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

Summary of Ph.D. Thesis

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Szeged, 2009
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-1β, 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 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 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.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AF6, acute lymphoblastic leukemia 1 fusion partner from chromosome 6</td>
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<td>AHR, airway hyperresponsiveness</td>
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<td>ALI, acute lung injury</td>
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<td>AP-1, activator protein-1</td>
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<td>BAL, bronchoalveolar lavage</td>
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<td>BPD, bronchopulmonary dysplasia;</td>
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<td>CASK, calcium/calmodulin-dependent serine protein kinase</td>
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<td>CECs, cerebral endothelial cells</td>
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<td>CLD, chronic lung disease</td>
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<td>DMEM, Dulbecco’s modified Eagle’s medium</td>
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<td>DXM, dexamethasone</td>
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<td>ECM, extracellular matrix</td>
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<td>ERK 1/2, extracellular signal-regulated kinase 1/2</td>
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<td>FCS, foetal calf serum</td>
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<td>GTPase, guanosine triphosphatase</td>
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<td>IFN-γ, interferon γ</td>
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<td>IgG, immunoglobulin G</td>
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<td>IL, interleukin</td>
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<td>IL-n, interleukin n</td>
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<td>LPS, lipopolysaccharide</td>
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<td>MMP, matrix metalloproteinase</td>
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<td>MMPn, matrix metalloproteinase n</td>
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<td>NF-κB, nuclear factor-κB</td>
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<td>PA, plasminogen activator</td>
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<td>PAI, plasminogen activator inhibitor</td>
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<td>PAI-n, plasminogen activator inhibitor-n</td>
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<td>PAR, protease-activated receptor</td>
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<td>PBS, phosphate-buffered saline</td>
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<td>PDTC, pyrrolidine dithiocarbamate</td>
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<tr>
<td>PKC, protein kinase C</td>
<td></td>
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<tr>
<td>pro-uPA, pro-urokinase-type plasminogen activator</td>
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<td>RDS, respiratory distress syndrome</td>
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<tr>
<td>SDS-PAGE, sodium dodecyl sulfate polyacryl-amide gel electrophoresis</td>
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<tr>
<td>SEM, standard error of mean</td>
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<tr>
<td>SOD, superoxide dismutase</td>
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<tr>
<td>TGF-β, transforming growth factor-β</td>
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<td>TIMP, tissue inhibitor of metalloproteinases</td>
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<td>TIMP-n, tissue inhibitor-n of metalloproteinases</td>
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<tr>
<td>TJ, tight junction</td>
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<td>TNF-α, tumour necrosis factor-α</td>
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<tr>
<td>tPA, tissue-type plasminogen activator</td>
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<tr>
<td>uPA, urokinase-type plasminogen activator</td>
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<tr>
<td>uPAR, urokinase-type plasminogen activator receptor</td>
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<tr>
<td>ZO, zonula occludens</td>
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<tr>
<td>ZO-n, zonula occludens-n</td>
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1. INTRODUCTION

Respiratory illnesses are the major cause of morbidity and mortality in childhood. Asthma, a chronic inflammatory disorder characterized by episodic and reversible airflow obstruction and airway hyperresponsiveness (AHR), has a prevalence of 8.5% in childhood and causes significant mortality. 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. The research in paediatric pulmonary diseases is a priority.

1.1. Pulmonary Inflammations

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. 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.

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (RDS), are characterized by an acute inflammatory process in the air spaces and lung parenchyma. The loss of barrier function of alveolar epithelial and pulmonary capillary endothelial cells results in respiratory failure in critically ill patients. 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.

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. These changes include fibrosis, increased amount of airway smooth muscle, and increased number of blood vessels.

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 $O_2$ concentration predisposes to this disease. The lung injury in bronchopulmonary dysplasia (BPD) includes re-epithelialization of denuded alveoli followed by the increased presence of fibroblasts and major areas of fibrosis. Elevated concentrations of cytokines, such as interleukins (ILs) IL-1β, IL-6, IL-8, and tumour necrosis factor-α (TNF-α) were observed in tracheal aspirates and serum of infants with RDS and contributed to the development of BPD.

Chronic inflammatory processes of the airways are important elements of several CLDs. Asthma is characterized by episodic wheezing, lung inflammation, and progressive irreversible airway dysfunction in some patients. Histological hallmarks of asthma include homing of T-helper 2 inflammatory cells into lung parenchyma, eosinophilia, and increased mucous cell metaplasia. Epithelial cell damage and infiltration of the bronchial wall by inflammatory cells can be observed in the airways of bronchial asthma patients. Other irreversible changes include increased deposition of extracellular matrix (ECM) proteins in
the bronchial wall, hyperplasia and hypertrophy of smooth muscle cells and an increased number of blood vessels. Allergen challenge and clinical asthma are associated with synthesis and release of pro-inflammatory cytokines, such as TNF-\(\alpha\), IL-1, or IL-13. These mediators can be found in bronchoalveolar lavage (BAL) fluid in status asthmaticus.

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. Mast cells, macrophages, dendritic cells, eosinophil, neutrophil and basophil granulocytes, T- and B-lymphocytes, and platelets are involved in asthma. Structural cells of the airways, such as epithelial cells, smooth muscle cells, endothelial cells, and fibroblasts may also be an important source of inflammatory mediators. Structural cells outnumber inflammatory cells in the airways, therefore they may become the major source of mediators. 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. Airway epithelial cells have a key role in translating inhaled environmental signals into an inflammatory response. Epithelial cells express various pattern recognition receptors including Toll-like receptors 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.

1.3. Cytokines and Other Inflammatory Mediators in the Lung

Inflammatory mediators have been implicated in the pathogenesis of asthma and CLDs. Their effects on the airways could account for the pathology of allergic diseases. 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 and chemokines play a role in maintaining chronic inflammation. Many inflammatory (macrophages, mast cells, eosinophils, lymphocytes) and structural (epithelial cells, airway smooth muscle cells, endothelial cells) cells can synthesise and release these proteins. TNF-\(\alpha\) axis is up-regulated in patients with refractory asthma, as evidenced by the increased expression of membrane-bound TNF-\(\alpha\), TNF receptor 1, and TNF-\(\alpha\) converting enzyme by monocytes. TNF-\(\alpha\) have multiple effects in asthmatic airways; it induces adhesion, migration, and activation of inflammatory cells through the epithelial and endothelial barriers. Effective anti-TNF therapy supports the view that TNF-\(\alpha\) contributes to the pathogenesis of asthma. Both TNF-\(\alpha\) and IL-1\(\beta\) activate pro-inflammatory transcription factors, nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) and activator protein-1 (AP-1), which switch on many inflammatory genes in the asthmatic airways. Other cytokines, such as interferon-\(\gamma\) (IFN-\(\gamma\)), IL-10, IL-12, and IL-18, play an inhibitory role in the allergic inflammatory process. During allergic inflammation activated inflammatory cells, e.g. macrophages and eosinophils, produce reactive oxygen species. Increased oxidative stress is related to disease severity. Oxidative stress may be detrimental in asthma through the reaction of superoxide anions with nitric oxide to form the reactive radical peroxynitrite, that may modify several target proteins.
1.4. Regulation of Proteolysis in the Lung
Proteolytic enzymes may also cause damage to the lung during inflammatory process. Extracellular endogenous proteases are released 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. Proteases are important in normal growth and development of the lung and in lung host defense. They can injure cells directly, degrade ECM components and break down extracellular and cell surface macromolecules under pathological circumstances. Proteases can react with airway receptors 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.
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 is an aspartic-protease. Proteases containing metal ions are metalloenzymes, such as matrix metalloproteinases (MMPs). Proteases act through PARs, 7-transmembrane proteins coupled to G proteins. Stimulation leads to increased intracellular Ca$^{2+}$ level and gene transcription. In alveolar epithelial cells it opens tight intercellular junctions, causes desquamation, and produces cytokines, chemokines, and growth factors. Lung antiproteases include α$_1$-antitrypsin, α$_2$-macroglobulin, secretory leucoprotease inhibitor, tissue inhibitor of metallo-proteases (TIMPs), plasminogen activator inhibitor (PAI)-1 and -2, and α$_1$-antichymotrypsin.

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. Endothelial cells, fibroblasts, epithelial cells, mast cells, monocytes/macrophages and smooth muscle cells are responsible for production of PAs. Plasmin system inhibitors can be also synthesised by platelets, neutrophils, 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.
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. 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 α$_2$-antiplasmin and α$_2$-macroglobulin. However, inflammatory mediators including IL-1, IL-2, IL-4, IL-6, TNF-α, IFN-γ, macrophage colony stimulating factor, transforming growth factor-β (TGF-β), 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. uPAR mediates cell attachment and movement, this process remains under the influence of uPA and PAI-1. The fibrinolytic system prevents fibrin deposition in the alveolar compartment of normal lung. Decreased alveolar fibrinolysis due to altered expression of components of fibrinolytic system takes a part in acute lung injury and subsequent fibrosis. The uPA/plasmin system plays a major role in airway remodelling in asthma. The changes in the balance of procoagulant and fibrinolytic activities also contribute to lung fibrosis in acute RDS and pneumonia.

Plasmin can degrade most of the protein components of ECM, either by direct removal of glycoproteins or by activation of MMPs. 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 serine protease inhibitors.

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 ECM components. The MMPs share three common domains, namely the pro-peptide, the catalytic domain and the haemopexin-like C-terminal domain. There are 4 major MMP groups: collagenases, gelatinases, stromelysins, and membrane-type MMPs. 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. 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. The stromelysins (MMP3, MMP10, MMP11) are able to cleave ECM proteins, but not the triple-helical fibrillar collagens. The biological processes involving MMPs depend on the balance between proteinases and their natural inhibitors. 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.

MMPs play a role in many physiological processes, such as embryo implantation, bone remodelling, or organogenesis, and in pathological conditions, e.g. tissue reorganization, wound healing, or invasion of cancer cells. The known targets of MMPs in the lung are ECM molecules, growth factors, chemokines, proteinases, or adhesion molecules.

MMPs also play a role in the pathogenesis of CLD of prematurity. Increased amounts of MMP2, and a higher ratio of MMP9 to TIMP1 have correlated with poor outcome in infants with BPD. The interactions between the surfactant proteins and MMPs and TIMPs play an important role in the lung maturation process in infants. Expression of several MMPs has also been associated with asthma. Increases in MMP1, MMP2, MMP3, MMP8, and MMP9 levels have been found in sputum and BAL from patients with asthma.

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. TJs are formed by a complex of integral membrane proteins and peripheral membrane proteins that interact with the cytoskeleton. Integral membrane proteins involved in TJ formation include occludin and members of
claudin family. Zonula occludens (ZO)-1 protein has strong interaction with transmembrane proteins occludin and claudins, as well as cytoplasmic proteins including ZO-2, ZO-3, actin, AF6, or cingulin. There are more than 20 different claudins and alveolar epithelial cells simultaneously express six or more claudin isoforms with different paracellular permeability characteristics. 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. Junctional complexes at cell-cell contact sites also regulate solute flow across cell monolayers (through TJs) and from one cell to another (through gap junctions). Disruption of the paracellular alveolar permeability barrier is a significant pathological consequence of ALI. 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. 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.

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 lipopolysaccharide (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-α, 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); Y27632 and PP-1 were from Tocris (Avonmouth, UK).
3.2. Cell Culture

Human alveolar epithelial A549 cell line, which retains features of type 2 alveolar epithelial cells, was cultured in DMEM containing 10% heat inactivated foetal calf serum (FCS) and antibiotics. Confluent epithelial monolayers were washed twice with phosphate-buffered saline (PBS) and kept in 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 (DXM), 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) isolated from brain cortical microvessels were cultured in DMEM supplemented with 10% FCS. CECs grown in the presence of endothelial cell growth factor-α and its cofactor heparin (100 µg/ml) exhibit epithelial-like morphology (type I), while in the absence of them CECs have 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.

3.4. Colorimetric Plasminogen Activator Assay

PA activity was measured in culture medium (3, 6, 24 h) and cell lysates (24 h) in the absence or presence of uPA-inhibitor amiloride (1 mM). Plasminogen activation and the resulting plasmin hydrolysis was monitored by a colorimetric assay. 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

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 CaCl₂ at 37 °C for 24 h. Proteolytic bands were visualized by staining the gels with Coomassie R-250.
3.6. Motility Assay
Subconfluent lung epithelial cells, cultured in 6 cm Petri dishes, were wounded with a razor blade. Then the cells were washed three times with PBS and further incubated in the presence or absence of TNF-α, 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 TJ 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, 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 (Biomed, 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 anti-β-catenin antibodies (5 µg) 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 β-catenin (Sigma) and fluorophore-labeled secondary antibodies (Jackson Laboratories). Immunoreaction was visualized by ECL plus chemiluminescence detection kit (GE Healthcare).

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-α
TNF-α induced PA activity has been measured both in the culture medium and the soluble fraction of cell lysates. In culture medium, strong activity at 50 kDa and weaker activity at 70 kDa could be detected corresponding to uPA and tPA, respectively. Almost exclusively uPA was detected in soluble cellular fraction. Treatment of A549 cells with TNF-α for 6 h
induced significant, concentration-dependent activation of both uPA and tPA activity in the culture medium and an induction of the uPA activity in the soluble fraction of cell lysates.

4.1.2. Effect of inhibitors on the induction of PA activity by TNF-α
Administration of DXM (10 mM) did not change the PA activity in the culture medium and soluble fraction; however it could significantly reduce the TNF-α induced activation of both uPA and tPA. The effect of signal transduction inhibitors on TNF-α induced uPA activity has been also checked. Neither src-kinase inhibitor PP1 nor superoxide dismutase (SOD) could inhibit the TNF-α induced uPA activity. However, the Rho inhibitor Y27632 and MMP inhibitor phenanthroline were able to significantly inhibit the activation of uPA in A549 human alveolar epithelial cells. Densitometrical analysis also proved that TNF-α induced uPA activation could be significantly inhibited by Y27632, phenanthroline, or DXM.

4.1.3. Effect 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 weaker activity at around 70 kDa (tPA). LPS also increased uPA activity, whereas IL-2 and IL-6 did not produce significant changes. The effect of inflammatory mediators on PA activity was also estimated using colorimetric PA assay. IL-1β administration (10 ng/ml) caused about 9-fold and 6-fold increases in total PA activity in culture medium at 3 h and 24 h, respectively, and similar increase in soluble cellular fraction at 24 h. Although IL-2 treatment (50 U/ml) for 24 h did not change total PA activity in cellular lysates, it induced significant increases in the activity in culture medium with a decreasing tendency between 3 h and 24 h. IL-6 treatment (1 ng/ml) decreased PA activity in cell lystate, but not in culture medium. LPS incubation significantly elevated PA activity in culture medium between 3 and 24 h, however PA level in cell fraction was only slightly elevated after 24 h. IFN-γ (100 ng/ml) increased PA activity after 3 h, but not at 6 or 24 h, in culture medium, and after 24 h in cell lysates. 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 amiloride-insensitive (tPA) activity was approximately 74-81% vs. 19-26% both in culture medium and cell lysates. 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.

4.2. MMP Activity in Epithelial and Endothelial Cells
Metalloprotease zymography showed a proteolytic band at 72 kDa which corresponds to MMP-2 (gelatinase A). The MMP-2 activity was significantly stronger in the culture medium compared to that in the cell lysate. TNF-α induced activation of MMP-2 could be partially blocked by co-incubation with 10 nM DXM. Rho kinase inhibitor Y27632 also reduced TNF-α induced activation of MMP-2. A faint proteolytic band corresponding to MMP-3 (stromelysin 1) could be also detected at 40-45 kDa in the culture medium. MMP activity was also investigated in CECs and 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.
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. A549 cells migrate further from the wound edge in the presence of 5.0 ng/ml TNF-α. This effect could be reduced to the control level by DXM and Y27632. DXM and Y27632 alone did not influence the migration of cells.

4.4. Integrity of Epithelial Cellular Barrier
TNF-α treatment led to a relocalisation of TJ transmembrane protein occludin, although the expression of occludin did not change. TNF-α treatment dose-dependently decreased β-catenin expression, whereas cadherin and α-catenin expression did not change. 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. This reduction may reflect recruitment of these proteins into insoluble membrane complexes.

5. DISCUSSION
The present study strengthens the views that cytokines promote fibrinolysis in alveolar epithelium and contribute to pathogenesis and repair of lung injury. New observations of the thesis have been discussed.

5.1. Cytokines in the Induction of Proteolytic Enzymes in the Airways
- It has been demonstrated that TNF-α strongly enhances the expression and release of uPA and tPA in human lung epithelial cells and DXM can inhibit these changes.
- Our data support the views that bacterial LPS and cytokines IL-1β, IL-2, and IFN-γ can regulate the production and synthesis of PAs in alveolar epithelial cells.
- It has been proved that uPA is responsible for approximately 75-80% of total PA activity in lung epithelial cells.
- The role of Rho kinase-dependent mechanisms in the regulation of cytokine-induced activation of uPA in alveolar epithelial cells has been established in the present study.

5.1.1. Effects of TNF-α and other inflammatory mediators on PA activity
Cytokines and bacterial LPS were able to induce significant PA activity. TNF-α and IL-1β proved to be the strongest, time- and dose-dependent PA inducers in cell lysates and supernatants of alveolar A549 cells. IL-1β produced greater increase in PA activity in cell lysate, whereas TNF-α did it in supernatant. These data are in accordance with a previous study indicating that inflammatory mediators including LPS and IFN-γ had much lower capacity than IL-1β and TNF-α to cause an up-regulation of PA activity in pulmonary epithelial cells after 24 h. 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. Increased uPA levels in the supernatant of IL-1β stimulated epithelial cells indicated activation of fibrinolysis through plasmin system and resulted in quicker and more efficient alveolar epithelial repair. Upregulation may be caused by accumulation of newly synthesized uPA since TNF-α is able to induce rapid accumulation of uPA mRNA. It has been confirmed that the majority of total PA activity is amiloride-sensitive in A549 cells. Our observation supports a predominant role for uPA in the proteolytic activity of
alveolar epithelial cells. Amiloride was shown to completely inhibit uPA in control and stimulated rat alveolar epithelial cells, while it did have no effect on tPA.

5.1.2. Cytokine-induced signalling and PA activity

Signal transduction pathways regulating epithelial proteolytic activity in response to inflammatory stimuli have been investigated and a role for Rho-dependent mechanisms in the regulation has been revealed. TNF-α and LPS induced increase in PA activity could be prevented by Y27632, a specific inhibitor of Rho kinase in alveolar epithelial cells. Y27632 proved to be effective in a murine model of intravenous LPS-induced acute lung injury; it attenuated lung oedema, neutrophil emigration, and cytoskeletal rearrangement of pulmonary endothelial cells. In contrast to the in vitro observations, inhaled nebulized LPS inhibited PA activity and increased PAI-1 production in BAL fluid of healthy human volunteers. The role of Rho-associated protein kinases in mediating the effect of TNF-α 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-α driven E-selectin gene expression. Recent results indicate that the Rho family of small GTP-binding proteins plays an important role in the expression of NF-κB-dependent genes. However, in our experiments inhibition of NF-κB by PDTC did not inhibit the TNF-α induced activation of uPA, which suggests that TNF-α may also exert its effects through alternative signaling pathways. Furthermore, TNF-α 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. However, our results using the ERK inhibitor U0126 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. The role of PKC in PA synthesis is well-known; treatment with phorbol myristate acetate, a PKC activator, caused time- and dose-dependent up-regulation of uPA synthesis by alveolar epithelial cells. However, we could not detect inhibition of TNF-α-induced uPA activation by the src-kinase inhibitor PP-1 and PKC inhibitor bisindolylmaleimide in A549 cells. Interestingly, PP-1 enhanced the TNF-α-induced uPA activation, which could be explained by the inhibitory effect of PP-1 on PAI-1 gene expression. Glucocorticoids have also been shown to suppress uPA activity probably due to interactions between the glucocorticoid receptor and another transcriptional activating system such as AP-1 and NF-κB. Since SOD did not affect TNF-α-induced uPA activation in our study, increased production of reactive oxygen species 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.

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. A recent Canadian study detected an association between allelic variants of human uPA gene PLAU located on 10q24 and asthma phenotype and confirmed that uPA might act as a regulator of asthma susceptibility. 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 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. Similar increase in epithelial uPA activity was seen in airway tissue samples of patients died in status asthmaticus. Local fibrinolytic activity of the lung tissue during inflammation might differ from the changes in BAL fluid. Intense PA activity was localized to areas of lung injury by in situ zymography after exposure to bleomycin in mice while the PA activity within lavage fluid was suppressed. On the other hand, inhalation of exogenous uPA significantly reduced subepithelial fibrosis, decreased AHR, up-regulated plasmin activity in BAL fluid, and reduced airway remodelling in a murine model of chronic asthma.

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. 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. 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. 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.

5.2. Regulation of Matrix Metalloproteinases in Alveolar Epithelial Cells

- TNF-α induced regulation of MMP-2 has been shown in lung epithelial cells.
- Treatment with DXM or Rho kinase inhibitor can prevent MMP-2 induction by TNF-α.

5.2.1. Effect 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 alveolar epithelial cells. An induction of MMP expression by inflammatory mediators has been reported recently, but the mechanisms of inhibition was not presented. MMPs are known to degrade the ECM, basal membrane components, and are involved in the regulation of TJ proteins, such as occludin. Similar data have been obtained in a study showing that MMP-9 is induced by TNF-α in pulmonary microvascular endothelial cells. Fibroblast migration, proliferation, and MMP production were also shown to be regulated by IL-1β and TNF-α. 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.

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 previous results 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. 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 fibrosarcoma cells. Our results suggest that Rho may be at least partially involved in the 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, MMP-2 is preferentially secreted from fibroblasts and airway epithelial cells, and MMP-9 is preferentially expressed by inflammatory cells. Previous studies 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 MMP1, MMP2, MMP3, MMP8, and MMP9 has been associated with asthma. Increase in MMP9 activity in the subepithelial basement membrane was accompanied by higher TGF-β level. 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. 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. The levels of MMP2, MMP8, MMP9 and TIMP-2 are elevated in tracheal aspirate from preterm infants with RDS. ECM components, including collagen and fibronectin, are increased in the lungs of infants who die from CLD.

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

- The present study has demonstrated that TNF-α treatment increases the migration potential of wounded alveolar epithelial cells.
- The inhibition of epithelial cell migration by dexamethasone or Rho inhibitor Y27632 can be partly explained by the reduced protease activity.

Cell migration is a highly integrated, multi-step process that plays an important role in the progression of various diseases including cancer. An increased proteolytic activity is often associated with an increased migratory potential mainly due to ECM degradation or proteolysis of cell surface molecules regulating cell-cell interactions. uPA facilitates cell migration by localizing proteolysis on the cell surface and by inducing intracellular signalling pathways. The role of uPA in human bronchial epithelial cell migration was confirmed when a monoclonal antibody raised against uPA showed 70% reduction in cell velocity.

It was also observed that uPA was only detected in migrating cells at the wound edges and located at crucial sites for cell/ECM interactions. 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 cell migration possible. 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. It has been also known that Rho family GTPases regulate actin cytoskeleton, cell adhesion, and play an important role in cell polarisation and directional migration.

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

- TNF-α-induced damage to the epithelial barrier integrity has been demonstrated in the present study.
• It has been revealed that relocalisation of occludin, decreased β-catenin expression, and modified recruitment of the proteins in adherens junction can contribute to the mechanism of barrier injury.

The structural changes of epithelial barrier may well correspond to the increased permeability seen in in vivo and in vitro studies. In a rat model of pneumonia, histologic evidence of alveolar epithelial injury 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. In another study, LPS injection (2 mg/kg) resulted in leakage of FITC-dextran from blood into BAL fluid in endotoxaemic mice. This decrease in barrier function was associated with up-regulation of inducible nitric oxide synthase expression and NF-κB, and decreased expression of the TJ proteins ZO-1, ZO-2, ZO-3, and occludin in lung after the injection of mice with LPS. Release of TNF-α during pneumonia or peritonitis increases the transport capacity of the alveolar epithelium. Increased uPA expression can also enhance paracellular permeability across epithelial monolayers after asbestos treatment. 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.

TNF-α can also induce barrier dysfunction in endothelium of the pulmonary vessels. TNF-α (1,000 U/ml) increased albumin permeability in bovine pulmonary artery endothelial cell monolayers, induced pulmonary endothelial F-actin depolymerization, intercellular gap formation, and barrier dysfunction. TNF-α activates PKC-α, then stabilization of actin fibers affects junctional proteins and altered cell-cell adherence results in the permeability response. The family of zinc- and calcium-dependent MMPs play an important role in remodelling of the airways in disease. Apical MMP9 significantly decreases immunostaining of TJ proteins and transepithelial electrical resistance in a model of well-differentiated human airway epithelia. MMP9 exerts its effects on the epithelium by cleaving one or more components of cell-cell junctions. Due to disruption of barrier function viruses gained access to the epithelial basolateral surface, which could increase infection efficiency.

6. CONCLUSIONS

The new observations of the present study indicate that cytokines promote fibrinolysis in alveolar epithelium and contribute toogenesis 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 DXM and Rho-kinase inhibitors. TNF-α induced structural change in the barrier integrity of epithelial monolayers was also 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.
ACKNOWLEDGEMENTS

I would like to express my sincere thanks to Prof. Sándor Túri M.D., Ph.D., D.Sc., 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 M.D., Ph.D., 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, M.D., Ph.D., 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.
PUBLICATIONS

PUBLICATIONS IN THE TOPIC OF PH.D. THESIS

Full papers:


Abstracts:


MANUSCRIPT IN THE TOPIC OF PH.D. THESIS

PRESENTATIONS RELATED TO THE TOPIC OF PH.D. THESIS
2. Szabó H., Bauer H.-C., Novák Z., Krizbai I., Proinflammatorikus hatás alveoláris epithél sejtvonalra (oral presentation in Hungarian). Meeting of the Paediatric Pulmonology Section of the Hungarian Paediatric Association (MGYT) and the Hungarian Respiratory Society (MTT), Pécs, November 9-11, 2000.

3. Szabó H., Novák Z., Krizbai I., TNF alfával kezelt légúti e pithél sejtvonal proteolytikus aktivitása (oral presentation in Hungarian). Meeting of the Paediatric Pulmonology Section of the Hungarian Paediatric Association (MGYT) and the Hungarian Respiratory Society (MTT), Kiskunhalas, November 8-10, 2001.


OTHER PUBLICATIONS NOT RELATED TO THE TOPIC OF PH.D. THESIS:


