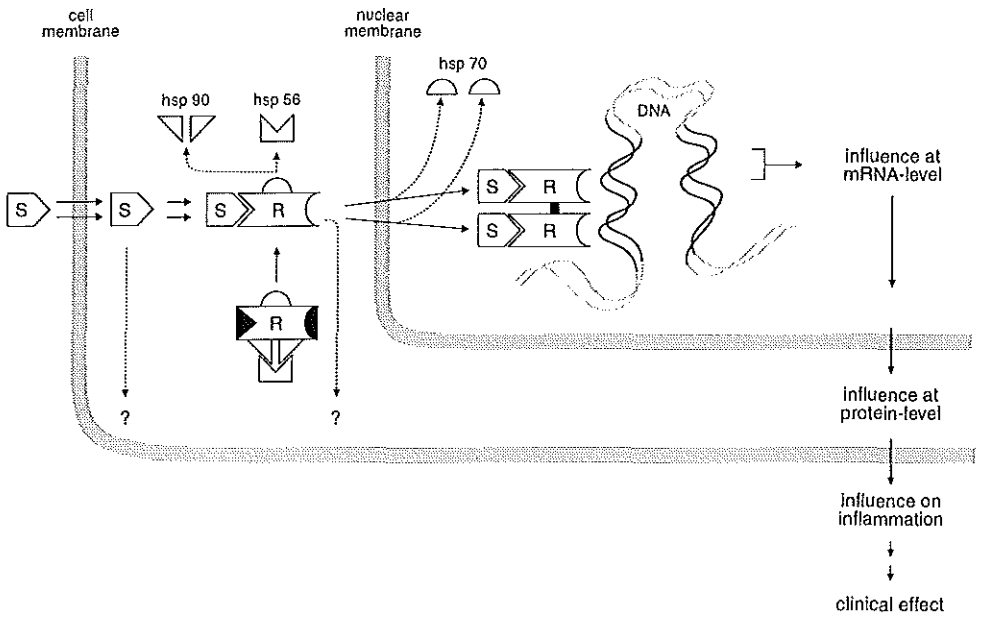

Figure 5. Hypothetical cellular events after glucocorticoid administration. Glucocorticoids (S) pass the cell membrane and bind to their receptors (R). The binding of glucocorticoids to their receptors is accompanied by the dissociation of some heat shock proteins (hsp 56 and hsp 90) from the receptor in the cytoplasm. Others (hsp 70) probably from the receptor in the nucleus. Furthermore, in the nucleus liganded glucocorticoid receptors form homodimers and interact with specific DNA sequences. Eventually, this interaction results in modulation of transcription (154).

The type 1 mechanism represents the classic model of GR action. In this model a receptor homodimer binds to a specific DNA sequence termed glucocorticoid responsive element (GRE) in the promoter region of steroid responsive target genes (Figure 5) (183). GR homodimer interacts with components of the basic transcription machinery, either directly, i.e. by physical contact between the GR transcription activation domains and basic transcription factors such as TFIIB, or indirectly, i.e. via "bridging" factors (184-186), such as the recently identified steroid receptor coactivator 1 (SRC-1) (187). This changes the rate of transcription, resulting in either induction, via a *positive* GRE, or repression, via a *negative* GRE, of the gene. The consensus sequence for GRE binding is the palindromic 15-base pair sequence GGTACAnnnTGTTCT, although for repression of transcription, the negative GRE has a more variable sequence

(ATYACnnTNTGATCn) (188). The rate of transcriptional regulation of a target gene by glucocorticoids is dependent on both the quality and quantity of GRE linked to the target gene and their position relative to the transcriptional start site (20). Thus an increased number of GRE, a high affinity binding of the hormone complex to the GRE and proximity to the TATA box increases steroid inducibility of a gene. Binding of the hormone-GR complex to the GRE, changes the sensitivity for DNase I of certain DNA sequences downstream of the DNA containing the TATA box (189-192). These findings indicate that there may be a change in the configuration of DNA or chromatin and this may expose previously masked areas, resulting in increased binding of other transcription factors (193).

Many effects of glucocorticoids are achieved by inhibition rather than by activation of target genes. This is especially true for the anti-inflammatory effects of glucocorticoids that involve the negative transcriptional regulation of immune genes, such as the collagenase gene and the IL-2 genes. Surprisingly, the promoters of these genes do not contain negative GRE sequences or any other GR binding sites, yet they are repressed by glucocorticoids (194,195). It has now been established that these genes are regulated by a different mechanism of GR action (194,196,197), referred to as the type 2 mechanism. The above mentioned genes are positively regulated by activating protein-1 (AP-1), a transcription factor composed of a dimer of two proto-oncogenes (c-jun and c-fos) (198,199). The liganded GR was shown to interact with AP-1 (147,200), modulating the effect of AP-1 on gene transcription. For example, in human lung and in peripheral blood monocytes, TNF-α and phorbol esters increase AP-1 binding to DNA and this is inhibited by glucocorticoids (201). It has recently been demonstrated that the GR and AP-1 can reciprocally repress one another's transcriptional activations by mechanisms involving direct protein-protein interactions that are independent of DNA binding (194,196,197). The interaction of the GR with the AP-1 molecule, which is strictly dependent on the presence of hormone (202), abolishes its transcription activity by preventing its interaction with the GRE.

AP-1 is not the only transcription factor that can be modulated by the liganded GR. A similar pattern of GR mediated transrepression has been reported for NF-κB, a heterodimer composed of structurally related DNA binding subunits, p50 and p65 (RelA) (203,204). NF-κB is also an activator of many immune system genes (203). NF-κB antagonizes GR action on GRE regulated promoters, probably through direct physical interaction (204). Furthermore, NF-κB is inhibited by GR in stimulating NF-κB responsive genes, such as the IκB inhibitory protein, which traps NF-κB in inactive cytoplasmic complexes (205-207). A 'functional antagonism' between the transcription factor NF-κB and the glucocorticoid receptor has been found in the regulation of the expression of the IL-6 gene (204). This antagonism resulted from physical association between the two proteins.

An interaction between the transcription factor CREB (cAMP-responsive element binding protein) and the GR has also been suggested. CREB, that binds to a cAMP-responsive element (CRE) on genes is thought to be activated by β-agonists via cyclic AMP formation and activaton of protein kinase A (207). β-Agonists increase CRE binding in human lung cells *in vitro* and at the same time reduce GRE binding. This suggests that a protein-protein interaction takes place between CREB and the GR within the nucleus (208). Such an interaction has been demonstrated recently in epithelial cells, causing a reduced binding of GR to GRE (209).

In addition to direct and indirect effects on transcription, glucocorticoids can also affect post-transcriptional events. They can alter the stability and hence steady-state levels of specific mRNA via enhanced transcription of specific ribonucleases that breakdown mRNA containing constitutive AU-rich sequences in the untranslated 3'-region, thus shortening the turnover time of mRNA (210). This is observed in the case of the cytokine IL-1β. Dexamethasone causes a reduction in IL-1β mRNA half-life, a moderate inhibition of translation of the IL-1β precursor and a profound inhibition of the release of IL-1β into the extracellular fluid (211,212).

1.3.3 Glucocorticoid regulated proteins

By influencing the transcription of several genes, glucocorticoids are able to modulate the expression a variety of mediators.

Phospholipase A_2 inhibitory proteins

Glucocorticoids inhibit the activity of phospholipase A_2 (PLA_2), the rate-limiting enzyme in the mobilization of arachidonic acid from membrane phospholipids (212). Thus glucocorticoids ultimately inhibit the formation of prostaglandins, thromboxane, leukotrienes and PAF (Figure 6). These steroids are thought to inhibit PLA_2 indirectly by the release of inhibitory proteins identified as lipocortins. Lipocortins are members of a superfamily of proteins characterized by their ability to bind calcium and anionic phospholipids, now known as the 'annexins' (213). Recent data show that lipocortin-mediated inhibition of PLA_2 is most probably due to Ca^{2+}-dependent binding of lipocortins to phospholipids, the substrate of PLA_2 (214). On the basis of *in vitro* experiments, regulation of the expression and activity of lipocortins has been postulated to be one of the important mechanisms by which glucocorticoids exert their anti-inflammatory actions *in vivo*. It has been demonstrated that neutralizing anti-lipocortin antibodies reverse anti-inflammatory and anti-pyretic actions of glucocorticoids *in vivo* (215). Autoantibodies against lipocortins have been found in some patients with rheumatic disease (216). A marked decrease in the number of lipocortin I binding sites has been observed in some patients suffering from autoimmune disease (215). An

elevated production of lipocortin I in response to glucocorticoids has been demonstrated in human peripheral blood monocytes, human alveolar macrophages, rat alveolar epithelial cells and in bovine bronchial epithelial cells (217-220). Whilst the lipocortin I gene does contain several potential GRE suggesting that its expression could be under the control of glucocorticoids, the evidence for a relationship between glucocorticoid administration and inducibility of lipocortin I synthesis is not present in all cell types (212). Others did not observe an induction of lipocortin I by dexamethasone in seven different cell types, including primary human macrophages (221). Furthermore, the synthesis of lipocortin I does not appear to be under glucocorticoid control in certain cell lines (222-223).

Another important mechanism by which glucocorticoids exert their anti-inflammatory actions, is the direct inhibitory effect on the transcription of enzymes involved in the formation of eicosanoids. Glucocorticoids inhibit the gene transcription of a cytosolic form of PLA_2 induced by cytokines and inhibit the gene expression of cytokine-induced cyclooxygenase 2 in monocytes (224,225). It has not been established yet whether glucocorticoids are also able to modulate the expression of 5-lipoxygenase.

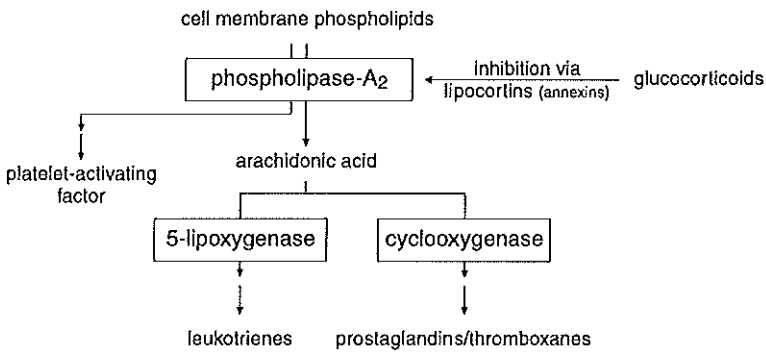

Figure 6. Interaction site of glucocorticoids.

Cytokines

Glucocorticoids inhibit the production of many cytokines thought to be important in chronic inflammation. These include IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, TNF-α and GM-CSF (226). Glucocorticoid inhibition of these, in general, pro-inflammatory cytokines may be mediated at several sites. Firstly, at the level of gene transcription directly, or as a result of direct protein-protein interaction, such as the 'functional antagonism' between the GR and additional transcription factors such as AP-1, and NF-κB (212). One mechanism of gene

transcription repression is mediated via interaction of GR with negative GRE. The IL-6 gene contains at least four negative GRE close to the promoter site and one close to the transcription start site (227). Several cytokines exert their cellular effects by inducing the production of transcription factors, which activate or repress target genes that are regulated in an opposing manner by GR. For example, TNF-α activates both AP-1 and NF-κB in human lung and this activation is opposed by glucocorticoids (201). Stimulation of T cells, which leads to the induction of IL-2 and IL-2 receptor, is partly mediated by AP-1 (228). This activation is also strongly inhibited by glucocorticoids. Alternatively, glucocorticoids may inhibit the production of cytokines by acting upon post-transcriptional events through increased breakdown of cytokine mRNA (229).

Adhesion molecules

Glucocorticoids inhibit leukocyte adhesion to endothelial cells via inhibition of the expression of the adhesion molecules endothelial leukocyte adhesion molecule-1 (ELAM-1) and ICAM-1 (230). The expression of these adhesion molecules is induced by cytokines, and glucocorticoids may lead indirectly to a reduced expression via their inhibitory effects on cytokines such as IL-1β and TNF-α. Glucocorticoids may also have a direct inhibitory effect on the expression of these adhesion molecules. The ELAM-1 gene lacks a GRE, but contains an AP-1 site. This site does not seem to participate in the regulation of the transcription of ELAM-1, however (231). ELAM induction is influenced predominantly by the transcription factor NF-κB (200). The mechanism of glucocorticoid mediated repression of ICAM-1 is still unclear. Protein-protein interaction similar to that found with the AP-1 dimer may be involved, but this remains to be established.

1.3.4 Modulation of bronchial epithelial functions

It has now become clear that inflammatory airway diseases such as asthma generally respond well to anti-inflammatory drugs. However, the most important target cells, responsible for the glucocorticoid- induced improvement in clinical parameters are not known yet. Most attention has been paid to the anti-inflammatory effects of glucocorticoids on mast cells, eosinophils, T lymphocytes and macrophages (132). Recent studies have now also suggested the involvement of bronchial epithelial cells in the anti-inflammatory actions of glucocorticoids (74,232).

In cultured human bronchial epithelial cells, GM-CSF release (basal and IL-1-induced) was inhibited by hydrocortisone, dexamethasone and budesonide (130,131). GM-CSF mRNA was also reduced, suggesting inhibition of transcription (130). In short term cultured bronchial epithelial cells obtained from asthmatic patients, hydrocortisone reduced GM-CSF, IL-6 and IL-8 release (134). Glucocorticoid treatment reversed epithelial cell-dependent increases in

the survival and activation of eosinophils, but not of neutrophils (133). Dexamethasone decreased TNF-α-induced RANTES mRNA and RANTES production in two human airway epithelial cell lines (17,110,111). Although it is not possible to relate these *in vitro* findings directly to airway inflammation, such as in asthma, it may be expected that the anti-inflammatory effects of glucocorticoids result, at least in part, from inhibition of inflammatory actions of epithelial cell-derived cytokines and chemokines. This may be especially relevant to GM-CSF and RANTES, as reduction of their production could lead to reduced eosinophil recruitment, activity and survival.

Dexamethasone exposure of the bronchial epithelial cell line NCI-H292 reduced protein and mRNA expression of ICAM-1, suggesting inhibition of transcription (233). This observation was not supported *in vivo*, as beclomethasone had no effect on the expression of ICAM-1 on bronchial epithelium from asthmatic patients (234). These results suggest that the anti-inflammatory effects of glucocorticoids do not depend upon a reduction in adhesion molecule expression in the bronchial epithelium.

Dexamethasone has been shown to increase NEP in airway epithelial cells (87,235), which is involved in the degradation of inflammatory peptides. Increased expression of NEP by glucocorticoids may therefore reduce neurogenic inflammation.

Inhaled glucocorticoids have been shown to decrease iNOS in the human bronchial epithelium, resulting in decreased NO production (236). NO may increase blood flow and plasma exudation at inflammatory sites. There is evidence that iNOS is expressed in airway epithelial cells in asthmatics but not in normal individuals (237). It has been hypothesized that the induction of iNOS in airway epithelial cells can be linked with the characteristic eosinophilic inflammation in asthma (238). Decreased expression of iNOS in epithelial cells by glucocorticoids may therefore be of importance in anti-inflammatory therapy in asthma.

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CHAPTER 2

AIM OF THE STUDY AND INTRODUCTION TO THE EXPERIMENTAL WORK

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The aim of the studies described in this thesis was to identify and characterize specific GR in human bronchial epithelial cells and to study the potential cellular mechanisms of anti-inflammatory actions of glucocorticoids in these cells.

In all types of leukocytes, the role of glucocorticoids during airway inflammation has been investigated extensively. Only recently, the anti-inflammatory effects of glucocorticoids in bronchial epithelial cells have become of interest, as these cells are now known to play an active role in airway inflammation. Since inhaled glucocorticoids mainly precipitate in the larger airways (1), we investigated the expression of GR in bronchial epithelial cells and studied the anti-inflammatory actions of glucocorticoids in these cells.

In **Chapter 3**, we first analyzed GR in human bronchial epithelial cell lines by a whole cell dexamethasone binding assay. The effect of the inflammatory mediators IL-1β, TNF-α and LPS was studied on the number and ligand affinity of GR. Furthermore, we investigated the binding of nuclear translocated GR to specific sites on DNA, termed glucocorticoid responsive elements (GRE).

Subsequently, the expression of GR in cultured human bronchial epithelial cells was studied (**Chapter 4**). Bronchial epithelial cells were isolated and cultured via the dissociation method using bronchial biopsies obtained after thoracotomy (2). After enzymatic dissociation from the bronchial tissue, the bronchial epithelial cells started growing in islets as illustrated in Figure 1. The whole cell dexamethasone binding assay used in bronchial epithelial cell lines was not suitable for use in our cultured normal human bronchial epithelial cells. The nonspecific binding was extremely high in these cells, often in the same range as the total binding. Similar high nonspecific binding was described by others in human alveolar macrophages (3). The high nonspecific binding, which interfered with reliable estimation of the specific binding sites, could be circumvented using a cytosol binding assay, in which first a cytosol was prepared from the cells followed by incubation with different concentrations of dexamethasone (4).

Besides studying the effects of inflammatory mediators on the characteristics of GR in human bronchial epithelial cells *in vitro*, we investigated whether inflammation *in vivo* also affected the GR characteristics. This was performed in COPD patients and smokers, as general airway inflammation is known to be present in COPD patients and smokers (**Chapter 4**).

To analyse the potential cellular mechanisms of anti-inflammatory actions of glucocorticoids in human bronchial epithelial cells, we studied both the effects of glucocorticoids on the induction (lipocortins) or inhibition

(chemokines) of proteins. In **Chapter 5** the expression of lipocortins in human bronchial epithelial cells and their inducibility by glucocorticoids were investigated. In addition, we studied the effects of inflammatory mediators on the lipocortin expression in these cells. In **Chapter 6** the effects of glucocorticoids and cytokines on MCP-1 release by human bronchial epithelial cells is described. MCP-1 release by these cells was compared to IL-8 release.

Finally, the conclusions of the different chapters are summarized and discussed in the light of the literature data in **Chapter 7**.

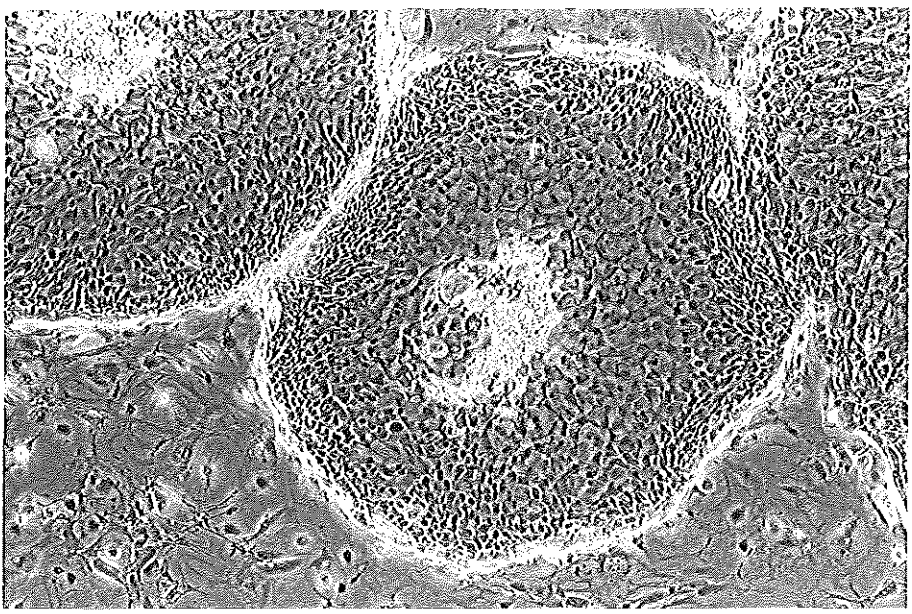

Figure 1. Phase contrast morphology of cultured human bronchial epithelial cells.

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CHAPTER 3

MODULATION OF GLUCOCORTICOID RECEPTORS IN HUMAN BRONCHIAL EPITHELIAL CELL LINES BY IL-1β, TNF-α AND LPS

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MODULATION OF GLUCOCORTICOID RECEPTORS IN HUMAN BRONCHIAL EPITHELIAL CELL LINES BY IL-1β, TNF-α AND LPS

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ABSTRACT

Bronchial epithelium plays a major role in the regulation of inflammatory reactions in the airways. It is thought to be a possible target for glucocorticoid therapy. Glucocorticoid responsiveness requires the presence of specific glucocorticoid receptors (GR). Until now, little is known about the presence of such receptors in the human bronchial epithelium.

In this study we demonstrated the expression of GR mRNA in two SV-40/adenovirus-transformed human bronchial epithelial cell lines, BEAS S6 and BEAS 2B. In a whole cell dexamethasone binding assay, BEAS S6 and BEAS 2B cells were found to possess $28.9 \pm 4.4 \times 10^3$ and $32.1 \pm 5.7 \times 10^3$ binding sites per cell, respectively, with dissociation constant (K_d) values of 8.2 ± 1.5 nM and 8.6 ± 2.4 nM, respectively. Using electrophoretic mobility shift assays we demonstrated the binding of nuclear translocated GR to specific sites on DNA, named glucocorticoid responsive elements (GRE).

LPS and IL-1β significantly increased the number of GR per cell (median = 312% and 171% of control, respectively, $p<0.05$), but significantly reduced the ligand affinity of these receptors, i.e. increased the K_d (median = 410% and 145% of control, respectively, $p<0.05$) in BEAS 2B cells.

These results indicate that the bronchial epithelium may be an actual target for glucocorticoid therapy. Inflammatory mediators as IL-1β and LPS modulate the number and ligand affinity of these GR. Therefore, the response of bronchial epithelium to glucocorticoid therapy may be modulated by airway diseases associated with inflammation.

INTRODUCTION

Until recently the bronchial epithelium was considered to be a passive barrier between the environment and the internal milieu of the lung (1). In addition to this barrier function, bronchial epithelial cells are now also known to produce various inflammatory mediators upon exposure to immunologic and nonimmunologic stimuli (1). Cultures of human bronchial epithelial cells exposed to toluene diisocyanate or to stimuli as acetylcholine and phorbol-12-myristate-13-acetate, release chemotactic arachidonic acid metabolites, such as 15-hydroxyeicosatetraenoic acid (15-HETE), prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (1,2). Other factors produced by cultured human bronchial epithelial cells upon exposure to stimuli or during recovery from injury are interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-6 (IL-6), granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) (3-6). Therefore, the bronchial epithelium is considered to play an essential role in initiating and perpetuating inflammatory and immunological reactions as observed in pulmonary diseases such as asthma.

Glucocorticoids are widely used in the treatment of several pulmonary diseases, e.g. bronchial asthma (7). They exert numerous biological effects on various cellular and metabolic processes. After entering the cell, glucocorticoids bind to an inactive cytosolic GR, which subsequently translocates to the nucleus. Within the nucleus, the complex binds as a dimer to specific sites on DNA, named glucocorticoid response elements (GRE), upstream of the promoter region in steroid-responsive genes. Transcription of the target gene is enhanced or repressed by the binding of the steroid-GR complex to the GRE (8,9). Recent studies showed that glucocorticoids are able to inhibit the release of bronchial epithelial cell-derived cytokines (4,10). Because bronchial epithelial cells seem to play an important role in airway inflammation and the function of these cells can be influenced by glucocorticoids, glucocorticoids may suppress airway inflammation by influencing bronchial epithelial cells. Such direct cellular effects of glucocorticoids require the presence of specific GR but the presence of these receptors has not been demonstrated yet by radioligand binding studies in human bronchial epithelial cells.

Here we report on the identification and characterization of specific GR in two human bronchial epithelial cell lines, BEAS 2B and BEAS S6. In comparison with the data on primary cultures of human airway epithelium, BEAS cell lines have been shown to be an appropriate model (11). These cell lines have been reported to exhibit positive immunofluorescent staining for cytokeratin, production of mucin-like glycoconjugates, and sensitivity to the differentiating effects of serum or transforming growth factor-β (11). In addition, we examined the role of inflammatory stimuli, such as IL-1β, TNF-α

and LPS, in modulating the number and ligand affinity of GR in BEAS cells.

MATERIALS AND METHODS

Cell lines and culture conditions

BEAS 2B and BEAS S6, two human bronchial epithelial cell lines, transformed by an adenovirus 12-SV-40 hybrid virus, were kindly provided by Dr. J. Lechner (Inhalation Toxicology Research Institute, Albuquerque, NM) (12). Cells were maintained in a serum-free, keratinocyte growth medium containing bovine pituitary extract (KGM; GIBCO, Paisly, UK) (13). Plastic cell culture plates (Falcon, Becton Dickinson, NJ) were precoated with a mixture of human fibronectin (10 µg/ml; Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands), collagen (Vitrogen 100, 30 µg/ml; Collagen Corp., Palo Alto, CA) and bovine serum albumin (10 µg/ml; Boehringer, Mannheim, Germany) (14,15). For GR experiments, cells were cultured for 1 passage in a mixture of Dulbecco's modified Eagles medium and Ham's F12 (DMEM/F12)(1:1) (GIBCO) supplemented with insulin (0.01 mg/ml; Sigma, St. Louis, MO), hydrocortisone (1 µg/ml; Pharma Chemie, Haarlem, the Netherlands), transferrin (0.01 mg/ml; Behring Marburg, Germany), epidermal growth factor (EGF) (10 ng/ml; Collaborative Research Inc., Lexington, MA), fetal calf serum (FCS) (1%), Na_2SeO_3 (50 nM), glutamine (1 mM; JT Baker bv., Deventer, The Netherlands) penicillin G sodium (100 U/ml; Gist-Brocades, Delft, The Netherlands) and streptomycin sulfate (0.1 mg/ml; Biochrom KG, Berlin, Germany).

Twenty four hours before performing GR binding experiments the medium was replaced by a basal medium of DMEM/F12 (1:1) with penicillin G sodium (100 U/ml) and streptomycin sulfate (0.1 mg/ml), but without hydrocortisone or other supplements to prevent influence of endogenous steroids on the number and affinity of GR. The effect of inflammatory mediators was studied by adding IL-1β (20 ng/ml; UBI, Lake Placid, NY), TNF-α (20 ng/ml; UBI) or lipopolysaccharide (LPS) (100 µg/ml; Difco Laboratories, Detroit MI) to the basal medium.

The CV-1 cell line and the malignant mesothelioma cell line Mero-14 were used as a negative and positive control, respectively, for GR mRNA experiments. The CV-1 cell line, derived from the kidney of a male adult African green monkey (American Type Culture Collection, Rockville, MD) was cultured on DMEM with 5% FCS. The malignant mesothelioma cell line Mero-14 was cultured as described by Versnel et al. (16).

RNA isolation and Northern blot analysis

Total RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform extraction procedure and stored at -80°C until used (17). Northern blotting and hybridization were performed as described earlier (18). Briefly, electrophoresis of 20 µg total RNA was performed on a 1 % agarose gel with formaldehyde. After blotting to nitrocellulose, hybridization was performed using ^{32}P labeled probes. Filters were washed twice for 20 min at 42°C with 3xSSC (20xSSC=3 M NaCl and 300 mM Nacitrate, pH 7.0) and exposed to a Fuji-RX film.

The intensity of the GR and the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) signals on the autoradiograph were scanned with a handscanner (Colorscanner 2^{24}, Highscreen, Würselen, Germany) at a resolution of 100 dots per inch. Computer software described by Koning et al. was used to analyze the intensity of the bands (19). Values were expressed as the ratio of GR mRNA intensity to GAPDH mRNA intensity.

Probes

The GR probe, kindly provided by dr. J. Trapman (Department of Pathology, Erasmus University, Rotterdam, The Netherlands) was a 368 bp *Hin*dIII-*Eco*RI fragment, corresponding to part of the N-terminal hypervariable region of the human GR (20). The GAPDH probe was a 0.7 kb *Eco*RI-*Pst*I fragment (21).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays were performed as described by Adcock et al. (22). BEAS 2B cells were cultured for 1 passage in DMEM/F12 supplemented with insulin, hydrocortisone, transferrin, EGF, FCS and Na_2SeO_3 after which nuclear protein was isolated (23). A double-stranded oligonucleotide encoding the consensus target sequence of GRE (5'-TCGACTGTACAGGATGTTCTAGCTACT-3') was endlabeled using [γ-^{32}P] ATP (Amersham, Den Bosch, the Netherlands) and T_4 polynucleotide kinase (Pharmacia Biotech, Roosendaal, The Netherlands). Ten micrograms of nuclear protein were incubated with 5,000 cpm of labeled oligonucleotide and 0.3 µg poly dIdC (Pharmacia Biotech) in 20 µl incubation buffer (4% glycerol, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM Tris-HCl, pH 7.5) for 20 min at 22°C. GR-GRE complexes were separated on a 6% polyacrylamide gel using 0.25xTBE running buffer (1xTBE=50 mM Tris base, 50 mM boric acid, 1 mM EDTA, pH 8.6). The retarded band was detected by autoradiography. Specificity was determined by addition of excess unlabeled double-stranded oligonucleotide.

Steroids

^{3}H-labeled dexamethasone (1,2,4,6,7 [^{3}H] dexamethasone; specific activity 81 Ci/mmol) was obtained from Amersham (Buckinghamshire, UK). Nonradioactive dexamethasone was kept in a stock solution of 2×10^{-3} M in ethanol (Duchefa bv., Haarlem, The Netherlands).

Glucocorticoid receptor assay

Bronchial epithelial cells were harvested with 0.02% EGTA, 1% polyvinylpyrrolidine and 0.025% trypsin (13). GR numbers and K_d's were determined according to established methods (24). Cells were washed with phosphate-buffered saline solution (PBS) and resuspended at a density of 4-5 x 10^6 cells/ml. Seven serial doubling dilutions (200 µl) were prepared in PBS to final ^{3}H-labeled dexamethasone concentrations of 32, 16, 8 , 4, 2, 1 and 0.5 nmol/L, respectively. For measurements of nonspecific binding, parallel serial doubling dilutions of ^{3}H-labeled dexamethasone

plus a 100-fold molar excess of nonradioactive dexamethasone were prepared. To each of the 14 tubes 200 µl of the cell suspension were added and cells were incubated for 90 min at 25°C in a waterbath under continuous shaking. Binding equilibrium was reached at all concentrations after 90 min of incubation at 25°C. Subsequently, three 100 µl aliquots of each incubation mixture (determination in triplicate) were transferred to polypropylene tubes. From the remaining incubation mixture 50 µl was used to establish the exact concentration of ^{3}H-labeled dexamethasone. To each 100 µl aliquot, 1 ml of ice-cold PBS was added and tubes were centrifugated for 5 min at 400 x g at 4°C. Supernatants were removed and pellets were resuspended in 1 ml of ice-cold PBS and incubated for 30 min on ice to reduce nonspecific binding. Thereafter, samples were centrifugated and the supernatants were removed. The cell pellets were transferred to scintillation vials and radioactivity was determined by liquid scintillation counting. Specific binding was calculated as the difference between the totally bound reactivity and the nonspecifically bound reactivity at each glucocorticoid concentration. Nonspecific binding was calculated from the aliquots containing the 100-fold molar excess non-radioactive dexamethasone, assuming that nonspecific binding was nonsaturable and linearly related to the concentration of free glucocorticoid.

Analysis

Binding curves were constructed from increasing concentrations of ^{3}H-dexamethasone and dissociation constant (K_d) values and receptor binding capacity were determined by Scatchard analysis of these data. Data were analysed with the radioligand binding analysis program 'Ebda/Ligand' by GA Pherson from Elsevier-BIOSOFT. The Wilcoxon Matched-Pair Signed-Ranks test was used to asses the equality of GR number and K_d distributions in cells treated with IL-1β, TNF-α or LPS and in untreated cells. A p-value of less than 0.05 was considered significant.

RESULTS

Expression of GR mRNA in BEAS cells

Total RNA was isolated from BEAS S6 and BEAS 2B cells cultured for 24 h in either KGM, or in DMEM/F12 supplemented with insulin, hydrocortisone, transferrin, EGF, FCS and Na_2SeO_3 or in DMEM/F12 without supplements. Hybridization of filters containing RNA from BEAS S6 and BEAS 2B cells with the GR probe revealed the presence of a 7.0 kb GR specific transcript. Approximately similar levels of GR mRNA expression were found in the two BEAS cell lines. Figure 1 shows the GR mRNA expression in BEAS S6 cells, cultured in KGM (lane a), in DMEM/F12 with hydrocortisone and other supplements (lane b) and in DMEM/F12 without supplements (lane c). RNA from the malignant mesothelioma cell line Mero-14 was used as a positive

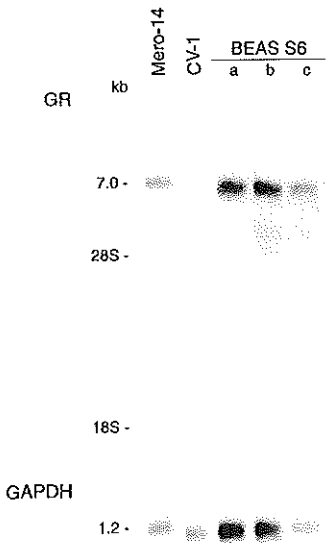

Figure 1. Northern blot analysis of 20 µg total RNA from the cell line BEAS S6. BEAS S6 cells were cultured for 24 h in a keratinocyte growth medium (lane a), in DMEM/F12 supplemented with insulin, hydrocortisone, transferrin, EGF, FCS and Na_2SeO_3 (lane b), and in DMEM/F12 without supplements (lane c). 20 µg of total RNA from the malignant mesothelioma cell line Mero-14 was used as a positive control (lane 1) and RNA from the CV-1 cell line as a negative control (lane 2). The filter was hybridized to both ^{32}P-labeled GR and GAPDH probes.

Table 1. GR mRNA expression in BEAS S6 cells

Culture medium	GR/GAPDH mRNA intensity
in keratinocyte growth medium	0.49
in DMEM/F12 with supplements	0.48
in DMEM/F12 without supplements	0.42

Ratio of GR mRNA intensity to GAPDH mRNA intensity in BEAS S6 cells cultured for 24 h in a keratinocyte growth medium, in DMEM/F12 supplemented with insulin, hydrocortisone, transferrin, EGF, FCS and Na_2SeO_3, and in DMEM/F12 without supplements.

control (17) and RNA form the CV-1 cell line as a negative control (21). The quantity of the mRNA applied was related to the constitutively expressed GAPDH mRNA. No effect of the different culture media on the GR mRNA content was observed (n=3). The ratio of GR mRNA intensity to GAPDH mRNA intensity in one representative experiment in BEAS S6 cells is shown in Table 1. A similar GR mRNA expression was found in BEAS 2B cells (data

not shown). GR mRNA expression was not altered after incubation for 4, 8, 24 or 48 h with IL-1β or TNF-α (5 or 20 ng/ml) or LPS (100 μg/ml) (data not shown).

Basal numbers and K_d of GR in BEAS cells

After demonstrating the presence of GR mRNA in BEAS cell lines, the number of GR and K_d values were determined. Using established methods to identify GR, we could demonstrate specific binding of ^{3}H-labeled dexamethasone by these cells in our final assay conditions. A typical binding curve for BEAS 2B is shown in Figure 2a. After Scatchard analysis of the data, the linear regression line obtained indicated a single class of GR for both cell lines (Figure 2b). BEAS 2B cells appeared to have 32.1 ± 5.7 x 10^3 binding sites per cell (n=3), and BEAS S6 cells were found to have 28.9 ± 4.4 x 10^3 binding sites per cell (n=4). K_d values as determined from their Scatchard plots, were 8.6 ± 2.4 nM and 8.2 ± 1.5 nM, respectively.

GR binding to its specific DNA recognition sequence

Using an electrophoretic mobility shift assay, the interaction between glucocorticoid-GR complexes isolated from the nucleus and GRE, can be measured. Nuclear extracts of BEAS 2B cells were incubated with a labeled dsDNA recognition sequence and the bound oligonucleotide was detected by its retardation in a nondenaturing gel. A clear GR/GRE binding was observed in nuclear extracts of BEAS 2B cells, (Figure 3, control lane). An excess (50-fold) of unlabeled GRE oligonucleotide produced a strong inhibition of band density (Figure 3, XS GRE oligo). No effect was observed of an excess of control unrelated oligonucleotide, corresponding to a part of the PDGF B-chain promoter region (Figure 3, XS irrelevant oligo).

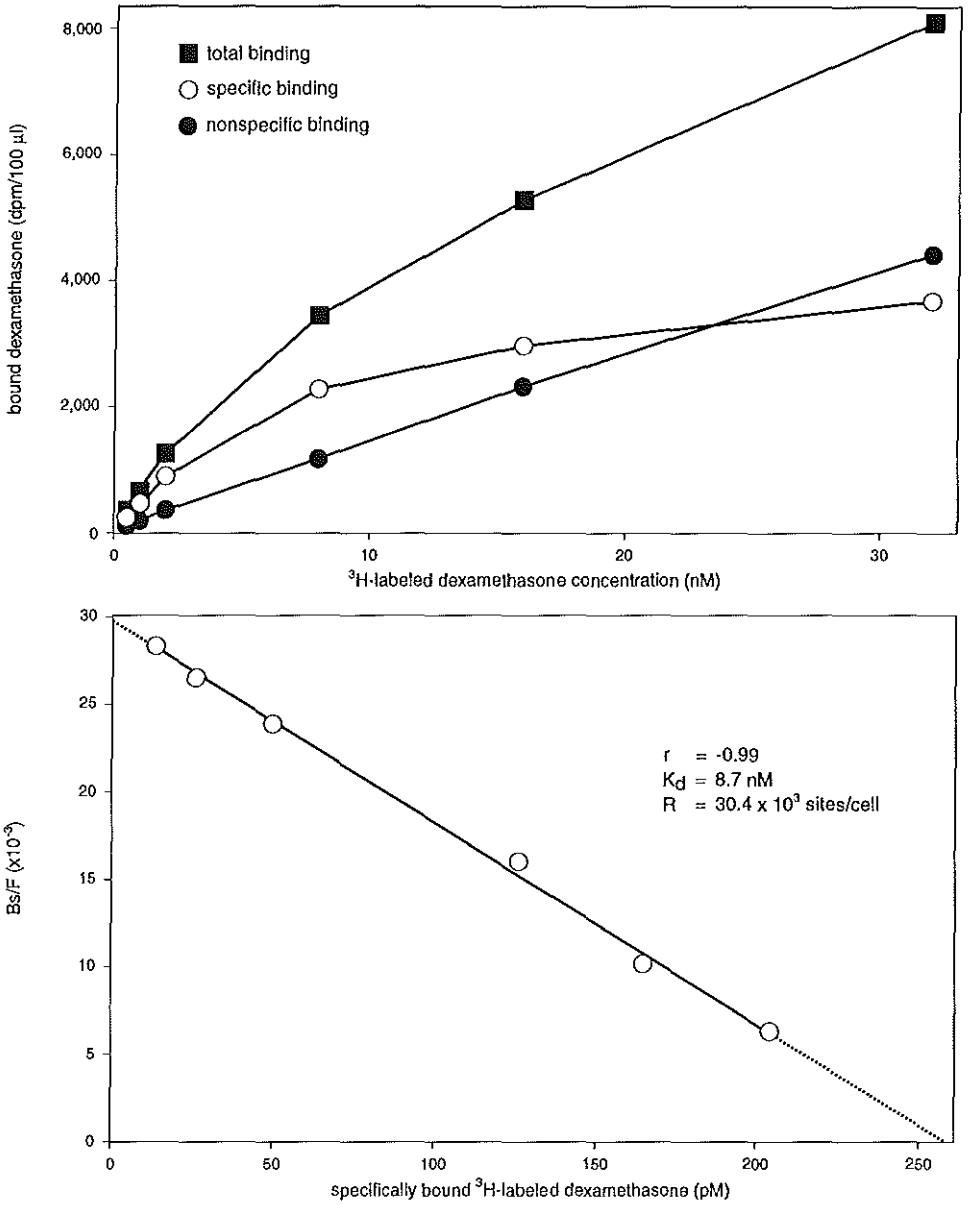

Figure 2. (A) Binding curve of ^{3}H-labeled dexamethasone for BEAS 2B cells. The specific binding is represented by the difference between the total and nonspecific binding. The data points represent mean values of quadruplicate determinations. (B) Scatchard plot of the specific binding of ^{3}H-labeled dexamethasone to BEAS 2B cells. On the ordinate is given the ratio of the amount of specifically bound ^{3}H-labeled dexamethasone (B_s) to the amount of free ^{3}H-labeled dexamethasone (F). One representative experiment out of three is shown. The K_d and the number of glucocorticoid receptors per cell (R) were calculated using the negative inverse of the slope and the x intercept, respectively.

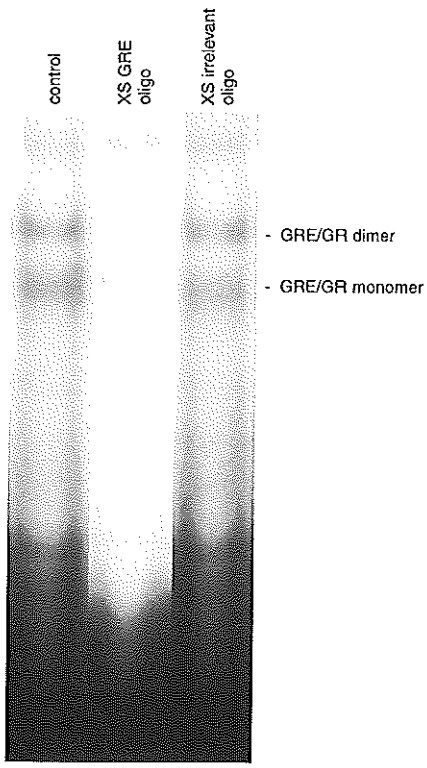

Figure 3. GR/GRE binding in BEAS 2B cells in an electrophoretic mobility shift assay. Effect of an excess (50-fold) of unlabeled GRE oligonucleotide (XS GRE oligo) and of an excess of control unrelated oligonucleotide corresponding to part of the PDGF B-chain promoter region (XS irrelevant oligo).

Effects of LPS, IL-1β and TNF-α on GR-binding affinity and number

To study the effect of inflammatory mediators on GR number and binding affinity, BEAS 2B cells were incubated with LPS, IL-1β, or TNF-α for 24 h. To exclude that LPS or IL-1β interfered with the ability of ^{3}H-dexamethasone to bind to the GR, LPS or IL-1β were added for 5, 15, 30 or 60 minutes to the bronchial epithelial cells and subsequently a ^{3}H-dexamethasone binding assay was performed. No effect on GR number or affinity was observed. Figure 4 shows a representative Scatchard plot of [^{3}H] dexamethasone radioligand-binding in BEAS 2B cells incubated with LPS or IL-1β for 24 h, or cultured without inflammatory stimuli. The radioligand-binding data of BEAS 2B treated with LPS or IL-1β ($n=5$) showed a significant increase in the K_d, i.e. a decrease in binding affinity for glucocorticoids (median = 410% and

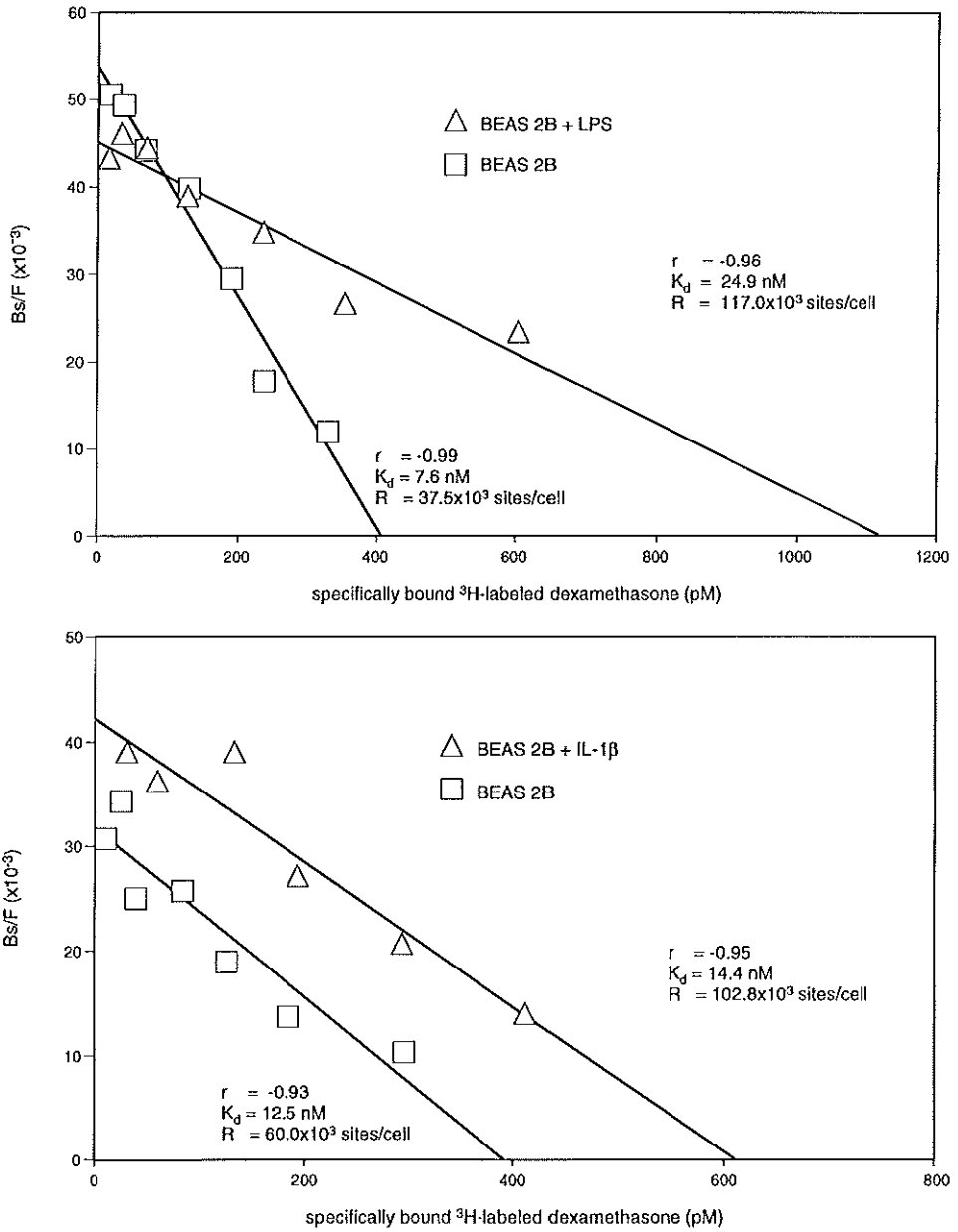

Figure 4. Scatchard plot of the specific binding of ^{3}H-labeled dexamethasone to BEAS 2B cells treated for 24 h with LPS (100 µg/ml)(A) or IL-1β (20 ng/ml)(B). On the ordinate is given the ratio of the amount of specifically bound ^{3}H-labeled dexamethasone (B_s) to the amount of free ^{3}H-labeled dexamethasone (F). One representative experiment out of five is shown. The K_d and the number of glucocorticoid receptors per cell (R) were calculated using the negative inverse of the slope and the x intercept, respectively.

145% of control, respectively, $p<0.05$), and a significant increase in GR number (median = 312% and 171% of control, respectively, $p<0.05$) (Figure 5). In contrast, treatment with TNF-α had no significant effect on GR K_d (85.5% of control) or GR number (107.5% of control) (Figure 5).

Data were analysed with a radioligand binding analysis program. When using a two-site model for ligand binding the Scatchard results were not affected.

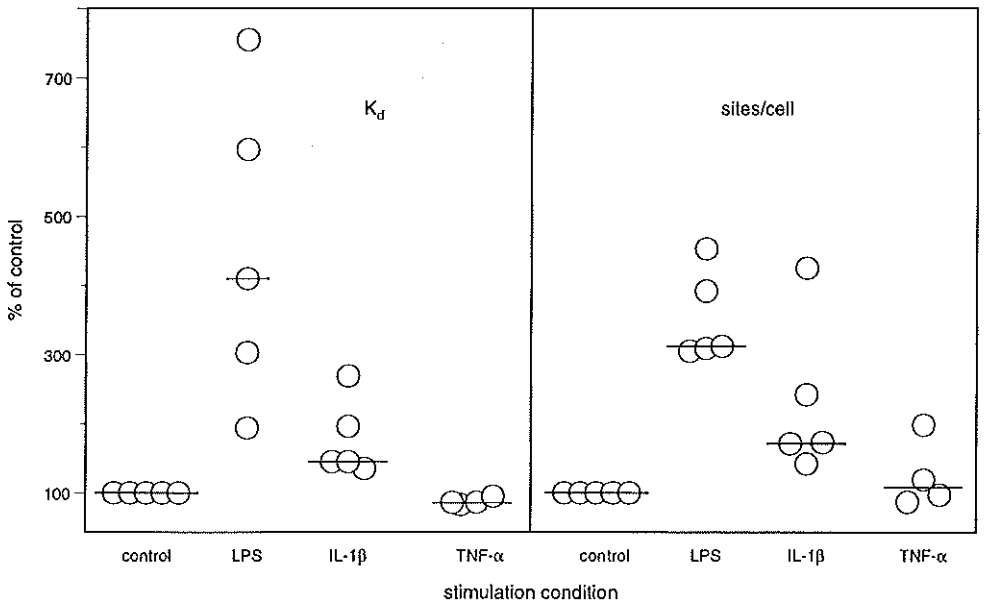

Figure 5. The effect of LPS, IL-1β and TNF-α on GR number and binding affinity. Incubation of BEAS 2B cells with LPS (100 μg/ml) or IL-1β (20 ng/ml) resulted in a significantly increased receptor number and a decreased binding affinity of the GR for dexamethasone ($n=5$, $p=0.05$) when compared with the medium control. Incubation with TNF-α (20 ng/ml) for 24 h had no effect on GR K_d or GR number. The solid bars indicate median values.

DISCUSSION

In this report we demonstrate the presence of GR mRNA and protein in two human bronchial epithelial cell lines, BEAS S6 and BEAS 2B. In nuclear extracts obtained from BEAS 2B cells cultured in hydrocortisone containing medium, electrophoretic mobility shift assays showed that nuclear translocated GR bind to their GRE. BEAS S6 and BEAS 2B cell lines were found to possess $28.9 \pm 4.4 \times 10^3$ and $32.1 \pm 5.7 \times 10^3$ specific binding sites per cell, respectively, with K_d values of 8.2 ± 1.5 nM and 8.6 ± 2.4 nM, respectively. Treatment of BEAS 2B cells with LPS or IL-1β resulted in a

significantly higher number of GR compared to control cells, whereas GR binding affinity for glucocorticoids was reduced.

We hypothesize that the clinical response to inhaled glucocorticoids, which mainly precipitate in the larger airways, results, at least partly, from the modulation of airway epithelial cell functions. From the K_d value of the GR in bronchial epithelial cells observed in our studies, we expect that effective *in vivo* glucocorticoid concentrations should be around 5-10 nM. A recent study by Van den Bosch et al showed that at least 90 minutes after inhalation of 1.6 mg budesonide, lung tissue concentrations ranged from 2.1 to 8.9 nM (25). Therefore, we can assume that inhalation of glucocorticoids may result in such concentrations of glucocorticoids in lung tissue that interaction with GR in epithelial cells will occur.

Human bronchial epithelial cells are considered to play an important role in airway inflammation (1-6). Inhaled glucocorticoids are used to suppress airway inflammation and, concomitantly, to improve clinical parameters. As inhaled glucocorticoids mainly precipitate in the larger airways, one of the mechanisms of action of these drugs may be the modulation of the functioning of bronchial epithelium. Cellular response to glucocorticoids demands the presence of functional GR. Only a few studies concern the presence of GR in bronchial cells, and the majority concerns animal studies (26-29). Previous *in vitro* binding experiments with ^{3}H-dexamethasone performed on lung samples from adult and fetal animals and from a human fetus showed nuclear localization of ^{3}H-dexamethasone in alveolar epithelial cells, but no significant nuclear localization in bronchial epithelial cells (26-29). In these studies autoradiographic data were confirmed with liquid scintillation counting for specific ^{3}H-dexamethasone binding in nuclear and cytosolic fractions prepared from lung tissue. Until now, one pilot study has been performed on adult human lung, in which the localization and expression of GR mRNA was studied by in situ hybridization and Northern blot analysis (30). Highest concentration of human GR mRNA was found in the alveolar walls with lesser amounts in the larger airway epithelium (29). However, in that study no data were presented on the number of receptors and their K_d values.

In the study presented here, it is shown for the first time with ^{3}H-dexamethasone binding assays that human bronchial epithelial cells possess GR and that these receptors are able to interact with GRE. Using the same ^{3}H-dexamethasone binding assay, we found in U937 cells, an immature monocytic cell line, and in the NHIK 3025 cell line, derived form a carcinoma of the human uterine cervix, 18.2×10^3 and 86.0×10^3 specific binding sites per cell, respectively. Their dexamethasone K_d values were 3.5 nM and 3.1 nM, respectively. The number of GR and the K_d values we found in bronchial epithelial cell lines were similar to those described for cultured dispersed adult rat lung cells (27). Cultured L-2 cells, a cell line that

originated from a type II epithelial cell of adult rat lung contained 58.2 x 10^3 nuclear binding sites per cell with a K_d of 8.0 nM (27). Peripheral blood mononuclear cells (PBMC) from normal human controls were found to possess 2.7 x 10^3 receptor sites per cell with a K_d of 6.7 nM (31). Compared to PBMC, BEAS cell lines contain a relatively high number of glucocorticoid binding sites. The number and quality of GR in target cells may determine the extent of glucocorticoid responsiveness (32,33). Even if the fact that they are 2-3 times larger than PBMC is taken into account, bronchial epithelial cells still possess a greater number of GR.

We demonstrated that both LPS and IL-1β, significantly increased the number of GR, but decreased the GR binding affinity in BEAS 2B cells. These results and the work by several other investigators indicate that cytokines produced during the course of an immune response may regulate GR number and affinity (31,34-36). This suggests that inflammation in itself may modulate cellular steroid responsiveness through locally produced inflammatory mediators. Inflammatory mediators can induce an increase in GR number, perhaps to control excess of inflammatory stimuli by sensitizing the cell to feedback inhibition by glucocorticoids. IL-1, TNF-α and IL-6 have all been shown to stimulate the hypothalamic-pituitary axis to secrete corticotropin-releasing hormone and adrenocorticotropin hormone which, in turn, induces glucocorticoid secretion from the adrenal cortex (37,38).

Northern blotting experiments showed that GR mRNA expression was not altered after incubation with LPS or IL-1β. Therefore, the increase in GR number after treatment with LPS or IL-1β could not be explained by an increase in GR mRNA. Increased translation or increased half-life time of GR mRNA could provide an explantion for the increase in GR number. An altered phosphorylation state of the GR after treatment with LPS or IL-1β is unlikely to account for the discrepancy between GR mRNA and protein expression. Studies performed by Moyer et al. showed no relation between enhanced transcriptional activity and phosphorylation of the GR (39).

The work by several other investigators indicates that cytokines produced during the course of an immune response may regulate both GR number and affinity (31,34-36). Kam et al. found that when normal T cells and the non-T cell fraction of PBMC were individually stimulated with IL-2 and IL-4, a significant reduction in GR binding affinity for glucocorticoids was observed only in the T cell population (31). An increase in GR number was observed both in T and non-T cells. The reduction in PBMC GR binding affinity with IL-2 and IL-4 was associated with a reduced T cell response to methylprednisolone. Sher et al. demonstrated that steroid resistant patients had a significantly reduced GR binding affinity for glucocorticoids, but an increased number of nuclear GR compared with steroid sensitive patients and normal patients (34). This defect was localized to T cells and reverted to normal after 48 h in culture. Incubation with IL-2 and IL-4 sustained these

abnormalities. Rakasz et al. have shown that glucocorticoid binding was increased by IL-1β, TNF-α and IL-6 in a B-cell line (CESS), in U937, and in a hepatoma cell line (HepG2) (35). IFN-γ has been shown to mediate increased GR expression in murine macrophages (36). The decrease in GR binding affinity for dexamethasone and the increase in GR number, which we found with IL-1β and LPS in BEAS 2B cells is in accordance with the effects of IL-2 and IL-4 on the GR, found in T cells by Kam et al. (31). The differences in the results obtained by the several investigators mentioned above could well be due to differences in cell type, methods of ligand binding, the ligands, and the cytokines or inflammatory mediators used for different periods of time.

The actual mechanisms by which inflammatory mediators induce a decrease in GR binding affinity for glucocorticoids remains to be established. Others have shown that altered expression of glucocorticoid-regulated proteins appears to be mediated via interaction of the modulatory domain of the GR with transcriptional factors, such as AP-1 (40-42). Overexpression of AP-1 interferes with the function of the modulatory domain of the GR. Because cytokines can induce elevated levels of AP-1, it has been suggested that this may provide a plausible explanation for the decreased ligand binding affinity of nuclear GR for glucocorticoids, induced by IL-2 and IL-4 in T cells (40-42). Perhaps, a similar explanation could be given for the decreased GR binding affinity induced by LPS or IL-1β in BEAS 2B cells. Another explanation for decreased GR binding affinity could be a modulating effect of LPS and IL-1β on the expression and phosphorylation of heat shock proteins, which are associated with the unliganded GR (43,44). Bacterial products and cytokines can regulate the expression and phosphorylation of heat shock proteins and this may modulate glucocorticoid binding to the GR (43,44). Further studies are necessary to clarify the exact mechanism of these LPS and IL-1β effects on GR.

In summary, we demonstrated that two human bronchial epithelial cell lines, BEAS S6 and BEAS 2B, possess a single class of specific GR, which display *in vitro* specific binding to its GRE. LPS and IL-1β significantly increased the number of GR and decreased the GR binding affinity in BEAS 2B cells. Treatment with TNF-α had no effect on GR K_d or receptor number. These results provide further evidence that the bronchial epithelium may be a direct target for glucocorticoid therapy in inflammatory airway diseases. Furthermore, this study suggests that inflammatory processes may influence the response of bronchial epithelium to glucocorticoid therapy via locally produced cytokines. As the response to glucocorticoids varies considerably among patients with asthma and chronic obstructive pulmonary disease, it is of great interest to look in these patients for correlations between the clinical response to glucocorticoids and the number of GR or their K_d in bronchial epithelial cells, in combination with the local expression of cytokines.

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CHAPTER 4

GLUCOCORTICOID RECEPTOR EXPRESSION IN HUMAN BRONCHIAL EPITHELIAL CELLS:
effects of smoking and COPD

CHAPTER 4

GLUCOCORTICOID RECEPTOR EXPRESSION IN HUMAN BRONCHIAL EPITHELIAL CELLS: effects of smoking and COPD

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ABSTRACT

The bronchial epithelium plays a major role in the regulation of inflammatory reactions in the airways. It is thought to be a target for inhaled glucocorticoid therapy. Previously, we identified specific glucocorticoid receptors (GR) in human bronchial epithelial cell lines. Furthermore, we found that inflammatory mediators modulated the number and binding affinity of GR and hence may affect glucocorticoid responsiveness.

Here we report on the expression of GR protein in cultured human bronchial epithelial cells (HBEC). We investigated whether in chronic obstructive pulmonary disease (COPD) patients and smokers, in whom general airway inflammation is present, GR characteristics are altered.

A significant difference was found between the dissociation constant (K_d) values in HBEC from smoking (K_d = 0.98 ± 0.08 nM; n = 6) and nonsmoking controls (K_d = 0.76 ± 0.10 nM, p = 0.03; n = 5), but no significant difference was found between the mean number of binding sites (70.5 ± 14.0 and 87.2 ± 16.7 fmol/mg protein, respectively, p = 0.3). Nor was any significant difference observed between nonsmoking COPD patients (79.7 ± 22.6 fmol/mg protein, K_d = 0.74 ± 0.19 nM; n = 3) and the values measured in nonsmoking controls (p = 0.6 and p = 0.6, respectively; n = 5). Furthermore, the mean number of binding sites and K_d values measured in nonsmoking (n = 3) and smoking COPD patients (p = 0.6 and p = 0.6 respectively; n = 5) did not differ significantly, nor was any significant difference observed between smoking controls (n = 6) and smoking COPD patients (p = 0.9 and p = 0.1, respectively; n = 5).

These results provide further evidence that the bronchial epithelium may be an actual target for inhaled glucocorticoid therapy. Our results are the

first indication that cultured HBEC from smokers possess GR with a lower binding affinity. This may result from the inflammation found in the airways from smokers.

INTRODUCTION

The bronchial epithelium has long been regarded as a passive barrier to protect the underlying tissue (1). Now, bronchial epithelial cells are also known to play an active role in airway inflammation. They are able to produce various inflammatory mediators including cytokines and eicosanoids (2,3). Bronchial epithelial cells recovered from asthmatic patients show increased gene expression for GM-CSF, IL-6 and IL-8 (3). Furthermore, increased expression of endothelin-1, TNF-α, IL-1β and MCP-1 has been demonstrated (4,5).

Glucocorticoids are widely used in the treatment of inflammatory pulmonary diseases, e.g. bronchial asthma and chronic obstructive pulmonary disease (COPD) (6). Recent studies showed that glucocorticoids are able to inhibit the release of some bronchial epithelial cell-derived cytokines (3,7). These effects of glucocorticoids combined with the above mentioned role of bronchial epithelial cells in airway inflammation, suggest that glucocorticoid therapy may suppress airway inflammation, at least partially, by modulating the function of bronchial epithelial cells. This hypothesis is supported by the observation that the greater part of inhaled glucocorticoids precipitate on the epithelium of the larger airways (8). A prerequisite for steroid responsiveness is the presence of specific glucocorticoid receptors (GR).

In a recent study we identified and characterized specific GR in two SV-40/adenovirus-transformed human bronchial epithelial cell lines, BEAS 2B and BEAS S6 (9). We also demonstrated that inflammatory stimuli such as IL-1β and LPS may modulate the number and the binding affinity of GR in BEAS 2B cells. Similar results were found by other investigators in human T cells, other cell lines and murine macrophages (10-12). These results indicate that inflammatory processes may influence the response of the bronchial epithelium to glucocorticoid therapy via locally produced cytokines.

Here, as an extension of our findings in cell lines, we report on the identification and characterization of specific GR in cultured human bronchial epithelial cells (HBEC). From the above mentioned *in vitro* findings concerning the effects of inflammatory mediators on GR, it may be expected that inflammation *in vivo* may also affect GR characteristics. Therefore, we also studied whether in cultured HBEC from smokers and COPD patients, in whom general inflammation has been described (13), the number or binding affinity of GR is altered.

MATERIALS AND METHODS

Patients

COPD patients (n=8) were selected according to ATS criteria (14). Nonallergic patients with complaints of chronic cough and sputum production were selected. Chronic cough means that coughing occurs on most days during at least three consecutive months in two consecutive years. FEV_1 was less than or equal to 70% of predicted normal values and a FEV_1/VC ratio was less than or equal to 70%. Controls (n=11) were subjects who denied any symptoms of asthma or COPD, and had normal lung function parameters. The COPD and control group contained 5 and 6 smoking individuals, respectively. From the nonsmokers, one individual stopped smoking 12 years ago. Smoking individuals were current smokers with a smoking history of at least 35 pack-years. The mean age of controls and COPD patients was 64.6 ± 9.1 years (range 50-77 years) and 63.1 ± 6.3 years (range 52-69), respectively.

Isolation and culture conditions of HBEC

Bronchial tissue was obtained from patients undergoing surgery for lung cancer and used immediately for culture of HBEC by a cell culture method described previously (15). Briefly, bronchial tissue distant from the tumor was cut into pieces and incubated either overnight at 4°C or 1 h at 37°C in 0.1% protease XIV (Sigma, St. Louis, MO). Subsequently, epithelial cells were gently scraped from the tissue samples, washed twice in culture medium and plated onto 35-mm dishes at a density of 2.5×10^5 cells/dish. HBEC were cultured in a 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F12 (DMEM/F12)(GIBCO, Paisly, UK), with supplements as described previously (15). Cells were characterized as epithelial cells by immunofluorescence staining using a mouse monoclonal antibody directed against a number of human cytokeratins (CK-1; DAKOpatts, Glostrup, Denmark). At least 99% of the isolated cells stained positive for cytokeratin.

When dishes were confluent, HBEC were passaged to 75 cm^2 flasks and used for experiments after confluence. Twenty four hours before performing GR binding experiments the medium was replaced by a basal medium of DMEM/F12 (1:1) with penicillin G sodium and streptomycin sulfate, but without hydrocortisone or other supplements to prevent influence of endogenous steroids on the number and affinity of GR.

Cell lines

BEAS 2B is a SV-40/adenovirus transformed human bronchial epithelial cell line, which was kindly provided by Dr. J. Lechner (Inhalation Toxicology Research Institute, Albuquerque, NM) (16). Cells were maintained in a complete keratinocyte growth medium (KGM) containing bovine pituitary extract, EGF, penicillin G sodium and streptomycin sulfate (GIBCO).

The COS-1 cell line was used as a negative control for GR Western blot experiments. The COS-1 cell line, derived from the kidney of a male adult African green

monkey (American Type Culture Collection, Rockville, MD) was cultured in DMEM with 5% FCS.

Preparation of cytosol

Flasks containing approximately 10 x 10^6 cells were washed twice with cold phosphate buffered saline (PBS) and HBEC were scraped in 0.5 ml of buffer A (40 mM Tris-HCl pH 7.4, 1 mM EDTA, 10% (v/v) glycerol, 10 mM dithiothreitol, 10 mM Na_2MoO_4) supplemented with 0.6 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM bacitracine and 0.5 mM leupeptin. The cells were lysed by freezing/thawing three times and the homogenate was centrifuged for 10 min at 400,000 x g (17). The supernatant was collected and stored at -80°C. Protein concentrations were determined according to the method of Bradford (18) and ranged from 1 to 2 µg/µl. Cytosol preparations were used for both Western blotting and binding assays.

Western blotting

Samples (20 µl cytosol containing 1 µg/µl protein) were mixed with 5 µl of 5 x sample buffer (1 x sample buffer= 50 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 10 mM dithiothreitol, 0.001% (w/v) bromophenol blue), boiled for 2.5 min and separated on a 7% SDS-PAGE gel. After electrophoresis the gels were blotted to nitrocellulose membrane in blotting buffer (16.5 mM Tris-HCl, pH 8.3, 150 mM glycine and 20% v/v methanol). The membranes were blocked for 1 h at RT with 1% block solution (Boehringer, Mannheim, Germany) and incubated for 1 h at room temperature with a commercially available GR polyclonal rabbit antibody, # 57 (PA1-511; Affinity Bioreagents, Neshanic Station, NJ), diluted 1:250 in 0.5% block solution. Membranes were washed twice with PBS/Tween 0.1% and twice with 0.5% block solution, then incubated with horseradish peroxidase-conjugated goat anti-rabbit-IgG (Sigma) for 1 h at RT. After 4 x 15 min washes (PBS/Tween 0.1%) proteins were detected with Boehringer-Mannheim's Chemiluminescence Western Blotting kit and membranes were exposed to X-ray film.

Steroids

^{3}H-labeled dexamethasone (1,2,4,6,7 [^{3}H] dexamethasone; specific activity 81 Ci/mmol) was obtained from Amersham (Buckinghamshire, UK). Nonradioactive dexamethasone was kept in a stock solution of 2 x 10^{-3} M in ethanol (Duchefa bv., Haarlem, the Netherlands).

Binding assays

GR numbers and K_d values were determined according to established methods (19). Six serial doubling dilutions (50 µl) were prepared in duplicate in PBS to final ^{3}H-labeled dexamethasone concentrations of 32, 16, 8 , 4, 2, and 1 nmol/l, respectively. For measurements of nonspecific binding, parallel dilutions of ^{3}H-labeled dexamethasone plus a 100-fold molar excess of nonradioactive dexamethasone were prepared. To each of the 24 tubes 150 µl of the cytosol preparation was added and the mixture

was incubated overnight at 4°C. Binding equilibrium was reached at all concentrations after incubation overnight at 4°C. Subsequently, 160 µl aliquots of each incubation mixture were transferred to albumin (0.1%) coated tubes, 750 µl of protamine dihydrochloride solution (0.5 mg/ml) was added and tubes were centrifuged for 15 min at 4000 rpm. From the remaining incubation mixture 20 µl was used to establish the exact concentration of ^{3}H-labeled dexamethasone. Tubes containing protein pellets were washed three times with 1 ml of incubation buffer (50 mM Tris-HCl pH 7.4, 1.5 mM EDTA, 1.5 mM DTT, 10% glycerol) and pellets were solubilized in Soluene-350 (Packard, Meriden, CT). Thereafter, samples were transferred to scintillation vials and radioactivity was determined by liquid scintillation counting. Specific binding was calculated as the difference between the totally bound radioactivity and the nonspecifically bound radioactivity at each glucocorticoid concentration. Nonspecific binding was calculated from the aliquots containing the 100-fold molar excess non-radioactive dexamethasone, assuming that nonspecific binding was nonsaturable and linearly related to the concentration of free glucocorticoid.

Analysis

Binding curves were constructed from increasing concentrations of ^{3}H-dexamethasone. GR number and K_d values were determined by Scatchard analysis of these data. Data were expressed as mean fmol/mg protein specific binding ± the standard error of the mean (SEM). The Mann-Whitney U test was used to assess the equality of GR number and K_d distributions in HBEC from patients with COPD and controls. A p-value of less than 0.05 was considered significant.

RESULTS

Expression of glucocorticoid receptor protein in normal HBEC

Cytosolic fractions were isolated from HBEC, BEAS 2B and COS-1 cells, and GR levels were examined by Western blotting. The GR polyclonal rabbit antibody recognized a prominent band at ~97 kDa in the HBEC and BEAS 2B preparations (Figure 1). This estimated molecular mass is consistent with that reported previously for the human GR (20). In the GR negative cell line COS-1, no immunoreactive protein was detectable.

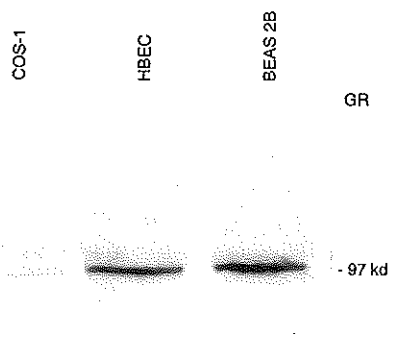

Figure 1. Western blot of 20 μg protein illustrating immunoreactive GR levels in cultured human bronchial epithelial cells (HBEC) and in the cell line BEAS 2B. One representative experiment out of three is shown. The COS-1 cell line (20 μg) was used as a negative control.

After demonstrating the presence of GR protein, the number of GR and K_d values were studied in cultured HBEC from controls (n = 11). Using established methods to identify GR, we could demonstrate specific binding of ^{3}H-labeled dexamethasone by these cells. A typical binding curve for cultured HBEC is shown in Figure 2A. After Scatchard analysis of the data, the linear regression line obtained indicated a single class of GR (Figure 2B).

GR binding in HBEC from COPD patients, smokers and controls

HBEC were isolated from bronchus tissue from 8 COPD patients and 11 controls. The COPD and control group contained 5 and 6 smoking individuals, respectively. Cells were cultured for 1 passage. Thereafter, cytosols were prepared and the number of glucocorticoid binding sites and K_d were determined. Results are presented in Table 1 and Figure 3. A significant difference was found between the K_d values in HBEC from smoking (K_d = 0.98 ± 0.08 nM) and nonsmoking controls (K_d = 0.76 ± 0.10 nM, p = 0.03), but no significant difference was found between the mean number of binding sites (70.5 ± 14.0 and 87.2 ± 16.7 fmol/mg protein, respectively, p = 0.3). Furthermore, values measured in nonsmoking and smoking COPD patients (p = 0.6 and p = 0.6, respectively) did not differ significantly. Nor was any significant difference observed in glucocorticoid binding sites or K_d between nonsmoking COPD patients and nonsmoking controls (p = 0.6 and p = 0.6, respectively) or between smoking COPD patients and smoking controls (p = 0.9 and p = 0.1, respectively).

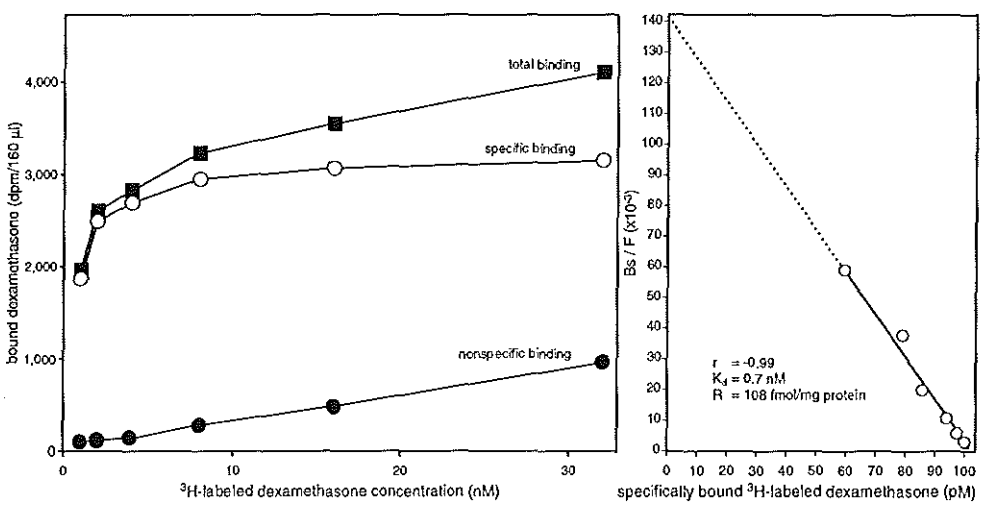

Figure 2. (A) Binding curve of ^{3}H-labeled dexamethasone for cultured HBEC. The specific binding is represented by the difference between the total and nonspecific binding. The data points represent mean values of duplicate determinations. (B) Scatchard plot of the specific binding of ^{3}H-labeled dexamethasone to HBEC. On the ordinate is given the ratio of the number of specifically bound ^{3}H-labeled dexamethasone (Bs) to the number of free ^{3}H-labeled dexamethasone (F). One representative experiment out of 11 is shown. The K_d and the number of glucocorticoid binding sites per mg protein (R) were calculated using the negative inverse of the slope and the x intercept, respectively.

Table 1. Glucocorticoid binding in HBEC from smoking and nonsmoking COPD patients and controls

	controls		COPD patients	
	nonsmoking (n=5)	smoking (n=6)	nonsmoking (n=3)	smoking (n=5)
R (fmol/mg protein)	87.2 ± 16.7	70.5 ± 14.0	79.7 ± 22.6	68.4 ± 12.8
K_d (nM)	0.76 ± 0.10	0.98 ± 0.08*	0.74 ± 0.19	0.71 ± 0.15

R: number of glucocorticoid binding sites
K_d: dissociation constant
*: significantly higher ($p<0.05$) than in nonsmoking controls

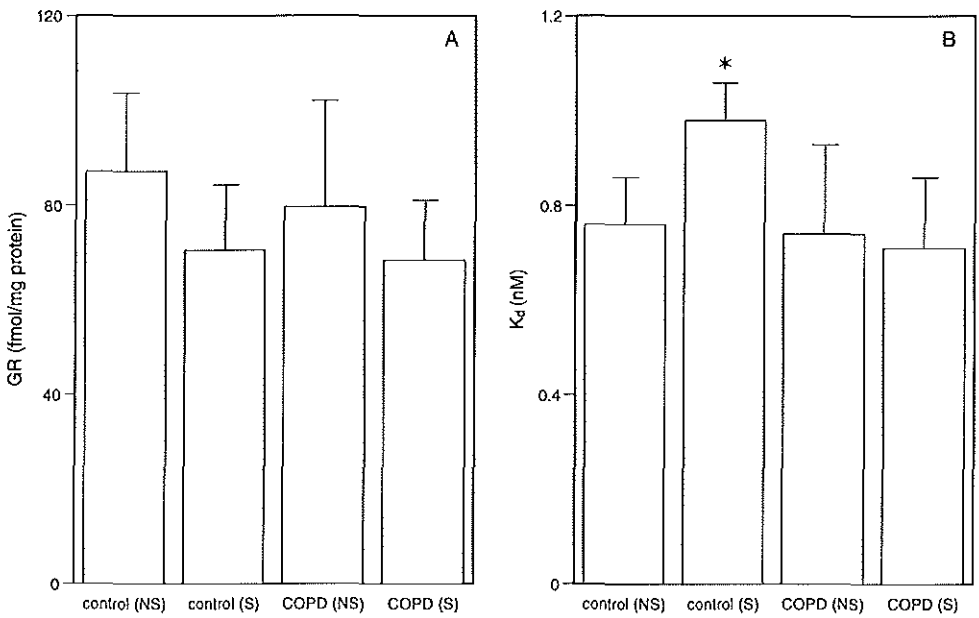

Figure 3. Total number of GR (A) and K_d values (B) in cultures of HBEC from nonsmoking controls (n=5), smoking controls (n=6), nonsmoking COPD patients (n=3) and smoking COPD patients (n=5). Results are expressed as mean ± SEM. * $p < 0.05$ for smoking controls versus nonsmoking controls. NS: nonsmoking; S: smoking.

DISCUSSION

In this study we identified and characterized specific GR in primary cultures of HBEC from COPD patients and controls. The presence of GR protein was demonstrated using Western blot analysis and ^{3}H-dexamethasone binding studies. A significant decrease was found in the binding affinity of GR in HBEC from smoking controls compared to nonsmoking controls, but no significant difference was found between the mean number of specific glucocorticoid binding sites. Furthermore, values measured in nonsmoking and smoking COPD patients did not differ significantly. Nor was any difference observed between the mean number of binding sites and the K_d values in HBEC from nonsmoking COPD patients and the values measured in nonsmoking controls or between the values measured in smoking COPD patients and smoking controls.

HBEC are considered to play an important role in airway inflammation (1-5). Inhaled glucocorticoids are used to suppress airway inflammation and, concomitantly, to improve clinical parameters. The finding that HBEC possess functional GR allows us to hypothesize that the clinical response to inhaled glucocorticoids, which mainly precipitate in the larger airways, results, at least partly, from the modulation of airway epithelial cell functions. From the K_d value of the GR in HBEC observed in our studies, we expect that effective *in vivo* glucocorticoid concentrations should be around 1 nM. In a recent study by Van den Bosch et al. it was shown that at least 90 minutes after inhalation of 1.6 mg budesonide, lung tissue concentrations ranged from 2.1 to 8.9 nM (21). Therefore, we can assume that therapeutical inhalation of glucocorticoids results in such concentrations of glucocorticoids in lung tissue that interaction with GR in HBEC will occur.

In a previous study we found that the SV-40/adenovirus transformed human bronchial epithelial cell line BEAS 2B contained a higher number of glucocorticoid binding sites compaired with peripheral blood mononuclear cells (PBMC) (8). The number and quality of GR in target cells may determine the extent of glucocorticoid responsiveness (22). HBEC were observed to contain less GR than the BEAS 2B cell line (87.4 ± 16.5 and 370 ± 14 fmol/mg protein, respectively). This difference may be caused by the SV-40/adenovirus transformation. HBEC contain a relatively high number of GR compared to PBMC, which contain approximately 30 fmol/mg protein (unpublised results). It would be interesting to investigate whether these differences in GR number result in differences in the functional response to glucocorticoids. As both epithelial cells and PBMC are able to produce IL-1β, the inhibition of IL-1β production by glucocorticoids may be a useful parameter to study this issue. Few data are available on the number of GR in other epithelial cells. A human epithelial duct cell line was found to contain 83-92 fmol GR/mg protein (23).

Previously, we demonstrated that the inflammatory mediators IL-1β and LPS increased the GR number and K_d in BEAS 2B cells (8). Similar results were found in other cell types (9-11). These results suggest that inflammatory processes may indirectly influence the response of bronchial epithelium to glucocorticoid therapy via production of cytokines by infiltrating cells. It has been suggested that in COPD, bronchial inflammation is responsible for the development of airway hyperreactivity, and chronic airflow limitation (12). Cigarette smoking causes an inflammatory reaction in the airways and can lead to the development of COPD (13). We found a significantly decreased binding affinity of the GR in smoking controls when compared to values found in nonsmoking controls. These results indicate that the inflammation present in the airways of smokers may be associated with altered GR binding. The finding that *in vivo* inflammation found in smokers affects GR characteristics in HBEC is in accordance with our above mentioned findings *in vitro*. We did not find any difference in the binding affinity of the GR in COPD patients when compared to values found in controls. Therefore, the airway inflammation in COPD does not seem to be associated with altered GR binding. Different inflammatory mediators may be involved in COPD and in smoking and it remains to be established which mediators actually alter GR characteristics.

The actual mechanisms by which inflammatory mediators, present in the airways of smoking controls can induce a decrease in GR binding affinity in HBEC remain to be established. Others have shown that altered expression of glucocorticoid-regulated proteins appears to be mediated via interaction of the modulatory domain of the GR with transcriptional factors, such as AP-1 (24-26). Overexpression of AP-1 interferes with the function of the modulatory domain of the GR. Because cytokines can induce elevated levels of AP-1, it has been suggested that this may provide a plausible explanation for the decreased ligand binding affinity of nuclear GR for glucocorticoids found in T cells after incubation with IL-2 and IL-4 (24-26). Perhaps, a similar explanation could be given for the decreased GR binding affinity in HBEC induced by inflammatory mediators in the airways of smoking controls. Another explanation for decreased GR binding affinity could be a modulating effect of inflammatory mediators in the airways of smoking controls on the expression and phosphorylation of heat shock proteins, which are associated with the unliganded GR (27,28). Bacterial products and cytokines can regulate the expression and phosphorylation of heat shock proteins and this may modulate glucocorticoid binding to the GR (27,28). Further studies are necessary to clarify the exact mechanism of the effects of these inflammatory mediators found in the airways of smoking controls on GR.

We did not find any difference in the number of GR between COPD patients and controls, nor between smoking and nonsmoking controls. However, it cannot be excluded that differences, present between the

groups *in vivo*, disappeared during isolation and culture of these cells. We tried to clarify this issue and tested cultures of HBEC at subsequent passages. Similar numbers of GR and K_d values were found in subsequent passages. Others have found that increased GR numbers and decreased GR binding affinity in T cells from steroid resistant asthmatic patients reverted to normal after 48 h in culture (29). It would be interesting to study GR number and affinify directly on the bronchial tissue or directly after cell isolation. However, performance of a ^{3}H-dexamethasone binding assay, the most sensitive method to determine GR number and affinity, directly after cell isolation is not possible, because of the low cell number obtained. We are currently working on a method to quantify GR number in small cell samples by flow cytometry using several GR specific antibodies.

To our knowledge, no other studies have been published on GR number and K_d values in HBEC from smoking and nonsmoking COPD patients and controls. Until now, most investigations have concentrated on the analysis of GR in PBMC, comparing asthmatics with healthy individuals (30,31). No differences in GR number or GR binding affinity were found between PBMC from asthmatics and controls (23,29). Recently, Kam et al. showed that PBMC from steroid resistant asthmatics had a significantly reduced GR binding affinity and an increased GR number when compared with normal subjects (9). Others have claimed that the clinical response to glucocorticoids can not be explained by abnormal GR number or affinity (32). They suggested that the ability of GR to bind to their DNA binding sites (GRE) is impaired. Further studies are necessary to clarify whether the clinical response to inhaled glucocorticoids is related to the number and binding affinity of GR and which cells are involved in this response. As only a subgroup of COPD patients responds to inhaled glucocorticoids, it is of interest to look in resistant patients for correlations between the clinical response to glucocorticoids and the number of GR and their K_d in bronchial epithelial cells (33).

In conclusion, we demonstrated that cultured HBEC possess a single class of specific GR and that the binding affinity of GR in HBEC from smoking controls was significantly decreased when compared to values found in nonsmoking controls. These results provide further evidence that the bronchial epithelium may be an actual target for glucocorticoid therapy. Furthermore, our findings are the first indication that altered GR characteristics are present in cultured HBEC from smokers. We hypothesize that this may result from the inflammation found in the airways from smokers.

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CHAPTER 5

EXPRESSION OF LIPOCORTINS IN HUMAN BRONCHIAL EPITHELIAL CELLS: effects of IL-1β, TNF-α, LPS and dexamethasone

CHAPTER 5

EXPRESSION OF LIPOCORTINS IN HUMAN BRONCHIAL EPITHELIAL CELLS: effects of IL-1β, TNF-α, LPS and dexamethasone

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ABSTRACT

In this study, we investigated the expression of lipocortin I and II (annexin I and II) in the human bronchial epithelium, both *in vivo* and *in vitro*. A clear expression of lipocortin I and II protein was found in the epithelium in sections of bronchial tissue. In cultured human bronchial epithelial cells we demonstrated the expression of lipocortin I and II mRNA and protein using Northern blotting, FACScan analysis and ELISA. No induction of lipocortin I or II mRNA or protein was observed after incubation with dexamethasone. Stimulation of bronchial epithelial cells with IL-1β, TNF-α or LPS for 24 h did not affect the lipocortin I or II mRNA or protein expression, although PGE_2 and 6-keto-$PGF_{1\alpha}$ production was significantly increased. This IL-1β- and LPS-mediated increase in eicosanoids could be reduced by dexamethasone, but was not accompanied by an increase in lipocortin I or II expression. In human bronchial epithelial cells this particular glucocorticoid action is not mediated through lipocortin I or II induction.

INTRODUCTION

The bronchial epithelium is considered to play an important role in initiating and perpetuating inflammatory and immunological reactions by production of a variety of inflammatory mediators. Therefore, it is thought that human bronchial epithelial cells (HBEC) may play a role in inflammatory pulmonary diseases such as asthma. Upon *in vitro* stimulation with inflammatory agents such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and lipopolysaccharide (LPS), HBEC are able to produce several cytokines, such as IL-1, interleukin-6 (IL-6), interleukin-8 (IL-8), granulocyte-macrophage colony-stimulating factor (GM-CSF) and monocyte chemoattractant factor-1

(MCP-1) (1-3). Furthermore, HBEC exposed to various stimuli *in vitro* release several arachidonic acid metabolites, including 15-hydroxyeicosatetraenoic acid (15-HETE), prostaglandin E_2 (PGE_2) and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) (4,5). In addition to these pro-inflammatory properties, HBEC could also be involved in anti-inflammatory reactions through the production of potential anti-inflammatory proteins, e.g. lipocortins.

Lipocortin I and II (annexin I and II) are members of the annexin family of Ca^{2+}-dependent phospholipid-binding proteins (6). Biological evidence suggests that (at least some) members of this family are glucocorticoid inducible proteins with anti-inflammatory properties (7). It has been proposed that lipocortins I and II mediate part of the immunosuppressive activity of glucocorticoids by inhibiting phospholipase A_2 (PLA_2) activity, hereby preventing eicosanoid production (8). In some recent studies, however, the induction of lipocortin I and II by glucocorticoids was not observed (9-11). Other biological functions for lipocortins have also been reported, such as the regulation of cell differentiation and growth, and a role in the central nervous system and neuroendocrine system (reviewed in 12).

Animal studies have suggested that the lung is a rich source of lipocortin I (13-16). In the human lung, lipocortin synthesis has been described in blood leukocytes and alveolar macrophages (17,18). In cultured human tracheal submucosal gland cells production of lipocortin-like proteins have been found (19). However, to our knowledge, no data are available on the presence of lipocortins in HBEC.

The aim of this study was to investigate the expression of lipocortin I and II in HBEC and in the human bronchial epithelial cell line BEAS 2B, and to examine whether these potential anti-inflammatory proteins could be induced by glucocorticoids. Furthermore, we investigated the effect of inflammatory agents such as IL-1β, TNF-α and LPS on the lipocortin expression. Finally, we studied whether the effects of dexamethasone on the IL-1β- and LPS-stimulated production of PGE_2 and 6-keto-$PGF_{1\alpha}$ correlated with an induction of lipocortin I and II.

MATERIALS AND METHODS

Isolation and culture conditions of HBEC

Bronchi were obtained from patients undergoing surgery for lung cancer. Only bronchial tissue distant from the tumor and having a normal macroscopic appearance was used. Freshly removed tissue samples were collected in cold sterile hepes-buffered RPMI (GIBCO, Paisly, UK), supplemented with penicillin G sodium (100 U/ml; Gist-Brocades, Delft, The Netherlands) and streptomycin sulfate (0.1 mg/ml; Biochrom KG, Berlin, Germany) for transport to the laboratory. Tissue samples were cut into pieces, washed with cold phosphate buffered saline (PBS), and incubated either

overnight at 4°C or 1 h at 37°C in hepes-buffered RPMI containing 0.1% protease XIV (Sigma, St. Louis, MO), penicillin G sodium (100 U/ml) and streptomycin sulfate (0.1 mg/ml). Subsequently, epithelial cells were gently scraped from the tissue samples, washed twice in culture medium and plated onto 35-mm dishes at a density of 2.5×10^5 cells/dish.

HBEC were cultured in a 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F12 (DMEM/F12)(GIBCO), supplemented with insulin (0.01 mg/ml; Sigma), hydrocortisone (1 µg/ml; Pharma Chemie, Haarlem, The Netherlands), transferrin (0.01 mg/ml; Behring Marburg, Germany), epidermal growth factor (EGF) (10 ng/ml; Collaborative Research Inc., Lexington, MA), fetal calf serum (FCS) (1%), Na_2SeO_3 (50 nM), glutamine (1 mM; JT Baker bv., Deventer, The Netherlands), penicillin G sodium and streptomycin sulfate (0.1 mg/ml). Cells were characterized as epithelial cells by immunofluorescence staining using a mouse monoclonal antibody directed against a number of human cytokeratins (CK-1; DAKOpatts, Glostrup, Denmark). At least 99% of the isolated cells stained positive for cytokeratin (n = 5).

Human bronchial epithelial cell line

BEAS 2B is a SV-40/adenovirus transformed human bronchial epithelial cell line, which was kindly provided by Dr J. Lechner (Inhalation Toxicology Research Institute, Albuquerque, NM) (20). Cells were maintained in a keratinocyte growth medium containing bovine pituitary extract, EGF, penicillin G sodium and streptomycin sulfate (KGM; GIBCO) (21). Plastic cell culture plates (Falcon, Becton Dickinson, NJ) were precoated as described by Lechner et al. with a mixture of human fibronectin (10 µg/ml; Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands), collagen (Vitrogen 100, 30 µg/ml; Collagen Corp., Palo Alto, CA) and bovine serum albumin (BSA) (10 µg/ml; Boehringer, Mannheim, Germany) in PBS (22). Before using the BEAS 2B cells for experiments, cells were cultured for 1 passage in DMEM/F12 with supplements, as described for HBEC cells.

Immunofluorescence and immunoperoxidase stainings of cells and tissue

Immunostainings were performed with a rabbit polyclonal antibody against lipocortin I, kindly provided by Dr R.B. Pepinsky and a mouse monoclonal antibody against lipocortin II (Oncogene Science Inc. Manhasset, NY). All antibody reagents were diluted in PBS containing (wt/vol) 0.5% BSA and 0.1% sodium azide. Normal mouse serum, normal rat serum and 2 subclass specific antibodies were used as negative controls.

Cells

For fluorescence activated cell scan (FACScan) experiments cells were detached with 0.02 % EDTA, fixed for 40 min at room temperature (RT) with Permea-Fix Reagent (Ortho Diagnostic Systems, Beerse, Belgium) and washed for 10 min with PBS/BSA (0.5%). The cells were then incubated with anti-lipocortin I or II antibodies for 30 min on ice. After washing with PBS/BSA, cells were incubated for another 30 min with either fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit-IgG against lipocortin I or FITC-conjugated goat anti-mouse-IgG against lipocortin II.

Subsequently, the cells were washed and staining was analyzed and quantified using a FACScan flow cytometer (Becton Dickinson).

Tissue

Bronchi specimens for immunohistochemistry, from 1 female and 2 male non-asthmatic patients, were quickly frozen in liquid nitrogen and stored at -80°C. Six µm frozen bronchi sections were fixed in acetone for 1 min at RT and incubated with anti-lipocortin I or II antibodies for 60 min in a humidified chamber. The sections were then washed twice with PBS/Tween (0.1%) and incubated for 60 min with horse radish peroxidase (HRP)-conjugated swine anti-rabbit-IgG or HRP-conjugated rabbit anti-mouse-IgG for detection of lipocortin I and II, respectively (DAKOpatts). After three washing procedures with PBS/Tween, peroxidase activity was measured using diaminobenzidine tetrahydrochloride (Sigma) as substrate.

Stimulation experiments with IL-1β, TNF-α and LPS in the presence of dexamethasone

All experiments were performed on days 7 through 10 of primary culture of HBEC. Twenty four h before treatment with cytokines and/or dexamethasone the medium was replaced by a basal medium consisting of DMEM/F12 without hydrocortisone or other supplements to prevent influence of endogenous steroids. IL-1β (20 ng/ml; UBI, Lake Placid, NY), TNF-α (20 ng/ml; UBI), LPS (10 or 100 µg/ml; Difco Laboratories, Detroit, MI) and/or dexamethasone (10^{-6} M; Duchefa bv., Haarlem, The Netherlands) were added to the basal medium. After 24 h, supernatants were collected. Total RNA was isolated as described below. Supernatants and RNA were stored at -20°C and -80°C, respectively, until analysis.

RNA isolation, Northern blot analysis and probes

Total RNA was extracted as described previously and stored at -80°C until used (23). Northern blotting and hybridization were performed as described previously (24). The lipocortin I and II probes were a 1.3 and a 0.9 kb *Eco*RI fragment, respectively, kindly provided by Dr B.P. Wallner (Biogen Research Corp., Cambridge, MA). The glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) probe was a 0.7 kb *Eco*RI-*Pst*I fragment (25). The IL-8 probe was a 1.3 kb *Eco*RI fragment, kindly provided by Dr T.J. Stoof (Department of Dermatology, VU Hospital, Amsterdam, The Netherlands). The intensity of the lipocortin I, lipocortin II, IL-8 and GAPDH mRNA signals on the autoradiograph were scanned with a handscanner (Colorscanner 2^{24}, Highscreen, Würselen, Germany) at a resolution of 100 dots per inch. Computer software described by Koning et al. was used to analyze the intensity of the bands (26). Values were expressed as the ratio of lipocortin to GAPDH mRNA intensity.

Analysis of extracellular lipocortin I in culture supernatants

Extracellular lipocortin I levels were kindly measured by dr Susan F. Smith, using a competitive ELISA (27).

Production of arachidonic acid metabolites

The arachidonic acid metabolites PGE_2 and 6-keto-$PGF_{1\alpha}$ were measured by radioimmunoassay as described previously (28). Arachidonic acid metabolite release was normalized to total RNA content.

Statistical analysis

The Mann-Withney U test was used to assess significant differences in PGE_2 and 6-keto-$PGF_{1\alpha}$ production, and in lipocortin I and II mRNA expression in cell cultures under different conditions of incubation; a p-value of less than 0.05 was considered significant.

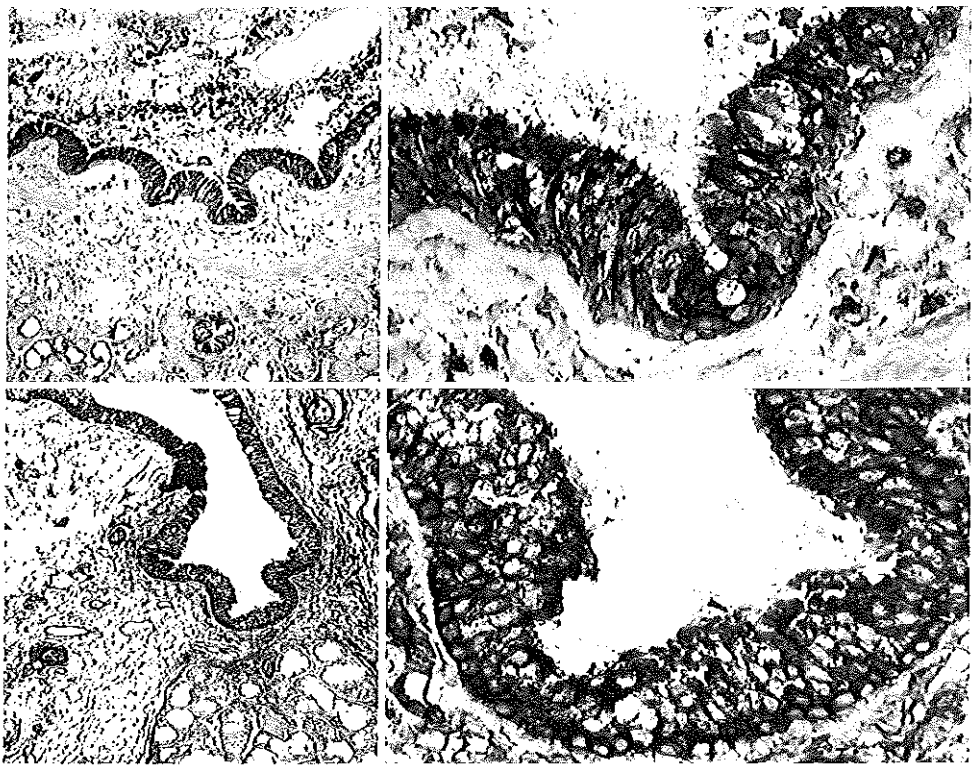

Figure 1. Immunoperoxidase staining for lipocortin I (upper panel) and II (lower pannel) of frozen sections of human bronchi. **Left:** magnification 6.3x. **Right:** magnification 40x.

RESULTS

Lipocortin I and II expression *in vivo*

Immunoperoxidase staining of bronchi sections (n=3) with anti-lipocortin I and II antibodies showed a strong expression in the bronchial epithelium (Figure 1). Similar staining patterns were observed for lipocortin I and II. Positive staining for lipocortin I and II was also found in the epithelial cells of the submucosal glands.

Lipocortin I and II expression *in vitro*

In cultured HBEC and in BEAS 2B cells lipocortin expression was studied using FACScan and Northern blot analysis. With FACScan analysis we found that more than 99% of BEAS 2B cells were positive for both intracellular lipocortin I and II (Figure 2). Fifty and 80% of cultured HBEC (n=3) were positive for intracellular lipocortin I and II, respectively. A representative experiment is shown in Figure 2. Northern blot analysis showed expression of lipocortin I and II mRNA, 1.4 and 1.6 kb respectively, in both cultured HBEC and BEAS 2B cells (Figures 3A and 3B).

Effects of IL-1β, TNF-α, LPS and dexamethasone on lipocortin I and II mRNA and protein expression

To investigate the effect of inflammatory agents and dexamethasone on lipocortin I and II mRNA and protein expression, HBEC and BEAS 2B cells were incubated with different concentrations of IL-1β, TNF-α, LPS and dexamethasone. Incubation for 24 h with IL-1β, TNF-α, LPS or dexamethasone did not significantly affect lipocortin I or II mRNA expression in cultured

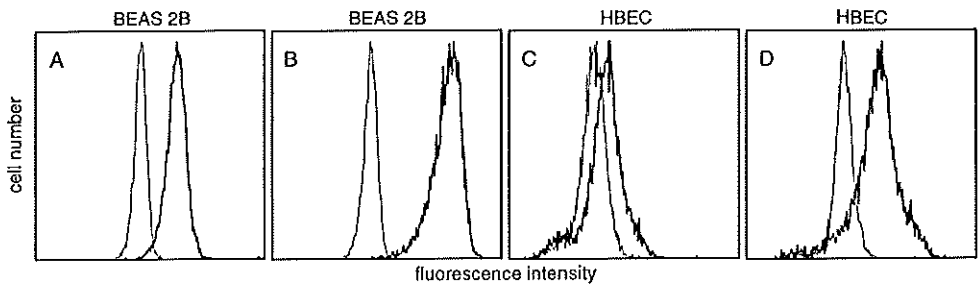

Figure 2. FACS analysis of intracellular lipocortin I (A,C) and II (B,D) expression in BEAS 2B cells and in HBEC. Representative histograms, indicating nonspecific fluorescence intensity (*thin line*) and lipocortin I or II expression (*bold line*) are shown.

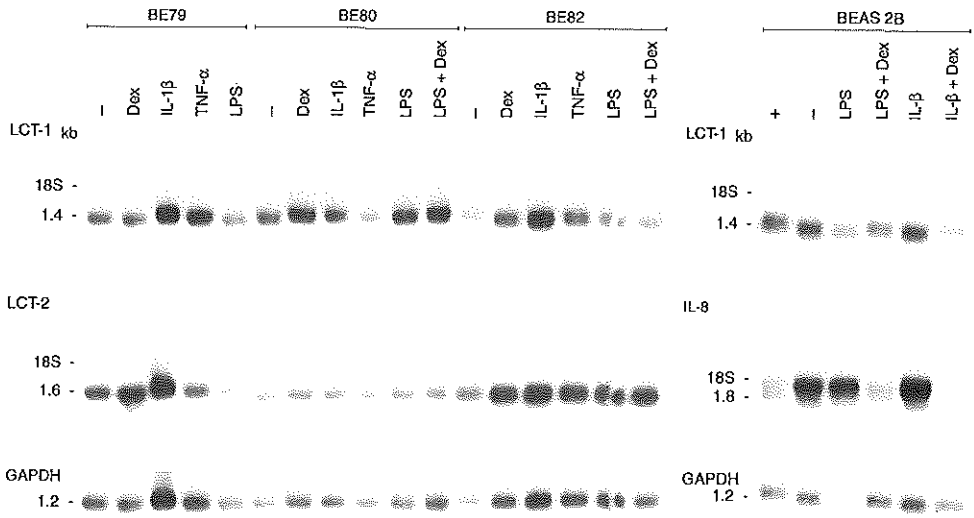

Figure 3. Effects of IL-1β, TNF-α, LPS and dexamethasone on lipocortin I and II mRNA expression. Northern blot analysis of HBEC and BEAS 2B cells. HBEC from 3 different patients were cultured for 24 h in a basal medium of DMEM/F12 (lanes 1,6,12), with 10^{-6} M dexamethasone (lanes 2,7,13), with 20 ng/ml IL-1β (lanes 3,8,14), with 20 ng/ml TNF-α (lanes 4,9,15), with 100 µg/ml LPS (lanes 5,10,16), or with 100 µg/ml LPS and 10^{-6} M dexamethasone (lanes 11,17). BEAS 2B cells were cultured for 24 h in DMEM/F12 supplemented with insulin, hydrocortisone, transferrin, EGF and FCS (lane 1), in a basal medium of DMEM/F12 (lane 2), with 10 µg/ml LPS (lane 3), with 10 µg/ml LPS and 10^{-6} M dexamethasone (lane 4), with 20 ng/ml IL-1β (lane 5), or with 20 ng/ml IL-1β and 10^{-6} M dexamethasone (lane 6). The filters were hybridized to ^{32}P-labeled lipocortin I (LCT-1), lipocortin II (LCT-2), IL-8 and GAPDH probes.

HBEC or in BEAS 2B cells (Figures 3A and 3B). IL-8 mRNA was used as a positive control, as it has been shown previously to increase upon stimulation with IL-1β or LPS, and to decrease upon incubation with dexamethasone. Figure 3B shows that IL-8 mRNA expression in BEAS 2B cells was decreased after incubation with dexamethasone, whereas lipocortin I mRNA expression was not increased. The results of the experiments (n=5), in which the effects of IL-1β, TNF-α, LPS and dexamethasone on the basal lipocortin I mRNA expression in cultures of HBEC were studied, is shown in Figure 4. Using FACScan analysis and ELISA no effect of IL-1β, TNF-α, LPS or dexamethasone was observed on the lipocortin I or II protein expression in HBEC or BEAS 2B cells (data not shown).

Effects of IL-1β, TNF-α, LPS and dexamethasone on PGE_2 and 6-keto-$PGF_{1\alpha}$ production, and correlation with lipocortin mRNA expression

The effect of IL-1β, TNF-α, and LPS on PGE_2 and 6-keto-$PGF_{1\alpha}$ production was measured, and we studied whether the expected inhibition of

stimulated eicosanoid production by dexamethasone correlated with an induction of lipocortin I and II. Incubation of HBEC (n=5) for 24 h with either IL-1β, or TNF-α or LPS significantly increased basal PGE_2 and 6-keto-$PGF_{1\alpha}$ production ($p < 0.01$) (Figure 4). PGE_2 and 6-keto-$PGF_{1\alpha}$ production in one representative culture of HBEC is shown in Table 1. The LPS- and IL-1β-mediated increases in PGE_2 and 6-keto-$PGF_{1\alpha}$ production were reduced by dexamethasone. Reduction of the LPS-mediated increase of PGE_2 by dexamethasone was statistically significant ($p < 0.05$). Dexamethasone also clearly reduced the IL-1β- mediated increase of both PGE_2 and 6-keto-$PGF_{1\alpha}$, but statistical significance could not be reached because of low sample number. Reduction of the IL-1β- and LPS-mediated PGE_2 and 6-keto-$PGF_{1\alpha}$ production by dexamethasone was not accompanied by an increase in lipocortin I mRNA (figure 4) or lipocortin II mRNA (data not shown).

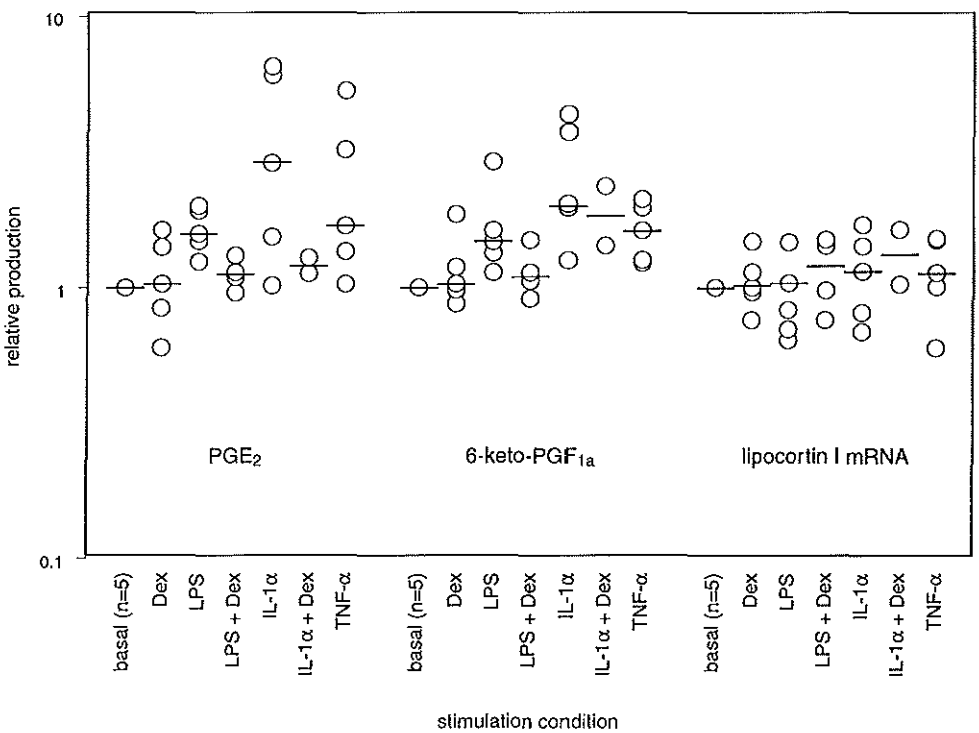

Figure 4. Effect of 24 h incubation with IL-1β (20 ng/ml), TNF-α (20 ng/ml), LPS (100 μg/ml) and dexamethasone (10^{-6} M) on the basal lipocortin I mRNA expression and the basal release of PGE_2 and 6-keto-$PGF_{1\alpha}$ in cultures of HBEC (n=5). PGE_2 and 6-keto-$PGF_{1\alpha}$ release and lipocortin I mRNA expression are expressed relative to the basal production. Lipocortin I mRNA expression was corrected for GAPDH mRNA expression. The solid bars indicate median values.

Table 1. PGE_2 and 6-keto-$PGF_{1\alpha}$ production in a representative culture of HBEC

stimulation condition [a]	PGE_2 [b]	6-keto-$PGF_{1\alpha}$ [b]
Basal	5.68	1.60
Dex	3.43	1.42
LPS	8.98	4.68
LPS + Dex	7.60	1.45
IL-1β	16.51	3.25
IL-1β + Dex	7.31	2.29
TNF-α	7.71	2.60

[a] HBEC were incubated for 24 h with IL-1β (20 ng/ml), TNF-α (20 ng/ml), LPS (100 μg/ml) and/or dexamethasone (Dex)(10^{-6} M).
[b] PGE_2 and 6-keto-$PGF_{1\alpha}$ production are expressed as $ng.ml^{-1}.mg^{-1}$ total RNA.

Analysis of the inducibility of lipocortin I and II mRNA by dexamethasone

To evaluate the effect of dexamethasone on lipocortin I and II mRNA expression more precisely we cultured HBEC and BEAS 2B cells for up to 5 days without glucocorticoids. The cells were then incubated with different concentrations of dexamethasone (10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M) and with 10^{-6} M dexamethasone for various lengths of times (2, 4, 6, 10, 24 and 48 h). Both the effects of dexamethasone on cells cultured in a medium of DMEM/F12 without supplements and on cells in a medium of DMEM/F12 supplemented with insulin, transferrin, EGF, Na_2SeO_3, and charcoal-stripped FCS, but without hydrocortisone were examined. Under none of these conditions dexamethasone affected the expression of lipocortin I or II mRNA (data not shown).

DISCUSSION

We demonstrated in this study that lipocortin I and II are expressed in cultured HBEC, a bronchial epithelial cell line and in the epithelium in bronchi sections. Immunoperoxidase stainings of human bronchi sections with anti-lipocortin I and II antibodies showed a clear expression in the bronchial epithelium and in the epithelial cells of the submucosal glands. In cultured HBEC and in the BEAS 2B cell line, we demonstrated the presence of lipocortin I and II mRNA and protein by Northern blotting and FACScan analysis, respectively. Lipocortin I and II mRNA and protein expression in the HBEC was not affected by incubation of the cells with IL-1β, TNF-α, LPS or dexamethasone. The HBEC were able to respond to these stimuli, as the production of PGE_2 and 6-keto-$PGF_{1\alpha}$ significantly increased upon incubation with IL-1β, TNF-α or LPS. This increased production was reduced by

dexamethasone. Reduction by dexamethasone of the stimulated production of PGE_2 and 6-keto$PGF_{1\alpha}$ was not accompanied by an increase in lipocortin I or II expression. Thus, in human bronchial epithelial cells this particular glucocorticoid action is not mediated through lipocortin I or II induction.

Our findings on the expression of lipocortin I in the human bronchus are in agreement with those in the airway epithelium of the rat, studied by Fava and Cohen (29). They demonstrated the presence of lipocortin I in the rat epithelium of nasal, tracheal and bronchial airways, and in the ductal epithelium of various glands. Production of lipocortin-like proteins in cultured human tracheal submucosal gland cells has been described by Jacquot et al. (19). In the fetal and neonatal human lung, lipocortin I immunostaining was found in the bronchiolar epithelium as early as 12 weeks, beginning with the largest airways, and by 24 weeks extending distally to the bronchioloalveolar portals (30). In bovine bronchial epithelial cells a differential expression of lipocortin I and II was found (16). Lipocortin I was expressed in the ciliated cells, whereas lipocortin II was expressed in the basal cells. We did not find such a differential expression of lipocortin I and II in human bronchi in our studies.

The bronchial epithelium is considered to play an important role in inflammatory and immunological reactions by production of a variety of inflammatory mediators as can be observed in inflammatory pulmonary diseases such as asthma. In addition to this role, the bronchial epithelium could also be involved in anti-inflammatory reactions through the production of anti-inflammatory proteins, e.g. lipocortins. In this study we found a high expression of lipocortin I and II in the human bronchial epithelium. A physiological role has been proposed for lipocortin I recently. Lipocortin I may function as a 'barrier' to inappropriate inflammatory and autoimmune responses at specific sites around the body (7). Following an inflammatory stimulus, stress-induced stimulation of the hypothalamic-pituitary-adrenal axis (HPA) axis may result in the release of cortisol, which in turn leads to the production of lipocortin I. The lipocortin-binding molecules on monocytes and neutrophils arriving at tissue sites are expected to become saturated, with resultant moderation of migratory and/or pro-inflammatory activities. Others have speculated from results in animal studies that epithelial cell damage and loss, as seen in asthmatics, could decrease availability of these protective properties of lipocortin I (16). Increased inflammation would thereby be predicted.

We did not observe an induction of lipocortin I or II by dexamethasone in cultures of HBEC or in the BEAS 2B cell line. An elevated production of lipocortin I in response to glucocorticoids has been demonstrated in human peripheral blood monocytes, human alveolar macrophages, rat alveolar epithelial cells and in bovine bronchial epithelial cells (15-18). Glucocorticoids increased amounts of lipocortin I in these cells in a dose dependent manner. However, Brönnegard et al. did not observe an induction of lipocortin I mRNA

by dexamethasone in seven different cell types, including primary human macrophages (11). The synthesis of lipocortin I does not appear to be under glucocorticoid control in certain cell lines and has been linked with cell differentiation events in some cases (7,31,32). Isacke et al. were unable to observe any effect of dexamethasone treatment in U-937 cells under a variety of conditions on the expression of lipocortin I or II, nor did dexamethasone induce their secretion (10). An induction by dexamethasone of the expression of mRNA of lipocortin I and II and the release of lipocortin I and V was observed in differentiated, but not in undifferentiated U-937 cells by Sollito et al. (32).

For the induction experiments we used both HBEC, just a few days after isolation and after culturing for 1 or two weeks. Other studies point to the importance of culture conditions in studying synthesis of lipocortins by cultured cells (31). In our experiments we have used charcoal-stripped FCS in the culture medium so that endogenous steroids, which might influence lipocortin synthesis, are removed. We have incubated epithelial cells for up to 5 days in steroid-free medium prior to the addition of dexamethasone, to ensure that the cells were in an unstimulated condition at the beginning of the experiment. As growth factors, such as EGF, are thought to induce lipocortin synthesis, we have also performed experiments, in which cells were cultured in a basal medium without growth factors. In all cases we found a clear mRNA and protein expression of lipocortin I and II in HBEC, but no induction of lipocortins by dexamethasone after incubation for various length of times with different concentrations. After culturing for 5 days in steroid free medium, lipocortin I mRNA was expressed constitutively at approximately 90% of the lipocortin I mRNA level in complete medium, containing hydrocortisone. Vishwanatha et al. found in bovine bronchial epithelial cells, that the constitutive expression of lipocortin I was 20% of the lipocortin I level in medium containing hydrocortisone after culturing for 5 days (16). These studies indicate that there is a difference between species in the inducibility of lipocortins by glucocorticoids.

In summary, we found a high expression of lipocortin I and II in the bronchial epithelium compared to the underlying layers in human bronchi sections and demonstrated that glucocorticoids do not increase the expression of lipocortin I and II in HBEC. Our study indicates, that decrease in PGE_2 and 6-keto-$PGF_{1\alpha}$ production in HBEC upon incubation with glucocorticoids is not mediated by increased expression of lipocortins.

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CHAPTER 6

MCP-1 RELEASE BY HUMAN BRONCHIAL EPITHELIAL CELLS: modulation by cytokines and glucocorticoids

CHAPTER 6

MCP-1 RELEASE BY HUMAN BRONCHIAL EPITHELIAL CELLS: modulation by cytokines and glucocorticoids

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ABSTRACT

Airway inflammation is characterized by an accumulation of activated leukocytes. The human bronchial epithelium is known to be an active participant in the initiation, modulation and perpetuation of this inflammatory process. Monocyte chemoattractant protein-1 (MCP-1) is a potent chemoattractant for predominantly monocytes. Previously, MCP-1 expression has been described in human bronchial epithelial cells. In this study we analyzed the effects of pro-inflammatory cytokines and glucocorticoids on MCP-1 release by human bronchial epithelial cells.

Primary cultures of human bronchial epithelial cells release low levels of MCP-1. IFN-γ induced a more than 10-fold increase in MCP-1 release, whereas IL-1β and TNF-α induced a 6-, 2-fold increase in MCP-1 release by human bronchial epithelial cells, respectively. The MCP-1 increase by IFN-γ was dose- and time-dependent. IFN-γ had no effect on the release of IL-8 in human bronchial epithelial cells, whereas IL-1β and TNF-α increased the IL-8 release in these cells. Dexamethasone decreased the cytokine-induced release of MCP-1 and IL-8 by human bronchial epithelial cells. The IFN-γ-, IL-1β- and TNF-α-induced release of MCP-1 was inhibited by 29, 48 and 32%, respectively, by the addition of dexamethasone.

Our findings indicate a role for human bronchial epithelial cells in the recruitment and activation of monocytes during airway inflammation through the release of MCP-1. The data suggest that MCP-1 and IL-8 release in human bronchial epithelial cells are differentially regulated, depending upon the presence of different cytokines. This indicates that the type of cellular infiltrate and the progress of airway inflammation is likely to depend on the kind of stimulatory cytokines present. One beneficial effect of glucocorticoid therapy in airway inflammatory diseases may be inhibition of the MCP-1

release by the bronchial epithelial cells.

INTRODUCTION

During airway inflammation, peripheral blood monocytes migrate out of the vascular space into the airways in response to several signals, including chemotactic factors produced at the inflammatory focus (1,2). Monocyte chemoattractant protein-1 (MCP-1), a member of the C-C branch of the chemokine family, may play a role in this process. MCP-1 has chemotactic properties for monocytes, basophils, T lymphocytes and NK cells (1). In addition, MCP-1 activates monocytes and basophils, and can induce leukocyte adhesion molecules on endothelial and vascular smooth muscle cells (3-6).

The bronchial epithelium is actively involved in the modulation and perpetuation of inflammatory reactions in the airways through the synthesis of arachidonic acid metabolites, such as prostaglandin E_2 (PGE_2) (7,8), cytokines including GM-CSF and IL-6 (9,10) and chemokines such as IL-8 (9,10), MCP-1 (13) and RANTES (12).

Using immunohistochemistry, increased expression of MCP-1 has been found in the bronchial epithelium from asthmatic subjects compared to normal subjects (14). In short term cultured human bronchial epithelial cells, MCP-1 mRNA release was observed to be stimulated by TNF-α (13). These data implicate the bronchial epithelium as a source of MCP-1. Therefore, we investigated the effect of cytokines involved in inflammation, such as IFN-γ, IL-1β, and TNF-α on the release of MCP-1 by human bronchial epithelial cells. The effect of these inflammatory mediators on the release of MCP-1 was compared with the effect on the release of IL-8. IL-8 is a member of the C-X-C branch of the chemokine family and is *in vitro* predominantly chemotactic for neutrophils (15). There is abundant evidence that bronchial epithelial cells can be induced to release IL-8 (16-18).

Glucocorticoids are the most effective drugs in the treatment of asthma and are also frequently applied in chronic obstructive pulmonary disease (COPD) (19). Chronic treatment with inhaled glucocorticoids reduces the number of mast cells, eosinophils, lymphocytes and monocytes (20). To investigate whether the anti-inflammatory effects of glucocorticoids can be mediated by inhibition of chemokine release, we examined the effect of dexamethasone on the release of MCP-1 by human bronchial epithelial cells.

MATERIALS AND METHODS

Cell culture

Bronchial tissue was obtained from patients undergoing surgery for lung cancer and used immediately for culture of human bronchial epithelial cells by a cell culture method described previously (18). Briefly, bronchial tissue distant from the tumor was cut into pieces and incubated either overnight at 4°C or 1 h at 37°C in 0.1% protease XIV (Sigma, St. Louis, MO). Subsequently, epithelial cells were gently scraped from the tissue samples, washed twice in culture medium and plated onto 25-mm^2 culture flasks or onto 6-well culture dishes. Human bronchial epithelial cells were cultured in a 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F12 (DMEM/F12) (GIBCO, Paisly, UK), with supplements as described previously (21). Cells were characterized as epithelial cells by immunofluorescence staining using a mouse monoclonal antibody directed against a number of human cytokeratins (CK-1; DAKOpatts, Glostrup, Denmark). At least 99% of the isolated cells stained positive for cytokeratin.

The human bronchial epithelial cell lines, BEAS 2B, transformed by an adenovirus 12-SV-40 hybrid virus, was kindly provided by Dr J. Lechner (Inhalation Toxicology Research Institute, Albuquerque, NM) (22) and was cultured as described previously (23).

Stimulation experiments with IFN-γ, IL-1β, TNF-α and dexamethasone

To study the effects of cytokines and dexamethasone, human bronchial epithelial cells or BEAS 2B cells were plated onto 6-well culture dishes (0.5×10^6 and 0.25×10^6 cells/well, respectively). After the cells reached 80 to 90% confluence, the culture medium was replaced by a basal medium (consisting of DMEM/F12 (1:1) with penicillin G sodium and streptomycin sulfate, but without other supplements) and IFN-γ (5 ng/ml; Boehringer, Ingelheim, Germany) IL-1β (20 ng/ml; UBI, Lake Placid, NY), TNF-α (20 ng/ml UBI) and/or dexamethasone (10^{-6} M; Duchefa bv., Haarlem, The Netherlands) were added. Supernatants were collected after 24 h (unless stated otherwise), centrifuged and stored at -80°C for subsequent analysis by an ELISA specific for MCP-1 or IL-8.

Analysis of MCP-1 and IL-8 release

Levels of immunoreactive MCP-1 in the culture medium were quantified in a previously described sandwich ELISA (24), using a novel, highly specific monoclonal antibody against MCP-1 (5D3-F7 mAb; IgG1κ) and a polyclonal rabbit anti-MCP-1 serum. The sensitiviy of the sandwich ELISA was ~30 pg/ml.

Levels of immunoreactive IL-8 in the culture medium were quantified using a commercially available ELISA (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands), according to the manufacturer's instruction. This ELISA was sensitive to ~300 pg/ml IL-8.

Statistical analysis

Data are expressed as mean ± SEM. The Mann-Whitney U test was used to assess significant differences in MCP-1 release between stimulated and unstimulated cultures of human bronchial epithelial cells. A p-value of less than 0.05 was considered significant.

RESULTS

Modulation of MCP-1 release by cytokines and dexamethasone

The release of MCP-1 by human bronchial epithelial cells in primary culture and its modulation by cytokines involved in inflammation was investigated. Unstimulated human bronchial epithelial cells released small amounts of MCP-1 (Figure 1A). MCP-1 release was significantly increased when human bronchial epithelial cells were stimulated for 24 h with IFN-γ, IL-1β or TNF-α ($p<0.05$) (Figure 1A). IFN-γ induced a more than 10-fold increase in MCP-1 release, whereas IL-1β and TNF-α only induced a 6- and 2-fold increase, respectively. Costimulation of human bronchial epithelial cells with cytokines and dexamethasone decreased the cytokine-induced release of MCP-1. By addition of dexamethasone, the IFN-γ-, IL-1β- and TNF-α-induced release of MCP-1 was inhibited by 29, 48 and 32%, respectively.

The effect of inflammatory mediators on the release of MCP-1 was compared with the effect on the release of IL-8, another chemokine produced by human bronchial epithelial cells. In contrast to MCP-1, IFN-γ had no effect on the IL-8 release by human bronchial epithelial cells (92% compared to unstimulated cells) (Figure 1B). IL-1β and TNF-α increased the IL-8 release (331 and 118% compared to unstimulated cells, respectively). By addition of dexamethasone the IL-1β- and TNF-α-induced release of IL-8 was inhibited by 27 and 14%, respectively.

Similar stimulation experiments were performed with BEAS 2B cells in order to investigate whether these cells are an appropriate model for MCP-1 studies in primary cultures of human bronchial epithelial cells. The results obtained with BEAS 2B cells were similar to the results found in primary cultures of human bronchial epithelial cells. Stimulation of BEAS 2B cells with IFN-γ (5 ng/ml) for 24 h resulted in an MCP-1 release of 700 ± 260% ($n=3$) of unstimulated cells.

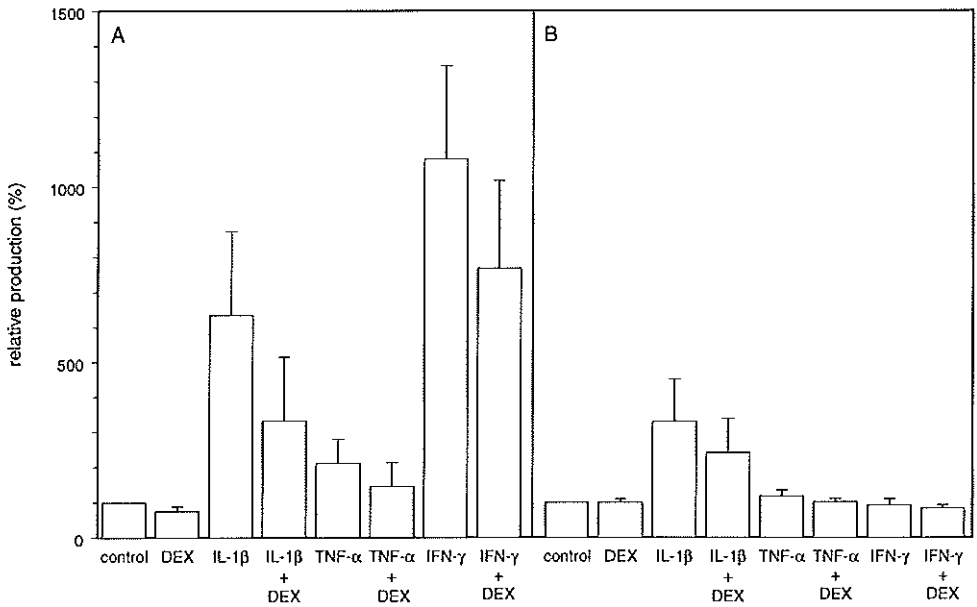

Figure 1. Modulation of MCP-1 and IL-8 release. Human bronchial epithelial cells were stimulated with IFN-γ (5 ng/ml), IL-1β (20 ng/ml) or TNF-α (20 ng/ml) in the presence or absence of dexamethasone (10^{-6} M) for 24 h, after which MCP-1 (A) or IL-8 (B) release were determined by ELISA. Data are expressed as percentage release compared to unstimulated cells (mean ± SEM; n=11 (MCP-1) and n=3 (IL-8).

Dose- and time-dependent MCP-1 release by IFN-γ

To determine whether the MCP-1 increase by IFN-γ was dose-dependent, human bronchial epithelial cells were stimulated with increasing concentrations of IFN-γ (0.25-10 ng/ml). IFN-γ increased MCP-1 release in a concentration dependent manner (Figure 2A). For time-course experiments human bronchial epithelial cells were stimulated with IFN-γ for 2, 4, 6, 8 and 24 h. The MCP-1 concentration in the supernatant increased with time up to 24 h by IFN-γ stimulation. The increase could be detected as early as 4 h after stimulation (Figure 2B).

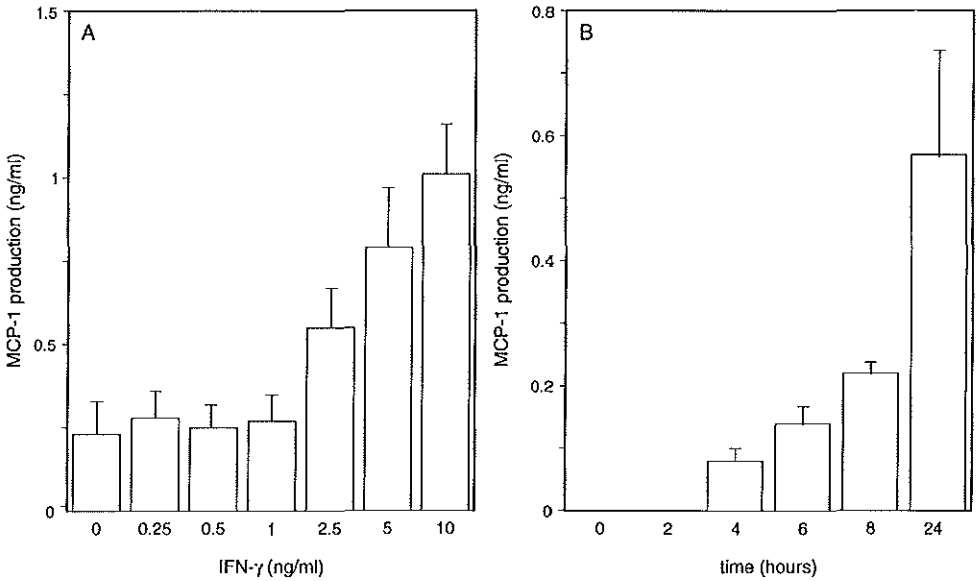

Figure 2. (A) Dose-response of IFN-γ on MCP-1 release by human bronchial epithelial cells. Cells were stimulated with increasing concentrations of IFN-γ for 24 h. (B) Time-course of IFN-γ induced MCP-1 release by human bronchial epithelial cells. Cells were stimulated with IFN-γ (5 ng/ml). Data are expressed as mean ± SEM (n=3) after substracting basal release of MCP-1 at each individual time-point.

DISCUSSION

In this study we analyzed the effects of pro-inflammatory cytokines and glucocorticoids on MCP-1 release by human bronchial epithelial cells. While basal release was low, pro-inflammatory cytokines significantly increased MCP-1 release by human bronchial epithelial cells. Incubation with IFN-γ resulted in a more than 10-fold increase in MCP-1 release, whereas IL-1β and TNF-α only induced a 6- and 2-fold increase in MCP-1 release by human bronchial epithelial cells, respectively. The increase in MCP-1 release by IFN-γ was dose- and time-dependent. IFN-γ, IL-1β and TNF-α are cytokines that are likely to be present in the lungs in airway inflammatory diseases such as asthma. IFN-γ is produced during viral infections (25), which are important triggers of asthmatic attacks (26). Increased levels of IFN-γ, produced by activated T lymphocytes, have been found in the bronchoalveolar lavage of patients with intrinsic asthma compared to control groups (27). Increased levels of IFN-γ, together with TNF-α were also observed in bronchoalveolar lavage of allergic patients after antigen challenge (28).

A variety of cell types including mononuclear phagocytes, endothelial

cells, and retinal pigment epithelial cells can produce MCP-1 in response to pro-inflammatory signals (29-32). Under these *in vitro* conditions IFN-γ was shown to be a less effective inducer of MCP-1 than IL-1 or TNF (31,32). Thus, among the cellular systems studied so far, bronchial epithelial cells are to our knowledge unique in that IFN-γ is the most potent inducer of MCP-1.

The effect of inflammatory mediators on the release of MCP-1 was compared with the effect on the release of IL-8, another chemokine produced by human bronchial epithelial cells. The expression of IL-8 by human bronchial epithelial cells and its modulation by cytokines and glucocorticoids has been described earlier by others (10,33). In contrast to the 10-fold increase in MCP-1 release, IFN-γ had no effect on the IL-8 release by human bronchial epithelial cells. IL-1β and TNF-α increased both MCP-1 and IL-8 release. These data suggest that MCP-1 and IL-8 release in human bronchial epithelial cells are differentially regulated depending upon the presence of different cytokines. This indicates that the type of cellular infiltrate and the progress of airway inflammation is likely to depend on the kind of stimulatory cytokines present. Differential regulation by IFN-γ of two chemokine genes has been described in synovial fibroblasts (34). In these cells, IFN-γ enhanced TNF-α and IL-1β-induced increase in RANTES mRNA, whereas the induction of IL-8 mRNA by TNF-α or IL-1β was inhibited by IFN-γ (34). It was suggested that IL-8 mRNA may be controlled at the level of mRNA stabilization as it contains several AUUUA sequences (35,36). The RANTES gene may be regulated by mechanisms other than (de)stabilization, since unlike IL-8, the 3'-untranslated region of RANTES mRNA does not possess AUUUA sequences (37). The MCP-1 mRNA, similarly to the RANTES mRNA, has a low (66 %) A+U content in its 3'-untranslated region compared to other proteins related to the inflammatory response. No AUUUA sequences are found in this region of the MCP-1 mRNA (2) in contrast to this region in IL-8 mRNA. Therefore, the differential effect of IFN-γ on MCP-1 and IL-8 release might be due to differences at the level of mRNA stabilization.

IL-1β and TNF-α, in contrast to IFN-γ, increased the release of both MCP-1 and IL-8 by human bronchial epithelial cells. IL-1 and TNF-α are considered as early response cytokines (38). The release of early response cytokines is crucial for induction of adhesion molecule expression, the initiation of cytokine cascades, the upregulation of specific chemokines and the recruitment of leukocyte subsets (38). IFN-γ is produced by activated T lymphocytes. Our findings and previous studies suggest that IFN-γ only increases the release of C-C chemokines, and not of C-X-C chemokines by human bronchial epithelial cells (11,12). This is consistent with the observation that IFN-γ also increases the RANTES release by human bronchial epithelial cells (11,12). Although the effect of IFN-γ was not studied on the release of other C-X-C chemokines, we hypothesize that IFN-γ may be a relatively specific stimulator for the influx of monocytes compared to IL-1β

and TNF-α, which can stimulate the recruitment of all types of leukocytes.

The exact mechanism of the induction of MCP-1 expression by cytokines remains to be clarified. The 5'-upstream transcriptional regulatory region of the human MCP-1 gene contains consensus sequences for TNF-α, IL-1β and IFN-γ responsive transcription factors, such as NF-κB and AP-1 (39-41). Previously, it was demonstrated that the consensus NF-κB element 5'-GGGAATTTCC-3', located between bp -2612 and -2603 of the MCP-1 5'-flanking region, was necessary for cytokine-induced reporter gene activity in transfected malignant cells (39). Mutation of the NF-κB site completely abrogated the response to IL-1 (39). A recent report demonstrated that the activation of NF-κB correlated with MCP-1 expression by human mesangial cells (42). AP-1 was found to be involved in the induction of MCP-1 by transforming growth factor-β in osteoblastic cells (40). Therefore, it is very likely that transcription factors such as NF-κB and AP-1 are involved in the cytokine mediated induction of MCP-1 release by human bronchial epithelial cells.

Dexamethasone decreased the cytokine-induced release of MCP-1 by human bronchial epithelial cells, but no reduction to basal levels was observed. IFN-γ and dexamethasone were added simultaneously to the cell cultures, and it would be of interest to study the inhibitory effect of dexamethasone, when cells are incubated with dexamethasone after or prior to the addition of cytokines. Until now, no studies have been performed on the molecular mechanism of dexamethasone-mediated inhibition of MCP-1. However, the presence of AP-1 and NF-κB sites in the MCP-1 gene suggest that the inhibitory effect of dexamethasone may be mediated through the antagonism by the glucocorticoid-receptor complex of the action of NF-κB and/or the action of AP-1.

In conclusion, IFN-γ is a potent stimulator of MCP-1 release by human bronchial epithelial cells, but does not affect IL-8 release. Therefore, IFN-γ may be a relatively specific stimulator of the influx of monocytes. We hypothesize that epithelial-derived MCP-1 is among other chemokines responsible for the submucosal accumulation of monocytes observed in inflammatory airway diseases. Part of the beneficial effect of glucocorticoid therapy in airway inflammatory diseases may be the inhibition of MCP-1 release by bronchial epithelial cells.

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CHAPTER 7

GENERAL DISCUSSION

CHAPTER 7

GENERAL DISCUSSION

The objectives of the studies described in this thesis were:

1. To characterize the expression of glucocorticoid receptors in human bronchial epithelial cells and to analyse the effect of inflammatory mediators on these receptors both *in vitro* and *in vivo*.

2. To investigate the cellular mechanism(s) of the anti-inflammatory actions of glucocorticoids in human bronchial epithelial cells.

7.1 Expression of glucocorticoid receptors in human bronchial epithelial cells

Glucocorticoids are the most effective drugs in the treatment of asthma and are also frequently applied in the treatment of COPD (1). Glucocorticoids are able to inhibit the release of bronchial epithelial cell-derived cytokines, which are now known to play an important role in airway inflammation (2,3). A prerequisite for glucocorticoid responsiveness is the presence of functional glucocorticoid receptors (GR). In chapters 3 and 4 the expression of GR was investigated in human bronchial epithelial cell lines and in primary cultures of bronchial epithelial cells. The expression of GR mRNA was demonstrated in two SV-40/adenovirus-transformed human bronchial epithelial cell lines, BEAS S6 and BEAS 2B. In a whole cell dexamethasone binding assay, BEAS S6 and BEAS 2B cells were found to possess $28.9 \pm 4.4 \times 10^3$ and $32.1 \pm 5.7 \times 10^3$ binding sites per cell, respectively, with K_d values of 8.2 ± 1.5 nM and 8.6 ± 2.4 nM, respectively. Using a cytosol radioligand binding assay, cultured human bronchial epithelial cells were found to contain a mean number of binding sites of 87.2 ± 16.7 fmol/mg protein with a K_d value of 0.76 ± 0.10 nM. In this binding assay, BEAS 2B cells were observed to contain a mean number of binding sites of 370 ± 14 fmol/mg with a K_d value of 0.8 ± 0.1 nM. The difference in the number of binding sites between cultured human bronchial epithelial cells and BEAS 2B cells may be caused by the SV-40/adenovirus transformation. Others have shown that Epstein-Barr Virus transformation of cultured human lymphocytes resulted in a 5-fold increase in glucocorticoid binding sites when compared to the non-transformed lymphocytes (4). However, the affinity of the GR was the same in both types of lymphocytes (4). Compared to peripheral blood mononuclear cells (PBMC), BEAS cells contain a relatively high number of glucocorticoid binding sites (2.7×10^3 and $32.1 \pm 5.7 \times 10^3$ binding sites per cell, respectively). The number and quality of GR in target cells may determine the extent of glucocorticoid responsiveness (5,6). Even if the fact is taken into account

that BEAS cells are 2-3 times larger than PBMC, BEAS 2B cells still possess a greater number of GR. These studies indicate that the bronchial epithelium may be an actual target for glucocorticoid therapy, especially in asthma in which epithelial cells may exhibit pro-inflammatory functions. Therefore, we hypothesize that glucocorticoid therapy may suppress airway inflammation, at least partly by modulating the pro-inflammatory function of bronchial epithelial cells.

In a previous study it was shown that inflammatory mediators, such as IL-2 and IL-4, may modulate the GR number and affinity in human lymphocytes (7). Therefore, we examined the effects of inflammatory stimuli on the expression of GR in human bronchial epithelial cells both *in vitro* (chapter 3) and *in vivo* (chapter 4). LPS and IL-1β significantly increased the number of GR per cell, but significantly reduced the ligand affinity of these receptors in BEAS 2B cells. Northern blotting experiments showed that the GR mRNA expression was not altered after incubation with LPS or IL-1β. Therefore, the increase in GR number after treatment with LPS or IL-1β could not be explained by an increase in GR mRNA level. Increased translation or increased half-life time of GR mRNA could provide an explanation for the increase in GR number. Studies using actinomycin D may resolve this question.

To study the effect of inflammation on GR expression *in vivo*, we analyzed the GR number and affinity in cultured human bronchial epithelial cells from healthy controls, smokers and COPD patients. In smoking individuals and in COPD patients general airway inflammation is known to be present (8). Bacterial colonization of the bronchi has been found in smoking subjects, and thus LPS is likely to be present (9). Excessive numbers of macrophages and neutrophils were detected in the airways from smokers and from patients with bronchitis (8). Therefore, increased expression of IL-1 can be expected in the bronchi of smokers and COPD patients. The GR binding affinity in smoking controls was significantly decreased compared with nonsmoking controls, but no significant difference was found between the mean number of binding sites. The finding that *in vivo* inflammation present in smokers affects GR characteristics in human bronchial epithelial cells is in accordance with our above mentioned findings *in vitro*. In contrast to the differences in binding affinity found in smoking controls, we did not find any difference in the binding affinity of the GR in COPD patients when compared to values found in controls. Therefore, the airway inflammation in COPD does not seem to be associated with altered GR binding. Different inflammatory mediators may be involved in COPD and in smoking and it remains to be established which mediators actually alter GR characteristics. No difference was observed in the number of glucocorticoid binding sites between COPD patients and controls, nor between smoking and nonsmoking controls. However, it cannot be excluded that differences, present between

the groups *in vivo*, disappeared during culture of these cells. The studies in chapters 3 and 4 indicate that inflammatory mediators modulate GR characteristics in human bronchial epithelial cells. We hypothesize that this plays an important role in glucocorticoid therapy.

Regulation of GR number

The GR studies in bronchial epithelial cell lines, described in chapter 3 and the work by several other investigators indicate that the GR number may be regulated by cytokines produced during an immune response (7,10-13). This suggests that inflammation in itself may modulate cellular responsiveness through locally produced inflammatory mediators. Inflammatory mediators can induce an increase in GR number, perhaps to control excess of inflammatory stimuli by sensitizing the cell to feedback inhibition by glucocorticoids. IL-1, TNF-α and IL-6 have all been shown to stimulate the hypothalamic-pituitary axis to secrete corticotropin-releasing hormone and adrenocorticotropin hormone which, in turn, induces glucocorticoid secretion from the adrenal cortex (14,15).

Several investigators have shown that GR levels are closely related to the magnitude of the GR-mediated response (16). This has been demonstrated *in vivo* in transgenic mice expressing GR antisense RNA. The level of GR was reduced in tissues where the transgene was expressed, leading to a decreased glucocorticoid response (17). Karl et al. described a human kindred with a microdeletion in the GR gene. This deletion resulted in a decrease of GR protein by 50% in affected members of this glucocorticoid-resistant family (18). Among the factors that alter the level of GR expression, glucocorticoids themselves appear to be the most potent regulators and have been shown to cause downregulation of the receptor in many cell lines and in tissues and cells from intact animals and healthy human subjects (19-22). In a recent study by Vachier et al. it was demonstrated that glucocorticoids downregulated GR mRNA expression in asthma (23).

A major issue in GR downregulation is the existence of critical sequences within the GR gene that are required for the glucocorticoid-induced transcriptional repression (24). A previous study indicated that intragenic *cis* elements might be important for ligand-induced downregulation (25). Others have shown by the use of deletion mutants, that there might be multiple intragenic signals within the coding region of the GR gene responsible for GR downregulation (21). An alternative mechanism that regulates GR expression, suggested by Brönnegard, is the signal transduction pathway involving cell surface receptors and the release of second messengers, such as cyclic adenosine 3',5'monophosphate (cAMP), diacylglycerol, inositol-1,4,5-triphosphate and calcium (24,26). The cAMP second messenger cascade appears to modulate GR expression by prolonging the half-life of GR mRNA transcripts, which enhances the concentration of GR within a given

cell. These findings suggest that a combination of glucocorticoids and cAMP-generating drugs may be of potential therapeutic benefit.

Regulation of GR affinity

GR affinity was shown to be regulated by inflammatory mediators both *in vitro* and *in vivo* (7,10 and chapters 3 and 4). The hormone binding affinity of the GR, which is determined by a number of different factors, plays an important role in the potency of the GR in transcriptional regulation. Point mutations within the region coding for the GR ligand-binding domain causing substitutions of amino acids can lead to altered hormone binding affinity of the receptor. Almost all such modifications of the GR ligand binding domain are known to cause reduction of either glucocorticoid binding affinity (27) or ligand/receptor complex stability (28) and are associated with various clinical syndromes of glucocorticoid hyposensitivity. The GR/heat shock protein (hsp) complex plays an important role in maintaining the GR in a ligand-friendly high affinity conformation (29-32). For normal hormone/receptor interaction proper assembly and folding of the complex are therefore essential. First, the unliganded GR must associate with the preformed hsp complex. As this is an energy-dependent process, artificially ATP-depleted cells contain only so-called "null" receptors that appear to be unable to associate with the hsp complex and to bind hormone (33,34). Previous findings in the rat also showed that hormone binding affinity falls and rises with cellular ATP levels (35). Second, the expression levels of hsp 90 can influence GR function. In this context, Picard (36) demonstrated severely impaired GR function in yeast mutants expressing low levels of hsp 90. High levels of hsp 90 are found in target tissues that are particularly glucocorticoid sensitive, *e.g.* in the thymus (37). Finally, the structural integrity of the hsp 90 molecule may also be important in establishing a proper conformation of the GR ligand-binding domain. Artificial hsp 90 deletion mutants that could still associate with the GR failed to maintain the steroid binding properties of the receptor (38,39). In a recent study, hsp 90 mutants were shown to reduce the transcriptional activity of GR in yeast (32). Despite the potential pathophysiological significance of naturally occurring hsp 90 mutants, such abnormalities have not yet been demonstrated *in vivo*.

The GR affinity may also be affected by other factors that do not normally participate in the GR/hsp complex. Recently, Kam et al. showed a ~6-fold reduction of glucocorticoid binding affinity in human lymphocytes treated with IL-2 and IL-4 (7). As only the nuclear fraction of GR was affected by this change in affinity, the interleukins most likely induced a nuclear factor which then interacted with the activated GR, influencing the receptor's affinity for the ligand. Nuclear factors that interfere with GR mediated transcription activation are referred to as dominant negative inhibitors. These molecules probably represent the most important

endogenous regulators of glucocorticoid sensitivity and can, theoretically, act through the following mechanisms (40):

1. steric hindrance of the GR by binding to DNA sequences overlapping a GRE;
2. formation of inactive complexes with the GR in solution or on the DNA;
3. competition with the GR for GRE binding sites; and
4. titration ("squelching") of accessory factors necessary for the interaction of the GR with the basic transcription complex.

AP-1, a transcription factor composed of dimers of Jun and Fos family proteins, is the most extensively studied dominant negative inhibitor of GR. Its activity is modulated by growth factors and cytokines via mitogen-activated protein kinases (41). Another dominant negative inhibitor is NF-κB, a heterodimer composed of structurally related DNA binding subunits, p50 and p65 (RelA) (42,43). NF-κB is also an activator of many immune system genes (42). NF-κB antagonizes GR action on GRE regulated promoters, probably through direct physical interaction. Furthermore, NF-κB is inhibited by GR in stimulating NF-κB responsive genes, by the IκB inhibitory protein, which traps NF-κB in inactive cytoplasmic complexes (43-45). A 'functional antagonism' between the transcription factor NF-κB and the glucocorticoid receptor has been found in the regulation of the expression of the IL-6 gene (43). This antagonism resulted from physical association between the two proteins. The transcription factor cAMP response element binding protein (CREB) represents another example of a protein that is thought to establish physical contact with the GR. CREB, that binds to a cAMP-responsive element on genes, may be activated by β-agonists via cAMP formation and activation of protein kinase A (46). β-Agonists increase CREB binding to CRE in human lung *in vitro* and at the same time reduce GR binding to GRE. This suggests that a protein-protein interaction takes place between CREB and the GR within the nucleus (47). Such a direct interaction between CREB and the GR has been demonstrated recently in epithelial cells and resulted in a reduced binding of the latter protein to GRE (48).

Inflammatory mediators, such as IL-1β and LPS can induce elevated levels of nuclear factors as AP-1 and NF-κB, which are activators of many immune system genes and interfere with the function of the modulatory domain of the GR. Therefore, we suggest that this may provide a plausible explanation for the decrease in GR binding affinity found in smoking individuals, in whom general airway inflammation is known to be present, and *in vitro* in BEAS 2B cells after treatment with IL-1β or LPS. Another explanation for decreased GR binding affinity could be a modulating effect of LPS and IL-1β on the expression and phosphorylation of hsp, which are associated with the unliganded GR (49,50). Bacterial products and cytokines

can regulate the expression and phosphorylation of hsp and this may modulate glucocorticoid binding to the GR (49,50).

No study mentioned above distinguished between the two different human GR isoforms, termed hGRα and hGRβ, that are generated by alternative splicing of the human GR pre-mRNA (51,52). The distinction between hGRα and hGRβ may be more relevant than previously thought (54). hGRβ differs from hGRα only in its COOH terminus with replacement of the last 50 amino acids of the latter with a unique 15 amino acid sequence. This difference renders hGRβ unable to bind glucocorticoids (51) and to be transcriptionally active (27). In a pilot study, it was demonstrated that budesonide downregulated both hGRα- and hGR-β mRNA *in vitro* and *in vivo* in human bronchial epithelial cells (54). In a recent study Bamberger et al. demonstrated that overexpression of hGRβ could antagonize the effects of hormone activated hGRα on a glucocorticoid responsive reporter gene (53). Thus, hGRβ potentially functions as a dominant negative inhibitor of hGRα activity. It was shown by ^{3}H-dexamethasone binding studies that hGRβ did not alter the affinity of hGRα for its hormonal ligand. In contrast to the GR inhibitors mentioned earlier, hGRβ binds specifically to GRE sequences *in vitro* (53). hGRβ mRNA (53) and protein (55) have a widespread tissue distribution, suggesting that this isoform might be physiologically relevant and may participate in defining the sensitivity of target tisues to glucocorticoids. The existence of at least two human GR isoforms that apparently exert opposite effects makes the picture of GR mediated transcription more complex. It demands careful reevaluation of previous, mostly nonligand binding studies, that did not distinguish between the two receptor forms. It will be of particular interest to determine whether the other inhibitors of GR function also interact with GRβ and/or with each other.

As far as our own results are concerned, only the studies not involving ligand binding have to be reevaluated. The GR probe, which we used in the mRNA experiments, corresponded to part of the N-terminal hypervariable region of the human GR. Thus, this probe recognized both isoforms of the human GR. We found no effect of IL-1β or LPS on the total GR mRNA levels, but the effect of the inflammatory mediators on each isoform separately remains to be established. The GR/GRE binding we observed in nuclear extracts of BEAS 2B cells may concern both the binding of hormone liganded hGRα and unliganded hGRβ to the GRE.

7.2 Cellular mechanisms of anti-inflammatory actions

To analyse the cellular mechanisms of the anti-inflammatory actions of glucocorticoids in human bronchial epithelial cells, we studied the effects of glucocorticoids on both the induction and inhibition of regulatory proteins

(lipocortins in chapter 5 and chemokines in chapter 6).

In chapter 5 we described the expression of lipocortin I and II in the human bronchial epithelium *in vivo* and *in vitro,* and we analyzed the inducibility of these proteins by glucocorticoids. Biological evidence suggests that lipocortin I and II are glucocorticoid inducible proteins with anti-inflammatory properties (56). It has been proposed that lipocortins mediate part of the immunosuppressive activity of glucocorticoids by inhibiting PLA_2 activity, hereby preventing eicosanoid production (57). Lipocortin I exists primarily as an intracellular protein, and lacks a leader sequence necessary for cellular release by classical mechanisms. However, lipocortin I can be secreted from cells and may occupy high affinity binding sites present on the surface of some cells (e.g. neutrophils and monocytes) (58,59). The nature of these binding sites is not clear at present, although they appear crucial to the action of lipocortin I and are absent after cell activation in inflammation (60). In the study of human bronchial sections described in chapter 5, we found a high expression of lipocortin I and II in the human bronchial epithelium compared to the underlying layers.

Lipocortin I may function as a 'barrier' to inappropriate inflammatory and autoimmune responses at specific sites around the body (56). Following an inflammatory stimulus, stress-induced stimulation of the hypothalamic-pituitary-adrenal axis may result in the release of cortisol, which in turn leads to the production of lipocortin I at discrete sites throughout the body including the site of inflammation. Neutrophils and monocytes trafficking to tissue sites then have to pass through regions of elevated lipocortin I concentration. The lipocortin-binding molecules on monocytes and neutrophils arriving at those tissue sites are expected to become saturated, resulting in moderation of migratory and/or pro-inflammatory activities (56). Lipocortin I appears to mimic many effects of glucocorticoids including inhibition of superoxide generation, chemotaxis, production of eicosanoids and suppression of phagocyte functions (61-63). Others have speculated from results in animal studies that epithelial cell damage and loss, as seen in asthmatics, could decrease availability of these protective properties of lipocortin I (64). Increased inflammation would thereby be predicted.

In the study described in chapter 5, no induction of lipocortin I or II mRNA or protein was observed after incubation with dexamethasone. Stimulation of cultured human bronchial epithelial cells with IL-1β, TNF-α or LPS for 24 h did not affect the lipocortin I or II mRNA or protein expression, although PGE_2 and 6-keto-$PGF_{1\alpha}$ production was significantly increased. This IL-1β- and LPS-mediated increase in eicosanoids could be reduced by dexamethasone, but was not accompanied by a detectable increase in lipocortin I or II expression. This study suggests that glucocorticoids do not increase the expression of lipocortin I and II in human bronchial epithelial cells. Induction of lipocortin I expression by glucocorticoids has been

reported in human peripheral blood monocytes, human alveolar macrophages, rat alveolar epithelial cells and bovine bronchial epithelial cells (64-67). However, others did not observe an induction of lipocortin I by glucocorticoids in seven different primary cell types, including human macrophages (68). Furthermore, the synthesis of lipocortin I does not appear to be under glucocorticoid control in certain cell lines (69). Flower et al. have stated that a potential source of error in all 'induction' experiments is the presence of a substantial amount of the protein on the surface of the cells (70). This pool is very sensitive to glucocorticoid action. The protein on the surface of the cell may easily be lost during cell preparation and bias the results. Recovery of the protein by washing of the cells with EDTA should be performed. Several studies have demonstrated that the glucocorticoids promote the release of lipocortin I from the intracellular compartment to the extracellular cell surface (58,71).

The production of lipocortin I by cells *in vitro* may also be influenced by factors such as the differentiation state. An early report suggested that U937 cells exposed to glucocorticoids did not synthesize lipocortin I (72). However, when the cells were brought in a differentiated state by the addition of phorbol 12-myristate 13-acetate (PMA), dexamethasone induced lipocortin I mRNA and the protein itself (69). For our induction experiments with dexamethasone we used both human bronchial epithelial cells just a few days after isolation and after culturing for one or two weeks in order to study lipocortin expression in different differentiation states. In all cases we found a clear mRNA and protein expression of lipocortin I and II in human bronchial epithelial cells, but no induction of lipocortin by dexamethasone. As culturing of cells may change their differentiation state, it would be of interest to perform induction experiments on directly isolated, uncultured bronchial epithelial cells. A suitable way to obtain bronchial epithelial cells would then be by bronchial brushing, as dissociation of the cells from the bronchial tissue would no longer be necessary.

In chapter 6 we investigated the effect of dexamethasone and inflammatory mediators on the release of MCP-1 by human bronchial epithelial cells. MCP-1, a member of the C-C branch of the chemokine family, is a potent chemoattractant for monocytes (73,74). In addition to being a chemotactic factor, MCP-1 activates monocytes (74) and basophils (73) and can induce leucocyte adhesion molecules on endothelial and vascular smooth muscle cells (75,76). Today, MCP-1 is considered to be a potent pro-inflammatory molecule.

In the study described in chapter 6, primary cultures of human bronchial epithelial cells were found to release low levels of MCP-1. MCP-1 expression was significantly increased when human bronchial epithelial cells were stimulated for 24 h with IFN-γ, IL-1β, or TNF-α. IFN-γ induced a more than 10-fold increase in MCP-1 release, whereas IL-1β and TNF-α only induced a

6- and 2-fold increase in MCP-1 release by human bronchial epithelial cells, respectively. The MCP-1 increase by IFN-γ was dose- and time-dependent.

IFN-γ, IL-1β and TNF-α are cytokines that are likely to be present in airway inflammatory diseases such as asthma. IFN-γ is produced during viral infections (77), which are important triggers of asthmatic attacks (78). Increased levels of IFN-γ, produced by activated T lymphocytes, have been found in the bronchoalveolar lavage of patients with intrinsic asthma compared to control groups (79). Also increased levels of IFN-γ, together with TNF-α were observed in bronchoalveolar lavage fluid of allergic patients after antigen challenge (80). IFN-γ may play an important role in airway inflammation as it has been reported to upregulate ICAM-1 expression on epithelial and endothelial cells, especially in combination with TNF-α (81,82). In future studies, it would be of interest to study the effect of IFN-γ in combination with TNF-α on the release of MCP-1 by human bronchial epithelial cells.

In the study described in chapter 6 the effect of inflammatory mediators on the release of MCP-1 was also compared with the effect on the release of IL-8 by human bronchial epithelial cells. IL-8 is a member of the C-X-C branch of the chemokine family and has been shown to be a chemotactic factor for mainly neutrophils (83). There is abundant evidence that bronchial epithelial cells can be induced to release IL-8 (84-86). In contrast to the 10-fold induction in MCP-1 release, IFN-γ had no effect on the IL-8 release by human bronchial epithelial cells. IL-1β and TNF-α increased both IL-8 and MCP-1 release. These data indicate that MCP-1 and IL-8 release in human bronchial epithelial cells are differentially regulated depending upon the presence of different cytokines. This suggests that the type of cellular infiltrate and the progress of airway inflammation is likely to depend on the kind of stimulatory cytokines present. IFN-γ has been reported to increase not only MCP-1, but also RANTES release, whereas no stimulatory effects were found on IL-8 release by human bronchial epithelial cells (87-89). Although the effect of IFN-γ was not studied on the release of other C-X-C chemokines, we hypothesize that IFN-γ may be a relative specific stimulator of the influx of monocytes compared to IL-1β and TNF-α, which can stimulate the recruitment of all type of leukocytes.

The exact mechanism of the induction of MCP-1 expression by cytokines remains to be clarified. The 5'-upstream transcriptional regulatory region of the human MCP-1 gene contains consensus sequences for both TNF-α, IL-1β and IFN-γ responsive transcription factors such as NF-κB and AP-1 (90-92). Therefore, it is likely that transcription factors such as NF-κB and AP-1 are involved in the cytokine mediated induction of MCP-1 release by human bronchial epithelial cells.

Dexamethasone decreased the cytokine-induced release of MCP-1 by human bronchial epithelial cells, but no reduction to basal levels was

observed. IFN-γ and dexamethasone were added simultaneously to the cell cultures, and it would be of interest to study the inhibitory effect of dexamethasone, when cells are incubated with dexamethasone after or prior to the addition of cytokines. No studies have been performed yet on the molecular mechanism of dexamethasone-mediated inhibition of MCP-1. However, the presence of AP-1 and NF-κB sites in the MCP-1 gene suggests that the inhibitory effect of dexamethasone may be mediated through the antagonism by the glucocorticoid-receptor complex of the action of NF-κB and/or the action of AP-1.

The results in chapter 6 show that IFN-γ is a potent stimulator of MCP-1 release by human bronchial epithelial cells, but does not affect IL-8 release. Therefore, IFN-γ may be relatively specific stimulator for the influx of monocytes. We hypothesize that epithelial-derived MCP-1 is among other chemokines responsible for the submucosal accumulation of monocytes observed in inflammatory airway diseases. One potential beneficial effect of glucocorticoid therapy in airway inflammatory diseases may be inhibition of MCP-1 release by bronchial epithelial cells.

From the results described in chapter 5 we conclude that the anti-inflammatory effects of glucocorticoids in bronchial epithelial cells do not seem to be mediated through the induction of anti-inflammatory proteins (lipocortins). Many effects of glucocorticoids are achieved by inhibition rather than by activation of target genes. This is especially true for the anti-inflammatory effects of glucocorticoids that involve the negative transcriptional regulation of genes involved in inflammation, e.g. chemokines. Many promoters of these genes do not contain negative GRE sequences or any other glucocorticoid receptor binding sites (93,94). Instead these genes are regulated by interaction of the GR with other transcription factors in the absence of specific DNA binding (51,94,95). Therefore, we hypothesize that the cellular mechanisms of the anti-inflammatory actions of glucocorticoids in human bronchial epithelial cells during airway inflammation mainly concerns the inhibition of genes involved in this inflammatory proces. Our hypothesis is supported by the recent work described by Barnes et al. They suggested that epithelial cells may be the key cellular target for inhaled glucocorticoids in asthma (96); it was stated that by inhibiting the transcription of several inflammatory genes in airway epithelium, inhaled steroids may reduce inflammation in the airway wall. It was suggested that this may be achieved by an inhibitory effect on NF-κB activation, as the genes of many inflammatory mediators produced in airway epithelial cells are primarily regulated by NF-κB. Therefore, by blocking NF-κB, glucocorticoids may thus control the airway inflammatory process.

7.3 Limitations of the study

7.3.1. Culture of bronchial epithelial cells

Various culture systems for respiratory epithelial cells can be used to study airway epithelial characteristics and functions. Each system has its advantages and limitations, which must be considered in the scope of specific questions. In monolayer cultures of dissociated cells, used in our experiments, only epithelial cells are present and the effects observed are caused by a pure epithelial response. The two major differentiated cell types usually disappear shortly after plating at low density: the goblet cells disappear first and the ciliated cells at a later stage (97-99). However, after two weeks of culture some ciliated cells can still be present. The expression of cellular differentiation in culture differs between species, and is largely dependent on the culture media supplements and the substratum. Besides the monolayer culture system, another culture systems exists where the cells are grown as cellular aggregates in suspension. In this culture system, the functional and structural properties and the differentiation state of the cells are better preserved. However, suspension cultures of pure airway epithelial cells are difficult to obtain and proliferation of the cells can not be observed (100). Very recently, a new method has been developed in which the cells are cultured at the air-liquid interface (101). First, the cells are cultured under submerged conditions, after which the culture is lifted to the air-liquid interface. A great advantage of the air-exposed cultures is the reappearance of cilia (101). The growth capacities of non-transformed respiratory epithelial cells are limited to approximately 25 population doublings with a doubling time of 1-3 days (100). This implies a culture span of 1 month and a maximum of 5 passages, irrespective of the tissue or species origin of the cells. The limited growth capacity is a major inconvenience to obtain large numbers of cells. Therefore, tumor and *in vitro* transformed human cell lines have been developed.

7.3.2. Determination of the number of glucocorticoid receptors

The most sensitive method to determine GR number and affinity is the radioligand binding assay. For a ^{3}H-dexamethasone binding assay 30 x 10^{6} cells are needed. In order to obtain enough bronchial epithelial cells from bronchial biopsies for cell culture followed by a ^{3}H-dexamethasone binding assay, it is necessary to take at least 4 or 5 biopsies. We initiated our studies with bronchial tissue from patients undergoing surgery for lung cancer, from which a large number of epithelial cells could be obtained. Although we carefully selected epithelium distant from the tumor, we would have liked to use biopsies from non-diseased individuals.

7.4 Major conclusions

The major conclusions of the work presented in this thesis are:

1. Cultured human bronchial epithelial cells possess a class of specific GR, which display *in vitro* specific binding to their GRE. Since human bronchial epithelial cells are considered to play an important role in airway inflammation and inhaled steroids mainly precipitate in the larger airways, the clinical response to inhaled glucocorticoids may result from the modulation of airway epithelial cell functions. Therefore, the bronchial epithelium may be an actual target for glucocorticoid therapy.
2. Inflammatory stimuli such as LPS and IL-1β significantly increase the number of GR and decrease the GR binding affinity in BEAS 2B cells. These data suggest that inflammatory processes may influence the response of bronchial epithelium to glucocorticoid therapy via locally produced inflammatory mediators.
3. The binding affinity of GR in cultured human bronchial epithelial cells from smoking individuals is significantly decreased when compared to values found in nonsmoking individuals. We hypothesize that this results from the inflammation found in the airways of smokers.
4. Lipocortin I and II are highly expressed in the bronchial epithelium, but glucocorticoids do not increase the expression of these lipocortins *in vitro*. The decrease in cytokine-induced PGE_2 and 6-keto-$PGF_{1\alpha}$ production found in human bronchial epithelial cells upon incubation with glucocorticoids is not mediated by increased expression of lipocortins.
5. IFN-γ is a potent stimulator of MCP-1 release by human bronchial epithelial cells, but does not affect IL-8 release. Therefore, IFN-γ may be a relatively specific stimulator for the influx of monocytes. Dexamethasone can downregulate MCP-1 release by human bronchial epithelial cells and we hypothesize that one potential beneficial effect of glucocorticoid therapy in airway inflammatory diseases is the inhibition of MCP-1 release by bronchial epithelial cells.

7.5 Clinical implications

In asthma and to a lesser extent in COPD, bronchial hyperreactivity plays an important role. Recent investigations have implied that a causal relationship exists between the presence of airway inflammation and the occurrence of bronchial hyperreactivity. During airway inflammation, inflammatory mediators are produced by different cell types. The studies described in this thesis support the notion that bronchial epithelial cells are

an active participant in airway inflammation. In some patients with COPD or asthma, glucocorticoids do not bring about the expected improvement in clinical symptoms (102). This clinical unresponsiveness may result from a defect in the cellular response to glucocorticoids (103,104). Investigations by Sher et al. showed that T lymphocytes from glucocorticoid resistant asthmatic patients had GR with a decreased binding affinity (10). They hypothesized that persistent inflammation may contribute to abnormalities in ligand binding in these patients. This hypothesis was supported by the observation that inflammatory mediators such as IL-2 and IL-4 decreased GR binding affinity in T lymphocytes (7). In the studies described in this thesis we observed that inflammatory stimuli such as IL-1β and LPS decreased GR binding affinity in human bronchial epithelial cell lines. In addition, we found in studies with smoking and control subjects, that GR binding affinity was decreased in bronchial epithelial cells from smokers, in whom general airway inflammation is known to be present. The decreased GR binding affinity found in bronchial epithelial cell lines and in T lymphocytes after incubation with inflammatory mediators resembles the decreased ligand binding found in T lymphocytes of glucocorticoid resistant patients. Therefore, we hypothesize that decreased cellular response of bronchial epithelial cells may also play a role in the clinical unresponsiveness to glucocorticoids.

7.6 Future directions

As the cellular response of bronchial epithelial cells might play a role in the clinical unresponsiveness to glucocorticoids, it would be of interest to look for correlations between the clinical response to glucocorticoids and GR characteristics in bronchial epithelial cells from patients with a decreased response to glucocorticoids. It would be of advantage when GR number and affinity could be studied directly on the bronchial tissue or directly after cell isolation, as culturing of the cells could perhaps change GR characteristics. In a previous study a method was developed to analyse GR number in small cell samples by flow cytometry using monoclonal antibodies against the rat GR, which were known to crossreact with the human GR (105). Another recently described method is a radioligand binding assay performed on cytocentrifuge preparations (106). These may be useful methods to study GR in small cell samples in future. For example GR characteristics could be studied in bronchial epithelial cells obtained by bronchial brushing or by dissociation from a single bronchial biopsy. In this way it would be possible to study GR in epithelial cells from different patient groups without cell culture.

Other investigators have claimed that the lack of clinical response to glucocorticoids can not be explained by abnormal GR number or affinity

(107). They showed that a reduced number of GR available for GRE binding may underlie the phenomenon of glucocorticoid resistant asthma. To study this reduced GRE binding, another study was recently performed in which the ability of the nuclear translocated transcription factors AP-1, NF-κB and CREB were examined for binding to their DNA binding sites and for interaction with GR in PBMC from patients with glucocorticoid sensitive and glucocorticoid resistant asthma (108). A significant reduction was observed in the interaction between GR and AP-1 in glucocorticoid resistant asthmatic patients, although interaction with other transcription factors known to be activated in inflammation (NF-κB and CREB), were unaffected (108). An increase in the basal level of AP-1 DNA binding was also detected in the nuclei of these patients. From these results it was suggested that either the ability of the GR to bind to GRE and AP-1 is altered in glucocorticoid resistant asthmatic patients or that increased levels of AP-1 prevent binding of the GR to DNA. Therefore, studies on the expression and interaction of additional transcription factors may elucidate the molecular basis of altered glucocorticoid responsiveness. To further understand the mechanisms regulating human glucocorticoid responsiveness, it will be necessary to determine the relative amounts of the two human GR isoforms and of the inhibitors of GR function, followed by an investigation whether their level of expression is subject to regulatory processes.

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SUMMARY

SUMMARY

The main subject of this thesis is the role of the human bronchial epithelium as an actual target for glucocorticoid therapy in airway inflammation. To gain more insight into the actions of glucocorticoids on human bronchial epithelial cells, glucocorticoid receptor (GR) expression was analyzed and the effect of inflammatory mediators on these receptors was investigated both *in vitro* and *in vivo*. Furthermore, the potential cellular mechanisms of the anti-inflammatory actions of glucocorticoids in human bronchial epithelial cells were studied.

Chapter 1 gives a summary of the present knowledge with regard to airway inflammation, the bronchial epithelium and glucocorticoids. Diseases characterized by airway inflammation, like asthma and chronic obstructive pulmonary disease (COPD) affect a substantial proportion of the population. Interactions between airway epithelial cell products and inflammatory cells may directly contribute to the pathogenesis of airway inflammation. These airway epithelial cell products may contribute to the recruitment, activation, differentiation and survival of eosinophils, lymphocytes, and monocytes/macrophages in airways of asthma and COPD patients. Glucocorticoids are powerful agents in the treatment of airway inflammatory diseases. The involvement of bronchial epithelial cells in the anti-inflammatory actions of glucocorticoids has been suggested. At present, the precise molecular interactions underlying the immunosuppressive effects of glucocorticoids are not yet clear. The modulation of the expression of a number of genes encoding known (e.g. cytokines) and probably also unknown proteins is thought to be their most important function. The presently known anti-inflammatory mechanisms of action of glucocorticoids are discussed in this chapter. *In chapter 2* the aim of the study and the nature of the experimental approach are described. The aim of the studies described in this thesis is to identify and characterize specific GR in human bronchial epithelial cells and to study the potential cellular mechanisms of anti-inflammatory actions of glucocorticoids in these cells.

In *chapter 3* the analysis of GR in two human bronchial epithelial cell lines, BEAS S6 and BEAS 2B is described. In a whole cell dexamethasone binding assay, BEAS S6 and BEAS 2B cells were found to possess $28.9 \pm 4.4 \times 10^3$ and $32.1 \pm 5.7 \times 10^3$ glucocorticoid binding sites per cell, respectively, with dissociation constant (K_d) values of 8.2 ± 1.5 nM and 8.6 ± 2.4 nM, respectively. In addition, the effect of inflammatory stimuli on GR characteristics was investigated. LPS and IL-1β significantly increased the number of GR per cell, but significantly reduced the ligand affinity of these receptors in BEAS 2B cells. The GR mRNA expression was not altered after incubation with LPS or IL-1β. Furthermore, nuclear translocated GR, isolated from human bronchial epithelial cell lines, were found to bind to glucocorticoid responsive elements (GRE).

Chapter 4 describes the study of GR expression in primary cultures of

human bronchial epithelial cells. Using a cytosol radioligand binding assay, cultured human bronchial epithelial cells were found to contain a mean number of glucocorticoid binding sites of 87.2 ± 16.7 fmol/mg protein with a K_d value of 0.76 ± 0.10 nM. To investigate whether inflammation *in vivo* also affected GR characteristics, we studied the GR number and binding affinity in human bronchial epithelial cells from smoking and nonsmoking COPD patients and controls. In COPD patients and in smokers general airway inflammation is known to be present. In smoking controls, GR binding affinity was significantly decreased compared to nonsmoking controls, but no significant difference was found between the mean number of binding sites. No differences were observed between COPD patients and controls. We conclude that different inflammatory mediators may be involved in COPD and in smoking. It remains to be established which mediators actually alter the GR characteristics.

To analyse the potential cellular mechanisms of the anti-inflammatory actions of glucocorticoids in human bronchial epithelial cells, the effects of glucocorticoids on the induction (*chapter 5*) or inhibition (*chapter 6*) of proteins were studied. In *chapter 5* it is shown that bronchial epithelial cells express lipocortin I and II both *in vivo* and *in vitro.* No induction of lipocortin I or II mRNA or protein was observed in human bronchial epithelial cells after incubation with dexamethasone. Stimulation of bronchial epithelial cells with IL-1β, TNF-α or LPS for 24 h did not affect the lipocortin I or II mRNA or protein expression, although PGE_2 and 6-keto-$PGF_{1\alpha}$ production were significantly increased. This IL-1β- and LPS-mediated increase in eicosanoids could be reduced by dexamethasone, but was not accompanied by an increase in lipocortin I or II expression.

In *chapter 6* the effects of glucocorticoids and cytokines on MCP-1 production by human bronchial epithelial cells is described. Primary cultures of human bronchial epithelial cells were found to express low levels of MCP-1. MCP-1 expression were significantly increased when human bronchial epithelial cells were stimulated with IFN-γ, IL-1β or TNF-α. IFN-γ induced a more than 10- fold increase in MCP-1 release, whereas IL-1β and TNF-α only induced a 6- and 2-fold increase, respectively. The effect of these inflammatory mediators on the expression of MCP-1 was compared with the effect on the expression of IL-8. IFN-γ had no effect on the release of IL-8 by human bronchial epithelial cells, whereas IL-1β and TNF-α increased the IL-8 release by these cells. Dexamethasone decreased the cytokine-induced release of MCP-1 by human bronchial epithelial cells, but no reduction to basal levels was observed.

In conclusion, the studies in this thesis show that the bronchial epithelium may be an actual target for glucocorticoid therapy and that the response of bronchial epithelium to glucocorticoid therapy may be modulated by airway inflammation. The anti-inflammatory effects of glucocorticoids in

bronchial epithelial cells do not seem to be mediated through the induction of lipocortins. Glucocorticoids were shown to downregulate the MCP-1 release by human bronchial epithelial cells. We hypothesize that the anti-inflammatory actions of glucocorticoids in human bronchial epithelial cells during airway inflammation mainly concern the expression of genes involved in the inflammatory process. In future studies it would be of interest to look for correlations between the clinical response to glucocorticoids and GR characteristics in bronchial epithelial cells from patients with a decreased response to glucocorticoids. Furthermore, the binding of GR to GRE and the interaction with additional transcription factors should be studied in these patients.

SAMENVATTING

SUMMARY IN DUTCH

SAMENVATTING (SUMMARY IN DUTCH)

In dit proefschrift staat de rol van het humane bronchusepitheel als doelwit voor glucocorticoïdtherapie bij luchtwegontsteking centraal. Om meer inzicht te verkrijgen in de invloed van glucocorticoïden op humane bronchusepitheelcellen werd de glucocorticoïdreceptor (GR) expressie geanalyseerd en het effect van ontstekingsmediatoren op deze receptoren onderzocht, zowel *in vitro* als *in vivo*. Tevens werden de mogelijke cellulaire mechanismen van het anti-inflammatoire effect van glucocorticoïden op humane bronchusepitheelcellen bestudeerd.

In *hoofdstuk 1* wordt een samenvatting gegeven van de huidige kennis over luchtwegontsteking, het bronchusepitheel en glucocorticoïden. Aandoeningen, die gekenmerkt worden door ontsteking van de luchtwegen, zoals astma en chronische obstructieve longaandoeningen (COPD), komen voor bij een aanzienlijk deel van de bevolking. Interacties tussen producten van luchtwegepitheelcellen en ontstekingscellen spelen waarschijnlijk een rol in de pathogenese van luchtwegontsteking. De producten van deze luchtwegepitheelcellen dragen mogelijk bij aan de recrutering, activatie, differentiatie en overleving van eosinofielen, lymfocyten, en monocyten/macrofagen in de luchtwegen van astma en COPD patiënten. Glucocorticoïden zijn krachtige middelen bij de behandeling van luchtwegaandoeningen. Er zijn aanwijzingen dat bronchusepitheelcellen betrokken zijn bij de anti-inflammatoire werking van glucocorticoïden. Tot op heden zijn de moleculaire interacties die ten grondslag liggen aan de immunosuppressieve effecten van glucocorticoïden nog onduidelijk. De modulatie van de expressie van vele genen die coderen voor bekende (b.v. cytokinen) en mogelijk onbekende eiwitten wordt gezien als hun meest belangrijke functie. De huidige bekende anti-inflammatoire werkingsmechanismen van glucocorticoïden worden besproken in dit hoofdstuk. In *hoofdstuk 2* wordt het doel van de studie en de experimentele benaderingswijze beschreven. Het doel van de studies beschreven in dit proefschrift is om specifieke GR te identificeren en karakteriseren in humane bronchusepitheelcellen en om de mogelijke cellulaire mechanismen van de anti-inflammatoire werking van glucocorticoïden in deze cellen te bestuderen.

In *hoofdstuk 3* wordt de analyse van GR in twee humane bronchusepitheelcellijnen, BEAS S6 en BEAS 2B, beschreven. Met behulp van een dexamethason bindingassay werden in BEAS S6 en BEAS 2B cellen respectievelijk $28.9 \pm 4.4 \times 10^3$ en $32.1 \pm 5.7 \times 10^3$ glucocorticoïdbindingplaatsen per cel gevonden, met dissociatie constantes (K_d) van respectievelijk 8.2 ± 1.5 nM en 8.6 ± 2.4 nM. Daarnaast werd de invloed van ontstekingsprikkels op de eigenschappen van de GR onderzocht. LPS en IL-1β verhoogden significant het aantal GR per cel, maar verlaagden significant de affiniteit van deze receptoren voor hun ligand in BEAS 2B cellen. De GR mRNA expressie veranderde niet na incubatie met LPS of IL-1β. Tevens bleken GR geïsoleerd uit kernen van humane bronchusepitheelcellijnen te kunnen binden aan

"glucocorticoid responsive elements" (GRE).

Hoofdstuk 4 beschrijft de studie van GR expressie in primaire kweken van humane bronchusepitheelcellen. Met behulp van een radioligand bindingassay werd in het cytosol van gekweekte humane bronchusepitheelcellen een gemiddeld aantal glucocorticoïdbindingplaatsen van 87.2 ± 16.7 fmol/mg eiwit gevonden, met een K_d waarde van 0.76 ± 0.10 nM. Om te onderzoeken of ontsteking *in vivo* ook kenmerken van de GR beïnvloedt, hebben wij het GR aantal en hun bindingsaffiniteit in humane bronchusepitheelcellen van rokende en niet-rokende COPD patiënten en controlepersonen bestudeerd. In COPD patiënten en in rokers wordt ontsteking van de luchtwegen aangetroffen. Bij rokende controlepersonen was de GR bindingsaffiniteit significant lager dan bij niet-rokende controlepersonen, maar er werd geen significant verschil gevonden tussen het gemiddelde aantal glucocorticoïdbindingplaatsen. Geen verschillen werden waargenomen tusssen COPD patiënten en controlepersonen. Wij concluderen dat mogelijk verschillende ontstekingsmediatoren betrokken zijn bij de ontsteking bij COPD en bij roken. Nader onderzoek zal moeten uitwijzen welke mediatoren daadwerkelijk de GR karakteristieken veranderen.

Om de mogelijke cellulaire mechanismen van de anti-inflammatoire werking van glucocorticoïden in humane bronchusepitheelcellen te analyseren, werden de effecten van glucocorticoïden op de inductie (*hoofdstuk 5*) of remming (*hoofdstuk 6*) van eiwitten bestudeerd. In *hoofdstuk 5* werd aangetoond dat bronchusepitheelcellen zowel *in vivo* als *in vitro* lipocortine I en II tot expressie brengen. Inductie van lipocortine I of II mRNA of eiwit werd niet waargenomen in humane bronchusepitheelcellen na incubatie met dexamethason. Stimulatie van bronchusepitheelcellen met IL-1β, TNF-α of LPS gedurende 24 uur had geen effect op de lipocortine I of II mRNA expressie, ofschoon de PGE_2 en 6-keto-$PGF_{1\alpha}$ productie significant werd verhoogd. Deze IL-1β en LPS gemedieerde toename in eicosanoiden kon verlaagd worden met dexamethason, maar ging niet gepaard met een toename in lipocortine I of II mRNA expressie.

In *hoofdstuk 6* worden de effecten van glucocorticoïden en cytokinen op de MCP-1 productie door humane bronchusepitheelcellen beschreven. Primaire kweken van humane bronchusepitheelcellen hebben een lage expressie van MCP-1. Deze MCP-1 expressie werd significant verhoogd wanneer humane bronchusepitheelcellen werden gestimuleerd met IFN-γ, IL-1β of TNF-α. IFN-γ induceerde een meer dan 10-voudige verhoging van de MCP-1 productie, terwijl IL-1β en TNF-α slechts een 6-, respectievelijk, 2-voudige verhoging gaven. Het effect van deze ontstekingsmediatoren op de productie van MCP-1 werd vergeleken met het effect op de productie van IL-8. IFN-γ had geen effect op de productie van IL-8 door humane bronchusepitheelcellen, terwijl IL-1β en TNF-α de IL-8 productie door deze cellen verhoogden. Dexamethason verlaagde de cytokine-geïnduceerde

productie van MCP-1 door humane bronchusepitheelcellen, maar verlaging tot het basale niveau werd niet waargenomen.

Concluderend kunnen we stellen dat het onderzoek in dit proefschrift laat zien dat het bronchusepitheel een doelwit voor glucocorticoïdtherapie kan zijn en dat de reactie van het bronchusepitheel op glucocorticoïdtherapie beïnvloed kan worden door luchtwegontsteking. De anti-inflammatoire effecten van glucocorticoïden op bronchusepitheelcellen lijken niet gemedieerd te worden door de inductie van lipocortinen. Glucocorticoïden verlaagden de MCP-1 productie door humane bronchusepitheelcellen. Onze hypothese is dat glucocorticoïden in humane bronchusepitheelcellen gedurende luchtwegontsteking met name van invloed zijn op de expressie van genen betrokken bij dit ontstekingsproces. Het zou interessant zijn om toekomstig onderzoek te richten op correlaties tussen de klinische respons op glucocorticoïden en GR karakteristieken in bronchusepitheelcellen van patiënten die verminderd reageren op glucocorticoïden. Bovendien zou in zulke patiënten de binding van GR aan GRE en de interacties met andere transcriptiefactoren moeten worden onderzocht.

ABBREVIATIONS

A549	:	epithelial cell line derived from human lung carcinoma
ACE	:	angiotensine-converting enzyme
AP-1	:	activator protein-1
BAL	:	bronchoalveolar lavage
BEAS	:	a human bronchial epithelial cell line derived from normal human bronchial epithelial cells tranformed by infection with adenovirus 12-SV40 hybrid virus
bp	:	basepair
BSA	:	bovine serum albumin
cAMP	:	cyclic adenosine 3',5' monophosphate
CD	:	cluster of designation/cluster of differentiation
COPD	:	chronic obstructive pulmonary disease
COS	:	monkey kidney cell line
CRE	:	cAMP responsive element
CREB	:	cAMP responsive element binding protein
CV-1	:	monkey kidney cell line
DEX	:	dexamethasone
DNA	:	deoxyribonucleic acid
dpm	:	disintegrations per minute
DTT	:	dithiothreitol
ECP	:	eosinophil cationic protein
EDRF	:	endothelium derived relaxing factor
e.g.	:	exempli gratie (for example)
EDTA	:	ethylene diamine tetra-acetic acid
EGF	:	epidermal growth factor
EGTA	:	ethylene glycol-bis (ß-aminoethyl ether)
ELAM	:	endothelial leukocyte adhesion molecule
ENA-78	:	neutrophil activating peptide from epithelial cells
EpDRF	:	epithelium derived relaxing factor
FACScan	:	fluorescence activated cell scan
FcεR	:	receptor for constant part of heavy chain of IgE
FCS	:	fetal calf serum
FEV_1	:	forced expiratory volume in 1 second
FITC	:	fluorescein isothiocyanate
g	:	unit of gravity
GAM	:	goat anti-mouse
GAR	:	goat anti-rabbit
GAPDH	:	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	:	granulocyte macrophage colony-stimulating factor
GR	:	glucocorticoid receptor
GRE	:	glucocorticoid responsive element
HBEC	:	human bronchial epithelial cell(s)
HeLa	:	name of the cells of the first human continuously cultured carcinoma cell line; acronym of the name of the patient (Henriette Lacks) from whose carcinoma the parent cells were isolated in 1951.
HETE	:	hydroxyeicosatetraenoic acid
hGR	:	human glucocorticoid receptor

HLA	:	human leucocyte antigen
hsp	:	heat shock protein
ICAM	:	intercellular adhesion molecule
IFN	:	interferon
Ig	:	immunoglobulin
IL	:	interleukin
iNOS	:	inducible nitric oxide synthase
kb	:	kilobase
K_d	:	dissociation constant
KGM	:	keratinocyte growth medium
LPS	:	lipopolysaccharide
LT	:	leukotriene
MBP	:	major basic protein
MCP	:	monocyte chemoattractant protein
Mero	:	malignant mesothelioma cell line
MHC	:	major histocompatibility complex
MIP	:	macrophage inflammatory protein
mRNA	:	messenger RNA
NAP	:	neutrophil activating protein
NEP	:	neutral endopeptidase
NF-κB	:	nuclear factor-κB
NHIK	:	cell line derived from a carcinoma of the human uterine cervix
NK cells	:	natural killer cells
NO	:	nitric oxide
NOS	:	nitric oxide synthase
PAF	:	platelet activating factor
PBMC	:	peripheral blood mononuclear cell
PBS	:	phosphate buffered saline
PDGF	:	platelet derived growth factor
PF-4	:	platelet factor-4
PGE_2	:	prostaglandin E_2
$PGF_{1\alpha}$	:	prostaglandin $F_{1\alpha}$
PLA_2	:	phospholipase A_2
PMA	:	phorbol 12-myristate 13-acetate
PMSF	:	phenylmethylsulfonyl fluoride
RANTES	:	regulated upon activation, normal T cell expressed, and presumably secreted.
RelA	:	p65 subunit of NF-κB
RNA	:	ribonucleic acid
SRC-1	:	steroid receptor coactivator-1
SSC	:	standard saline citrate
TDI	:	toluene diisocyanate
TF	:	transcription factor
TGF	:	transforming growth factor
T_h	:	T helper
TNF	:	tumor necrosis factor
U937	:	a human immature monocytic cell line
VC	:	vital capacity

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Ma bohème

Je m'en allais, les poings dans mes poches crevées;
Mon paletot aussi devenait idéal;
J'allais sous le ciel, Muse! et j'étais ton féal;
Oh! là là! que d'amours splendides j'ai rêvées!

Mon unique culotte avait un large trou.
- Petit-Poucet rêveur, j'égrenais dans ma course
Des rimes. Mon auberge était a la Grande-Ourse.
- Mes étoiles au ciel avait un doux frou-frou

Et je les écoutait, assis au bord des routes,
Ces bons soirs de septembre où je sentais des gouttes
De rosée à mon front, comme un vin de vigueur;

Où, rimant au milieu des ombres fantastiques,
Comme des lyres, je tirais les élastiques
De mes souliers blessés, un pied près de mon coeur!

Arthur Rimbaud