**Department of Paediatrics, University of Szeged**
**Head: Prof. Sándor Túri, MD, PhD, DSc**

# REGULATION OF PROTEOLYTIC ACTIVITY IN LUNG INFLAMMATION: CYTOKINE-INDUCED CHANGES IN PULMONARY EPITHELIAL CELLS

Ph.D. Thesis

Hajnalka Szabó, M.D.

Supervisor: Prof. Dr. Sándor Túri

**CONTENTS**

## SUMMARY

Due to the long-term effects of acute and chronic lung diseases of infancy and childhood research exploring the pathomechanism and treatment of paediatric pulmonary diseases is a priority. A large number of chronic lung diseases, e.g. bronchopulmonary dysplasia or asthma bronchiale, is associated with alveolar and bronchial inflammation. Proteolytic changes play a significant role in the damage to the alveolocapillary barrier. The role of proteolytic pathways in the epithelial damage has been investigated in the present study in order to find potential protective mechanisms which could reduce the injury caused by proinflammatory cytokines.

Tumour necrosis factor-α, interleukin-1 β, interleukin-2, interferon-γ, and bacterial lipopolysaccharide were able to induce significant increase in plasminogen activator activity in A549 human alveolar epithelial cells. Tumour necrosis factor-α had time- and dose-dependent effects both on urokinase and tissue-type of plasminogen activator induction. This cytokine could also significantly activate matrix metalloproteinase 2 in A549 cells and increased migration potential of wounded epithelial cells. All of these inductions change could be inhibited by dexamethasone and an inhibitor of Rho-kinase. Tumour necrosis factor-α treatment also caused damage to the integrity of epithelial cellular barrier, it led to relocalisation of the tight junction transmembrane protein occludin, and to a dose-dependent decrease in the expression of adherens junction protein β-catenin.

The present study indicates that cytokines promote fibrinolysis in alveolar epithelium and contribute to pathogenesis and repair of lung injury. Tumour necrosis factor-α is responsible for the induction of proteolytic activity and the injury of alveolocapillary barrier, while glucocorticoids and Rho-kinase inhibitors may have potential role in future therapeutic approaches. Data obtained on in vitro models may contribute to detection of the signal transduction pathways regulating epithelial proteolytic activity in response to inflammatory stimuli and to identification of potential therapeutic target molecules.

**PUBLICATIONS IN THE TOPIC OF PH.D. THESIS**

Full papers:

I. Krizbai, I.A., Bauer H., Anberger A., Hennig B., Szabó H., Fuchs R., Bauer H.C., Growth factor induced morphological, physiological and molecular characteristics in cerebral endothelial cells. *European Journal of Cell Biology* **79,** 594-600, 2000 ($IF_{2000}$: 2.801)

II. Szabó H., Novák Z., Bauer H., Szatmári E., Farkas A., Wejksza K., Orbók A, Wilhelm I., Krizbai I.A., Regulation of proteolytic activity induced by inflammatory stimuli in lung epithelial cells. *Cellular and Molecular Biology* **51,** OL729-OL735, 2005 ($IF_{2005}$: 1.018)

III. Szabó H., Novák Z., Farkas A., Krizbai I.A., Gyulladásos mediátorok indukálta proteolitikus aktivitás szabályozása légúti epitélsejtekben. *Allergológia és Klinikai Immunológia* **8,** 192-196, 2005 ($IF_{2005}$: -)

IV. Szabó É., Szabó H., A bronchopulmonális dysplasiáról egy esetünk kapcsán. *Tüdőgyógyászat* **3,** 24-26, 2009 ($IF_{2007}$: -)

**V.** Szabó H., Kádár L., Bronchoalveolaris lavage gyermekkorban. *Tüdőgyógyászat* **3,** 32-34, 2009 ($IF_{2007}$: -)

Abstracts:

VI. Szabó H., Bauer H., Novák Z., Szatmári E., Krizbai I.A., Molecular mechanism of the damage of the alveolocapillary barrier in response to inflammatory stimuli. *1st European Respiratory Society Lung Science Conference, Inflammation and Respiratory Disease,* 141, 2003 ($IF_{2003}$: -)

VII. Szabó H., Bauer H., Novák Z., Szatmári E., Farkas A., Wejksza K., Krizbai I.A., Activation of proteolytic mechanisms in response to inflammatory stimuli: a possible role in the development of the alveolocapillary barrier damage. *European Respiratory Journal* **22 (Suppl. 45.),** 458S, 2003 ($IF_{2003}$: 2.999)

VIII. Szabó H., Farkas A.E., Orbók A., Wilhelm I., Novák Z., Krizbai I.A., Regulation of plasminogen activator activity by inflammatory mediators in lung epithelial cells. *European Respiratory Journal* **26 (Suppl. 49.),** P2242, 2005 ($IF_{2005}$: 2.275)

**ABBREVIATIONS**

5-LOX, 5-lipoxygenase
AHR, airway hyperresponsiveness
ALI, acute lung injury AP-1,
activator protein-1 BAL,
bronchoalveolar lavage
BPD, bronchopulmonary distress syndrome
BSA, bovine serum albumin
C5a, complement component 5a
cAMP, 3'-5'-cyclic adenosine monophosphate
CCR2, chemokine (C-C motif) receptor 2
CCR3, chemokine (C-C motif) receptor 3
CCR4, chemokine (C-C motif) receptor 4
CCL7, 11, 17, chemokine (C-C motif) ligand 7,11,17
CECs, cerebral endothelial cells
CF, cystic fibrosis
CLD, chronic lung disease
CM, culture medium
COPD, chronic obstructive pulmonary disease
COX, cyclooxygenase
DMEM, Dulbecco's modified Eagle's medium
DXM, dexamethasone
ECGF-α, endothelial cell growth factor-α
ECM, extracellular matrix
ECs, endothelial cells
EGF, endothelial growth factor
ERK 1/2, extracellular signal-regulated kinase 1/2
FcεRII, low affinity immunoglobulin E receptors on human eosinophil cells
FCS, fetal calf serum
FGF, fibroblast growth factor
GM-CSF, granulocyte-macrophage colony-stimulating factor GTPase, guanosine triphosphatase
IFN-γ, interferon γ Ig,
immunoglobulin IgE,
immunoglobulin E
IGF-1, insulin-like growth factor 1
IL, interleukin
IL-1α, interleukin 1α IL-1β, interleukin 1β
IL-3, interleukin 3
IL-4, interleukin 4
IL-5, interleukin 5
IL-6, interleukin 6
IL-8, interleukin 8
IL-9, interleukin 9
IL-10, interleukin 10
IL-11, interleukin 11
IL-12, interleukin 12
IL-13, interleukin 13
IL-16, interleukin 16 IL-18, interleukin 18 IP,
immunoprecipitate LPS,
lipopolysaccharide
LTB4, leukotriene B4
LTC4, leukotriene C4
LTD4, leukotriene D4
LTE4, leukotriene E4
MCP-1, monocyte chemotactic protein 1 MCP-3, monocyte chemotactic protein-4 MCP-4, monocyte chemotactic protein-4 M-CSF, macrophage colony-stimulating factor MDC, monocyte-derived chemokine MMPs, matrix metalloproteinases MT-MMP, membrane-type matrix
metalloproteinase
NF-κB, nuclear factor-κB
NO, nitric oxide
$NO_2$, nitrogen dioxide
$O_2$, oxygen
PA, plasminogen activator
PAF, platelet-activating factor
PAI-1, plasminogen activator inhibitor-1
PAI-2, plasminogen activator inhibitor-2
PAIs, plasminogen activator inhibitors
PARs, protease-activated receptors PAR-2, protease-activated receptor 2 PBS, phosphate-buffered saline
PDGF, platelet-derived growth factor
PDTC, pyrrolidine dithiocarbamate
PGD2, prostaglandin GD2
Phen, phenanthroline
PKC, protein kinase C
PMN, polymorphonuclear leucocytes pro-MMP, pro-matrix metalloproteinase pro-uPA, pro-urokinase-type plasminogen activator
RANTES, regulated on activation T-cell expressed and secreted
RDS, respiratory distress syndrome SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM, standard error of mean
SOD, superoxide dismutase
Sol, soluble cellular fraction after Triton X-100 treatment
TARC, thymus and activation regulated chemokine
TGF-β, transforming growth factor-β
Th0, T-helper 0 cells
Th1, T-helper 1 cells
Th2, T-helper 2 cells
TIMPs, tissue inhibitors of metalloproteinases
TJs, tight junctions
TLR, Toll-like receptors
TNF-α, tumour necrosis factor-α
tPA, tissue-type plasminogen activator
Tr, regulatory T cells
uPA, urokinase-type plasminogen activator
uPAR, urokinase-type plasminogen activator receptor
ZO- 1, zonula occludes-1
ZO-2, zonula occludes-2
ZO-3, zonula occludes-3

# 1. INTRODUCTION

Respiratory illnesses are the major cause of morbidity and mortality in childhood. Asthma, a chronic inflammatory disorder of the airways is characterized by episodic and reversible airflow obstruction and airway hyperresponsiveness (AHR), has a prevalence of 8.5% in childhood and is responsible for the death of 200 children annually in the United States (Moorman et al., 2007). In addition to genetic factors, pre- and postnatal environmental exposures, acute and chronic lung diseases of infancy and childhood have long-term effects on lung structure and function which persist and adversely affect lung function and respiratory health into adulthood, therefore research in paediatric pulmonary diseases is a strategic priority (Castro et al., 2009).

## 1.1. Pulmonary Inflammations

Inflammation is an important response that defends the body against invasion from microorganisms and the effects of external toxins. Acute inflammation in the respiratory tract is an immediate defense reaction to inhaled allergens, pathogens or noxious agents which is accompanied by increased mucus secretion in order to protect the mucosal surface of the airways (Barnes, 2006). The acute response is followed by a repair process that restores the tissue back to normal involving proliferation of damaged epithelial cells and fibrosis. The repair process may become chronic in response to continued inflammation, resulting in remodelling, i.e. structural changes in the airways (Barnes, 2006).

Acute lung injury (ALI) and its more severe form, the acute respiratory distress syndrome (RD S), are characterized by an acute inflammatory process in the air spaces and lung parenchyma. The loss of barrier function of the alveolar epithelial and pulmonary capillary endothelial cells results in respiratory failure in critically ill patients. A complex network of pro- and anti-inflammatory cytokines and chemokines play a major role in mediating, amplifying, and perpetuating inflammatory-induced lung injury from sepsis, pneumonia, aspiration, and shock. Lung-protective ventilatory strategy reduces not only the number of neutrophils and the concentration of pro-inflammatory cytokines in injured lung, but also the mortality in patients with ALI (Goodman et al., 2003).

Chronic inflammation in the airways may persist for many years, sometimes even in the absence of the causal mechanisms. The molecular and cellular mechanism involves both

long-lived immunologic memory cells and structural cells in the airways. The structural changes may result in irreversible narrowing of the airways with the reduction in air flow in asthma and cystic fibrosis (CF). These changes include fibrosis, increased amount of airway smooth muscle, and increased number of blood vessels (Barnes, 2003). The allergic inflammation in asthma is driven by exposure to allergens through immunogobulin E (IgE)-dependent mechanisms, resulting in a characteristic pattern of inflammation. In asthma, the inflammatory response is activated inappropriately. Allergens, such as house dust mite and pollen proteins, induce an eosinophilic inflammation and the acute response becomes converted into a chronic inflammation which may have structural consequences in the lung. Other lung diseases, such as CF and bronchiectasis, are characterized by a neutrophilic pattern of inflammation, driven in part by chronic bacterial infection.

Chronic lung disease (CLD) of infancy most commonly occurs in low birth weight preterm infants treated for RDS, but any disorder that results in ALI or requires treatment with positive-pressure mechanical ventilation and high concentration of inspired oxygen ($O_2$) predisposes to this disease (Allen et al., 2003). Therefore pneumonia, sepsis, meconium aspiration syndrome, pulmonary hypoplasia, persistent pulmonary hypertension, apnoea, tracheo-oesophageal fistula, congenital diaphraghmatic hernia, congenital heart disease, and congenital neuromuscular disorders can also be responsible for CLD developing in infants (Allen et al., 2003; Szabó and Szabó, 2009). Bronchopulmonary dysplasia (BPD) is clearly distinct from the multiple CLDs of later life and its definition was proposed based on the severity of the disease, need for $O_2$ therapy, and point of assessment (Jobe and Bancalari, 2001). Infants who had been treated with oxygen for >28 days but no longer required oxygen at 36 weeks' postmenstrual age might also have residual lung disease. A hallmark of the lung injury in BPD is re-epithelialization of denuded alveoli followed by the increased presence of fibroblasts and major areas of fibrosis. In recent clinical studies, elevated concentrations of some cytokines including interleukin 1 β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8) were observed in tracheal aspirates and serum of infants with RDS and they contributed to the development of BPD (Groneck and Speer, 1995; Kazzi et al., 2001; Choi et al., 2006).

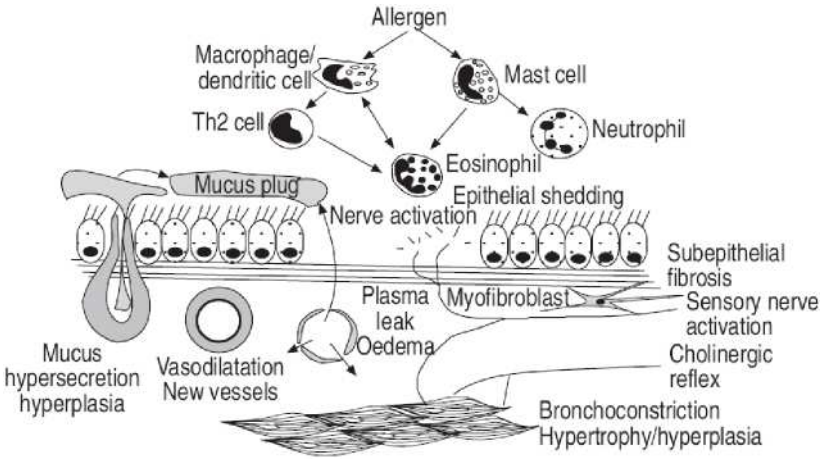
Chronic inflammatory processes of the pulmonary airways are important elements of several CLDs, such as asthma bronchiale. Asthma is characterized by episodic wheezing, lung inflammation, and progressive irreversible airway dysfunction in some patients.

Patients suffering from asthma display AHR in response to methacholine and can develop asphyxia during an acute exacerbation of their disease. Histological hallmarks of asthma include homing of T-helper 2 (Th2) inflammatory cells into the lung parenchyma, eosinophilia, and increased mucus metaplasia. Epithelial cell damage and infiltration of the bronchial wall by inflammatory cells (T cells, eosinophils, and monocytes) can be observed in the airways of bronchial asthma patients. Other irreversible changes may include increased deposition of extracellular matrix (ECM) proteins in the bronchial wall, hyperplasia and hypertrophy of smooth muscle cells, mucous cell metaplasia and an increased number of blood vessels. Allergen challenge and clinical asthma are associated with synthesis and release of several pro-inflammatory cytokines, e.g. tumour necrosis factor-á (TNF-á), IL-1, or interleukin 13 (IL-13) (Townley and Horiba, 2003). Despite an increase of anti-inflammatory mediators in bronchoalveolar lavage (BAL) fluid in status asthmaticus, the net inflammatory activity was found to be pro-inflammatory due to the presence of IL-1β and TNF-á (Tillie-Leblond et al, 1999).

### 1.2. Inflammatory and Structural Cells in the Airway Inflammations

Inflammation is characterised by an infiltration with inflammatory cells depending on the type of process (Fig. 1.). Mast cells, macrophages, dendritic cells, eosinophil, neutrophil and basophil granulocytes, T- and B-lymphocytes, and platelets are involved in asthma, although the precise role of each cell type is not yet certain (Barnes, 2006). Mast cells are important in the initiation of acute bronchoconstrictor responses to allergen or to indirect stimuli including exercise, hyperventilation or fog. Mast cells are activated by allergens through an IgE-dependent mechanism and release several pro-inflammatory cytokines, neurotrophins, chemokines, growth factors, and tryptase. Macrophages, derived from blood monocytes, traffic into the airways in asthma (Fig. 1.) and may be activated by allergen via low affinity IgE receptors (FcεRII) (Barnes, 2006). Their products include a large variety of pro-inflammatory (TNF-α, IL-1β, IL-6) and anti-inflammatory (interleukin 10 and 12; IL-10 and IL-12, respectively) cytokines. Macrophages may also act as antigen-presenting cells which process allergen for presentation to T-lymphocytes (Barnes, 2003). Dendritic cells are specialised macrophage-like cells which able to induce a T-lymphocyte mediated immune response during the development of asthma. Dendritic cells form a network in the epithelium in the respiratory tract and act as very effective antigen-presenting cells. They take up allergens, process them and migrate to local lymph nodes.

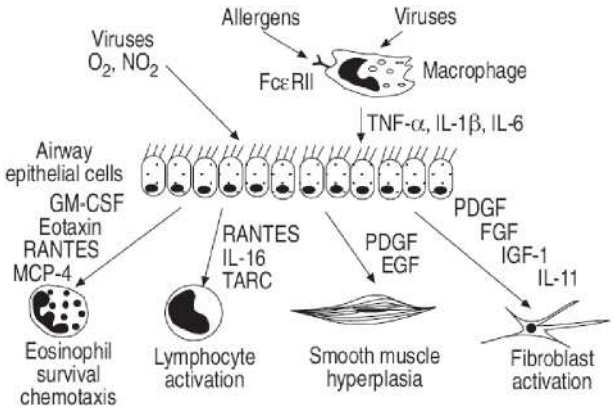
**Fig. 1.** *The pathophysiology of asthma is complex with participation of several interacting inflammatory cells which result in acute and chronic inflammatory efects on the airway (Barnes, 2003) (for abbreviations see page 5).*




Eosinophil infiltration is a characteristic feature of allergic inflammation (Fig. 1.). There is a correlation between eosinophil counts in peripheral blood or BAL fluid and the severity of AHR (Barnes, 2003; Szabó és Kádár, 2009). Eosinophils are linked to the development of AHR and airway epithelial damage through the release of basic proteins and oxygen-derived free radicals. Eosinophil recruitment involves adhesion of eosinophils to vascular endothelial cells, their migration into the submucosa and subsequent activation. Eosinophils also contribute to the structural changes in chronic asthma through the secretion of growth factors, such as transforming growth factor-β (TGF-β). Neutrophils are not predominant in mild-to-moderate asthma, but they may be recruited to the airways in severe asthma, therefore IL-8 levels are increased in induced sputum of the patients who die suddenly of asthma. T-lymphocytes release specific cytokines which result in the recruitment and survival of eosinophils and in the maintenance of mast cells in the airways (Fig. 1.). T-lymphocytes, similar to murine Th2 cells, express interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-9 (IL-9), and IL-13. This programming of T-lymphocytes is due to antigen-presenting dendritic cells, which may migrate from the epithelium to regional lymph nodes or interact with lymphocytes resident in the airway mucosa. Early infections or exposure to bacterial lipopolysaccharide (LPS) might promote T-helper 1 (Th1)-mediated responses to predominate and a lack of infection in childhood may favour Th2 cell expression thus atopic diseases. B-lymphocytes secrete IgE in allergic diseases, and the process is initiated by IL-4 responsible for switching B-cells to IgE production.

Structural cells of the airways, including epithelial cells, smooth muscle cells, endothelial cells, and fibroblasts may also be an important source of inflammatory mediators, such as cytokines and lipid mediators in asthma (Fig. 2). Structural cells outnumber inflammatory cells in the airways, therefore they may become the major source of mediators responsible for chronic inflammation in asthma. Bronchial epithelial cells represent an essential component of the innate immune system. They are in direct contact with inhaled materials, including pollutants, allergens, proteases, microbes, and other factors that are relevant to the development of human asthma (Janssen-Heininger et al., 2009). Airway epithelial cells may have a key role in translating inhaled environmental signals into an inflammatory response and they are probably the major target cell for inhaled glucocorticoids (Walsh et al., 2003). Epithelial cells express various pattern recognition receptors including Toll-like receptors (TLR), and protease-activated receptors (PARs), which recognize microbial motifs and allergens. The activation of epithelial cells through these diverse pathways results in the production of chemokines and cytokines, which may attract inflammatory dendritic cells to the lung and induce the maturation of these cells. The epithelial cells express the most potent chemokines, such as RANTES (regulated on activation T-cell expressed and secreted), eotaxins 1–3, and monocyte chemotactic protein-4 (MCP-4).

**Fig. 2.** *Airway epithelial cells may play an active role in asthmatic inflammation through the release of many inflammatory mediators, cytokines, chemokines and growth factors (Barnes, 2003) (for abbreviations see page 5).*

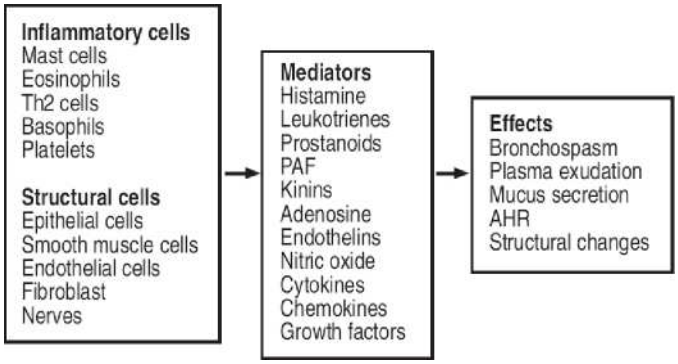



Many inflammatory mediators released in asthma have constrictor effects on airway smooth muscle cells (Barnes, 1998). Chronic exposure to pro-inflammatory cytokines, such as IL-1β, downregulates the response of airway smooth muscle to β2-adrenergic agonists. Characteristic hypertrophy and hyperplasia are the result of stimulation of airway smooth muscle cells by various growth factors, such as platelet-derived growth factor (PDGF), or endothelin-1 released from inflammatory cells. The smooth muscle cells also have the capacity to release multiple cytokines, chemokines, and lipid mediators in asthma.

### 1.3. Cytokines and Other Inflammatory Mediators in the Lung

Inflammatory mediators have been implicated in the pathogenesis of asthma and CLDs. They may have a variety of effects on the airways which could account for the pathology of allergic diseases (Fig. 3.). Mediators such as histamine, prostaglandins, leukotrienes and kinins contract airway smooth muscle, increase microvascular leakage, increase airway mucus secretion, and attract other inflammatory cells. These vasoactive mediators are important in acute and subacute inflammatory responses and asthma exacerbations, while cytokines play a dominant role in maintaining chronic inflammation in allergic diseases.

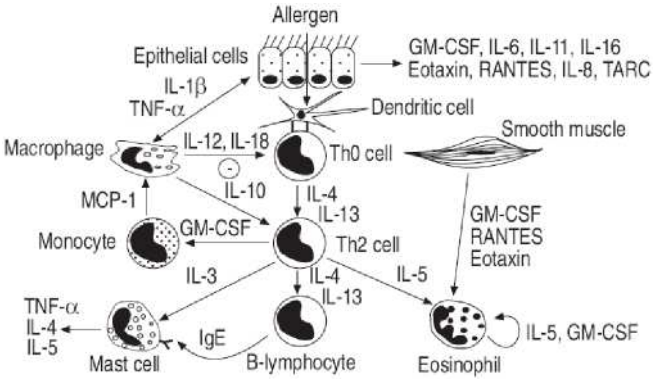
**Fig. 3.** *Various cells and mediators are involved in asthma and lead to several efects on the airways (Barnes, 2003) (for abbreviations see page 5).*




Lipid mediators, such as leukotriene C4 (LTC4), leukotriene D4 (LTD4), and leukotriene E4 (LTE4), are potent constrictors of human airways. LTD4 antagonists protect against exercise- and allergen-induced bronchoconstriction, and improve lung function and symptoms in asthmatic patients. Platelet activating factor (PAF) is a potent inflammatory mediator that mimics some features of asthma including eosinophil recruitment and activation, and induction of AHR. Prostaglandins have various effects on airway function, however, inhibition of prostaglandin synthesis with cyclooxygenase (COX) inhibitors is effective only in a minority of asthma patients. On the other hand, prostaglandin GD2 (PGD2), a bronchoconstrictor produced by mast cells, activates a novel chemoattractant receptor expressed on Th2 cells, eosinophils, and basophils and mediates chemotaxis. This mechanism provides a link between mast cell activation and allergic inflammation.

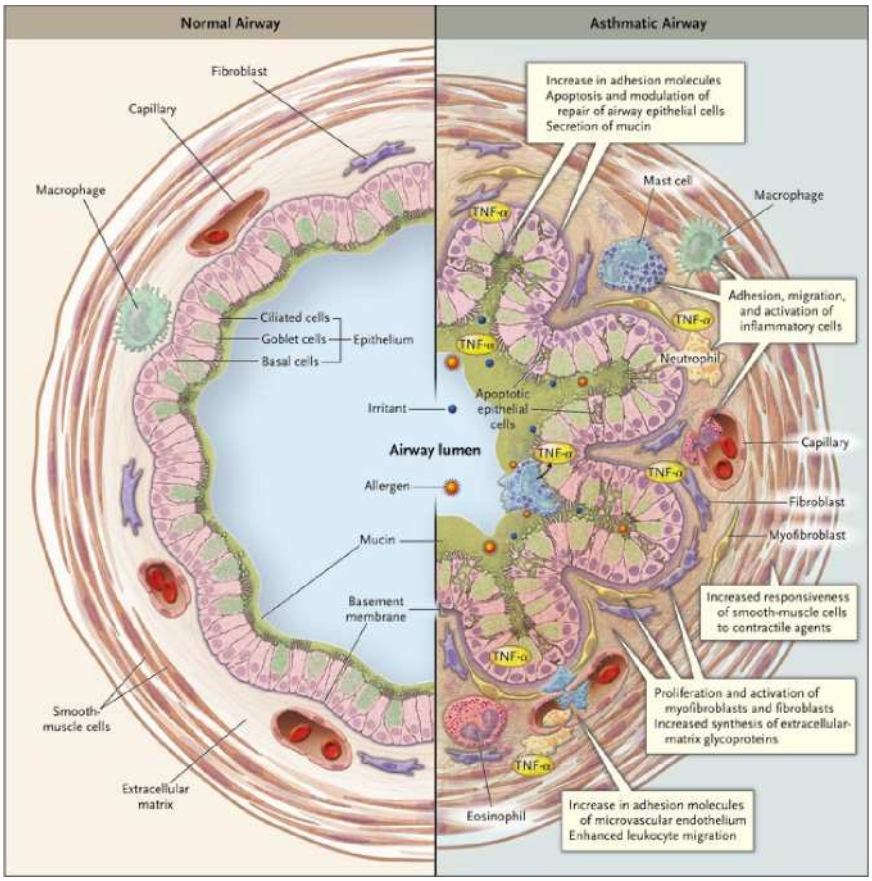
Cytokines are important mediators in chronic inflammation. Many inflammatory (macrophages, mast cells, eosinophils and lymphocytes) and structural (epithelial cells, airway smooth muscle and endothelial cells) cells are capable of synthesising and releasing these proteins (Fig. 4.).

**Fig. 4.** *The cytokine network in asthma (Barnes, 2003). Many inflammatory cytokines are released from inflammatory and structural cells in the airway and orchestrate and perpetuate the inflammatory response (for abbreviations see page 5).*




Almost every cell located in the airways is capable of producing cytokines under certain conditions. Lymphokines secreted by T-lymphocytes include interleukin 3 (IL-3) responsible for the survival of mast cells, whereas IL-4 and IL-13 play a key role in the allergic response of B-lymphocytes. IL-5 has a crucial role in the differentiation, survival and priming of eosinophils. IL-1β, IL-6, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) are released from various cells, including macrophages and epithelial cells and may be important in amplifying the inflammatory response.

Fig. 5. *The role of TNF-α in the allergen- and irritant-induced changes in asthmatic airways (Erzurum, 2006; for abbreviations see page 5).*



TNF-α axis is upregulated in patients with refractory asthma, as evidenced by the increased expression of membrane-bound TNF-α, TNF receptor 1, and TNF-α converting enzyme by peripheral blood monocytes (Berry et al., 2006). TNF-α have multiple effects in asthmatic airways (Erzurum, 2006); it induces adhesion, migration, and activation of inflammatory cells through the epithelial and endothelial barriers (Fig. 5). Effective anti-TNF therapy supports the view that TNF-α contributes to the pathogenesis of refractory asthma (Berry et al., 2006). Both TNF-α and IL-1β activate pro-inflammatory transcription factors, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which switch on many inflammatory genes in the asthmatic airways. Other cytokines, such as interferon-γ (IFN-γ), IL-10, IL-12, and IL-18, play a regulatory role and inhibit the allergic inflammatory process.

Chemokines are low molecular weight (tipically 8 to 10 kDa) chemotactic cytokines that regulate leukocyte trafficking across celullar barriers. Over 50 different chemokines are now recognised and they activate more than 20 different surface receptors. Several chemokines, including eotaxin- 1, eotaxin-2, eotaxin-3, RANTES (regulated on activation T-cell expressed and secreted), and monocyte chemotactic protein 4 (MCP-4), activate a CCR3 common receptor on eosinophils. The increased expression of eotaxins, monocyte chemotactic protein 3 (MCP-3), MCP-4, and CCR3 in the airways of asthmatic patients is correlated with increased AHR. RANTES activates CCR3 receptor which is mainly expressed in eosinophils, however it is also present in Th2 cells and mast cells. MCP-1 activates CCR2 receptor on monocytes and T-lymphocytes. MCP-1 levels are increased in BAL fluid of patients with asthma. CCR4 are selectively expressed on Th2 cells and are activated by monocyte-derived chemokine (MDC) and thymus and activation regulated chemokine (TARC). Epithelial cells of patients with asthma express TARC, which may then recruit Th2 cells. Increased concentrations of TARC are also found in BAL fluid of asthmatic patients, whereas MDC is only weakly expressed in the airways.

During allergic inflammation there is increased oxidative stress, as activated inflammatory cells, such as macrophages and eosinophils, produce reactive oxygen species. Increased oxidative stress is related to disease severity. One of the mechanisms whereby oxidative stress may be detrimental in asthma is through the reaction of superoxide anions with nitric oxide (NO) to form the reactive radical peroxynitrite, that may then modify several target proteins. The level of NO in the exhaled air of patients with asthma is higher than the level of NO in that of normal subjects. Endothelins are potent peptide vasoconstrictors and bronchoconstrictors. Endothelin-1 levels are increased in the sputum of patients with

asthma. Endothelins induce airway smooth muscle cell proliferation and promote a profibrotic phenotype and may contribute to the chronic inflammation of asthma.

### 1.4. Regulation of Proteolysis in the Lung

Proteolytic enzymes are a major group capable of causing damage to the lung during inflammatory process (Greenlee et al., 2007; Shetty et al., 2008). Extracellular endogenous proteases are released both by circulating inflammatory cells and resident lung cells, while exogenous proteases derive from mites and molds. The majority of endogenous proteases that cause lung injury is generated by neutrophils, although macrophages, T-lymphocytes, eosinophils, basophils, mast cells, type II alveolar epithelial cells or fibroblasts also release proteases in smaller quantities. Protease classification is based on the essential amino acid at the active site. Serine-proteinases include neutrophil elastase, cathepsin G, proteinase-3, granzymes, chymase, and plasminogen activators (PAs). Cathepsin L and B are cysteine-proteinases, cathepsin D belongs to aspartic-proteases. Proteases containing metal ions are metalloenzymes, including matrix metalloproteinases (MMPs): gelatinases or collagenases.

Proteases are important in normal growth and development of the lung as well as in lung host defense. They can injure cells directly, degrade components of the ECM and break down extracellular and cell surface macromolecules under pathological circumstances. Proteases can react with receptors in the airways to generate leukocyte infiltration and to amplify the response to allergens. They contract bronchial smooth muscle and cause it to proliferate. Proteases can promote maturation, proliferation, and collagen production of fibroblast precursors and mature fibroblasts. Proteolytic enzymes can also degranulate eosinophils and mast cells.

Proteases act through PARs, 7-transmembrane proteins coupled to G proteins, which are distributed on the cells of the airways. Stimulation leads to increased intracellular $Ca^{2+}$ level and gene transcription and in alveolar epithelial cells it opens tight intercellular junctions, causes desquamation, and produces cytokines, chemokines, and growth factors. The number of PAR-2 is increased on the epithelial cells of patients with asthma.

Antiprotease defenses in the lung limit the action of protease to the desired physical location for a short, specified period under normal physiologic conditions. Most lung tissue injury is likely the consequence of proteases generated locally, because the enzymes coming from the bloodstream are inhibited by the high serum levels of antiproteases. Lung

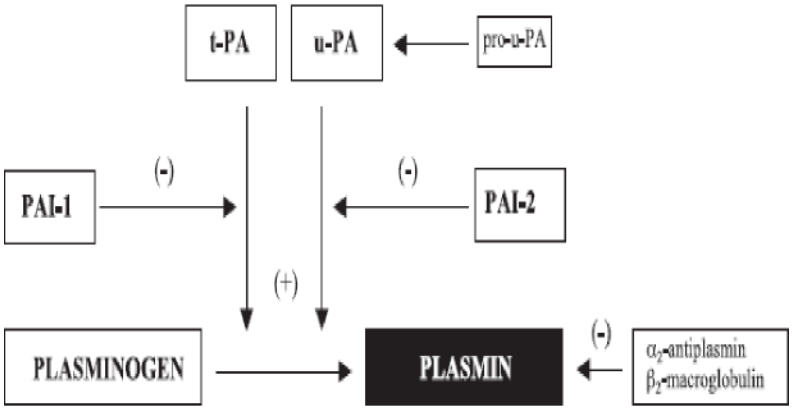
antiproteases include $\alpha_1$-antitrypsin, $\alpha_2$-macroglobulin, secretory leucoprotease inhibitor, tissue inhibitor of metalloproteases (TIMPs), plasminogen activator inhibitor-1 and -2 (PAI-1 and -2), and $\alpha_1$-antichymotrypsin.

Activation of proteolytic enzymes is associated with the acute and chronic phases of inflammatory response. Involvement of proteases in the remodelling of different tissues has been demonstrated in pulmonary emphysema, wound healing, rheumatoid arthritis, colitis, multiple sclerosis, and atherosclerosis. Increased proteolytic activity contributes to the pathogenesis of asthma via its influence on the function and migration of inflammatory cells, on matrix degradation and possible damage of the alveolocapillary barrier. The most studied protease families in this respect are PAs and MMPs.

*1.4.1. The plasmin system in the lung*

Components of the plasmin system, such as tissue-type PA (tPA), urokinase-type PA (uPA), and inhibitors PAI-1 and PAI-2 are synthesised by airway cells (Fig. 6). Endothelial cells, fibroblasts, epithelial cells, mast cells, monocytes/macrophages and smooth muscle cells are responsible for production of PAs. Plasmin system inhibitors are synthesised by endothelial cells, platelets and megakaryocytes, neutrophils, monocytes/macrophages, smooth muscle cells, and fibroblasts. Inflammatory mediators affect the expression of PAs and PAIs, and the plasmin system, in turn, can also actively influence the production of mediators and growth factors, extending pathological structural changes in the airway.

**Fig. 6.** *The regulation of the plasmin system (Kucharewicz et al., 2003) (for abbreviations see page 5).*




Plasminogen is converted into plasmin, its active form, by uPA or tPA. uPA is secreted as an inactive precursor form (pro-uPA) that binds with high affinity to a specific cell surface glycosylphosphatidylinositol-anchored receptor named uPAR (Kucharewicz et al., 2003). The binding of pro-uPA to uPAR activates uPA and enhances the generation of plasmin at

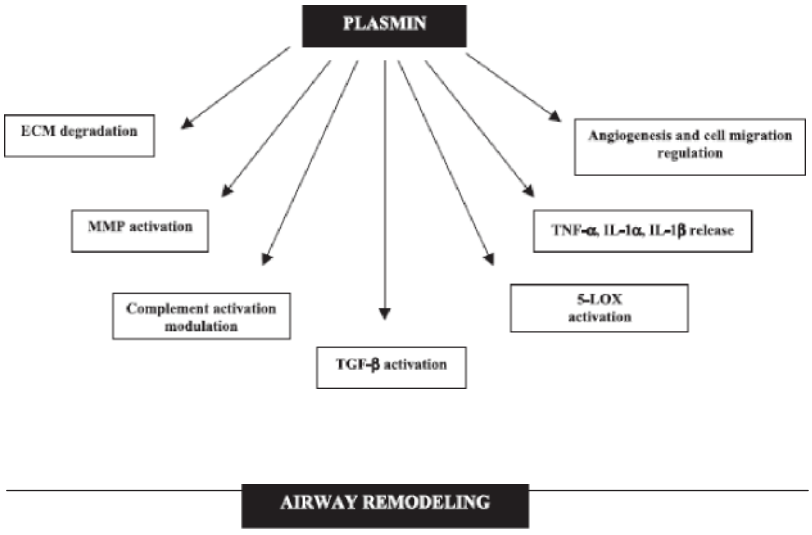
the cell surface, promoting matrix degradation, the activation of MMPs, and growth factors. PAI-1 is the physiological inhibitor of PAs, while local activity of plasmin remains also under the control of $\alpha_2$-antiplasmin and $\alpha_2$-macroglobulin (Fig. 6.). However, mediators of inflammatory response, such as cytokines including IL-1, IL-2, IL-4, IL-6, TNF-$\alpha$, IFN-$\gamma$, GM-CSF, macrophage colony stimulating factor (M-CSF), TGF-$\beta$, and tryptase, may also affect the expression of plasmin system components.

The plasmin system also influences cellular functions without involving proteolytic activity. Plasmin may enhance inflammation by inducing neutrophil aggregation, platelet degranulation, and the release of arachidonic acid derivatives. It has been demonstrated that uPAR mediates cell attachment and movement and this process remains under the influence of uPA and PAI-1. PAI-1 interrupts binding of the uPA/uPAR complex to integrins and blocks uPA-integrin-dependent cell growth. Moreover, uPAR is necessary for adequate recruitment of inflammatory cells, mice deficient in uPAR demonstrate an increased susceptibility to pulmonary infections.The fibrinolytic system prevents fibrin deposition in the alveolar compartment of normal lung. Decreased alveolar fibrinolysis due to altered expression of components of the fibrinolytic system is implicated in the pathogenesis of acute lung injury and subsequent fibrosis (Shetty, 2008). The uPA/plasmin system plays a significant role in the pathogenesis of airway remodelling in asthma (Kucharewicz, 2003); moreover exogenous uPA inhalation has been recently considered as a therapeutic strategy in chronic asthma (Kuramoto, 2009). Genome scan for asthma in the affected families has proved an association between uPA and asthma, atopy, and AHR (Bégin, 2007). The changes in the balance of procoagulant and fibrinolytic activities also contribute to lung fibrosis in acute RDS and in various types of pneumonia (de Benedetti, 1992, Schultz, 2006).

The plasmin system has been implicated not only in clot lysis, but also in a number of physiological and pathological processes, such as angiogenesis, cell migration, and tumor metastasis (Fig. 7.). Plasmin can degrade most of the protein components of ECM, either by direct removal of glycoproteins or by activation of MMPs (Kucharewicz et al., 2003). It converts inactive MMPs to active forms, which are responsible for ECM proteolysis, and prevents neutralisation of MMP by blocking inhibitors TIMPs. The action of MMPs is blocked by PAI-1 and other serine protease inhibitors. The binding of pro-uPA to uPAR activates uPA, enhances plasmin generation, promotes matrix degradation, and activates MMPs and growth factors. In addition to interacting with uPA, PAI-1 binds to vitronectin

leading to its stabilization and it also regulates the binding of integrins and cell migration. Plasmin can regulate complement activation by degrading the C5a component, thereby reducing the ability of mast cells to produce PAI-1. C5a upregulates PAI-1 expression and inhibits plasmin generation, preventing its own degradation (Kucharewicz et al., 2003). Subsequently, this may lead to overwhelming inhibition of fibrinolysis and tissue fibrosis. Tryptase released from activated mast cells increases synthesis type I collagen, stimulates fibroblast proliferation and its collagenolytic activity. It also stimulates the proliferation of epithelial cells, bronchial smooth muscle cells, and endothelial cells. Tryptase also activates uPA, which activates plasminogen and MMPs, and thereby modulates cell migration by fibrinogen cleavage. Plasmin can stimulate 5-lipoxygenase (5-LOX) pathway and increased levels of cysteinyl-leukotrienes and leukotriene B4 (LTB4) have been shown in BAL fluid of patients with pulmonary fibrosis and asthma. Leukotrienes exert bronchoconstrictive and proinflammatory effects, while mice lacking 5-LOX produce more antifibrotic mediators and show reduced airway inflammation (Barnes, 2003). Eosinophil cell infiltration may be an important factor responsible for airway remodelling. Eosinophils release both antifibrotic (IL-2, INF-γ, collagenase) and fibrogenic (TGF-β, IL-4, IL-6, TNF-α, major basic protein, eosinophil cationic protein) mediators. TGF-β derived from eosinophils, lymphocytes, macrophages or epithelial cells, can stimulate fibroblast proliferation and collagen synthesis. Asthmatic patients exhibit greater TGF-β expression in the airway submucosa, and subepithelial fibrosis corresponds to the severity of disease (Barnes, 2003). Plasmin activates TGF-β-dependent fibrotic repair and to promote fibrosis (Fig. 7.).

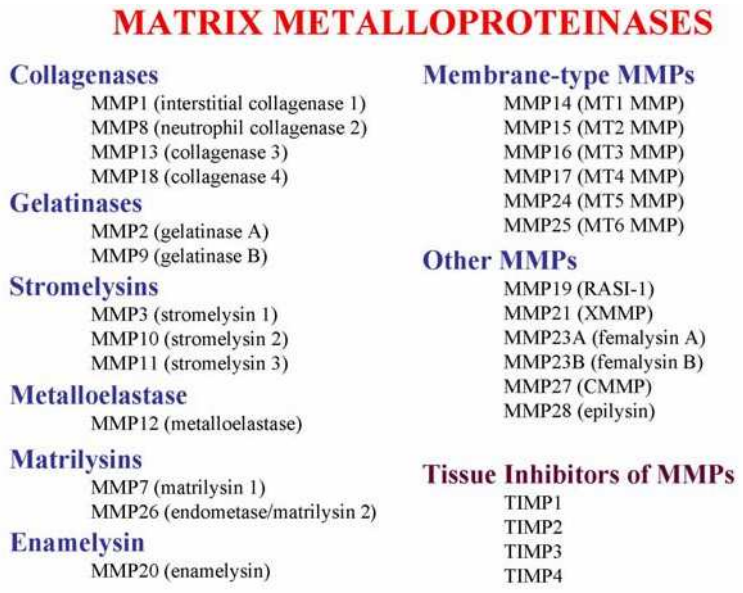
Fig. 7. *Potential elements of plasmin action on airway remodelling (Kucharewicz et al., 2003) (for abbreviations see page 5).*



*1.4.2. Matrix metalloproteinases in lung inflammations*

The MMP family consists of approximately 25 zinc-dependent endopeptidases that are involved in the remodelling of several components of the ECM (Greenlee et al., 2007; Hu et al., 2007). MMPs play a role in many physiological processes including embryo implantation, bone remodelling, and organogenesis. They also participate in pathological conditions, such as the reorganization of tissues during inflammation, wound healing or in invasion of cancer cells. The MMPs share three common domains, namely the pro-peptide, the catalytic domain and the haemopexin-like C-terminal domain which is linked to the catalytic domain by a flexible hinge region. Most MMPs are secreted from the cells as inactive zymogens requiring the cleavage of an amino terminal peptide of 10 kDa for activation. The most commonly used traditional classification is based on the substrate specificity and cellular localisation of the MMP. The major groups are the collagenases, the gelatinases, the stromelysins, and the membrane type MMPs (MT-MMPs) (Fig. 8.).

**Fig. 8.** *Class ification of MMPs and their tissue inhibitors (Greenle et al., 2007; Hu et al., 2007)*




The collagenases (MMP1, MMP8, MMP13, MMP18) are the only known mammalian enzymes capable of degrading triple-helical fibrillar collagens, the major components of bone and cartilage, into fragments. The main substrates of the gelatinases (MMP2, MMP9) are type IV collagen and gelatin, and these enzymes also have an additional domain inserted into the catalytic domain. This gelatin-binding region is positioned before the zinc-binding motif, and it forms a separate folding unit which does not disrupt the structure of the catalytic domain. The stromelysins (MMP3, MMP10, MMP1 1) are able to cleave ECM proteins, but are unable to cleave the triple-helical fibrillar collagens.

The biological processes involving MMPs depend on the balance between proteinases and their natural inhibitors (Greenlee et al., 2007; Hu et al., 2007). MMPs are inhibited by specific endogenous tissue inhibitors, such as TIMP-1, TIMP-2, TIMP-3, and TIMP-4. Plasmin prevents neutralisation of MMP by blocking TIMPs. The action of MMPs is also inhibited by PAI-1 and other serine protease inhibitors.

In the lung, ECM molecules, growth factors, chemokines, proteinases, and cell surface proteins, such as adhesion molecules, are the known targets of MMPs. Many members of the MMP family are up-regulated in the lung during pathological conditions such as allergic inflammation, tissue injury and repair, remodelling, and host defense against pathogens. Although small amounts of MMP2 and MMP14 are present in the lining fluid of the lung under normal conditions, other MMPs such as MMP7, MMP8, MMP9, and MMP12 are up-regulated in pulmonary diseases.

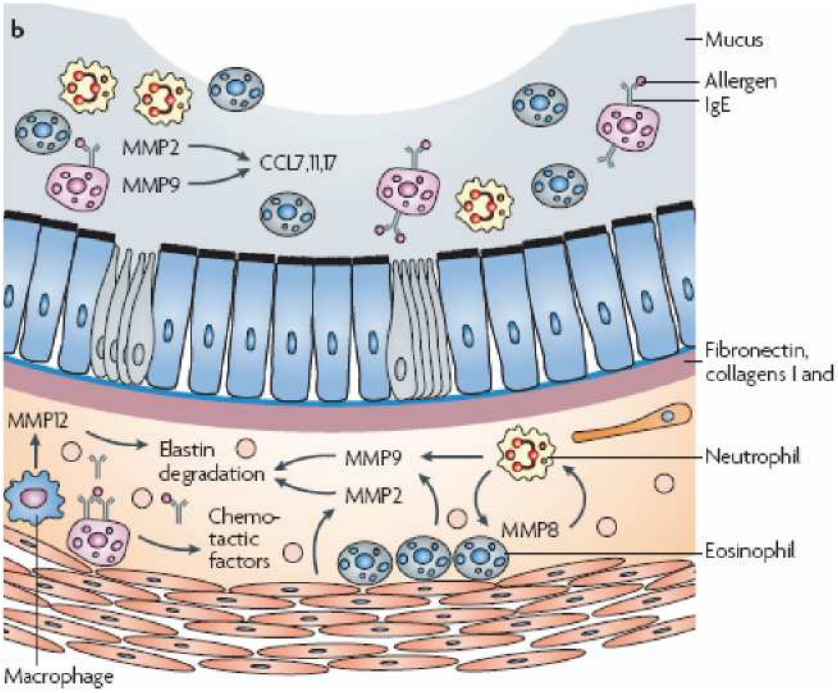
The role of MMP in the lung development and pathology has been investigated in MMP null animals. Knockout models showed developmental lung defects in MMP14 and MMP2 null mice, whereas gene deletion in MMP3, MMP7, MMP8, MMP9, or MMP12 did not result in abnormal lung development (Greenlee et al., 2007). Deletion of MMP2 increases susceptibility of mice to lethal asphyxiation in an asthma model. Absence of MMP3 or MMP12 has been shown to be advantageous, while the lack of of MMP7 or MMP8 was disadvantageous in a mouse model of ALI. Mice have developed exaggerated allergic inflammation in the absence of MMP2 or MMP8 in an acute model of asthma. MMP9 deletion resulted in mixed effects in the acute lung injury or allergy models. Knocking out MMP9 or MMP12 was beneficial in a mouse model of smoking-related lung disease.

MMPs also play a role in the pathogenesis of BPD and CLD of prematurity (Cederquist et el., 2001; Greenlee et al., 2007). Increased amounts of MMP2, and a higher ratio of MMP9 to TIMP1 during the first 3 days of life have correlated with poor outcome in infants with BPD. The infants who developed CLD were born earlier and had lower birth weights, thus an induction in airway MMPs may present an association rather than the cause. Premature infants have a higher concentration of MMP2, and lower surfactant levels, which results in a lack of adequate protection from the oxidative damage. BAL sampling from infants on mechanical ventilation showed a negative correlation between MMP9 activation and TIMP1 concentration and development of BPD. The interactions between the surfactant

proteins and MMPs and TIMPs in the alveolar space most likely play an important role in the lung maturation process in infants. Predominant proinflammatory cytokines (e.g. TNF-α, IL-1β, and the neutrophil chemotactic factor IL-8) in the lung during the first week of life may contribute to prolonged pulmonary inflammation and fibrosis in BPD. Disodium cromoglycate, an anti-inflammatory drug in asthma, could significantly inhibit TNF-α and IL-8 levels and improve the conditions of infants at risk for BPD (Viscardi et al., 1997).

Expression of several MMPs has also been associated with asthma. Increases in MMP 1, MMP2, MMP3, MMP8, and MMP9 levels have been found in sputum and BAL from patients with asthma. MMP9 activity in BAL correlated with the severity of asthma. In asthma, MMP9 is expressed in bronchial epithelium and submucosa, and it is also produced by eosinophils, macrophages, and neutrophils. The involvement of MMPs in the pathogenesis of asthma is summarized in Fig. 9. (Hu et al., 2007). The immune complexes, formed after allergen binding to specific cytophilic IgE molecules, stimulate mast cells to degranulate chemotactic factors for eosinophils, macrophages and neutrophils. These leukocytes and resident epithelial and smooth muscle cells produce MMPs, which cleave various substrates, including elastin (leading to structural destruction and loss of elasticity) and chemokines (resulting in enhanced chemotactic activity to the airway lumen). Smooth muscle cell proliferation, fibrosis of the lamina reticularis and mucus hypersecretion by proliferating goblet cells lead to narrowing of the airway lumen and inhibited air flow.

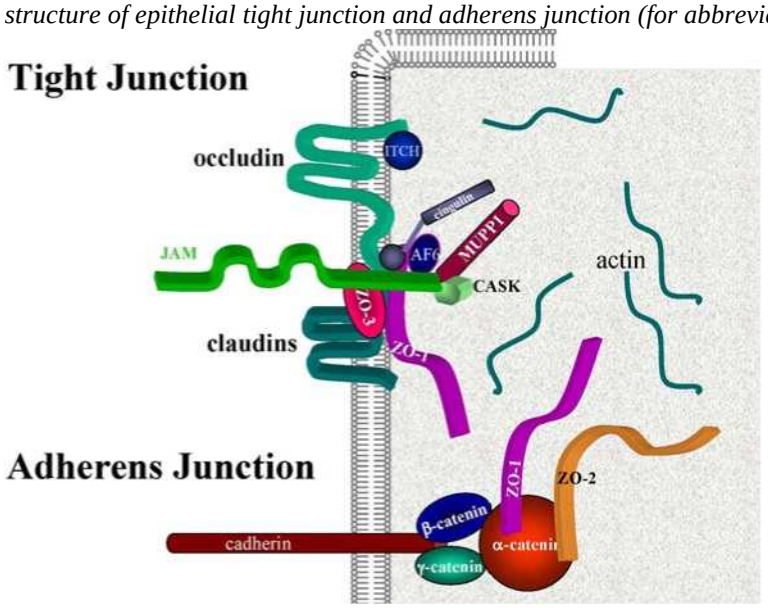
**Fig. 9.** *Role of MMPs in asthma (Hu et al., 2007). (for abbreviations see page 5).*



## 1.5. Alveolocapillary Barrier in Pulmonary Epithelial Cells

Barrier function is an important characteristics of the airway epithelium. Tight junctions (TJ), specialized structures between adjacent cells, are responsible for the establishment and maintenance of a milieu in the alveolar space (Fig. 10.). TJs are formed by a complex of several integral membrane proteins and peripheral membrane proteins that interact with the cytoskeleton (Han et al., 2004). Integral membrane proteins involved in TJ formation include occludin and members of claudin family. Zonula occludens- 1 (ZO- 1) protein has strong interaction with transmembrane proteins occludin and claudins, as well as several cytoplasmic proteins including zonula occludens-2 and -3 (ZO-2 and ZO-3), actin, AF6, or cingulin (Mitic et al., 2000). Adherens junctions are protein complexes that occur at cell-cell junctions more basal than TJs and their cytoplasmic face is linked to the actin cytoskeleton. They appear as bands encircling the cell (zonula adherens) or as spots of attachment to the ECM (adhesion plaques). TJs serve both as a fence differentiating the plasma membrane into apical and basolateral domain and as a barrier limiting the passive diffusion of solutes across the paracellular pathway (Han et al., 2004).

**Fig. 10.** *Schematic structure of epithelial tight junction and adherens junction (for abbreviations see page 5).*




Junctional complexes at cell-cell contact sites are also control points for regulating solute flow across cell monolayers (through tight junctions) and from one cell to another (through gap junctions) (Boitano et al., 2004). Disruption of the paracellular alveolar permeability barrier is a significant pathological consequence of ALI (Crandall and Matthay, 2001). The damage of the alveolocapillary barrier may also play an important role in a large number of

CLDs including asthma bronchiale. Cytokines and other inflammatory mediators participate in the process (Townley and Horiba, 2003). Chronic inflammation is often associated with increased proteolytic activity which contributes to the pathogenesis of asthma through the migration of inflammatory cells, matrix deposition and degradation. Loss of epithelial barrier function as a consequence of proteases associated with allergens or environmental pollutants results in the enhanced access of antigen to dendritic cells.

Almost 99% of the large internal surface area of the lung is lined by two morphologically distinct epithelial cells. Type I cells are large squamous cells; whereas type II cells are smaller cuboidal cells which synthesize, secrete, and recycle surfactant components and mediate repair to the injured alveolar epithelium. Both TJs and gap junctions couple type I and type II cells, providing barrier functions and pathways for intercellular communication (Crandall and Matthay, 2001). Transmembrane proteins in the claudin family act in concert with other transmembrane and peripheral proteins to form the physical basis for TJs. There are more than 20 different claudins and type I and II epithelial cells simultaneously express up to six or more claudin isoforms with different paracellular permeability characteristics (Mitic et al., 2000). In contrast to the mature alveolus formed by heterogeneous epithelium containing both cell types, a more uniform monolayer of fetal type II cells forms a tight seal important for alveolar development, since accumulated fetal lung fluid induces mechanical distension and differentiation of fetal alveolar epithelial cells (Boitano et al., 2004). Under in vitro conditions treatment with glucocorticoid and 3'-5'-cyclic adenosine monophosphate (cAMP) is necessary for induction of type II fetal alveolar epithelial cells. These cells cultured on permeable supports formed high-resistance (> 1,700 ~ × $cm^2$) monolayers, consistent with the ability of fetal type II cells to form tight junctions, while undifferentiated alveolar cells formed leaky ones (< 300 ~ × $cm^2$) (Gonzales et al., 2002).

A better understanding of how alveolar epithelial cell polarity develops and is maintained, as well as how cell polarity is reestablished after injury, is likely to be important for understanding lung injury and repair (Crandall and Matthay, 2001).

## 2. AIMS

The role of proteolytic pathways in the epithelial damage has been investigated in order to find potential protective mechanisms which could reduce the epithelial damage to inflammatory cytokines. The specific objectives of the present study were:

- To investigate the effects of TNF-α and other inflammatory mediators including interleukins, interferon, and bacterial LPS on the PA activity in human alveolar epithelial cells
- To reveal signal transduction pathways regulating epithelial proteolytic activity in response to inflammatory stimuli
- To investigate MMP activity in epithelial and endothelial cells and reveal TNF-α induced regulation
- To determine TNF-α induced changes in the migration potential of alveolar epithelial cells
- To check possible mechanisms of TNF-α induced damage to the barrier integrity of epithelial monolayers

## 3. MATERIALS AND METHODS

### 3.1. Materials

Dulbecco's modified Eagle's medium (DMEM), *E. coli* LPS, cytokines (TNF-á, IL-1β, IL-2, IL-6, IFN-γ), amiloride, colorimetric substrate (D-Val-Leu-Lys-*p*-nitroanilide), and other fine chemicals were obtained from Sigma (St. Louis, MO, USA), Plasminogen was purchased from Roche (Mannheim, Germany), whereas Y27632 and PP-1 were from Tocris (Avonmouth, United Kingdom).

### 3.2. Cell Culture

Human alveolar epithelial A549 cell line retains features of type 2 alveolar epithelial cells and have been extensively used as a model system (Shetty et al., 2008). A549 cells were cultured in DMEM containing 10% heat inactivated fetal calf serum (FCS) and antibiotics. Confluent epithelial monolayers were washed twice with phosphate buffered saline (PBS) and kept is serum-free DMEM before the experiments. Cells were treated with different concentrations of TNF-α (0.1 – 10.0 ng/ml), LPS (0.5-1 μg/ml), IL-1β (10 ng/ml), IL-2 (50 U/ml), IL-6 (1 ng/ml), or IFN-γ (1-100 ng/ml) for 3 to 24 hours. Dexamethasone, PP-1, and Y27632 were used in the concentration of 10 μM, pyrrolidinedithiocarbamate (PDTC) in 100 μM, bisindolylmaleimide in 0.5 μM.

Cerebral endothelial cells (CECs) were isolated from murine brain cortical microvessels as described (Tontsch and Bauer, 1989). Culture medium consisted of DMEM supplemented with 10% FCS, with or without endothelial cell growth factor-α (ECGF-α) and heparin (100 μg/ml) (Krizbai et al., 2000). CECs grown in the presence of ECGF-α and its cofactor heparin exhibit an epithelial-like morphology (type I), while in the absence of them, CECs develop an elongated spindle-like shape (type II) which is accompanied by actin filament reorganization. TGF-β (1 ng/ml) was added to the cell culture medium for 48 h.

### 3.3. Plasminogen Activator Activity Determination by Zymography

Epithelial cells were homogenized on ice in a buffer containing 20 mM Tris (pH=7.4), 150 mM NaCl, and 0.5% Triton X-100. Homogenates were centrifuged at 10,000 *g* for 5 min and equal amounts of protein (25 μg) or equal volumes (25 μl) of culture medium were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. After the electrophoresis the gels were washed in 20 mM Tris (pH=7.4) 2.5% Triton X-100 three times for 20 min to remove SDS and then in tridistilled water three times for 10 min. The gels were layered on agarose gel containing 2% casein and 5 μg/ml plasminogen and incubated at 37°C overnight in a wet chamber. Proteolytic activities were visualized as clear bands, uPA and tPA were identified on their molecular weights around 50 kDa and 70 kDa, respectively. Densitometry was performed using the NIH Image software. One representative of 3 independent experiments is shown.

### 3.4. Colorimetric Plasminogen Activator Assay

PA activity was measured in culture medium (3 h, 6 h, 24 h) and cell lysates (24 h) in the absence or presence of uPA-inhibitor amiloride (1 mM) using a coupled assay to monitor plasminogen activation and the resulting plasmin hydrolysis of a colorimetric substrate (Marshall *et al.,* 1990; Ghosh *et al.,* 2000). Samples were incubated in 200 μl buffer containing 50 mM Tris (pH=7.4), plasminogen (0.3 mM), and D-Val-Leu-Lys *p*nitroanilide (0.3 mM). A standard curve was established using human uPA. The amount of *p*-nitroaniline was detected by measuring the absorbance of the samples at 405 nm.

### 3.5. Matrix Metalloproteinase Activity Measurement

For the assessment of the MMP activity cells were homogenized in a buffer containing 20 mM Tris (pH=7.4), 150 mM NaCl, 0,5% Triton X-100. Homogenates were centrifuged at

10,000 × *g* for 5 min. Protein samples were loaded on a 10 % acrylamide gel containing 2 mg/ml casein and subjected to SDS-PAGE under non-reducing conditions. Following electrophoresis the gels were washed 3 x 20 min in 20 mM Tris (pH=7,4) 2.5% Triton X - 100 to remove SDS followed by 3 x 10 min wash in tridistilled water. The gels were incubated in 20 mM Tris (pH = 7.4) and 5 mM $CaCl_2$ at 37 °C for 24 h. Proteolytic bands were visualized by staining the gels with Coomassie R-250.

### 3.6. Motility Assay

Wound assays were performed according to Sato and Rifkin (1989). Briefly, subconfluent lung epithelial cells, cultured in 6 cm Petri dishes, were wounded with a razor blade. After wounding cells were washed three times with PBS and further incubated in the presence of absence of TNF-á, dexamethasone, or Y27632 for two days. Cells which had migrated in the denuded area from the wound edge were visualized by methylene blue staining.

### 3.7. Occludin Immunostaining

To characterize the morphology of tight junction protein occludin, A549 cell cultures were washed in PBS and were fixed in ethanol for 30 min at 4 °C. After a blockade with 3% bovine serum albumin (BSA), the cells on coverslips were incubated with anti-occludin primary antibody (1:200 dilution; Zymed Laboratories, USA) for 90 min, and washed three times in PBS. It was followed by incubation with secondary antibody Cy5-labelled anti-rabbit IgG (1:400 dilution) for 30 min. Preparations were mounted in Gel Mount (Biomeda, USA) and immunostaining was studied by a Nikon Eclipse 2000 fluorescent photomicroscope (Japan).

### 3.8. Immunoprecipitation and Immunoblotting of Junctional Proteins

A549 cells grown in Petri dishes were treated with 0.1-10.0 ng/ml TNF-α for 6 h. The cells were washed twice with PBS and lysed in 0.6 ml of a lysis buffer (20 mM Tris-Cl, 150 mM NaCl, 5 mM 2-mercaptoethanol, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS). The lysates were centrifuged at 15,000 × *g* for 10 min, then precleared with protein G sepharose (GE Healthcare). Supernatants were incubated with 5 μg of anti-βcatenin antibodies for 4 h at 4 °C, than the immunocomplexes were precipitated with protein G sepharose beads overnight at 4 °C.The precipitates were washed four times in the lysis buffer and boiled for 5 min in 100 μl of electrophoresis sample buffer to elute bound

proteins. Protein samples were resolved on 9% polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked in PBS containing 5% nonfat dry milk, incubated with primary antibodies for occludin, pan-cadherin (Santa Cruz), α- and βcatenin (Sigma) and fluorophore-labeled secondary antibodies (Jackson Laboratories). Immunoreaction was visualized by ECL plus chemiluminescence detection kit (GE Healthcare) and scanned with an imaging system.

### 3.9. Statistics

All data are means ± standard error (SEM). Statistical analysis was performed using analysis of variance followed by Dunn's test for densitometrical data, and by Student's *t* - test for colorimetry PA data. $P<0.05$ values were considered significant changes.

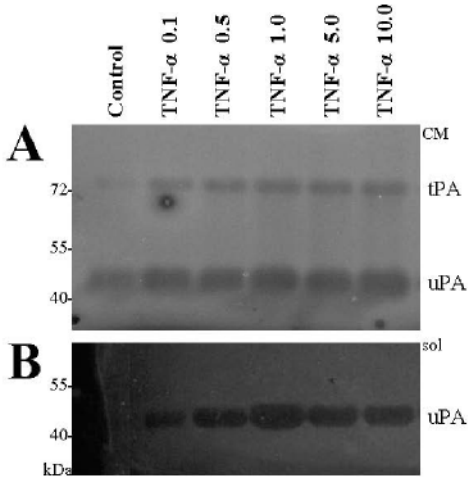
## 4. RESULTS

### 4.1. Effect of Inflammatory Mediators on Plasminogen Activator Activity

*4.1.1. Concentration- and time-dependent induction of PA activity by TNF-á*

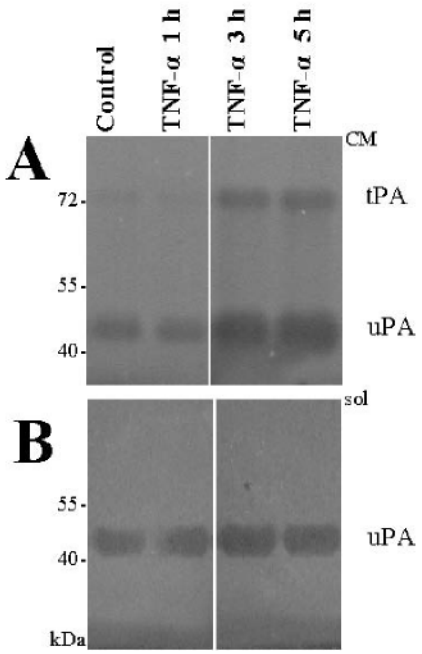
TNF-á induced PA activity has been measured both in the culture medium and the soluble fraction of cell lysates. Strong plasminogen activator activity could be detected at 50 kDa, and weaker activity at around 70 kDa in the culture medium (CM). These activities correspond to uPA and tPA, respectively (Fig. 1 1A). Almost exclusively uPA was detected in Triton X-100 soluble cellular fraction (Sol) (Fig. 1 1B). Treatment with TNF-á for 6 h induced strong, concentration-dependent activation of both uPA and tPA activity. The activation could be already observed at concentration of 0.1 ng/ml (Fig. 11).

**Fig. 11.** *Concentration-dependent efect of TNF-á on PA activity. A549 cells were treated with 0.1-10.0 ng/ml TNF-á for 6 h.* **A.** *Activity in culture medium,* **B.** *PA activity in Triton X-100 soluble cellular fraction. (for abbreviations see page 5).*



Incubation of A549 cells with 5 ng/ml TNF-á induced an activation of both uPA and tPA activity in the culture medium after 3 h treatment. An induction of the uPA activiy could be detected in the soluble fraction of cell lysates as well (Fig. 12).

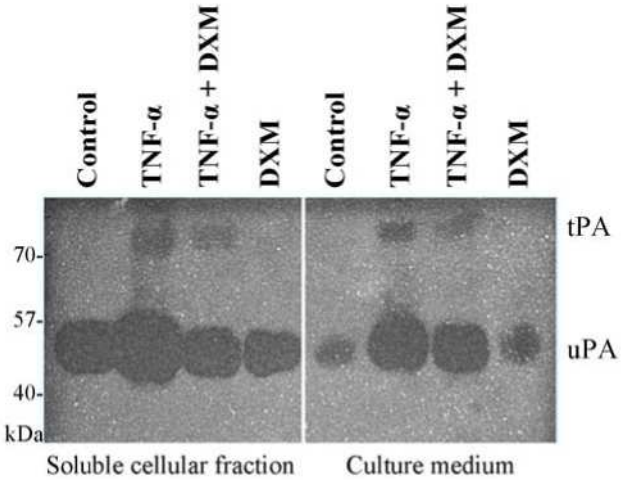
***Fig. 12.*** *Time-dependent efect of TNF-á on PA activity. A549 cells were treated with 5.0 ng/ml TNF-á for 1-5 h. A. PA activity in culture medium. B. PA activity in Triton X-100 soluble cellular fraction (for abbreviations see page 5).*




*4.1.2. Efect of inhibitors on the induction of PA activity by TNF-á*

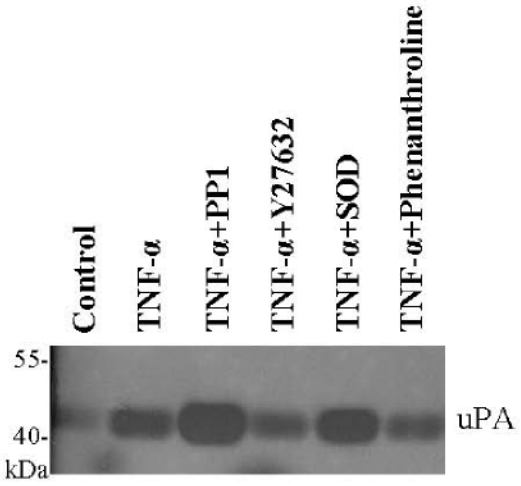
Administration of dexamethasone (DXM, 10 mM) did not change the PA activity in the culture medium and soluble cell lysates. However, the synthetic glucocorticoid could significantly reduce the TNF-á induced activation of both uPA and tPA (Fig. 13).

**Fig. 13.** *Efect of DXM on TNF-á induced PA activity. A549 cells were treated with 5.0 ng/ml TNF-á with or without 10 mM DXM for 16 h (for abbreviations see page 5).*



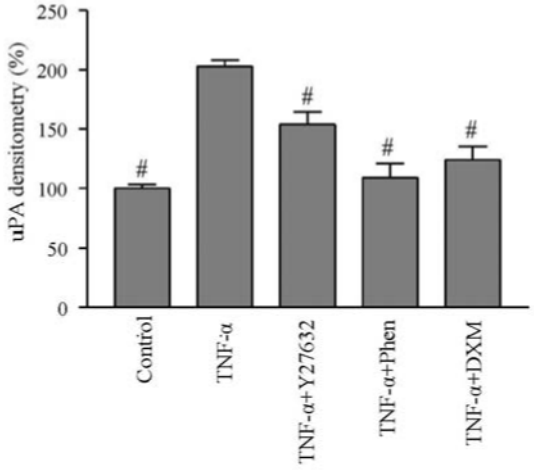
The effect of signal transduction inhibitors on TNF-á induced uPA activity has been also checked. Neither the *src*-kinase inhibitor PP1 nor superoxide dismutase (SOD) could inhibit the TNF-á induced uPA activity (Fig. 14). However, the Rho inhibitor Y27632 and MMP inhibitor phenanthroline were able to significantly inhibit the activation of uPA in A549 human alveolar epithelial cells (Fig. 14).

**Fig. 14.** *Efect of inhibitors on TNF-á induced uPA activity. A549 cells were treated with 5.0 ng/ml TNF-á for 16 h with or without the following inhibitors: PP1 (10 μM), Y27632 (10 μM), SOD (400 U/ml), Phenanthroline (200 μM) (for abbreviations see page 5).*




Densitometrical analysis also proved that TNF-α induced uPA activation could be significantly inhibited by Y27632, phenanthroline (Phen), or DXM (Fig. 15).

**Fig. 15.** *Densitometrical analysis of uPA activation by TNF-á in A549 cells. Epithelial cells were treated with 5.0 ng/ml TNF-á alone or in the presence of 10 mM Y27632, 200 mM phenanthroline, or 10 mM DXM. Data are shown as means±SEM, n= 3. #P< 0.05 vs. the value measured in TNF-á-treated cells (for abbreviations see page 5).*

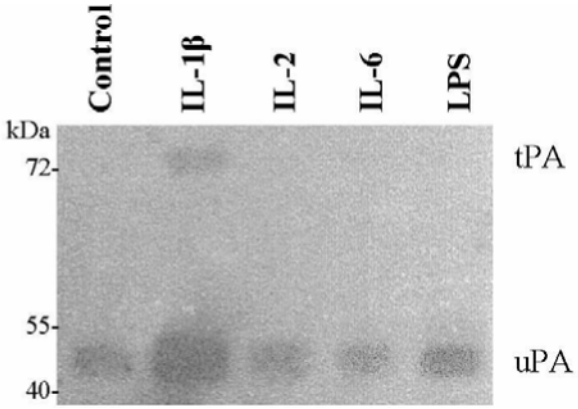



*4.1.3. Efect of bacterial LPS and various cytokines on the PA activity*

Treatment with cytokines IL-1β, IL-2, IL-6, or bacterial LPS for 24 h resulted in changes in PA activity in A549 epithelial cells. Strong PA activity could be detected at 50 kDa

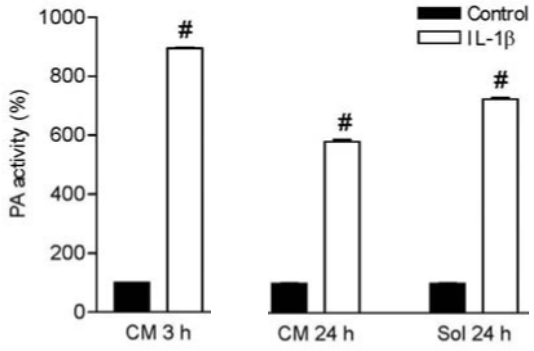
(uPA) by zymography in the soluble fraction of cell lysates after IL-1β treatment, as well as a weaker activity at around 70 kDa (tPA) (Fig. 16). LPS could also increase uPA activity, whereas IL-2 and IL-6 did not produce significant changes (Fig. 16).

**Fig. 16.** ***Efect of interleukins and bacterial LPS on PA activity.*** *A549 cells were treated with IL-1β (10 ng/ml) , IL-2 (50 U/ml), IL-6 (1 ng/ml) or LPS (1 μg/ml) for 24 h (for abbreviations see page 5).*




The effect of inflammatory mediators on PA activity was also estimated using colorimetric PA assay. IL-1β administration (10 ng/ml) caused approximately 9-fold and 6-fold increases in total PA activity in culture medium at 3 h and 24 h, respectively (Fig. 17). The 7-fold PA increase in soluble cellular fraction at 24 h correlated to densitometry result.

**Fig. 17.** ***Efect of IL-1β on PA production of A549 cells.*** *Epithelial cells were treated with 10 ng/ml IL-1β for 3-24 h. All data are means±SEM, n= 3. #P< 0.05 vs. the value measured in control cells at the same time (for abbreviations see page 5).*




Although IL-2 treatment (50 U/ml) for 24 h did not change total PA activity in soluble cellular fraction, it induced significant (1.5 to 3.0-fold) increases in the activity in culture medium with a decreasing tendency between 3 h and 24 h (Fig. 18).

IL-6 treatment (1 ng/ml) for 24 h decreased PA activity in cell lysate, but not in culture medium (Fig. 19).

**Fig. 18.** *Efect of IL-2 on the PA production of A549 cells. Epithelial cells were treated with 50 U/ml IL-2 for 3-24 h. All data are means±SEM, n= 3. #P< 0.05 vs. the value measured in control cells at the same time. (for abbreviations see page 5).*

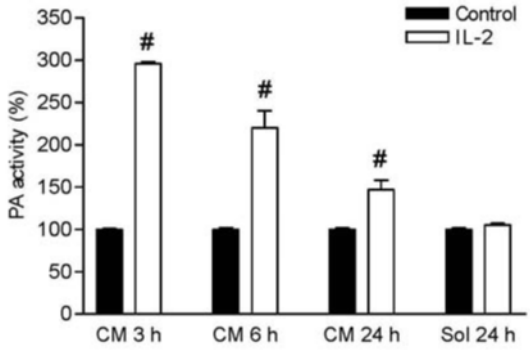
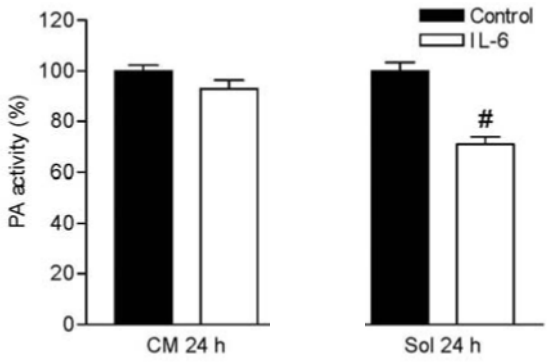
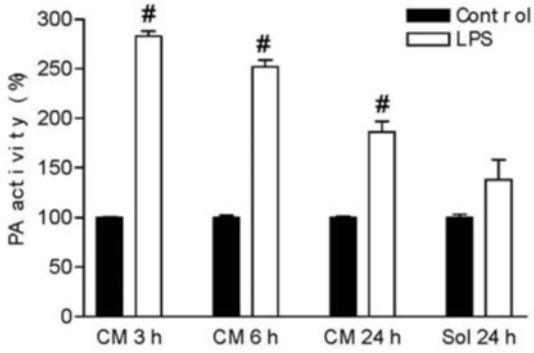



**Fig. 19.** *Efect of IL-6 on the PA production of A549 cells. Epithelial cells were treated with 1 ng/ml IL-6 for 24 h. All data are means±SEM, n= 3. #P< 0.05 vs. the value measured in control cells at the same time (for abbreviations see page 5).*



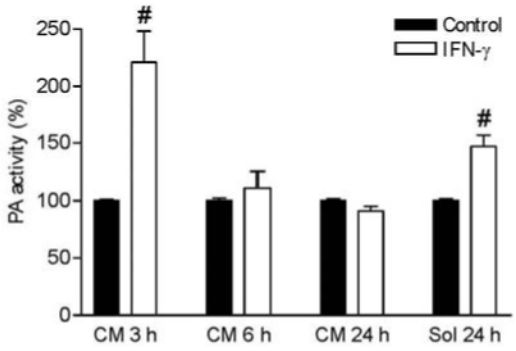

Bacterial LPS incubation significantly elevated PA activity in culture medium between 3h and 24 h, however PA level in cell fraction was only slightly elevated after 24 h (Fig. 20)

**Fig. 20.** *Efect of LPS on the PA production of A549 cells. Epithelial cells were treated with 1.0 μg/ml LPS for 3-24 h. All data are means±SEM, n= 3. #P< 0.05 vs. the value measured in control cells at the same time. (for abbreviations see page 5).*



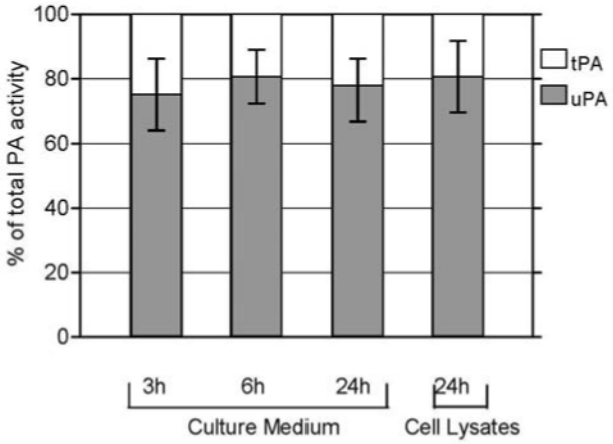

Dose of 100 ng/ml IFN-γ increased PA activity after 3 h, but not at 6 h or 24 h, in culture medium, however PA induction was seen after 24 h in cell lysates (Fig. 21).

**Fig. 21.** *Efect of IFN-γ on the PA production of A549 cells. Epithelial cells were treated with 100 ng/ml IFN-γ for 3-24 h. All data are means±SEM, n= 3. #P< 0.05 vs. the value measured in control cells at the same time (for abbreviations see page 5).*




Quantitative assays of PA activity were also performed in the absence or presence of uPA-inhibitor amiloride (1 mM). The ratio of amiloride-sensitive (uPA) and amilorideinsensitive (tPA) activity was approximately 74-81% *vs.* 19-26% both in culture medium and cell lysates (Fig. 22). Although slight time-dependent changes could be detected, this observation indicates that uPA is the predominant PA in cultured lung epithelial cells. Similar composition of PAs was seen in A549 epithelial cells treated with inflammatory mediators (data not shown).

**Fig. 22.** *Composition of PA production in untreated A549 cells: the ratio of amiloride-sensitive (uPA) and amiloride-insensitive (tPA) activity. All data are means±SEM, n=3 (for abbreviations see page 5).*

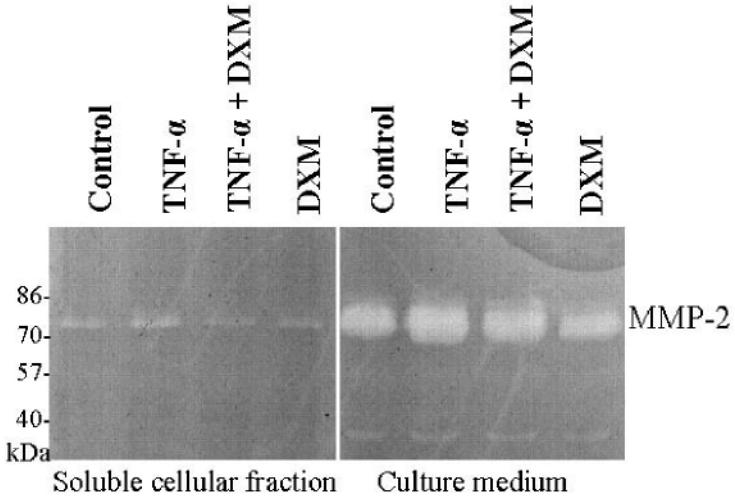



### 4.2. MMP Activity in Epithelial and Endothelial Cells

Metalloprotease zymography showed a proteolytic band at 72 kDa which corresponds to MMP-2 or gelatinase A (Fig. 23). The MMP-2 activity was significantly stronger in the culture medium compared to that in the cell lysate. TNF-á induced activation of MMP-2

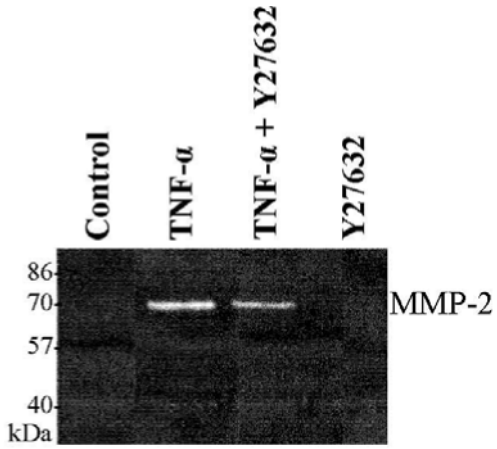
could be partially blocked by co-incubation with 10 nM DXM (Fig. 23). A faint proteolytic band at 40-45 kDa could be also detected in the culture medium. This band may correspond to MMP-3 or stromelysin 1.

**Fig. 23.** *Efect of DXM on TNF-á induced MMP activity. A549 cells were treated with 5.0 ng/ml TNF-á with or without 10 mM DXM for 16 h (for abbreviations see page 5).*



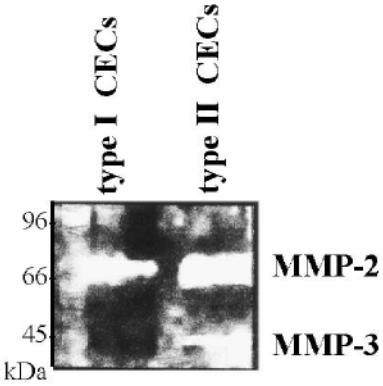

Similarly, Y27632 (10 ěM), a Rho inhibitor, could also reduce TNF-á induced activation of MMP-2 (Fig. 24) .

**Fig. 24.** *Efect of Y27632 on TNF-á induced MMP activity. A549 cells were treated with 5.0 ng/ml TNF-á with or without 10 μM Y27632 for 16 h (for abbreviations see page 5).*




We have also investigated the MMP activity in CECs of various morphological features. Type I endothelial cells exhibit epithelial-like morphology (cobblestone), whereas type II endothelial cells have spindle-like shape. Interestingly, only MMP-2 activity could be detected in type I cells with epithelial characteristics, whereas strong MMP-3 activity was also seen in type II cells (Krizbai et al., 2000).

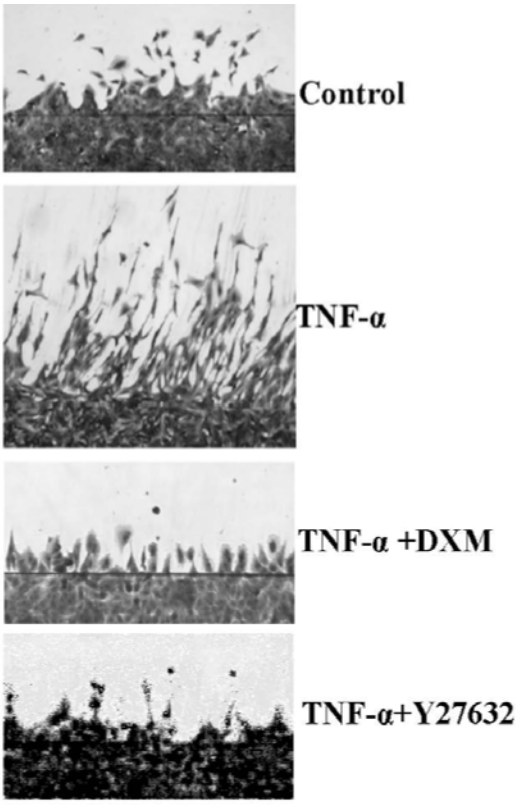
**Fig. 25.** *MMP activity in type I and type I cerebral endothelial cells (for abbreviations see page 5).*




### 4.3. Migration Potential of Epithelial Cells

After wounding the subconfluent lung epithelial cell layer with a razor blade, A549 cells moved from the wound edge into the denuded area. Microscopic evaluation of the migration revealed that in the presence of 5.0 ng/ml TNF-á A549 cells migrate further from the wound edge. This increased migratory capacity was reduced to the control level by DXM and Y27632 (Fig. 26). DXM and Y27632 alone did not influence the migration of A549 cells (data not shown).

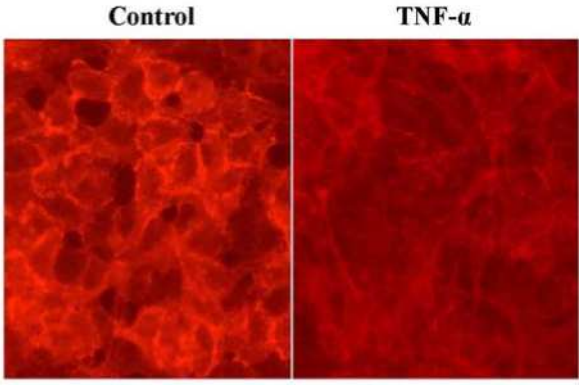
**Fig. 26.** *Efect of TNF-á on the migratory potential of A549 cells. Epithelial cells were wounded with a razor blade and cells were allowed to migrate in the denudated area for 24 h. A549 cells were treated with 5.0 ng/ml TNF-á, and 10 mM DXM, or 10 mM Y27632 (for abbreviations see page 5).*



### 4.4. Integrity of Epithelial Cellular Barrier

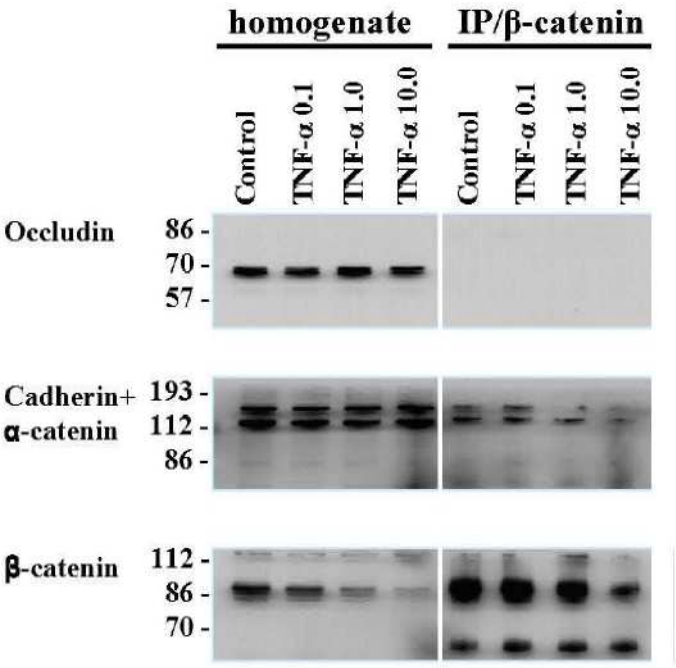
TNF-α treatment led to a relocalisation of the tight junction transmembrane protein occludin (Fig. 27.), although the expression of occludin did not change (Fig. 28.).

**Fig. 27.** *Efect of TNF-á on the morphology of tight junction protein occludin. A549 cells were treated with 5.0 ng/ml TNF-á for 6 h (for abbreviations see page 5).*




TNF-α treatment dose-dependently decreased β-catenin expression, whereas cadherin and α-catenin expression did not change (Fig. 28.). However, immunoprecipitation study with β-catenin antibody detected reduced amounts of cadherin and α-catenin in the β-catenin immunoprecipitates at higher TNF-α dose (Fig. 28.). This reduction may reflect recruitment of these proteins into insoluble membrane complexes.

**Fig. 28.** *Concentration-dependent efect of TNF-á on the expression of tight junction proteins. A549 cells were treated with 0.1 -10.0 ng/ml TNF-á for 6 h (for abbreviations see page 5).*



## 5. DISCUSSION

The present study strengthens the views that cytokines promote fibrinolysis in alveolar epithelium and contribute to pathogenesis and repair of lung injury.

### 5.1. Cytokines in the Induction of Proteolytic Enzymes in the Airways

*5.1.1. Efects of TNF-α and other inflammatory mediators on PA activity*

The results of the present study show that TNF-á and other cytokines are important regulators of the production and secretion PAs and MMPs in human alveolar epithelial cells. We have demonstrated that TNF-α strongly enhances the expression and release of uPA, tPA and MMP-2 in human lung epithelial cells and dexamethasone can inhibit these changes (Szabó et al., 2005). Our data also support a role for LPS and cytokines IL-1β, IL-2, and IFN-γ in the regulation of the production and synthesis of PAs in A549 cells (Szabó et al., 2009). It has been confirmed that uPA is responsible for approximately 75-80% of total PA activity in lung epithelial cells.

In accordance with our data, both IL-1β and TNF-α induced time- and dose-dependent increases in PA activity of cell lysates and supernatants of alveolar epithelial monolayers (Marshall et al., 1992). IL-1β produced greater increase in the PA activity in cell lysate, whereas TNF-α did it in supernatant. The effect of IL-1β on increased cell-surface plasmin generation is mediated in part by increased expression of uPAR which can be prevented by protein kinase C (PKC) inhibitors (Hasegawa et al., 1997). Decreased intracellular iron availability can prevent the IL-1β induced elevation in uPA expression in A549 cells (Hasegawa et al., 1999). Increased levels of uPA in the supernatant of IL-1β stimulated A549 epithelial cells indicated activation of fibrinolysis through plasmin system and resulted in quicker and more efficient alveolar epithelial repair (van Leer et al., 2005). Upregulation of uPA activity may be caused by an accumulation of newly synthesized uPA since Northern blot analyses demonstrated that TNF-á is able to induce a relatively rapid accumulation of uPA mRNA (Marshall et al., 1992).

It has been demonstrated in the present study that approximately 75-80% of total PA activity is amiloride-sensitive in cultured A549 cells. This observation supports a predominant role for uPA in the proteolytic activity of alveolar epithelial cells. Amiloride

was previously shown to completely inhibit uPA in control and stimulated rat alveolar epithelial cells, while it did have no effect on tPA (Marshall et al., 1990).

Cytokines and bacterial LPS were able to induce significant PA activity and DXM and Y27632, a specific inhibitor of Rho-associated protein kinases, were efficient inhibitor elevations in PA activity. TNF-α and IL-1β proved to be the strongest PA inducer in A549 cells. These data are in accordance with a previous study indicating that inflammatory mediators including LPS and IFN-γ had much lower capacity than cytokines IL-1β and TNF-α to cause an up-regulation of PA activity in pulmonary epithelial cells after 24 h of treatment (Marshall et al., 1992).

Rho-dependent mechanisms contributed to the TNF-α-induced activation of PAs and bacterial LPS induced increase in PA activity could also be prevented by Y27632, a specific inhibitor of Rho kinase in alveolar epithelial cells (Szabó et al., 2005a,b and 2009). Y27632 also proved to be effective in a murine model of intravenous LPS-induced acute lung injury; it attenuated lung edema, neutrophil emigration, and cytoskeletal rearrangement of pulmonary endothelial cells (Tasaka et al., 2005). In contrast to the LPS-induced PA release by cultured A549 cells *in vitro,* inhaled nebulized LPS inhibited PA activity and increased PAI-1 production in BAL fluid of healthy human volunteers (Maris et al., 2005). Chronic inhalation of LPS to mice caused all of the classic features of asthma including airflow obstruction, reversible airway inflammation, persistent airway hyperreactivity and lung tissue remodelling through the modification of pulmonary fibrinolytic system (Savov et al., 2003). The development of chronic LPS-induced airway disease is associated with fibrin deposition and enhanced expression of PAI-1 in the airways, concomitantly with an enhanced expression of MMP-9 (Savov et al., 2003).

The effect of LPS and cytokines on pulmonary epithelial cells differed from that on kidney epithelial cells, because intravenous administration of LPS dramatically reduced uPA release both proximal and distal tubules of murine kidney, whereas tPA synthesis was increased in glomerular cells, and up-regulation of PAI-1 was observed throughout the kidney (Moll et al., 1994). In cultured human kidney glomerular cells, IL-1β enhanced the release of tPA but not uPA, TNF-α induced both PAs, whereas IFN-γ had no significant effect (Iwamoto et al., 1990).

In the present study, IL-2 treatment induced a persistent elevation in PA activity in lung epithelial cells. Similarly, IL-2 infusion resulted up to 5-fold time-dependent increase in

plasma tPA values in of cancer patients (Baars et al., 1992). IL-6 did not affect total PA activity in alveolar epithelial cells, however IL-6 mediated increase in tPA activity was demonstrated in human endothelial cells infected by dengue virus (Huang et al., 2003). Cytokines regulate protease activity not only in alveolar epithelial cells, but also in neighboring cells in lung tissue. The secretion of uPA and PAI-1 was enhanced by IL-1β, IL-2, and TNF-α in human lung microvascular endothelial cells (Takahashi et al., 1998). IFN-γ treatment could increase both uPA and tPA activities in A549 lung epithelial cells in accordance with a previous study (Marshall et al., 1992) in which IFN-γ elevated total PA activity in the same cell line. In contrast to these observations, IFN-γ could inhibit IL-1α stimulated uPA expression in human endothelial cells (Wojta et al., 1992).

*5.1.2. Cytokine-induced signalling and PA activity*

Signal transduction pathways regulating epithelial proteolytic activity in response to inflammatory stimuli have been investigated. We demonstrated that inhibition of Rhokinase significantly reduces the activation of uPA and MMPs in A549 cells which shows that Rho-dependent mechanisms are involved in the regulation. The role of Rho-kinases in mediating the effect of TNF-á is supported by the finding that down-regulation of Rho signalling by coexpression of dominant-negative Rho mutants (i.e RhoA, RhoB and Rac) impairs TNF-á driven E-selectin gene expression (Nubel et al., 2004). Recent results indicate that the Rho family of small GTP-binding proteins plays an important role in the expression of NF-κB-dependent genes (Zhao and Pothoulakis, 2003). However, in our experiments inhibition of NF-κB by PDTC did not inhibit the TNF-á induced activation of u-PA suggesting that TNF-á may exert its effects through alternative signaling pathways as well. Furthermore, TNF-á is able to induce a rapid, sustained increase in p38 and extracellular signal-regulated kinase 1/2 (ERK1/2) activity in pulmonary endothelial cells which can be inhibited by Y-27632 indicating that Rho acts upstream of mitogen-activated protein kinases in signalling (Nwariaku et al., 2003). However, our results using the ERK inhibitor U0 126 show that this mechanism is not involved in the regulation of uPA activity in lung epithelial cells.

It has been shown that IL-1β induced transcriptional activation of the uPAR gene involves PKC-dependent mechanisms (Hasegawa et al., 1997). The role of PKC in PA synthesis is well-known; treatment with phorbol myristate acetate, a PKC activator, resulted in time-and dose-dependent up-regulation of uPA synthesis by alveolar epithelial cells (Marshall et

al., 1990). However, we could not detect inhibition of TNF-á-induced uPA activation by the Src-kinase inhibitor PP-1 and PKC inhibitor bisindolylmaleimide in A549 cells. Interestingly, PP-1 enhanced the TNF-á-induced uPA activation, which could be explained by the inhibitory effect of PP-1 on PAI-1 gene expression (Pontrelli et al., 2004). Glucocorticoids has also been shown to suppress uPA activity probably by due to interactions between the glucocorticoid receptor and another transcriptional activating system such as AP-1 and NF-κB (Hasegawa et al., 1997).

Inflammatory stimuli may lead to increased production of reactive oxygen species in airways, and lung tissue is protected against the oxidants by a variety of antioxidant mechanisms. Since SOD did not affect TNF-á-induced uPA activation in our study, oxygen radicals probably does not play significant role in this phenomenon. It is noteworthy that the MMP inhibitor phenanthroline inhibited uPA activity by 30% which could reflect a close connection between MMPs and PA system (Koolwijk et al., 2001).

*5.1.3. Role of PA activity in the pathogenesis of pulmonary diseases*

Increased proteolytic activity can be seen in the lung in chronic inflammation, during asthma, and after various mechanical or toxic stimuli. A recent Canadian study detected an association between allelic variants of human uPA gene *PLA U* located on 1 0q24 and asthma phenotype and confirmed that uPA might act as a regulator of asthma susceptibility (Bégin et al., 2007). At the cellular level, an increase in pericellular proteolysis may release chemotactic mediators and favor adhesion and migration of eosinophils, fibroblasts, and smooth muscle cells through the ECM in the lung tissue.

Mechanical stimulation, as would occur during bronchoconstriction, up-regulated the gene expression of uPA, tPA, and PAI-1, it increased uPA levels and uPA-dependent plasmin generation in cultured bronchial epithelial cells (Chu et al., 2006). Similar increase in epithelial uPA activity was seen in airway tissue samples of patients died in status asthmaticus (Chu et al., 2006). Inhaled mineral particles can also induce the production and release of uPA which may exert opposite effects at various stages of the inflammatory and fibrotic processes in lung tissue and contribute to the pathogenesis of silica-induced pulmonary fibrosis and chronic inflammatory lung disease (Lardot et al., 1998). An elegant *in situ* zymography study demonstrated that local microenvironmental fibrinolytic activity of the lung tissue during inflammation might differ from the changes in BAL fluid (Nishiuma et al., 2004). Intense PA activity was localized to areas of lung injury after

exposure to bleomycin in mice while the PA activity within lavage fluid was completely supressed (Nishiuma et al., 2004). On the other hand, inhalation of exogenous uPA significantly reduced subepithelial fibrosis, decreased airway hyperresponsiveness, up-regulated plasmin activity in BAL fluid, and reduced airway remodelling in a murine model of chronic asthma (Kuramoto et al., 2009).

BPD was associated with higher concentrations of IL-1β, IL-6, IL-8, IL-10, TNF-α and IFN-γ and lower concentrations of IL-17 (Ambalavanan et al., 2009). The overall cytokine pattern suggests that BPD and death may be associated with impairment in the transition from the innate immune response mediated by neutrophils to the adaptive immune response mediated by T lymphocytes (Ambalavanan et al., 2009). Pulmonary PAI-2 and trypsin-2 concentrations are also elevated in BPD (Cederquist et al., 2003, 2006).

Fibroblast proliferation and components of the ECM, including collagen and fibronectin, are markedly increased in the lungs of infants who die from CLD of prematurity (Kotecha, 1996). The pro-inflammatory cytokines, IL-1β and IL-6 and mediators which reflect neutrophil recruitment and activation, including soluble intercellular adhesion molecule, IL-8 and neutrophil elastase, were increased in BAL fluid obtained from infants who developed CLD (Kotecha, 1996). Fibrosis is thought to be mediated by the pro-fibrotic cytokines including TGF-β and both active and total TGF-β were increased in BAL fluid. Furthermore, both type I procollagen and TGF-β were increased qualitatively in lung tissue obtained at autopsy from infants who died from respiratory failure. Excessive release of TNF-α may contribute to the pathogenesis of BPD in very preterm infants and the risk of developing BPD may be associated with host genetic factors that regulate TNF production, however it is not the most commonly studied variant with guanine to adenine transition at position -308 nucleotides (Chauhan et al., 2009). Inhaled beclomethasone-treated infants with moderately elevated baseline IL-8 levels received less systemic glucocorticoid therapy and had a lower incidence of BPD than nontreated infants (Gupta et al., 2000).

### 5.2. Regulation of Matrix Metalloproteinases in Alveolar Epithelial Cells

#### *5.2.1. Efect of TNF-α and other cytokines on MMP induction*

We have investigated MMP activity in epithelial and endothelial cells and demonstrated strong TNF-α induced regulation of MMP-2 in lung epithelial cells. An induction of MMP expression by inflammatory mediators such as TNF-á has been reported recently, but the

mechanisms of inhibition was not presented (Hetzel et al., 2003). MMPs are known to degrade the ECM, basal membrane components, and are involved in the regulation of TJ proteins, such as occludin. (Wachtel et al., 1999). Similar results have been obtained in a study showing that MMP-9 is induced by TNF-α in pulmonary microvascular endothelial cells (Partridge et al., 1993). Fibroblast migration, proliferation, and MMP production were also shown to be regulated by IL-1β and TNF-α (Sasaki et al., 2000).

The proinflammatory cytokines IL-1, IL-6, and TNF-α were shown to up-regulate MMPs both in vitro and in vivo. IL-1β is produced in a biologically inactive form and can be activated by cleavage with MMP9. When IL-1β production is ectopically expressed in the lung epithelium, it causes pulmonary inflammation, spontaneous overexpression of MMP9 and MMP12, disruption of elastin fibers in alveolar septa, and fibrosis in airway walls, a pathology resembling that of emphysema (Lappalainen et al., 2005; Greenlee et al., 2007).

*5.2.2. MMPs in signalling and pulmonary diseases*

In this study, we provide evidence that DXM is able to reduce the activation of MMP-2. This finding is in line with the results of Araya et al. (2001) demonstrating that DXM inhibits the irradiation-induced increase in MMP-2 in lung epithelial cells. An explanation could be that MMP-2 gene contains putative binding sites for a variety of transcription factors which are regulated by glucocorticoids. In contrast, neutrophil MMP-9 activity in the BAL fluid was poorly inhibited by glucocorticoids (Cundall et al., 2003). The role of Rho in the regulation of MMP-2 is less well understood. It has been shown that Rac1, one member of Rho-related small GTPases, is a mediator of MMP-2 activation in HT1080 fibrosarcoma cells (Zhuge and Xu, 2001). Our results suggest that Rho may be at least partially involved in te regulation of MMP-2 in lung epithelial cells.

MMPs have been reported to play a critical role in the pathogenesis of acute and chronic lung diseases and in airway wall remodelling in chronic inflammatory processes of the respiratory system. In the lung, two gelatinases, MMP-2 and MMP-9, are known to be produced by a variety of cells in vitro. MMP-2 is preferentially secreted from fibroblasts and various epithelial cells including airway epithelial cells, and MMP-9 is preferentially expressed by inflammatory cells (Gibbs et al., 1999). Hayashi and colleagues (1996) have demonstrated that type IV collagen and MMP-2 showed co-localization in disrupted epithelial basement membrane. Furthermore, an increase in MMP-2 activity has been reported in epithelial lining fluid obtained from patients with acute RDS.

Expression of several MMPs (including MMP1, MMP2, MMP3, MMP8, and MMP9) has been associated with asthma. Increase in MMP9 activity in the subepithelial basement membrane is found to be accompanied by higher TGF-β. These studies suggest that in patients with severe asthma, neutrophils play a key role in lung remodelling because they express both MMP9 and TGF-β, which are involved in breakdown and repair of tissue, respectively. BAL fluid has been shown to possess increased MMP activity (Mautino et al., 1997) and allergen exposure is able to induce an increase in MMP9, MMP12, and MMP14 activity (Cataldo et al, 2002). Especially activation of MMP9 (Han et al., 2003) may have relevance to chronic structural airway changes in asthma through its ability to degrade proteoglycans and thus potentially enhance airway fibrosis and smooth muscle proliferation and its ability to release and activate latent, matrix-bound growth factors. Furthermore, recent results by Prikk et al (2003) suggest that MMP-8 and its activation has an important role in the airway destruction, healing, remodelling, and treatment response in asthma. An increased MMP2 activity in induced sputum and BAL fluid samples from patients with asthma bronchiale was evidenced as well (Maisi et al., 2002).

MMPs have important, mainly protective, roles in asthma. In particular, MMP8 deficiency leads to enhanced granulocytic inflammation after allergen exposure, presumably because MMP8 has a role in the apoptosis of granulocytes. Both MMP2 and MMP9 are essential for the movement of inflammatory cells into the airway lumen, because they cleave chemotactic factors in the luminal fluid, which results in enhanced chemotactic activities. In addition, MMP9 is also important for the movement of dendritic cells into the lumen. Corticosteroids decrease the inflammation and therefore also MMP production. Inhibition of MMPs with doxycycline, with a hydroxamate MMPI or with TIMP2 has been reported to decrease airway damage, hyperresponsiveness and inflammation.

The amount of MMP2 at birth successfully predicted the outcome of BPD in preterm infants and birth weight was significantly correlated with amount of MMP2 suggesting that the stage of development at birth is the biggest factor in whether an infant develops BPD. The levels of MMP-2, -8, -9 and inhibitor TIMP-2 are elevated in tracheal aspirate from preterm infants suffering from RDS (Cederquist et al., 2001). ECM components, including collagen and fibronectin, are increased in the lungs of infants who die from CLD (Kotecha, 1996)

### 5.3. Migration Potential of Tumor Necrosis Factor-α Induced Epithelial Cells

In our study the TNF-α induced increased changes of PA and MMP-2 activity were associated with an increased migration potential of A549 alveolar epithelial cells which was inhibited by both DXM and Rho inhibitor Y27632. This inhibition can be explained at least partially with the reduced protease activity after DXM or Y27632 treatment.

Cell migration is a highly integrated, multi-step process that plays an important role in the progression of various diseases including cancer, atherosclerosis and arthritis. An increased proteolytic activity is often associated with an increased migratory potential mainly due to ECM degradation ot proteolysis of cell surface molecules regulating cell-cell interactions. Wound healing, which describes the migration of cells toward a point of injury, could be detected by the conventional scratch assay. uPA facilitates cell migration by localizing proteolysis on the cell surface and by inducing intracellular signalling pathways. (Kiian et al., 2003). The implication of uPA in human bronchial epithelial cell migration was studied by incubating culture with a monoclonal antibody raised against uPA and these experiments led to a 70% reduction in cell velocity (Legrand et al., 2001).

Both serine proteases and MMPs have been implicated in the complex integrated events underlying cell migration. Membrane and soluble proteinases with integrins and other adhesion proteins and with intracellular signalling systems also play a role in the regulation of proteolysis in cell migration (Murphy and Gavrilovic, 1999). An important role of MMP-2 and MMP-9 in the migration of lung epithelial cells has been shown by several studies (Murphy and Gavrilovic, 1999; Buckley et al, 2001; Zhuge and Hu, 2001). Legrand et al. (2001) observed that uPA was only detected in migrating cells at the wound edges and located at crucial sites for cell/ECM interactions. A significant dose-dependent increase was observed in cell migration velocity after treatment with plasmin or MMP-9. Moreover, addition of exogenous plasmin led to a twofold increase of activated MMP-9 in migrating cells, while the addition of uPA antibody led to an inhibition of activated MMP-(Legrand et al., 2001). The action of uPA in the migration of human bronchial epithelial cells is mediated by the generation of plasmin, which in turn activates MMP-9, thus making possible cell migration. It has been also known that Rho family GTPases regulate actin cytoskeleton, cell adhesion, and play important role in cell polarisation and directional migration (Jo et al., 2002; Fukata et al., 2003).

### 5.4. Tumor Necrosis Factor-α and the Damage to Pulmonary Alveolocapillary Barrier

In the present study, TNF-α induced damage to the barrier integrity of epithelial monolayers was demonstrated. Cytokine treatment led to a relocalisation of the TJ transmembrane protein occludin, decreased β-catenin expression and modified recruitment of the proteins in adherens junction. These structural changes may well correspond to the increased barrier permeability seen in in vivo and in vitro studies (Partridge et al., 1993; Wachtel et al., 1999; Lacherade et al., 2001).

Alveolar liquid and protein clearance was measured in a model of Pseudomonas aeruginosa pneumonia in rats (Rezaiguia et al., 1997). Histologic evidence of alveolar epithelial injury and increased alveolar liquid clearance was seen 24 h after intratracheal instillation of bacteria. This increase was inhibited either by anti-TNF-α neutralizing antibody or by amiloride, an inhibitor of uPA. TNF-α (5 μg) instilled in normal rats could also increase alveolar liquid clearance by similar degree. Gram-negative pneumonia upregulates net alveolar epithelial fluid clearance by a TNF-α-dependent mechanism. In another study, LPS injection (2 mg/kg) resulted in leakage of FITC-dextran (4 kDa) from blood into BAL fluid in endotoxemic mice within 20 h (Han et al., 2004). This decrease in barrier function was associated with upregulation of inducible NO synthase expression and NF-κB activation in lung tissue. Expression of the TJ proteins, ZO-1, ZO-2, ZO-3, and occludin, as assessed by immunoblotting and/or immunofluorescence, decreased in lung after the injection of mice with LPS (Han et al., 2004). Growth factors and cytokines increase the rate of fluid transport across the alveolar epithelium. Alveolar fluid clearance may already be upregulated in the presence of clinical conditions that predispose to acute lung injury. Release of TNF-α in the presence of either pneumonia or peritonitis increases the transport capacity of the alveolar epithelium (Rezaiguia et al., 1997). Also, the extent of interstitial edema may limit the transport capacity of the alveolar epithelium. Moreover, if alveolar epithelial injury is so severe that the barrier properties of the epithelium have been substantially altered, then increasing the transport capacity of individual alveolar epithelial cells will not be effective until the epithelial barrier has been restored.

TNF-α can also induce barrier dysfunction in endothelium of the pulmonary vessels. TNF-α (1,000 U/ml) increased albumin permeability in postconfluent bovine pulmonary artery endothelial cell monolayers, induced pulmonary endothelial F-actin depolymerization, intercellular gap formation, and barrier dysfunction (Goldblum et al., 1993). Barrier

opening can occur independently of actin-myosin-mediated contraction, similar to the studies using thapsigargin (Siflinger-Birnboim and Johnson, 2003). TNF-α activates PKC-α, then stabilization of actin fibers affects junctional proteins and altered cell-cell adherence results in the permeability response.

Asbestos increases paracellular permeability across cultured human lung epithelial monolayers (Peterson and Kirschbaum, 1998). Plasma proteins, including fibrinogen, will cross the altered lung epithelium and form fibrin in the distal airways. Alveolar epithelial cells express significant amounts of uPA that activates plasmin and leads to increased fibrin degradation. Asbestos increases uPA expression by lung epithelial cells and the fibrin degradation products cross asbestos-exposed epithelial monolayers more readily. Fibrin degradation products are biologically active compounds that act as chemoattractants for neutrophils, inactivate surfactant, and increase cytokine production. Altered lung epithelial permeability is central in both initiating and perpetuating this pathophysiological sequence.

The family of zinc- and calcium-dependent MMPs play an important role in remodeling of the airways in disease. Proinflammatory cytokines increases lymphocyte-derived MMP9 levels in the airway lumen of asthmatics, whereas the level of the MMP9 inhibitor TIMP 1 is decreased leading to increased protease activity. Apical MMP9 significantly decreases immunostaining of TJ proteins and transepithelial electrical resistance in a model of well-differentiated human airway epithelia (Vermeer et al., 2009). Due to disruption of barrier function viruses gained access to the epithelial basolateral surface, which increased infection efficiency. MMP9 exerts its effects on the epithelium by cleaving one or more components of cell-cell junctions and airway remodeling associated with asthma may be directly regulated by MMP9.

Although several pharmacologic treatments might be successful in upregulating alveolar fluid clearance in the setting of clinical acute lung injury (Crandall and Matthay, 2001), there are potential problems: (i) the lack of sufficient functional epithelial barrier would blunt the efficacy of any fluid transport-enhancing therapy, under some conditions, (ii) the alveolar epithelium cannot respond to beta adrenergic agonists, (iii) the degree of epithelial injury may be so severe that relentless alveolar flooding may overwhelm any transport capacity of the epithelium, and (iv) endogenous factors might maximally upregulate alveolar epithelial fluid transport. Thus, exogenous delivery of an alveolar fluid transport-

enhancing therapy might be ineffective because of the presence of endogenous factors that have already upregulated clearance. On the other hand, increased macromolecular transport across the alveolar epithelial barrier may also have potential therapeutic advantages in case of pulmonary drug delivery systems (Crandall and Matthay, 2001). Administration of some macromolecule drugs (e.g., proteins) via the pulmonary route by inhalation of aerosolized drugs has been well documented to provide high bioavailability. However, this could be only a theoretical use of cytokine-induced epithelial barrier opening.

## 6. CONCLUSIONS

The new observations of the present study indicate that cytokines promote fibrinolysis in alveolar epithelium and contribute to pathogenesis and repair of lung injury. It has been demonstrated that TNF-α enhances the expression and release of uPA, tPA and MMP-2 in human lung epithelial cells. TNF-α-induced proteolytic activity can be inhibited by dexamethasone and Rho-kinase inhibitors. TNF-α induced structural changes in the barrier integrity of epithelial monolayers was demonstrated in alveolar epithelial cells.

Data obtained on in vitro models may contribute to detection of the signal transduction pathways regulating epithelial proteolytic activity in response to inflammatory stimuli and to identification of potential therapeutic target molecules.

## 7. BIBLIOGRAPHY


Allen J., Zwerdling R., Ehrenkranz R., Gaultier C., Geggel R., Greenough A., Kleinman R., Klijanowicz A., Martinez F., Ozdemir A., Panitch H.B., Nickerson B., Stein M.T., Tomezsko J., Van Der Anker J., American Thoracic Society, Statement on the care of the child with chronic lung disease of infancy and childhood, *Am J Respir Crit Care Med.,* 168: 356-396, 2003.

Ambalavanan N., Carlo W.A., D'Angio C.T., McDonald S.A., Das A., Schendel D., Thorsen T., Higgins R.D., Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network, Cytokines associated with bronchopulmonary dysplasia or death in extremely low birth weight infants, *Pediatrics,* 123: 1132-1141, 2009.

Araya J., Maruyama M., Sassa K., Fujita T., Hayashi R., Matsui S., Kashii T., Yamashita N., Sugiyama E., Kobayashi M., Ionizing radiation enhances matrix metalloproteinase-2 production in human lung epithelial cells, *Am. J. Physiol. Lung Cell. Mol. Physiol.,* 280: L30-L38, 2001

Baars J.W., de Boer J.P., Wagstaff J., Roem D., Eerenberg-Belmer A.J.M., Nauta J., Pinedo H.M, Hack C.E., Interleukin-2 induces activation of coagulation and fibrinolysis: resemblance to the changes seen during experimental endotoxaemia, *Br. J. Haematol.,* 82: 295-301, 1992.

Barnes P.J., Cytokine-directed therapies for the treatment of chronic airway diseases, *Cytokine Growth Factor Rev.,* 14: 511-522, 2003a.

Barnes P.J., Pathophysiology of asthma, *Eur. Respir. Mon.,* 23: 84–113, 2003b.

Barnes P.J., Biology and assessment of airway inflammation, *Kendig 's Disorders of the Respiratory Tract in Children, 7th edition, Chapter nr. 5.* Philadelphia, Saunders Elsevier, pp.65-74, 2006.

Barnes P.J., Pharmacology of airway smooth muscle, *Am. J. Respir. Crit. Care Med.* 158: S123–S132, 1998.

Bégin P., Tremblay K., Daley D., Lemire M., Claveau S., Salessa C., Kacel S., Montpetit A., Becker A., Chan-Yeung M., Kozyrskyj A.L., Hudson T.J., Laprise C., Association of urokinase-type plasminogen activator with asthma and atopy, *Am. J. Respir. Crit. Care Med.*, 175: 1109-1116, 2007.

Berry M.A., Hargadon B., Shelley M., Parker D., Shaw D.E., Green R.H., Bradding D., Brightling C.E., Wardlaw A.J., Pavord I.D., Evidence of a role of tumor necrosis factor α in refractory asthma, *N. Engl. J. Med.*, 354: 697-708, 2006.

Boitano S., Safdar Z., Welsh D.G., Bhattacharya J., Koval M., Cell-cell interactions in regulating lung functions, *Am. J. Physiol. Lung Cell. Mol. Physiol.*, 287: L455-L459, 2004.

Buckley S., Driscoll B., Shi W., Anderson K., Warburton D., Migration and gelatinases in cultured fetal, adult, and hyperoxic alveolar epithelial cells, *Am. J. Physiol. Lung Cell. Mol. Physiol.*, 281: L427-L434, 2001.

Castro M., Ramirez M.I., Gern J.E., Cutting G., Redding G., Hagood J.S., Whitsett J., Abman S., Raj J.U., Barst R., Kato G.J., Gozal D., Haddad G.G., Prabhakar N.R., Gauda E., Martinez F.D., Tepper R., Wood R.E., Accurso F., Teague W.G., Venegas J., Cole F.S., Wright R.J., Strategic plan for prediatric respiratory diseases research. An NHLBI Working Group Report, *Proc. Am. Thorac. Soc.*, 6: 1-10, 2009.

Cataldo D.D., Tournoy K.G., Vermaelen K., Munaut C., Foidart J.M., Louis R., Noel A., Pauwels RA., Matrix metalloproteinase-9 deficiency impairs cellular infiltration and bronchial hyperresponsiveness during allergen-induced airway inflammation, *Am. J. Pathol.*, 161: 491-498, 2002.

Cederqvist K., Haglund C., Heikkilä P., Sorsa T., Tervahartiala T., Stenman U.-H., Andersson S., Pulmonary trypsin-2 in the development of bronchopulmonary dysplasia in preterm infants, *Pediatrics*, 112: 570-577, 2003.

Cederqvist K., Sirén V., Petäjä J., Vaheri A., Haglund C., Andersson S., High concentrations of plasminogen activator inhibitor-1 in lungs of preterm infants with respiratory distress syndrome, *Pediatrics*, 117: 1226-1234, 2006.

Cederqvist K., Sorsa T., Tervahartiala T., Maisi P., Reunanen K., Lassus P., Andersson S., Matrix metalloproteinases-2, -8, and -9 and TIMP-2 in tracheal aspirates from preterm infants with respiratory distress, *Pediatrics*, 108: 686-692, 2001.

Chauhan, M., Bombell, S., McGuire, W., Tumour necrosis factor (--308A) polymorphism in very preterm infants with bronchopulmonary dysplasia: a meta-analysis, *Arch. Dis. Child. Fetal Neonatal*, 94: F257-F259, 2009.

Choi C.W., Kim B.I., Kim H.S., Park J.D., Choi J.H., Son D.W., Increase of interleukin-6 in tracheal aspirate at birth: a predictor of subsequent bronchopulmonary dysplasia in preterm infants, *Acta Paediatr.*, 95: 3 8-43, 2006.

Chu E.K., Cheng J., Foley J.S., Mecham B.H., Owen C.A., Haley K.J., Mariani T.J., Kohane I.S., Tschumperlin D.J., Drazen J.M., Induction of the plasminogen activator system by mechanical stimulation of human bronchial epithelial cells, *Am. J. Respir. Cell. Mol. Biol.*, 35: 628-638, 2006.

Crandall E.D., Matthay M.A., Alveolar epithelial transport: basic science to clinical medicine, *Am. J. Respir. Crit. Care Med.*, 163: 1021–1029, 2001.

Cundall M., Sun Y., Miranda C., Trudeau J.B., Barnes S., Wenzel S.E., Neutrophil-derived matrix metalloproteinase-9 is increased in severe asthma and poorly inhibited by glucocorticoids, *J. Allergy Clin. Immunol.*, 112: 1064-1071, 2003.

De Benedetti E., Nicod L., Reber G., Vifian C., De Moerloose P., Procoagulant and fibrinolytic activities in bronchoalveolar fluid of HIV-positive and HIV-negative patients, *Eur. Respir. J.*, 5: 411-417, 1992. Erzurum S.C., Inhibition of tumor necrosis factor α for refractory asthma, *New Engl. J. Med.*, 354: 754-758, 2006.

Fukata M., Nakagawa M., Kaibuchi K., Roles of Rho-family GTPases in cell polarisation and directional migration, *Curr. Opin. Cell Biol.*, 15: 590-597, 2003.

Ghosh S., Brown R., Jones J.C.R., Ellerbroek S.M., Stack M.S., Urinary-type plasminogen activator (uPA) expression and uPA receptor localization are regulated by $\alpha 3\beta 1$ integrin in oral keratinocytes, *J. Biol. Chem.*, 275: 23869-23876, 2000.

Gibbs D.F., Warner R.L., Weiss S.J., Johnson K.J., Varani J.. Characterization of matrix metalloproteinases produced by rat alveolar macrophages, *Am. J. Respir. Cell. Mol. Biol.*, 20: 1136-1144, 1999

Goldblum S.E., Ding X., Campbell-Washington J., TNF-α induces endothelial cell F-actin depolymerization, new actin synthesis, and barrier dysfunction, *Am. J. Physiol. Cell. Physiol.*, 264: C894-C905, 1993.

Gonzales L.W., Guttentag S.H., Wade K.C., Postle A.D., Ballard P.L., Differentiation of human pulmonary type II cells in vitro by glucocorticoid plus cAMP, *Am. J. Physiol. Lung Cell. Mol. Physiol.*, 283: L940-L951, 2002..

Goodman R.B., Pugin J., Lee J.S., Matthay M.A., Cytokine-mediated inflammation in acute lung injury, *Cytokine Growth Factor Rev.*, 14: 523–535, 2003.

Groneck P., Speer C.P., Inflammatory mediators and bronchopulmonary dysplasia, *Arch. Dis. Child. Fetal Neonatal,* 73: F1-F3, 1995.

Greenlee K.J., Werb Z., Kheradmand F., Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted, *Physiol. Rev.*, 87: 69-98, 2007.

Gupta G.K., Cole C.H., Abbasi S., Demissie S., Njinimbam C., Nielsen H.C., Colton T., Frantz I.D. 3rd, Effects of early inhaled beclomethasone therapy on tracheal aspirate inflammatory mediators IL-8 and IL-1ra in ventilated preterm infants at risk for bronchopulmonary dysplasia, *Pediatr. Pulmonol.*, 30: 275-281, 2000.

Han X., Fink M.P., Uchiyama T., Yang R., Delude R.L., Increased iNOS activity is essential for pulmonary epithelial tight junction dysfunction in endotoxemic mice, *Am. J. Physiol. Lung Cell. Mol. Physiol.*, 286: L259-L267, 2004.

Han Z., Junxu Z., Zhong N., Expression of matrix metalloproteinases MMP-9 within the airways in asthma, *Respir. Med.*, 97: 563-567, 2003.

Hasegawa T., Sorensen L., Dohi M., Rao N.V., Hoidal J.R., Marshall B.C., Induction of urokinase-type plasminogen activator receptor by IL-1β. *Am. J. Respir. Cell. Mol. Biol.*, 16: 683-692, 1997.

Hasegawa T., Sorensen L., Ooi H., Marshall B.C., Decreased intracellular iron availability suppresses epithelial cell surface plasmin generation. Transcriptional and post-transcriptional effects on u-PA and PAI-1 expression, *Am. J. Respir. Cell Mol. Biol.*, 21: 275-282, 1999.

Hayashi T., Stetler-Stevenson W.G., Fleming M.V., Fishback N., Koss M.N., Liotta L.A., Ferrans V.J., Travis W.D., Immunohistochemical study of metalloproteinases and their tissue inhibitors in the lungs of patients with diffuse alveolar damage and idiopathic pulmonary fibrosis, *Am. J. Pathol.*, 149: 1241-1256, 1996.

Hu J., Van den Steen P.E., Sang Q.-X.A., Opdenakker C., Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases, *Nat. Rev. Drug Discov.*, 6: 480-498, 2007.

Huang Y.-H., Lei H.-Y., Liu H.-S., Lin Y.-S., Chen S.-H., Liu C.-C., Yeh T.-M., Tissue plasminogen activator induced by dengue virus infection of human endothelial cells, *J. Med. Virol.*, 70: 610-616, 2003.

Iwamoto T., Nakashima Y., Sueishi K., Secretion of plasminogen activator and its inhibitor by glomerular epithelial cells, *Kidney Int.*, 37: 1466-1476, 1990.

Janssen-Heininger, Y.M.W., Poynter M.E., Aesif S.W., Pantano C., Ather J.L., Reynaert N.L., Ckless K., Anathy V., van der Velden J., Irvin C.G., van der Vliet A., Nuclear factor κB, airway epitelium, and asthma. Avenues for redox control, *Proc. Am. Thorac. Soc.*, 6: 249-255, 2009.

Jo M., Thomas K.S., Somlyo A.V., Somlyo A.P., Gonias S.L., Cooperativity between the Ras-ERK and Rho-Rho kinase pathways in urokinase-type plasminogen activator-stimulated cell migration, *J. Biol. Chem.*, 277: 12479-12485, 2002.

Jobe, A.H., Bancalari, E., Bronchopulmonary dysplasia, *Am. J. Respir. Crit. Care Med.*, 163: 1723-1729, 2001.

Kawkitinarong K., Linz-McGillem L., Birukov K.G., Garcia J.G.N., Differential regulation of human lung epithelial and endothelial cell barrier function by thrombin, *Am. J. Respir. Cell Mol. Biol.*, 31: 517-527, 2004.

Kazzi S.N., Romero R., McLaughlin K., Ager J., Janisse J., Serial changes in levels of IL-6 and IL-1β in premature infants at risk for bronchopulmonary dysplasia, *Pediatr. Pulmonol.*, 31: 220-226, 2001.

Kiian I., Tkachuk N., Haller H., Dumler I., Urokinase-induced migration of human vascular smooth muscle cells requires coupling of the small GTPases RhoA and Rac1 to the Tyk2/PI3-K signalling pathway, *Thromb. Haemost.*, 89: 904-914, 2003.

Koolwijk P., Sidenius N., Peters E., Sier C.F., Hanemaaijer R., Blasi F., van Hisbergh V.W., Proteolysis of urokinase-type plasminogen activator receptor by metalloproteinase-12: implication for angiogenesis in fibrin matrices, *Blood*, 97: 3123-3131, 2001.

Kotecha S., Cytokines in chronic lung disease of prematurity, *Eur. J. Pediatr.*, 155 Suppl 2: S14-S7, 1996.

Krizbai I.A., Bauer H., Amberger A., Hennig B., Szabó H., Fuchs R., Bauer H.-C., Growth factor-induced morphological, physiological and molecular characteristics in cerebral endothelial cells, *Eur. J. Cell Biol.*, 79: 594-600, 2000.

Kucharewicz I., Kowal K., Buczko W., Bodzenta-Lukaszyk A., The plasmin system in airway remodeling, *Thromb. Res.*, 112: 1-7, 2003.

Kuramoto E., Nishiuma T., Kobayashi K., Yamamoto M., Kono Y., Funada Y., Kotani Y., Sisson T.H., Simon R.H., Nishimura Y., Inhalation of urokinase-type plasminogen activator reduces airway remodeling in murine asthma model, *Am. J. Physiol. Cell Mol. Physiol.*, 296: L337-L346, 2009. Lacherade

J.-C., Van de Louw A., Planus E., Escudier E., D'Ortho M.-P., Lafuma C., Harf A., Delclaux C., Evaluation of basement membrane degradation during TNF-α-induced increase in epithelial permeability, *Am. J. Physiol. Lung Cell Mol. Physiol.*, 281: L134-L143, 2001.

Lardot C.G., Huaux F.A., Brockaert F.R., Declerck P.J., Delos M., Fubini B., Lison D.F., Role of urokinase in the fibrogenic response of the lung to mineral particles, *Am. J. Respir. Crit. Care Med.*, 157: 617-628, 1998.

Legrand C., Polette M., Tournier J.M., de Bentzmann S., Huet E., Monteau M., Birembaut P., uPA/plasmin system-mediated MMP-9 activation is implicated in bronchial epithelial cell migration, *Exp. Cell Res.*, 264: 326-336, 2001.

Maisi P., Prikk K., Sepper R., Pirila E., Salo T., Hietanen J., Sorsa T., Soluble membrane-type 1 matrix metalloproteinase (MT1-MMP) and gelatinase A (MMP-2) in induced sputum and bronchoalveolar lavage fluid of human bronchial asthma and bronchiectasis, *APMIS*, 110: 771-782, 2002.

Maris N.A., de Vos A.F., Bresser P., van der Zee J.S., Meijers J.C., Lijnen H.R., Levi M., Jansen H.M., Van der Poll T., Activation of coagulation and inhibition of fibrinolysis in the lung after inhalation of lipopolysaccharide by healthy volunteers, *Thromb. Haemost.*, 93: 1036-1040, 2005.

Marshall B.C., Sageser D.S., Rao N.V., Emi M., Hoidal J.R., Alveolar epithelial cell plasminogen activator. Characterization and regulation, *J. Biol. Chem.*, 265: 8198-8204, 1990.

Marshall B.C., Xu Q.P., Rao N.V., Brown B.R., Hoidal J.R., Pulmonary epithelial cell urokinase type plasminogen activator. Induction by interleukin-1 β and tumor necrosis factor-α, *J. Biol. Chem.*, 267: 11462-11469, 1992.

Mautino G., Oliver N., Chanez P., Bousquet J., Capony F., Increased release of matrix metalloproteinase-9 in bronchoalveolar lavage fluid and by alveolar macrophages of asthmatics, *Am. J. Respir. Cell. Mol. Biol.*, 17: 583-591, 1997.

Mitic L.L., Van Itallie C.M., Anderson J.M., Molecular physiology and pathophysiology of tight junctions, I. Tight junction structure and function: lessons from mutant animals and proteins, *Am. J. Physiol. Gastrointest. Liver Physiol.*, 279:250-254, 2000.

Moll S., Schifferli J.A., Huarte J., Lemoine R., Vassalli J.D., Sappino A.P., LPS induces major changes in the extracellular proteolytic balance in the murine kidney, *Kidney Int.*, 45: 500-508, 1994.

Moorman J.E., Rudd R.A., Johnson C.A., King M., Minor P., Bailey C., Scalia M.R., Akinbami L.J., Centers for Disease Control and Prevention, National surveillance for asthma--United States, 1980-2004. *MMWR Surveill. Summ.* 56:1-54, 2007.

Murphy G., Gavrilovic J., Proteolysis and cell migration: creating a path? *Curr. Opin. Cell Biol.*, 11: 614-621, 1999.

Nishiuma T., Sisson T.H., Subbotina N., Simon R.H., Localization of plasminogen activator activity within normal and injured lungs by in situ zymography, *Am. J. Respir. Cell Mol. Biol.*, 31: 552-558, 2004.

Nubel T., Dippold W., Kleinert H., Kaina B., Fritz G., Lovastatin inhibits Rho-regulated expression of E - selectin by TNF-α and attenuates tumor cell adhesion, *FASEB J.*, 18: 140-142, 2004.

Nwariaku F.E., Rothenbach P., Liu Z., Zhu X., Turnage R.H., Terada L.S., Rho inhibition decreases TNF-induced endothelial MAPK activation and monolayer permeability, *J. Appl. Physiol.*, 95: 1889-1895, 2003.

Partridge C.A., Jeffrey J.J., Malik A.B., A 96-kDa gelatinase induced by TNF- contributes to increased microvascular endothelial permeability, *Am. J. Physiol. Lung Cell. Mol. Physiol.*, 265: L438-L447, 1993.

Peterson M.W., Kirschbaum J., Azbestos-induced lung epithelial permeability: potential role of nonoxidant pathways, *Am. J. Physiol. Lung Cell. Mol. Physiol.*, 275: L262-L268, 1998.

Pontrelli P., Ranieri E., Ursi M., Ghosh-Choudhury G., Gesualdo L., Paolo Schena F., Grandaliano G., Jun-N-terminal kinase regulates thrombin-induced PAI-1 gene expression in proximal tubular epithelial cells. *Kidney Int.*, 65: 2249-2261, 2004.

Prikk K., Maisi P., Pirila E., Reintam M.A., Salo T., Sorsa T., Sepper R., Airway obstruction correlates with collagenase-2 (MMP-8) expression and activation in bronchial asthma, *Lab. Invest.*, 82: 1535-1545, 2002.

Rezaiguia S., Garat C., Delclaux C., Meignan M., Fleury J., Legrand P., Matthay M.A., Jayr C., Acute bacterial pneumonia in rats increases alveolar epithelial fluid clearance by a tumor necrosis factor-alphadependent mechanism, *J. Clin. Invest.*, 99: 325-335, 1997.

Sasaki M., Kashima M., Ito T., Watanabe A., Izumiyama N., Sano M., Kagaya M., Shioya T., Miura M., Differential regulation of metalloproteinase production, proliferation and chemotaxis of human lung fibroblasts by PDGF, interleukin-1 β and TNF-α, *Mediat. Inflamm.*, 9: 155-160, 2000.

Sato Y., Rifkin D.B., Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-beta 1 -like molecule by plasmin during co-culture, *J. Cell Biol.*, 109: 309-3 15, 1989.

Savov J.D., Brass D.M., Berman K.G., McElvania E., Schwartz D.A., Fibrinolysis in LPS-induced chronic airway disease, *Am. J. Physiol. Lung Cell Mol. Physiol.*, 285: L940-L948, 2003.

Schultz M.J., Haitsma J.J., Zhang H., Slutsky A.S., Pulmonary coagulopathy as a new target in therapeutic studies of acute lung injury or pneumonia – a review, *Crit. Care Med.,* 34: 871-877, 2006.

Shetty S., Padijnayayveetil J., Tucker T., Stankowska D., Idell S., The fibrinolytic system and the regulation of lung epithelial cell proteolysis, signaling, and cellular viability, *Am. J. Physiol. Lung Cell Mol. Physiol.,* 295: L967-L975, 2008.

Siflinger-Birnboim A., Johnson A., Protein kinase C modulates pulmonary endothelial permeability: a paradigm for acute lung injury, *Am. J. Physiol. Lung Cell Mol. Physiol.,* 284: L435-L45 1, 2003. Szabó É., Szabó H., A bronchopulmonális dysplasiáról egy esetünk kapcsán, *Tüdőgyógyászat,* 3: 24-26, 2009.

Szabó H., Novák Z., Bauer H., Szatmári E., Farkas A., Wejksza K., Orbók A., Wilhelm I., Krizbai I.A., Regulation of proteolytic activity induced by inflammatory stimuli in lung epithelial cells, *Cell. Mol. Biol. (Noisy-le-grand),* 51 Suppl:. OL729-OL735, 2005.

Szabó H., Novák Z., Farkas A., Krizbai I.A., Gyulladásos mediátorok indukálta proteolitikus aktivitás szabályozása légúti epitélsejtekben, *Allergológia és Klinikai Immunológia,* 8: 192-196, 2005b.

Szabó H., Kádár L., Bronchoalveoláris lavage gyermekkorban, *Tüdőgyógyászat,* 3: 32-34, 2009.

Szabó H., Novák Z., Bauer H., Bauer H.-C., Farkas A., Wilhelm I., Túri S., Krizbai I.A., LPS- and cytokine-induced regulation of proteolytic activity in cultured lung epithelial cells, manuscript, 2009

Takahashi K., Uwabe Y., Sawasaki Y., Kiguchi T., Nakamura H., Kahiwabara K., Yagyu H., Matsuoka T., Increased secretion of urokinase-type plasminogen activator by human lung microvascular endothelial cells, *Am. J. Physiol.,* 275: *(Lung Cell. Mol. Physiol., 19:),* L47-L54, 1998.

Tasaka S., Koh H., Yamada W., Shimizu M., Ogawa Y., Hasegawa N., Yamaguchi K., Ishii Y., Richer S.E., Doerschuk C.M., Ishizaka A., Attenuation of endotoxin-induced acute lung injury by the Rho-associated kinase inhibitor, Y-27632, *Am. J. Respir. Cell. Mol. Biol.,* 32:.504-510, 2005.

Tillie-Leblond I., Pugin J., Marquette C.H., Lamblin C., Saulnier F., Brichet A., Wallaert B., Tonnel A.B., Gosset P., Balance between proinflammatory cytokines and their inhibitors in bronchial lavage from patients with status asthmaticus, *Am. J. Respir. Crit. Care Med.,* 159: 487-494, 1999.

Tontsch U., Bauer H.-C., Isolation, characterization, and long-term cultivation of porcine and murine cerebral capillary endothelial cells, *Microvasc. Res.,* 37: 148-161, 1989.

Townley R.G., Horiba M., Airway hyperresponsiveness: a story of mice and men and cytokines, *Clin. Rev. Allergy Immunol.,* 24: 85-1 10, 2003.

van Leer C., Stutz M., Haeberli A., Geiser T., Urokinase plasminogen activator released by alveolar epithelial cells modulates alveolar epithelial repair in vitro, *Thromb. Haemost.,* 94: 1257-1264, 2005.

Viscardi R.M., Hasday J.D., Gumpper K.F., Taciak V., Campbell A.B., Palmer T.W., Cromolyn sodium prophylaxis inhibits pulmonary proinflammatory cytokines in infants at high risk for bronchopulmonary dysplasia, *Am. J. Respir. Crit. Care Med.,* 156: 1523-1529, 1997.

Vermeer P.D., Denker J., Estin M., Moninger T.O., Keshavjee S., Karp P., Kline J.N., Zabner J., MMP9 modulates tight junction integrity and cell viability in human airway epithelia, *Am J. Physiol. Lung Cell. Mol. Physiol.,* 296: L75 1 -L762, 2009.

Wojta J., Zoellner H., Galicchio M., Hamilton J.A., McGrath K., γ-Interferon counteracts interleukin-1α stimulated expression of urokinase-type plasminogen activator in human endothelial cells in vitro, *Biochem. Biophys. Res. Commun.,* 188: 463-469, 1992.

Wachtel M., Frei K., Ehler E., Fontana A., Winterhalter K., Gloor S.M., Occludin proteolysis and increased permeability in endothelial cells through tyrosine phosphatase inhibition, *J. Cell Sci.,* 112: 4347-4356, 1999.

Wagers S.S., Norton R.J., Rinaldi L.M., Bates J.H.T., Sobel B.E., Irvin C.G., Extravascular fibrin, plasminogen activator, plasminogen activator inhibitors, and airway hyperresponsiveness, *J. Clin. Invest.,* 114: 104-111, 2004.

Wagers S.S., Haverkamp H.C., Bates J.H.T., Norton R.J., Thompson-Figueroa J.A., Sullivan M.J., Irvin C.G., Intrinsic and antigen-induced airway hyperresponsiveness are the result of diverse physiological mechanisms, *J. Appl. Physiol.,* 102: 221-230, 2007.

Walsh G.M., Sexton D.W., Blaylock M.G., Corticosteroids, eosinophils and brochial epithelial cells: new insight into the resolution of inflammation in asthma, *J. Endocrinol.,* 178: 3 7-43, 2003.

Zhao D., Pothoulakis C., Rho GTPases as therapeutic targets for the treatment of inflammatory diseases, *Expert Opin. Ther. Targets,* 7: 583-592, 2003.

Zhuge Y., Xu J., Rac1 mediates type I collagen-dependent MMP-2 activation. role in cell invasion across collagen barrier, *J. Biol. Chem.,* 276: 16248-16256, 2001.

## 8. ACKNOWLEDGEMENTS

I would like to express my sincere thanks to Prof. Sándor Túri MD, PhD, DSc, Director of Department of Paediatrics at the University of Szeged, for his continuous help and encouragement during my clinical and research activities, as well as for his scientific supervision in the Doctoral School of Clinical Medicine of the University of Szeged.

I am grateful to István Krizbai MD, PhD, Principal Investigator at the Institute of Biophysics of the Hungarian Academy of Sciences, for the scientific ideas and his support during the planning and execution of cell culture and laboratory studies. I also thank all team members of his laboratory at the Biological Research Center for their helpful suggestions and assistance in the experimental work.

I am indebted to Zoltán Novák, MD, PhD, Head of Pulmonology Division of Department of Paediatrics, for his invaluable help in the practice and research in Paediatric Pulmonology and for his constructive advices regarding my thesis.

I would like to thank Dr. Hannelore Bauer and Dr. Hans-Christian Bauer for their scientific help and support during my visit in their laboratory.

I am very thankful to all colleagues and friends at both facilities of the Department of Paediatrics, their support is greatly appreciated.