MATRIX PROTEINASES IN LUNG INJURY IN THE PRETERM INFANT

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ACADEMIC DISSERTATION

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TO MY FAMILY

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

III Cederqvist K, Haglund C, Heikkilä P, Hollenberg MD, Karikoski R, Andersson S. High expression of pulmonary proteinase-activated receptor 2 in acute and chronic lung injury in preterm infants. *Pediatr Res* 57: 831–836, 2005

IV Cederqvist K, Janer J, Tervahartiala T, Sorsa T, Haglund C, Salmenkivi K, Stenman U-H, Andersson S. Up-regulation of trypsin and mesenchymal-MMP-8 during development of hyperoxic lung injury in the rat. *Pediatr Res* (in press)

ABBREVIATIONS

ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BM	basement membrane
BPD	bronchopulmonary dysplasia
ECM	extracellular matrix
HAT	human airway trypsin-like protease
IFMA	immunofluorometric assay
IL	interleukin
kD	kilodalton
L/S ratio	lecithin/sphingomyelin ratio
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
PAR	proteinase-activated receptor
RDS	respiratory distress syndrome
SC	secretory component of immunoglobulin-A
SLPI	secretory leukocyte proteinase inhibitor
TAF	tracheal aspirate fluid
TATI	tumor-associated trypsin inhibitor
TIMP	tissue inhibitor of metalloproteinases
TNF-α	tumor necrosis factor-α
tPA	tissue-type plasminogen activator
uPA	urokinase type plasminogen activator

ABSTRACT

During inflammation, excess production and release of matrix proteinases, including matrix metalloproteinases (MMPs) and serine proteinases, may result in dysregulated extracellular proteolysis leading to development of tissue damage. Injurious inflammatory pulmonary reaction may play an important role in the pathogenesis of bronchopulmonary dysplasia (BPD). The aims of the present study were to evaluate involvement of MMPs and serine proteinase trypsin in acute and chronic lung injury in preterm infants and to study the role of MMPs and trypsin in development of acute lung injury by means of an animal model of hyperoxic lung injury.

Samples of tracheal aspirate fluid (TAF) were collected during the early postnatal period from preterm infants with respiratory distress. Molecular forms of MMP-2, -8, and -9, and their specific inhibitor, tissue inhibitor of metalloproteinases (TIMP)-2, were identified by Western blotting and their relative levels by densitometry. Concentrations of trypsinogen-1, and -2, and tumor-associated trypsin inhibitor (TATI) were measured by immunofluorometry. Expression of trypsin-2 and proteinase-activated receptor 2 (PAR_2) in lung tissue was studied by immunohistochemistry performed on autopsy lung specimens from fetuses, from preterm infants with respiratory distress syndrome (RDS) or BPD, and from newborn infants who had died for nonpulmonary reasons. In the experimental study, rats were exposed to >95% oxygen for 24, 48, and 60 h, or room air. Expression of trypsin, MMP-2, MMP-8, and MMP-9 was studied in samples of bronchoalveolar lavage fluid (BALF) by zymography and Western blotting. Immunohistochemistry for trypsin and MMP-8 was performed on pulmonary samples.

Higher MMP-8 and lower TIMP-2 appeared in TAF from preterm infants with more severe acute respiratory distress.

Preterm infants subsequently developing BPD had higher MMP-8 levels during the early postnatal period than did those who survived without it. Low TIMP-2 levels during the early postnatal period were associated with poor respiratory outcome. We found that high pulmonary concentrations of trypsinogen-2 early postnatally were associated with the severity of acute lung injury and subsequent development of BPD. Immunohistochemistry revealed the expression of trypsin-2 in bronchial epithelium; in preterm infants with prolonged RDS, also in alveolar epithelium. Since trypsin-2 is potent activator of PAR_2, a G-protein coupled receptor involved in inflammation, we next studied the expression of PAR_2 in the lung. We observed that PAR_2 co-localized with trypsin-2 in bronchoalveolar epithelium, and a higher level of PAR_2 immunoreactivity was detectable in bronchoalveolar epithelium of preterm infants with prolonged RDS than in newborn infants without lung disorders. In experimental animals, a rapid increase in BALF levels of trypsin and MMP-8 appeared early in the course of hyperoxic lung injury. By immunohistochemistry, strong expression of trypsin was detectable in alveolar epithelium, and MMP-8 was predominantly localized in macrophages at 48 and 60 hours of hyperoxia.

In conclusion, high levels of MMP-8 and trypsin-2 in TAF early postnatally are associated with the severity of acute lung injury and subsequent development of BPD in preterm infants. In the injured preterm lung, trypsin-2 co-localizes with PAR_2 in bronchoalveolar epithelium, suggesting that PAR_2 activated by high levels of trypsin-2 may be involved in lung inflammation associated with development of BPD. The marked increase in pulmonary expression of MMP-8 and trypsin early in the course of experimental hyperoxic lung injury suggests that these enzymes play a role in the development of acute lung injury.

REVIEW OF THE LITERATURE

1. LUNG INJURY

1.1. Normal lung development

The normal development of the human lung can be divided into the following 5 stages (reviewed in Bland and Coalson 2000): 1) Embryonic stage (0 to 7 weeks), development of airways to the level of bronchopulmonary segments; 2) Pseudoglandular stage (5 to 17 weeks), a phase characterized by branching of the airway and arterial tree down to the preacinar level; 3) Canalicular stage (16 to 26 weeks), a phase when prospective gas-exchanging tissue is formed as the peripheral cuboidal cells start to differentiate into type I and type II cells, and primitive interstitium is canalized by multiplication of capillaries; 4) Saccular stage (24 to 36 weeks), during which peripheral airspaces expand at the expense of the intervening interstitium, and secondary crests containing a double capillary layer start to grow into the airspace from the saccular walls, a process called septation; and 5) Alveolar stage (from week 36 to 2 years postnatally), the time of alveolarization, characterized by extension and thinning of the secondary crests and fusion of capillaries into a single medial layer to form alveoli (Figure 1). The process of alveolar formation, especially septation, is a critical phase of lung development coordinated by multiple interactions between epithelial and interstitial cells, microvascular lung components, and extracellular matrix (ECM) (Roth-Kleiner and Post 2005).

1.2. Lung injury in the preterm infant

Within the first hours of life preterm infants are at risk of developing respiratory distress syndrome (RDS) which is primarily due to deficiency of the surfactant system resulting in failure of airspace expansion (Northway 2000). In preterm infants, RDS is the most common cause of acute lung injury and respiratory failure, and these infants often need ventilatory support and treatment with supplemental oxygen. Bronchopulmonary dysplasia (BPD) is a chronic lung disease that develops in preterm infants exposed to multiple injurious factors including baro- and volutrauma, oxygen toxicity, pulmonary inflammatory response, and perinatal infection (Jobe and Bancalari 2001, Speer 2003).

When BPD was first described in 1967 by Northway *et al*., the infants of 31 to 34 weeks of gestation had severe RDS, and were treated with prolonged mechanical ventilation and high inspired oxygen concentrations. With the advent of antenatal glucocorticoids and postnatal surfactant therapy, risk for severe RDS has decreased, and development of BPD is now infrequent in infants of more than 1200 g birth weight or with gestations exceeding 32 wk (Lemons *et al*. 2001, Bancalari *et al*. 2003). Concurrently, the survival rate of smaller and more immature infants has markedly increased, and BPD continues to be a major cause of mortality and morbidity in

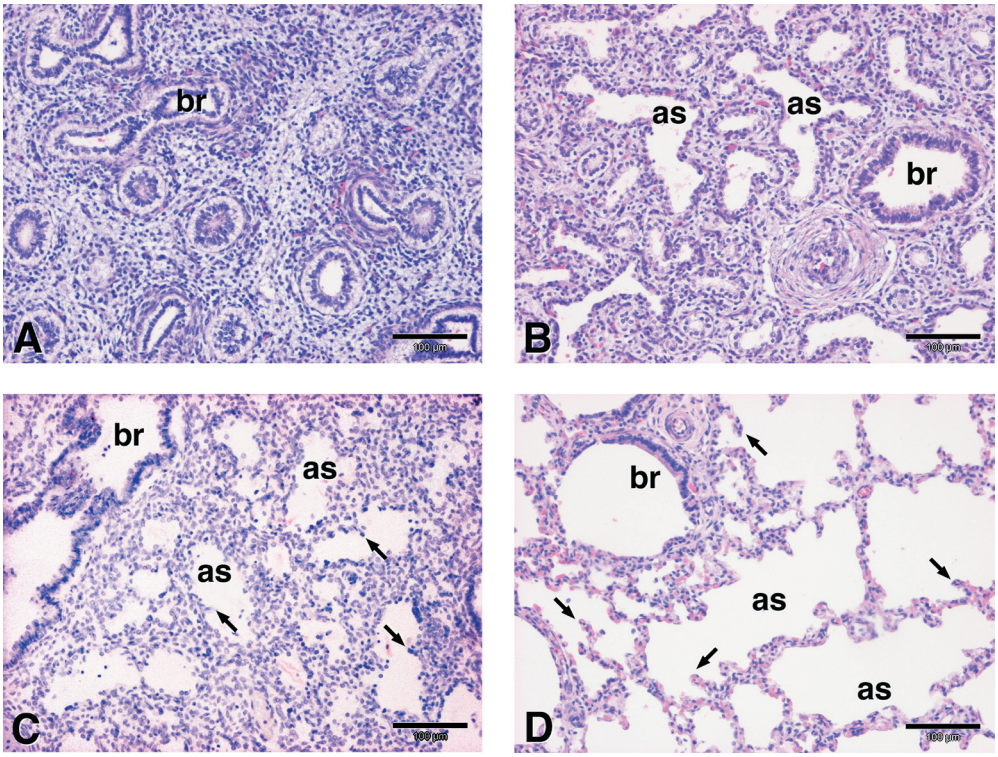

Figure 1. Human lung in the **(A)** pseudoglandular (fetus, gestational age 14 weeks), **(B)** canalicular (fetus, gestational age 21 weeks), **(C)** saccular (newborn, gestational age 26 weeks, age at death 2 hours), and **(D)** alveolar stage (newborn, gestational age 40 weeks, age at death 1 day). Arrows indicate secondary crests; as, airspace; br, bronchiole.

very low birth weight (VLBW; birth weight ≤1500 g), and especially extremely low birth weight (ELBW; ≤1000 g) infants born at <28 weeks of gestation (Stevenson *et al*. 1998, Lemons *et al*. 2001).

With the change in clinical presentation of BPD, the criteria defining BPD have been revised. The most widely used definitions in the literature are the need for supplemental oxygen at the postnatal age of 28 days with radiographic changes (Bancalari *et al*. 1979), and the definition by Shennan *et al*. in 1988. The latter suggested that the requirement of supplemental oxygen at 36 gestational weeks with characteristic radiographic changes was a better predictor of abnormal pulmonary outcome in VLBW infants with gestational ages of 30 weeks or less (Shennan *et al*. 1988). Using the 36 weeks´ definition, approximately 44% of the surviving ELBW infants nowadays develop BPD (Ehrenkranz *et al*. 2005). In 2000, a severity-based consensus definition of BPD was suggested with differing criteria for infants born at gestational ages of greater or less than 32 weeks (Jobe and Bancalari 2001).

1.2.1 Pathology of RDS and BPD

In lungs of preterm infants who have died of RDS, the autopsy shows atelectasis, hyaline membranes in the terminal airways and airspaces, and necrosis and desquamation of alveolar and bronchiolar epithelial cells, as well as alveolar and interstitial edema (reviewed by Coalson 2000).

In preterm infants with RDS, BPD appears to proceed as a continuous process from acute lung injury of the first few postnatal days through proliferative and reparative phases to chronic disease (Cherukupalli *et al*. 1996). The proliferative and reparative phases occur during the first 2 weeks following acute lung injury, and are characterized by regeneration of alveolar epithelium by alveolar type II cells, high numbers of inflammatory cells in the airspaces, and increasing numbers of myofibroblasts surrounding the airspaces (Toti *et al*. 1997).

Before the use of antenatal glucocorticoids and postnatal surfactant, the pathology of fully evolved BPD was characterized by altered inflation pattern of atelectasis and overinflation, severe airway epithelial injury with squasmous metaplasia, hypertrophy of airway smooth muscle, widespread fibrosis, and vascular hypertensive lesions (Coalson 2000). The introduction of surfactant treatment and the prenatal use of antenatal glucocorticoids, coupled with advances in mechanical ventilation, have resulted in the new BPD pathology of the more immature lung showing enlarged airspaces with decreased alveolar and capillary development; whereas interstitial hypercellularity and fibroproliferation are variable, and milder airway lesions are mainly detectable in infants with severe disease (Coalson 2003).

1.2.2 Role of inflammation in lung injury in the preterm infant

Multiple factors are involved in the development of BPD, including immaturity, barotrauma and volutrauma, oxidative stress, pulmonary inflammation, antenatal and postnatal infection, and patent ductus arteriosus (Jobe and Bancalari 2001, Bancalari *et al*. 2003, Saugstad 2003, Speer 2003). Growing evidence suggests that an injurious inflammatory pulmonary reaction followed by dysregulated reparative processes play an important role in the pathogenesis of BPD (Speer 2003). The inflammatory process is triggered early by initial trauma caused by various possible factors such as resuscitation, initiation of mechanical ventilation, and oxygen toxicity. Some very small preterm infants, however, initially have no RDS, or only mild respiratory disease, but after a few days or weeks show progressive deterioration in lung function and increased ventilatory and oxygen requirements (Lemons *et al*. 2001, Bancalari *et al*. 2003). In these infants, the initial hits may be exposure to chorioamnionitis or an early postnatal systemic or pulmonary infection, or both (Watterberg *et al*. 1996, Jobe 2003).

The inflammatory pulmonary reaction is characterized by early accumulation of inflammatory cells, production of multiple cytokines and chemokines, increased expression of adhesion molecules on endothelial and epithelial cells, secretion of proteinases and toxic oxygen radicals, and increased microvascular permeability (Speer 2003).

Within the first few days of life, neutrophils and macrophages accumulate in the lungs of preterm infants with RDS, and greater numbers of these cells have been demonstrated in airway aspirates from infants who subsequently develop BPD than from those who survive without it (Merritt *et al*.

1983, Ogden *et al*. 1984, Groneck *et al*. 1994, Murch *et al*. 1996). In preterm infants who recover from RDS, the neutrophil counts in airway aspirates decline rapidly to normal by the end of the first week, whereas in those who develop BPD, the number of neutrophils and macrophages remains elevated for weeks (Ogden *et al*. 1984, Groneck *et al*. 1994, Murch *et al*. 1996).

In lung lavage fluids from preterm infants developing BPD increased levels of chemotactic factors such as interleukin (IL)-8, anaphylatoxin C5a, and macrophage inflammatory protein (MIP)-1α, as well as proinflammatory cytokines including IL-1ß, tumor necrosis factor (TNF)-α, IL-6, are detectable during the first and second postnatal weeks (Groneck *et al*. 1994, Kotecha *et al*. 1996, Murch *et al*. 1996).

During this inflammatory process, production and release of potent proteinases by activated inflammatory cells and lung resident cells are increased, and these enzymes may play an important role in the development of tissue injury (Speer 2003). The role of proteolytic injury in the development of BPD was first suggested by Merritt *et al.*, who in 1983 demonstrated in airway aspirates from preterm infants who subsequently developed BPD an imbalance between neutrophil elastase and its inhibitor α_1-antitrypsin (also called α_1-proteinase inhibitor).

The involvement of inflammation in disrupted lung alveolarization in these infants is supported by findings in animal models of BPD (Warner *et al*. 1998, Coalson *et al*. 1999, Wagenaar *et al*. 2004). At birth, the murine lung is at the saccular stage of lung development, with alveolarization taking place during the first 2 weeks of life. Prolonged exposure of newborn mice and rats to hyperoxia interferes with the process of septation and results in chronic lung injury with pathological changes similar to those seen in infants with BPD (Warner *et al*. 1998, Wagenaar *et al*. 2004). In these animals, the pulmonary inflammatory response develops during the first week of exposure, and persists into the second week; it is characterized by influx of neutrophils and macrophages, by interstitial and alveolar edema, and by induced synthesis of various cytokines, chemokines, and proteinases (Warner *et al*. 1998, Wagenaar *et al*. 2004). The role of inflammation in the development of BPD has also been evidenced in the immature baboon model of BPD, which is characterized by preterm birth at the canalicular stage of lung development, comparable to the 24- to 26-week human infant, by use of antenatal glucocortics and postnatal prophylactic surfactant, coupled with appropriate oxygenation and positive pressure ventilation (Coalson *et al*. 1999).

1.3. Experimental hyperoxic lung injury in adult rat

Hyperoxia-induced lung injury (exposure to >95% oxygen) is a widely used model of acute lung injury and acute respiratory distress syndrome (ARDS). In the rat, hyperoxic lung injury is characterized by damage to the alveolar–capillary barrier with subsequent increased pulmonary vascular permeability, progressive inflammation, and pulmonary edema. The early stages of oxygen toxicity begin with an initiation phase, occurring within the first 40 hours of hyperoxia exposure, in which are few demonstrable morphologic changes (Crapo *et al*. 1980). The first signs of increased alveolar-capillary permeability are detected at 48 hours (Royston *et al*. 1990). Lung inflammation evident by infiltration of neutrophils into the lung interstitium occurs after 48 hours (Nishio *et al*. 1998). Increased pulmonary expression of intercellular adhesion molecule-1 (ICAM-1) plays an

important role in subsequent neutrophil transmigration into the airspace (Nishio *et al*. 1998) where high numbers of these cells as well as alveolar macrophages can be detected after 60 hours of oxygen exposure (Barry and Crapo 1985, Narasaraju *et al*. 2003). Severe tissue injury resulting from direct oxidative cell damage by increased reactive oxygen species and excessive inflammation leads to interstitial and alveolar edema at 60 hours and eventually to death usually within 72 hours of exposure (Crapo *et al*. 1980, Royston *et al*. 1990, Pagano and Barazzone-Argiroffo 2003).

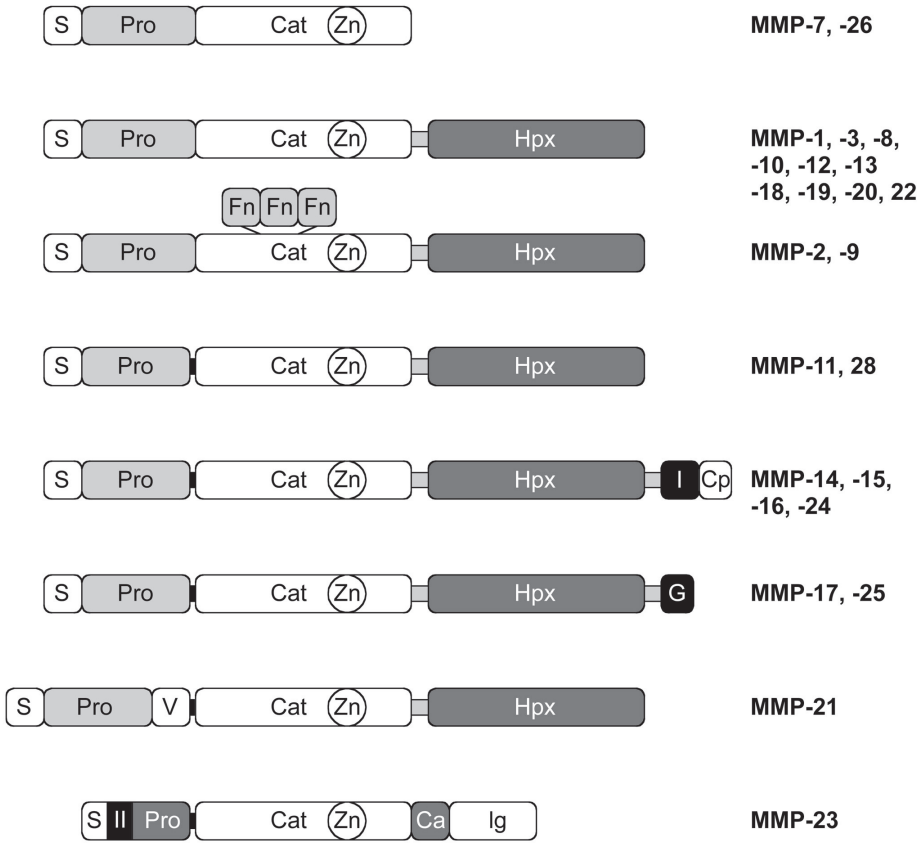

Figure 2. Domain structure of MMPs. The domain organization of MMPs is as indicated: S, signal peptide; Pro, propeptide; Cat, catalytic domain; Zn, active-site zinc; Hpx, hemopexin domain; Fn, fibronectin domain; I, type I transmembrane domain; II, type II transmembrane domain; G, GPI anchor; Cp, cytoplasmic domain; V, vitronectin insert; Ca, cysteine array region; and Ig, IgG-like domain. A furin-cleavage site is depicted as a black band between propeptide and catalytic domains. Modified from Visse and Nagase (2003).

2. MATRIX METALLOPROTEINASES (MMPS) AND THEIR INHIBITORS

The matrix metalloproteinases (MMPs) are a family of structurally and functionally related endopeptidases capable of degrading almost all components of the ECM and basement membranes (BM) (Birkedal-Hansen *et al*. 1993). In addition to their role in destruction and remodeling of the ECM, MMPs are important regulators of the functions of various biologically active molecules such as proinflammatory cytokines, chemokines, growth factors, and serine proteinase inhibitors (Vu and Werb 2000, Stamenkovic 2003, Parks *et al*. 2004). Thus, MMPs are involved in many physiological and pathological processes including embryonic development, inflammation, immunity, and cancer (Vu and Werb 2000, Stamenkovic 2003, Parks *et al*. 2004). The catalytic activity of MMPs is controlled at several points, e.g., at the level of transcription, by activation of the secreted zymogens (proMMPs), and through inhibition of the active enzyme by several endogenous inhibitors, of which the tissue inhibitors of metalloproteinases (TIMPs) are the most important (Nagase and Woessner 1999).

In humans, to date, the number of MMPs is 23 (Nagase *et al*. 2006). All MMPs share a basic domain structure comprising 1) a signal peptide that targets them for secretion, 2) a propeptide with a cysteine residue which ligates the catalytic zinc ion for preservation of latency, and 3) a catalytic domain containing the zinc-binding site (Birkedal-Hansen *et al*. 1993, Nagase and Woessner 1999). Most of the MMPs also have a hinge region and a carboxy-terminal hemopexin-like domain, both of which are lacking in MMP-7, -23, and -26. In addition, some domains are restricted to subgroups of MMPs, such as gelatin-binding domains present in the catalytic domain of the gelatinases (MMP-2 and MMP-9). These domains contain repeats of fibronectin motifs which facilitate enzyme binding to gelatin. Of the six membrane-type MMPs (MT-MMPs), four (MMP-14, -15, -16, and -24) have transmembrane and intracellular domains, whereas two (MMP-17 and -25) have glycosylphosphatidylinosi tol (GPI) anchors, which target them to the cell surface (Nagase *et al*. 2006) (Figure 2).

2.1. Members of the MMP family

MMPs are divided into 6 subgroups based on their structural characteristics and substrate specificity: collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11), matrilysins (MMP-7 and MMP-26), MT-MMPs (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, MMP-25), and other MMPs (MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27, MMP-28) (Birkedal-Hansen *et al*. 1993, Nagase *et al*. 2006).

2.1.1. Collagenases

MMP-1, MMP-8, and MMP-13 (collagenase-1, -2, and -3) belong to the collagenase subgroup of the MMPs. A unique feature of these enzymes is their capacity to cleave native interstitial collagens I, II, and III at a specific site of the alpha chain. This cleavage results in generation of ¾ amino terminal and ¼ carboxy terminal fragments, which, at body temperature, denature rapidly into gelatin, and are subsequently degraded by

gelatinolytic MMPs. MMP-1 prefers type III collagen, whereas MMP-8 preferentially cleaves type I and II collagens, and MMP-13 cleaves type II collagen more efficiently than other fibrillar collagens (Birkedal-Hansen *et al*. 1993, Knäuper *et al*. 1996). Collagenases can also cleave various other components of ECM, as well as nonmatrix proteins such as serine proteinase inhibitors, α_2-macroglobulin, and certain inflammatory mediators (McQuibban *et al*. 2002, Balbin *et al*. 2003, Owen *et al*. 2004).

MMP-1 and MMP-13 are mainly expressed *in vivo* during the active ECM remodeling associated with physiological situations such as fetal bone development and tissue repair, as well as various pathological conditions including inflammatory diseases and malignant tumors (Inada *et al*. 2004, Ala-aho and Kähäri 2005, Pardo and Selman 2005).

As for MMP-8, during early differentiation in the bone marrow, neutrophils synthesize MMP-8 and store it in a latent 75 to 85-kD form in specific intracellular granules. This highly glycosylated isoform of MMP-8 is released in latent form from activated neutrophils upon degranulation (Hasty *et al*. 1986). A significant part of the released enzyme is subsequently associated with the cell surface of neutrophils, where it is detectable in both latent and active form (Owen *et al*. 2004). The mesenchymal MMP-8 isoform is a less-glycosylated 50 to 55-kD form of MMP-8, which is expressed by several types of cells such as stimulated chondrocytes, rheumatoid synovial fibroblasts, oral carcinoma cells, and chorionic cells of human fetal membranes (Cole *et al*. 1996, Hanemaaijer *et al*. 1997, Moilanen *et al*. 2003, Arechavaleta-Velasco *et al*. 2004).

Activation of MMP-8 by proteinases such as trypsin-2, chymotrypsin, cathepsin G, MMP-3, and MMP-14 converts the latent forms of neutrophil-derived MMP-8 (Knäuper *et al*. 1990, Knäuper *et al*. 1993, Holopainen *et al*. 2003, Moilanen *et al*. 2003) and mesenchymal cell-derived MMP-8 (Moilanen *et al*. 2003) into active forms of 65 to 75-kD and 45 kD, respectively. MMP-8 can also be activated *in vitro* by myeloperoxidase-derived reactive oxygen species, and this activation mechanism does not necessarily involve change in molecular mass (Saari *et al*. 1990).

In addition to its collagenolytic properties, MMP-8 is capable of degrading α_1-antitrypsin and of processing chemokines, and it is also involved in the generation of apoptotic signals for inflammatory cells, suggesting that MMP-8 may play a significant role in the regulation of inflammation (Balbin *et al*. 2003, Owen *et al*. 2004, Gueders *et al*. 2005).

2.1.2. Gelatinases

MMP-2 (gelatinase A) and MMP-9 (gelatinase B) were originally described as gelatin-binding proteins (Vartio and Vaheri 1981, Vartio *et al.* 1982). They degrade several ECM proteins such as denatured collagens or gelatins, type IV and type V collagens, elastin, fibronectin, and vitronectin, as well as a number of non-matrix proteins including proteinase inhibitors, certain cytokines and chemokines, and latent growth factors and growth factor binding proteins (Björklund and Koivunen 2005, Chakrabarti and Patel 2005). Both MMP-2 and MMP-9 proteolytically process the inactive proforms of transforming growth factor-beta (TGF-β) and IL-1β into their corresponding active forms (Schonbeck *et al*. 1998, Yu and Stamenkovic 2000). MMP-9 also processes IL-8 into a truncated variant with increased activity, thus causing more effective neutrophil chemotaxis and activation (Opdenakker *et al*. 2001). In addition, MMP-9 degrades α_1-antitrypsin and in this way protects neutrophil elastase activity (Liu *et al*. 2000).

In general, MMP-2 is produced constitutively and by a variety of cell types including fibroblasts, endothelial cells, macrophages, and various malignant cells (Chakrabarti and Patel 2005). It is secreted in a latent 72-kD form, and its main activation occurs on the cell surface and is mediated by membrane-type MMPs (Strongin *et al*. 1995, Visse and Nagase 2003). MMP-14 (membrane-type 1 MMP) activation of MMP-2 occurs by the formation of a molecular complex containing MMP-2, MMP-14, and TIMP-2 which concentrates the components on the cell surface. An adjacent free and active MMP-14 subsequently cleaves proMMP-2 to the 64-kD intermediate form. This intermediate form is then processed to the fully active 62-kD form due to autoproteolytic cleavage at a second site (Strongin *et al*. 1995, Murphy *et al.* 1999).

In contrast to MMP-2, the expression of MMP-9 is highly inducible by growth factors and proinflammatory cytokines in many cell types including alveolar macrophages, alveolar and bronchial epithelial cells, fibroblasts, endothelial cells, and malignant cells (Atkinson and Senior 2003, Björklund and Koivunen 2005). Neutrophils synthesize MMP-9 during maturation in the bone marrow, and the MMP-9 is stored in latent form in intracellular tertiary granules before its release into the extracellular space after neutrophil activation. MMP-9 is released from cells as 92-kD proMMP-9, but also to a significant degree in complexed forms, including complexes with TIMP-1, with itself (200-kD homodimer), and with neutrophil gelatinase-associated lipocalin (120-kD complex) (Westerlund *et al*. 1996, Opdenakker *et al*. 2001). Part of the secreted MMP-9 is consequently bound to the cell surface, where it is significantly resistant to inhibition by TIMP-1 (Owen *et al*. 2003, Björklund and Koivunen 2005). MMP-9 can be proteolytically activated to a 75 to 82-kD active form by trypsin-2, neutrophil elastase, plasmin, and uPA (Ferry *et al*. 1997, Mazzieri *et al*. 1997, Sorsa *et al*. 1997, Liu *et al*. 2005). In addition, reactive oxygen species can activate MMP-9 (Westerlund *et al*. 1996). Moreover, the mere binding of proMMP-9 to gelatin or type IV collagen induces its enzymatic activity, without removal of the prodomain (Bannikov *et al*. 2002).

2.2. Regulation of MMPs

In healthy resting adult tissues many MMPs are expressed at low levels or not expressed at all. However, several factors such as proinflammatory cytokines, growth factors, hormones, and cell–cell and cell–matrix interactions can rapidly stimulate the gene expression of most MMPs (Birkedal-Hansen *et al*. 1993, Nagase and Woessner 1999).

Because neutrophil-derived MMP-8 and MMP-9 are not synthesized *de novo* by mature neutrophils, they are not regulated transcriptionally. When activated by proinflammatory mediators, degranulating neutrophils release the MMP-8 and MMP-9 stored in their intracellular granules. The secreted enzymes are rapidly bound to neutrophil plasma membrane, where they are expressed in both pro and active forms, and are often colocalized on the leading edge of a polarized neutrophil (Owen *et al*. 2003, 2004). The membrane-bound MMP-8 and MMP-9 have a spectrum of catalytic activity and a catalytic efficacy similar to those of the soluble forms of these enzymes; in contrast to the soluble forms, the membrane-bound enzymes are substantially resistant to inhibition by TIMP-1 and TIMP-2 (Owen *et al*. 2003, Owen *et al*. 2004). Cell-surface localization of MMP may be an important mechanism to maintain a high local enzyme concentration, and to target their catalytic activity to specific substrates in the

pericellular environment (Parks and Shapiro 2001, Stamenkovic 2003, Parks *et al*. 2004).

Most of the MMPs are secreted as proforms, which are maintained in a catalytically inactive state by interaction between the cysteine residue of propeptide domain and the zinc ion of the active site. Disruption of this interaction is required for activation of proMMPs, and this can occur by various nonproteolytic agents such as organomercurials and reactive oxygen species, or by cleavage of the propeptide by tissue and plasma proteinases such as trypsin, plasmin, neutrophil elastase, and mast cell tryptase, as well as by MMPs (Birkedal-Hansen *et al*. 1993, Ferry *et al*. 1997, Mazzieri *et al*. 1997, Sorsa *et al*. 1997, Holopainen *et al*. 2003, Moilanen *et al*. 2003, Liu *et al*. 2005). The main activation of proMMP-2 takes place on the cell surface and is mediated through a trimolecular complex of MMP-2/TIMP-2/MMP-14 (Strongin *et al*. 1995). In addition, certain MMPs such as MT-MMPs, MMP-11, MMP-23, and MMP-28 possess a furin cleavage site, and are likely to be activated intracellularly by furin-like pro-protein convertases and are secreted or bond to the cell surface as active enzymes (Nagase *et al*. 2006).

2.2.1. Inhibition of MMPs

The activity of MMPs is inhibited by both endogenous and synthetic MMP inhibitors, being physiologically inhibited by specific tissue inhibitors of metalloproteinases (TIMPs) as well as by the broad-spectrum proteinase inhibitor α_2-macroglobulin, the major inhibitor of MMPs in plasma (Baker *et al*. 2002).

TIMPs. TIMPs are relatively small 20 to 30-kD proteins that bind MMPs in a 1:1 stoichiometric ratio and in a reversible manner. To date, four TIMPs have been characterized in humans (TIMP-1, -2, -3, and -4). The TIMPs inhibit active forms of all MMPs without major selectivity, although TIMP-1 is a poor inhibitor of certain MT-MMPs and MMP-19 (Lambert *et al*. 2004). In addition, the TIMPs are also able to make complexes with proMMPs: TIMP-1 preferentially binds to proMMP-9, and TIMP-2 with proMMP-2, which is important in the cell-surface activation of proMMP-2. TIMP-3 makes complexes with both proMMP-2 and proMMP-9, whereas TIMP-4 has the ability to bind proMMP-2 (Lambert *et al*. 2004). Expression of TIMP-1 and TIMP-3 can be induced by a variety of cytokines and growth factors, whereas TIMP-2 is constitutively expressed in many cell types (Baker *et al*. 2002). In the healthy adult mouse, TIMP-2 is expressed at high levels in all tissues (Nuttall *et al*. 2004). It directly suppresses endothelial cell growth and angiogenesis, and this function is independent of its ability to inhibit MMP activity (Stetler-Stevenson and Seo 2005). Other TIMPs also show MMP-independent functions such as regulation of cell growth and apoptosis (Lambert *et al*. 2004).

Synthetic inhibitors of MMPs. Numerous synthetic MMP inhibitors have been developed, most of which target the catalytic site of the MMPs and act by chelating the active site zinc ion (Birkedal-Hansen *et al*. 1993). The MMP inhibitors can be divided into 3 major classes: peptidomimetic inhibitors, non-peptidomimetics, and tetracycline analogues. Some of them have been evaluated in clinical trials, mainly as a treatment option for cancer or rheumatoid arthritis, but the results have been disappointing (Coussens *et al*. 2002, Ala-aho and Kähäri 2005). The only MMP inhibitor in clinical use is low-dose doxycycline as adjunctive medication in human periodontal disease (Golub *et al*. 1998). Chemically modified tetracyclines (CMTs) are tetracycline analogues devoid of antimicrobial activity, which inhibit MMPs

by chelating the active site zinc ion, rendering the MMPs more susceptible to degradation, as well as by down-regulating MMP mRNA expression (Golub *et al*. 1998). Tetracyclines also inhibit neutrophil elastase and are capable of scavenging reactive oxygen species and inhibiting cytokine expression (Nieman and Zerler 2001). Studies in animal models of septic shock and ARDS have suggested that prophylactic treatment with CMT-3 may reduce morbidity in patients at risk for sepsis or ARDS (Carney *et al*. 1999, Nieman and Zerler 2001, Steinberg *et al*. 2005).

3. MATRIX SERINE PROTEINASES AND THEIR INHIBITORS

Serine proteinases comprise a large family proteolytic enzymes characterized by a serine residue at their active site. These enzymes regulate various biological functions, including coagulation, fibrinolysis, immune/ inflammatory responses, digestion of dietary proteins, and degradation of ECM and BM.

Inflammatory cell-derived serine proteinases. Inflammatory cells such as neutrophils, monocytes, and mast cells are significant stores of serine proteinases. Mast cell granules contain chymase and tryptase, both of which are released from mast cells during inflammation and allergic reactions (Sommerhoff 2001). Neutrophil elastase, cathepsin G, and proteinase 3 are produced during neutrophil development and stored in the primary granules of mature neutrophils. When the neutrophils are activated, these proteinases are released by degranulation into the extracellular space (Weiss 1989), although some of the elastase and cathepsin G remain bound to the neutrophil plasma membrane, where the enzymes are active and resistant to inhibition by antiproteinases (Owen *et al*. 1995). Neutrophil serine proteinases have a broad substrate specificity, and when released into the extracellular space in excessive amounts during inflammation they have the potential to cause tissue injury (Hiemstra *et al*. 1998, Lee and Downey 2001, Shapiro 2002). Neutrophil elastase and cathepsin G can cause ECM destruction either directly or indirectly by activating proMMPs such as MMP-9 (Watanabe *et al*. 1993, Ferry *et al*. 1997), as well as enhancing MMP activity by degrading and inactivating TIMPs (Itoh and Nagase 1995). Moreover, neutrophil elastase may mediate inflammation by inducing the secretion of proinflammatory cytokines (Nakamura *et al*. 1992).

The activity of neutrophil serine proteinases is controlled by serine proteinase inhibitors known as serpins, such as α_1-proteinase inhibitor (also known as α_1-antitrypsin), α_1-antichymotrypsin, secretory leukocyte proteinase inhibitor (SLPI), and elafin (Wewers *et al*. 1988, Weiss 1989, Hiemstra 2002, Sallenave 2002), as well as by α_2-macroglobulin which reacts with a large variety of proteinases (Wewers *et al*. 1988). SLPI and elafin are produced locally at mucosal sites, while the liver is the main source of α_1-proteinase inhibitor, α_1-antichymotrypsin, and α_2-macroglobulin (Sallenave 2002). Several studies have indicated that elafin, SLPI, and α_1-proteinase inhibitor may affect the inflammatory response also by mechanisms distinct from those associated with the antiproteolytic activity against leukocyte-derived serine proteinases (Churg *et al*. 2001, Hiemstra 2002, Sallenave 2002).

Fibrinolytic serine proteinases. The serine proteinases of the fibrinolytic system consist of plasminogen/plasmin and specific plasminogen activators (PA) (Vassalli *et al*. 1991). Inhibition of these fibrinolytic serine proteinases may occur either at the level of the PA, by specific plasminogen activator inhibitors (PAI-1 and PAI-2), or at the level of plasmin, mainly by α_2-antiplasmin (Lijnen 2001). In the vasculature, tissue-type PA (tPA) bound to fibrin generates plasmin for thrombolysis. Cell surface-associated plasmin, generated by the activity of receptor-bound urokinase type PA (uPA) on cell bound plasminogen, is believed to be involved in the degradation of the ECM. It activates several latent MMPs, notably MMP-3 and MMP-9 (Mazzieri *et al*. 1997, Murphy *et al*. 1999, Liu *et al*. 2005), and is also capable of directly degrading ECM components such as fibronectin, vitronectin, or laminin (Danø *et al*. 1985).

3.1. Trypsinogens and their inhibitors

Pancreatic trypsinogen is a well-characterized digestive serine proteinase. Several trypsinogen isoforms have been identified in humans (Scheele *et al*. 1981, Wiegand *et al*. 1993) and in several animal species including rat (Brodrick *et al*. 1980, Lütcke *et al*. 1989). The human pancreas produces three trypsinogen isoenzymes which differ in isoelectric point, namely trypsinogen-1 (cationic trypsinogen), trypsinogen-2 (anionic trypsinogen), and a minor isoform, trypsinogen-3 (mesotrypsinogen) (Scheele *et al*. 1981, Rinderknecht *et al*. 1984). In addition, trypsinogen-4, a splicing variant of trypsinogen-3, has been identified in the human brain (Wiegand *et al*. 1993).

Extrapancreatic trypsinogen. Trypsinogen-1 and -2 were detected and characterized for the first time outside the gastrointestinal tract in the cyst fluid of human ovarian tumors (Stenman *et al*. 1988). These tumor-associated trypsinogens are identical to the pancreatic trypsinogen-1 and -2 in amino acid sequences, in immunoreactivity, and in molecular size (25 kD and 28 kD, respectively), but display minor differences in substrate specificity and susceptibility to inhibition by protease inhibitors (Koivunen *et al*. 1989). Trypsinogen expression has more recently been observed in several tumors (Kawano *et al*. 1997, Fujimura *et al*. 1998) as well as in cancer cell lines (Koivunen *et al*. 1991). Trypsinogen is now known to be widely expressed in normal human extrapancreatic tissues. At the mRNA and protein level, trypsinogen occurs in vascular endothelial cells (Koshikawa *et al*. 1997) and in the epithelial cells of the skin, esophagus, stomach, small intestine, lung, kidney, liver, and extrahepatic bile duct, as well as in leukocytes in the spleen and in brain nerve cells (Koshikawa *et al*. 1998). Despite its wide distribution in various tissues, however, little is known about the regulation of trypsinogen expression.

3.1.1. Regulation of trypsin activity

Activation. During the digestive process, trypsinogens are secreted from the pancreas into the lumen of the duodenum, where they are activated by enterokinase (enteropeptidase). In humans, enterokinase is not known to occur in tissues other than the intestine. In the rat, expression of enterokinase has also been observed in the stomach, colon, and brain (Yahagi *et al*. 1996). *In vitro*, trypsinogen can be activated by lysosomal hydrolases (Figarella *et al*. 1988), and incubation of stimulated leukocytes with trypsinogen has been shown to convert trypsinogen to trypsin (Hartwig *et al*. 1999). At present it is, however, unclear how extrapancreatic trypsinogens are activated *in vivo*.

Inhibition. Activity of trypsin is controlled by several inhibitors such as α_1-antitrypsin, α_2-macroglobulin (Ohlsson 1988), and pancreatic secretory trypsin inhibitor (PSTI), also called tumor-associated trypsin inhibitor (TATI) (Huhtala *et al*. 1982). PSTI/TATI, a highly specific inhibitor of trypsin with a molecular weight of 6 kD, was originally found in the pancreas, but has subsequently been identified in a variety of human tissues, including the gastrointestinal tract and lung (Shibata *et al*. 1987). Although the main role of PSTI/TATI is thought to be protection of the tissue from the destructive activity of trypsin, PSTI/TATI may also play a role in maintaining mucosal integrity and in stimulating epithelial repair (Marchbank *et al*. 1998).

3.1.2. Functions of trypsin

Human trypsin is a potent matrix-degrading proteinase, which directly degrades various components of the ECM and BM, including fibronectin and collagen types I, II, and IV (Koivunen *et al*. 1991, Koshikawa *et al*. 1992, Moilanen *et al*. 2003, Stenman *et al*. 2005). Moreover, trypsin-2 efficiently activates latent proforms of MMP-1, -3, -8, -9, and -13 *in vitro* at very low concentrations (Sorsa *et al*. 1997, Moilanen *et al*. 2003), and partially activates MMP-2 (Sorsa *et al*. 1997). Human trypsin-1 and -2 are also activators of the proform of u-PA (Koivunen *et al*. 1989).

The wide distribution of trypsin expression in the intact epithelium of several human tissues suggests that it functions in common homeostatic processes. Trypsin is a potent activator of proteinase-ativated receptor 2 (PAR_2), which is extensively expressed in the epithelial cells and is believed to be involved in various physiological and pathophysiological functions (Dery *et al*. 1998, Steinhoff *et al*. 2005).

In the intestine, human α-defensin 5 (HD5) is a key contributor to microbial defense (Ouellette and Selsted 1996). It is stored as a propeptide in healthy ileal mucosa; to be fully functional, it must be processed proteolytically after its secretion (Cunliffe *et al*. 2001). Recently, it was found in that Paneth cells of the human terminal ileum produce trypsinogen-2 and -3 and the pro-HD5, and that trypsin is the cleaving enzyme for HD5 *in vivo* (Ghosh *et al.* 2002). Therefore, as a prodefensin convertase in human Paneth cells, trypsin is believed to be involved in the innate immunity of the human small intestine.

3.2. Serine proteinase signaling by activation of proteinase-activated receptors (PAR)

The serine proteinases act, at least in part, via proteinase-activated receptors, a family of G-protein-coupled seven-transmembrane receptors that are expressed in a variety of tissues, including lung, gastrointestinal tract, cardiovascular system, skin, and nervous system (Dery *et al*. 1998, Steinhoff *et al*. 2005). Four members of the PAR family have been cloned and characterized in humans and murines, thus far (Vu *et al*. 1991, Nystedt *et al*. 1994, 1995, Ishihara *et al*. 1997, Xu *et al*. 1998). The general mechanism by which proteinases activate PARs is the same: They cleave at specific sites within the extracellular amino terminus of the receptor (Dery *et al*. 1998). The newly exposed amino terminus itself acts as a tethered ligand which binds to conserved regions in the second extracellular loop of the cleaved receptor, resulting in receptor activation and initiation of multiple signaling cascades with diverse consequences such as hemostasis, inflammation, pain transmission, and repair (Macfarlane *et*

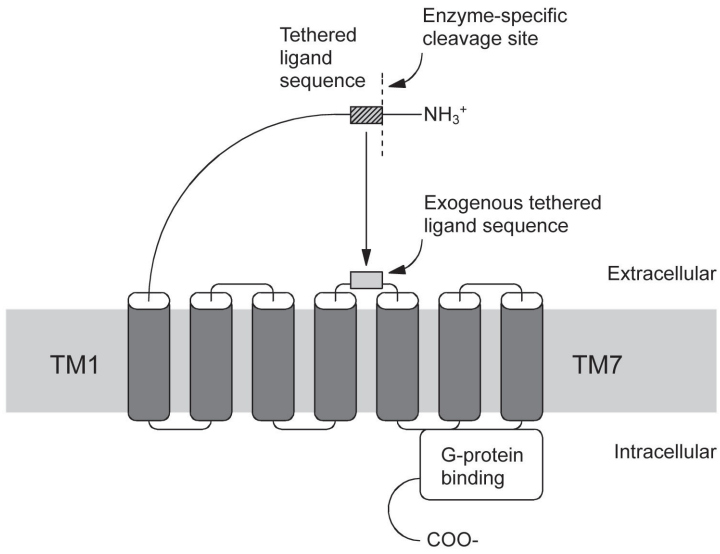

Figure 3. Mechanism of activation of proteinase-activated receptors (PARs). The "tethered ligand" sequence (hatched box) which is revealed following enzyme-specific cleavage, binds to a site on the second extracellular loop (grey box), to initiate G-protein-mediated cell signalling. Synthetic peptide mimetics of the PARs tethered ligand sequences are able to activate PARs by binding to the receptor (grey box) without proteolytic cleavage of the aminoterminus. Modified from Cocks and Moffatt (2000).

al. 2001, Hollenberg and Compton 2002, Ossovskaya and Bunnett 2004, Steinhoff *et al*. 2005) (Figure 3).

Of the serine proteinases, thrombin activates PAR_1, PAR_3, and PAR_4–but not PAR_2 (Vu *et al*. 1991, Ishihara *et al*. 1997, Xu *et al*. 1998), which is cleaved by trypsin (Nystedt *et al*. 1994). Although PAR_1, PAR_3, and PAR_4 are considered predominantly to be thrombin receptors, they can also be activated by trypsin (Hollenberg and Compton 2002), and PAR_4 by cathepsin G (Sambrano *et al*. 2000). All PARs except PAR_3 can also be activated by short synthetic peptides (PAR-activating peptides) that mimic the tethered ligand region of the receptor (Macfarlane *et al*. 2001). Activation of PARs by proteinases is irreversible, and once cleaved, the receptors are endocytosed and trafficked to lysosomes for degradation (Ossovskaya and Bunnett 2004). In addition to the activating cleavage, cell-surface proteolysis may disable PARs by removing or destroying the tethered ligand, and thus prevent receptor activation. Neutrophil elastase and cathepsin G can disarm PAR_1 and PAR_2, and in this way dampen signaling by activating proteinases (Dulon *et al*. 2003, Ossovskaya and Bunnett 2004).

3.2.1. PAR_2

Pancreatic trypsin, and extrapancreatic trypsin-2, as well as human recombinant trypsin-1 and -2 very potently activate PAR_2 (Nystedt *et al*. 1994, Alm *et al*. 2000, Grishina *et al*. 2005). PAR_2 is also activated by mast cell tryptase, although considerably less potently than by trypsin (Corvera *et al*. 1997, Alm *et al*. 2000). The coagulation factors FVIIa and FXa also function as activators when anchored by tissue factor to the cell surface (Camerer *et al*. 2000).

Like trypsin, PAR_2 is highly expressed in the lung, skin, kidney, gastrointestinal tract, and brain, where it is found in epithelial and endothelial cells, myocytes, fibroblasts, inflammatory cells, and neurons (D'Andrea *et al*. 1998). It is involved in both cytoprotective and proinflammatory responses (Cocks *et al*. 1999, Steinhoff *et al*. 2005). It plays a protective role in the gastric mucosa (Kawabata 2003), while in the intestine and lung it appears to play both pro- and anti-inflammatory roles (Cocks *et al*. 1999, Kawabata 2003).

The role of PAR_2 in inflammation has been suggested by its up-regulation by inflammatory mediators such as TNF-α and IL-α (Nystedt *et al*. 1996). Its activation leads to increased vascular permeability and leukocyte margination and infiltration (Vergnolle *et al*. 1999, Vergnolle 1999), as well as to the production of proinflammatory cytokines and MMP-9 (Vliagoftis *et al*. 2000, Asokananthan *et al*. 2002). The proinflammatory effects of PAR_2 activation are at least in part mediated by a neurogenic mechanism (Steinhoff *et al*. 2000). Recent studies using PAR_2 knockout mice suggest a critical role for this receptor in inflammation of the lung, joints, and intestine (Ferrell *et al*. 2003, Hansen *et al*. 2005, Kelso EB *et al*. 2006, Su *et al*. 2005).

4. MATRIX PROTEINASES IN THE LUNG

Controlled remodeling and degradation of ECM by matrix proteinases, especially MMPs, are essential for lung development and growth as well as for its function as the organ of ventilation (Chua *et al*. 2005, Ryu *et al*. 2005). In addition to matrix degradation and turnover, these enzymes function in the lung as regulators of inflammation, innate immunity, apoptosis, and repair (Lee and Downey 2001, Li *et al*. 2002, Shapiro 2002, McMillan *et al*. 2004, Parks *et al*. 2004).

In the healthy adult lung, proteinase inhibitors are present in higher concentrations than are matrix-degrading proteinases, and thus prevent the deleterious effects on tissue. However, during lung inflammation, the release of large amounts of proteolytic enzymes by inflammatory as well as by activated lung resident cells may result in dysregulated extracellular proteolysis leading to development of tissue damage (Lee and Downey 2001, Chua *et al*. 2005). Moreover, MMPs and serine proteinases are involved in the chemotaxis of various inflammatory cells and in growth factor bioavailability and may thus have a major impact on the overall level of inflammation and play a role in fibroproliferative lung repair following injury (Parks *et al*. 2004, Chua *et al*. 2005). Increased pulmonary levels of matrix-degrading proteinases and an imbalance between these enzymes and their inhibitors are associated with lung injury in preterm infants as well as with several acute and chronic inflammatory lung diseases in adults (Merritt *et al*. 1983, Ricou *et al*. 1996, Lee and Downey 2001, Prikk *et al*. 2002, Chakrabarti and Patel 2005).

4.1. MMPs and tissue inhibitors of metalloproteinases (TIMPs) in the lung

4.1.1. MMPs and TIMPs in lung development

In contrast to the adult human lung, which exhibits a high antiproteinase to proteinase ratio, the fetal human lung is characterized by a dominant proteolytic profile enabling rapid remodeling of the ECM during lung development (Ryu *et al*. 2005). During fetal and early postnatal lung development, several MMPs and TIMPs are expressed (Fukuda *et al*. 2000, Masumoto *et al*. 2005, Ryu *et al*. 2005). In the developing rabbit lung, MMP-1, -2, -9, and -14 and TIMP-2 are immunohistochemically localized in epithelial cells, and MMP-2 and TIMP-2 also in mesenchymal cells (Fukuda *et al*. 2000). In the mouse fetal lung, high levels of RNA have been demonstrated for MMP-2 and -14, as well as for TIMP-2, and -3 (Nuttall *et al*. 2004). By *in situ* hybridization, MMP-14 and TIMP-2 are localized in epithelial and mesenchymal cells, but MMP-2 only in mesenchymal cells (Kheradmand *et al*. 2002). During human fetal lung development, expression of MMP-1 and -9, as well as TIMP-1, -2, and -3 has been detected immunohistochemically in lung epithelial cells and vascular endothelial and smooth muscle cells, but expression of MMP-2 only in vascular endothelial and smooth muscle cells (Masumoto *et al*. 2005). Expression of MMP-1, -2, -8, and -9 has recently been studied during lung development of the fetal baboon, showing an increase in protein levels of MMP-1 and -9 with advancing gestation, and an increase in MMP-8 level during early gestation and then a decrease during the late phase of lung development, whereas it showed MMP-2 levels to remain high throughout gestation (Tambunting *et al*. 2005).

Several studies have demonstrated an increase in type IV collagenolytic activity during late fetal lung development as well as during the early phase of postnatal lung growth (Arden and Adamson 1992, Arden *et al*. 1993, Fukuda *et al*. 2000). This has been attributed mainly to increased levels of active MMP-2 (Arden *et al*. 1993, Fukuda *et al*. 2000), although MMP-9 may also be involved (Fukuda *et al*. 2000, Hosford *et al*. 2004). The significance of MMP-9 appears to be limited, however, since mice deficient in MMP-9 have normal branching morphogenesis and develop normal adult lungs (Atkinson and Senior 2003). In contrast, MMP-2 null mice and mice deficient in its activator MMP-14 exhibit abnormal branching morphogenesis and alveolarization (Kheradmand *et al*. 2002, Atkinson *et al*. 2005). Among the TIMPs, TIMP-3 may play an important role in regulating MMP activity during lung development, as demonstrated by impaired branching morphogenesis and progressive air space enlargement in TIMP-3 null mice, a phenomenon believed to be at least in part caused by excessive fibronectin fragmentation (Gill *et al*. 2003).

4.1.2. MMPs and TIMPs in lung injury in the preterm infant

After the beginning of this thesis project, several investigators have reported data describing the presence of MMPs and TIMPs in tracheal aspirates from ventilated preterm infants during the early postnatal period (Danan *et al*. 2002, Ekekezie *et al*. 2004) and in their BALF samples (Schock *et al*. 2001, Sweet *et al*. 2001, Curley *et al*. 2003, 2004, Sweet *et al*. 2004). Increased levels of MMP-8 and -9 were detected in preterm infants from pregnancies complicated by chorioamnionitis (Curley *et al*. 2003, Curley *et al*. 2004). A significant positive correlation has been observed between protein carbonyl concentrations and MMP-9 and TIMP-1

concentrations in BALF (Schock *et al*. 2001) suggesting that MMP-9 and TIMP-1 can be up-regulated by oxidative stress in preterm infants early postnatally. During the first few days of life, low MMP-2 (Danan *et al*. 2002, Ekekezie *et al*. 2004), high MMP-8 (Sweet *et al*. 2001), low TIMP-1 and -2 (Ekekezie *et al*. 2004), and a high MMP-9/TIMP-1 ratio (Ekekezie *et al*. 2004) have been associated with later development of BPD. In addition, Ekekezie *et al.* (2004) showed that the ratio of MMP-9 to TIMP-1 remains significantly higher during the first two postnatal weeks in preterm infants who subsequently develop BPD than in those who survive without it, but with no difference in levels of MMP-9 between groups.

In autopsy lung specimens from preterm infants with RDS or in different phases of BPD, immunohistochemical expression of MMP-1, TIMP-1, and -2 appears in alveolar type-II epithelial cells as well as in alveolar macrophages. In addition, in the chronic phase of BPD, immunoreactivity for MMP-1, TIMP-1, and -2 also appears in fibroblasts of fibrotic foci (Dik *et al*. 2001).

4.1.3. MMPs and TIMPs in animal models of BPD

Prolonged exposure of newborn mice and rats to hyperoxia results in chronic lung injury characterized by decreased alveolarization, progressive inflammation, edema, and increased septal and interstitial thickness (Warner *et al*. 1998, Wagenaar *et al*. 2004). In lungs of neonatal rats exposed to >95% of hyperoxia from postnatal day 4 to 14, decreased expression of both MMP-9 mRNA and protein occurred, and, conversely, an increase in TIMP-1 mRNA and protein when compared with animals raised in room air (Hosford *et al*. 2004). In neonatal rats exposed to hyperoxia from birth, several studies have, during the first few postnatal days, shown increased expression and activity of MMP-2 (Devaskar *et al*. 1994, Buckley and Warburton 2002, Wagenaar *et al*. 2004) and MMP-9 (Buckley and Warburton 2002), as well as increased expression of MMP-12 when compared with control rats (Wagenaar *et al*. 2004). During hyperoxia, no changes in the expression of TIMP-2 have been observed either at protein (Buckley and Warburton 2002) or mRNA level (Hosford *et al*. 2004).

Recently, a study of MMPs and TIMPs in an extremely premature-baboon model of BPD demonstrated significantly higher protein levels of MMP-9 and a higher ratio of MMP-9 to TIMP-1 in lungs of these baboons when compared with gestational controls (Tambunting *et al*. 2005). In baboons with BPD, pulmonary levels of MMP-8 were significantly lower, with no differences detectable in levels of MMP-2 (Tambunting *et al*. 2005).

4.1.4. MMPs and TIMPs in acute lung injury in adults

Elevated levels of MMP-2 and especially of MMP-9 occur in BALF from patients with ARDS (Ricou *et al*. 1996, Torii *et al*. 1997, Pugin *et al*. 1999). Although TIMP-1 in BALF is increased in ARDS (Ricou *et al*. 1996, Torii *et al*. 1997), the ratio of MMP-9 to TIMP-1 in BALF remains elevated in late phases of prolonged ARDS (Ricou *et al*. 1996). Thus, an imbalance between MMP-9 and TIMP-1 may play a role in the pathogenesis of ARDS.

Increased activities for MMP-2 and MMP-9 in BALF and lung exist in various animal models of hyperoxic lung injury (Pardo *et al*. 1996, Pardo *et al*. 1998, Melendez *et al*. 2000, Gushima *et al*. 2001). In addition, collagenolytic activity in BALF is increased in rats exposed to 100% oxygen for 60 hours (Pardo *et al*. 1996). During hyperoxic lung injury, MMP-2 and MMP-9 mRNA and

protein are mainly localized in alveolar epithelial cells and alveolar macrophages (Pardo *et al*. 1996, Gushima *et al*. 2001). Exposure to hyperoxia elevates expression of TIMP-1, but not TIMP-2 mRNA (Pardo *et al*. 1998, Melendez *et al*. 2000). Similarly to MMP-2 and MMP-9, TIMP-1 and -2 are mainly localized in alveolar macrophages and alveolar epithelial cells during hyperoxia (Pardo *et al*. 1998, Melendez *et al*. 2000).

A novel anti-inflammatory role for neutrophil-derived MMP-8 has recently been observed in lipopolysaccharide (LPS)-mediated acute lung injury in mice (Owen *et al*. 2004). A greater accumulation of neutrophils in the alveolar space occurred 24 hours after intratracheally administrated LPS in MMP-8 -/- mice than in wild-type controls. The investigators later show that the BALF from MMP-8 -/- mice has more neutrophil chemotactic activity as well as more macrophage inflammatory protein-1α (MIP-1α) than does BALF from wild-type mice after LPS exposure (Owen *et al*. 2005). *In vitro*, membrane-bound MMP-8 totally degraded MIP-1α, reducing its neutrophil chemotactic activity, whereas soluble MMP-8 cleaved MIP-1α only partially (Owen *et al*. 2005).

MMP-7 is constitutively expressed in intact human airway epithelium, and its expression is induced in injured epithelium, where it plays an important role in re-epithelisation by facilitating epithelial-cell migration (Dunsmore *et al.* 1998). In addition, epithelial-derived MMP-7 regulates chemokine mobilization and transepithelial efflux of neutrophils in bleomycin-injured murine lungs by causing shedding of the ectodomain of syndecan-1 (Li *et al*. 2002).

Deficiency in TIMP-1, as recently shown, aggravates acute bleomycin-induced lung injury as determined by enhanced pulmonary neutrophilia, hemorrhage, and vascular permeability (Kim *et al*. 2005). Levels of MMP-9 were higher in BALF from TIMP-1 null mice, with no difference in BALF chemotactic activity between genotypes. TIMP-1 may thus play an important role during acute lung injury in preserving the alveolar-capillary barrier from proteolytic damage (Kim *et al*. 2005).

In addition to TIMP-1, TIMP-2 plays an important role in regulating the intensity of lung inflammatory injury (Mulligan *et al*. 1993, Gibbs *et al*. 1999, Gipson *et al*. 1999). In rat models of both macrophage-dependent and neutrophil-dependent acute lung injury, intratracheally administered human recombinant TIMP-2 (Mulligan *et al*. 1993, Gibbs *et al*. 1999) inhibits pulmonary damage. Affecting the generation of the powerful chemoattractant complement anaphylatoxin C5a in the lung may be one mechanism by which TIMP-2 regulates lung inflammation (Gipson *et al*. 1999).

4.1.5. MMPs and TIMPs in chronic inflammatory airway diseases in adults

Sustained, elevated expression of MMP-2 or MMP-9, or both and altered expression of TIMPs exists in chronic pulmonary diseases such as asthma, chronic obstructive pulmonary disease, and idiopathic pulmonary fibrosis (Chakrabarti and Patel 2005). Smokers with emphysema show higher levels of MMP-8 and MMP-9 in BALF than do smokers without emphysema (Betsuyaku *et al*. 1999). BALF from adult patients with asthma or bronchiectasis contains elevated levels and a higher degree of activation of MMP-8 than does BALF from healthy controls, and in these diseases, MMP-8 localized at both the protein and mRNA level in bronchial epithelial cells, macrophages, and neutrophils (Prikk *et al*. 2001a, Prikk *et al*. 2002). In addition, the level and activation degree of MMP-8 in BALF were higher in patients with severe asthma than in those with well-

controlled asthma, and an inverse correlation existed with airflow obstruction, indicating a direct connection between MMP-8 activity and deterioration in lung function (Prikk *et al*. 2002).

The role of MMP-8 in asthma-related acute airway inflammation has recently been studied in an ovalbumin-sensitized mouse model of asthma using MMP-8 -/- and WT mice (Gueders *et al*. 2005). In this model, MMP-8 deficiency was, following allergen exposure, associated with increased numbers of neutrophils in BALF, and enhanced migration of neutrophils as well as eosinophils in the airway walls. Assessment of cell apoptosis demonstrated significantly reduced neutrophil apoptosis in MMP-8 -/- mice compared to WT mice, suggesting that the anti-inflammatory effect of MMP-8 is in part due to a role in the regulation of neutrophil apoptosis (Gueders *et al*. 2005).

Variable effects of MMP-9 on allergen-induced airway inflammation have been recently observed in MMP-9 knockout animals. Vermaelen *et al.* (2003) demonstrated reduced infiltration of inflammatory cells into the airways after allergic challenge in MMP-9 -/- mice. The study of McMillan *et al.* (2004), using an exposure protocol based on high allergen concentrations, showed in contrast, increased inflammatory cell infiltrate in both bronchial mucosa and BALF from MMP-9 null mice.

4.2. Serine proteinases and their inhibitors in the lung

4.2.1. Inflammatory cell-derived serine proteinases and their inhibitors

The major sources of serine proteinases in the lung are inflammatory cells such as neutrophils, mast cells, and macrophages (Lee and Downey 2001, Sommerhoff 2001). Neutrophil elastase has several detrimental effects on lung tissue: It is capable of degrading almost all components of ECM, it is directly cytotoxic to endothelial cells, and it has various proinflammatory effects (Lee and Downey 2001, Shapiro 2002).

Increased levels of neutrophil elastase together with reduced functional activity of its inhibitor α_1-antitrypsin have been detected in the airway secretions from preterm infants with RDS and from those who subsequently develop BPD (Merritt *et al*. 1983, Speer *et al*. 1993). During inflammation, α_1-antitrypsin is inactivated both oxidatively by reactive oxygen species released from inflammatory cells (Carp and Janoff 1980) and proteolytically by several MMPs, including MMP-8 and -9 (Liu *et al*. 2000, Owen *et al*. 2004). In the developing lung, elastic fibers provide structural support for alveolar septal development (Roth-Kleiner and Post 2005). Thus, in preterm infants with BPD elastolytic damage may be a significant factor in the impaired lung development. In neonatal rats, α_1-antitrypsin protects against the toxic effects of oxygen on pulmonary parenchymal and vascular development (Koppel *et al*. 1994). Furthermore, in a randomized, controlled trial, prophylactic administration of α_1-antitrypsin intravenously into preterm infants with RDS has reduced the incidence of pulmonary hemorrhage and the use of inhaled steroids and bronchodilators, although the reduction in risk for BPD at 36 gestational weeks failed to reach statistical significance (Stiskal *et al*. 1998).

An imbalance between neutrophil elastase and its inhibitors in the lung has also been implicated in adult inflammatory lung diseases, such as emphysema, chronic obstructive pulmonary disease, and ARDS (Moraes *et al*. 2003).

Serine proteinase tryptase is a major mast cell-derived proteinase believed to be involved in airway inflammation and

remodeling associated with fibroproliferative lung diseases such as asthma and chronic obstructive pulmonary disease (Sommerhoff 2001, Cairns 2005). The increased numbers of mast cells appearing in autopsy lung samples from preterm infants with long-standing BPD suggests that, in the development of BPD, tryptase may also play a role (Lyle *et al*. 1995). Tryptase induces proliferation of pulmonary fibroblasts and airway smooth muscle cells, and induces secretion of proinflammatory mediators by bronchial epithelial cells (Sommerhoff 2001, Cairns 2005). It is also capable of digesting 72-kD gelatinase into a 62-kD form, as well as degrading fibronectin, suggesting that it may play a role in the degradation of ECM (Lohi *et al*. 1992). Many of the cellular effects of tryptase may be mediated through PAR_2 cleavage and activation (Akers *et al*. 2000, Berger *et al*. 2001, Cairns 2005).

4.2.2. Trypsin and trypsin-like proteinases

In the lung, in addition to inflammatory cells, resident cells are also an important source of serine proteinases. Extrapancreatic trypsin has been identified at both the mRNA and protein level in normal adult bronchial epithelium (Koshikawa *et al*. 1998, Cocks *et al*. 1999), and recently, trypsin was identified in rat lung (Towatari *et al*. 2002). Little is known, however, about the role of trypsin in the lung. Trypsin colocalizes with PAR_2 in the epithelium of human bronchioles, and has been suggested to be the physiologic activator of pulmonary epithelial PAR_2 (Cocks *et al*. 1999). By activating PAR_2, trypsin may play a cytoprotective and, on the other hand, also a harmful role (Cocks *et al*. 1999, Ossovskaya and Bunnett 2004). In addition, if dysregulated, trypsin may cause pulmonary tissue destruction through direct degradation of ECM and BM proteins (Koivunen *et al*. 1991, Koshikawa *et al*. 1992, Moilanen *et al*. 2003, Stenman *et al*. 2005), as well as through activation of various proMMPs (Sorsa *et al*. 1997, Moilanen *et al*. 2003). In BALF from adult patients with chronic inflammatory airway diseases the levels of trypsin-2-α_1-antitrypsin complex correlate with the activation level of MMP-9 and of type I collagenolytic activity (Prikk *et al*. 2001b).

A novel serine proteinase called human airway trypsin-like protease (HAT) has been characterized in adult human lung (Yamaoka *et al*. 1998) and immunolocalized in ciliated cells of the bronchial epithelium (Takahashi *et al*. 2001). HAT is synthesized as a membrane-bound precursor with a molecular size of 48 kD which is then proteolytically converted at the cell surface into a 27-kD mature form, and then released into the mucous membrane (Yamaoka *et al*. 1998). On the basis of its structure, the HAT precursor is believed to be a member of the type II transmembrane serine proteinase family (Hooper *et al*. 2001). HAT may play a physiological role within the airway epithelium, but it may also become involved in airway remodeling by stimulating fibroblast proliferation via activation of PAR_2 (Matsushima *et al*. 2006).

4.2.3. PAR_2

All PARs have been immunolocalized in human lung epithelial and smooth muscle cells (D'Andrea *et al*. 1998, Knight *et al*. 2001, Asokananthan *et al*. 2002). In the mammalian lung, immunoreactive PAR_2 is also expressed by fibroblasts, sensory neurons, bronchial glands, endothelial cells, and vascular smooth muscle cells (D'Andrea *et al*. 1998, Akers *et al*. 2000, Ricciardolo *et al*. 2000), as well as by migrated inflammatory cells such as macrophages, neutrophils, mast cells, and eosinophils (Steinhoff *et al*. 2005).

The endogenous proteinases responsible for activating PAR_2 in the lung await

largely determination. Trypsin may act as a physiologic activator of epithelial PAR_2 (Cocks *et al*. 1999). Other possible activators of PAR_2 in the lung include mast cell tryptase (Cairns 2005), HAT (Matsushima *et al*. 2006), as well as certain nonmammalian proteinases such as dust mite allergens (Ossovskaya and Bunnett 2004).

In the lung, PAR_2 may regulate both cytoprotective and proinflammatory pathways (Cocks *et al*. 1999, Ossovskaya and Bunnett 2004). In support of this protective role are observations from *in vitro* studies performed on isolated airway preparations from the mouse and rat demonstrating that activation of PAR_2 causes an epithelium-dependent bronchorelaxation, and that this effect is mediated by release of prostaglandin E_2 (Cocks *et al*. 1999). In addition, intranasal administration of synthetic PAR_2 activating peptide protects against bronchoconstrictor challenges in the rat (Cocks *et al*. 1999). Conversely, activation of PAR_2 induces contraction of guinea pig and human intrapulmonary bronchi and bronchioles, the main site of resistance to air flow (Ricciardolo *et al*. 2000, Schmidlin *et al*. 2001). Thus, the effect of PAR_2 activation on airway tone depends on the species and experimental model used.

Several studies have indicated that PAR_2 plays an important role in the pulmonary inflammation, as it is markedly up-regulated *in vitro* by early pro-inflammatory cytokines TNF-α and IL-1α (Nystedt *et al*. 1996). Activation of PAR_2 in human respiratory epithelial cell lines induces release of important inflammatory mediators including IL-6, IL-8, and prostaglandin E_2 (Asokananthan *et al*. 2002), as well as synthesis and release of MMP-9 (Vliagoftis *et al*. 2000). In addition, PAR_2 activation induces proliferation of human lung fibroblasts and airway smooth muscle cells (Akers *et al*. 2000, Berger *et al*. 2001). These *in vitro* findings suggest that PAR_2 may participate in airway remodeling and in the fibroproliferation associated with pulmonary inflammatory diseases. Accordingly, increased expression of PAR_2 appears in the bronchial epithelium of asthma patients (Knight *et al*. 2001). Involvement of PAR_2 in airway inflammation and hyperreactivity has further been supported by studies using mice which lack or overexpress human PAR_2 (Schmidlin *et al*. 2002, Su *et al*. 2005). Recently, Su *et al.* (2005) showed in wild-type mice that intratracheal instillation of the PAR_2 activating peptide, but not the PAR_1 or PAR_4 activating peptides, induces airway constriction, acute lung inflammation, and protein-rich pulmonary edema in wild-type mice, but in PAR_2 -/- mice no responses to PAR_2 activating peptide were detectable.

AIMS OF THE STUDY

The aims of the present study were to

- identify the presence, molecular forms, and degree of activation of MMP-2, MMP-8, and MMP-9, and their specific inhibitor TIMP-2 in the lungs of preterm infants by evaluating their levels in tracheal aspirate fluid (TAF) samples, and to examine the involvement of these MMPs and TIMP-2 in lung injury in the preterm infant.

- study the presence, molecular forms, and concentrations of trypsin and its specific inhibitor TATI in lungs of preterm infants, and to evaluate the role of trypsin in the development of BPD.

- study the protein expression and possible co-localization of trypsin-2 and PAR_2 in the human lung during the perinatal period and in acute and chronic lung injury in the preterm infant.

- evaluate the role of MMP-2, MMP-8, and MMP-9, and of trypsin in the development of acute lung injury by use of animal model of hyperoxic lung injury.

MATERIALS AND METHODS

1. SUBJECTS AND EXPERIMENTAL ANIMALS

1.1. Patients in tracheal aspirate fluid (TAF) studies (I, II)

With the approval of the Ethics Committee of the Hospital for Children and Adolescents, University Central Hospital, Helsinki, a total of 48 preterm infants with respiratory distress were enrolled between December 1993 and October 1997 for Studies I and II (Table 1). These preterm infants were treated according to the standard protocols in the neonatal intensive care unit of the Hospital for Children and Adolescents, University Central Hospital. All were intubated at birth because of failure to establish spontaneous ventilation, and during the study period all underwent mechanical ventilation. Those with major anomalies were excluded. BPD was defined as need for supplemental oxygen at the age of 36 gestational weeks, in association with chest radiographic findings typical for BPD (Shennan *et al*. 1988).

Table 1. Characteristics of preterm infants in TAF studies (Studies I and II)

PARAMETER	STUDY	
	I 16 infants	II 32 infants
PRENATAL		
Proteinuric preeclampsia	3 (19)	11 (34)
Premature rupture of membranes ≥ 24 h	7 (44)	12 (38)
Chorioamnionitis [1]	5 (31)	9 (28)
Antenatal betamethasone	12 (75)	27 (84)
AT BIRTH		
Cesarean section	12 (75)	21 (66)
Gender (M/F)	8/8	21/11
Gestational age (wks)	27.0 ± 2.0	27.3 ± 2.0
Birth weight (g)	875 ± 246	926 ± 295
Apgar score at 1 min	6 ± 3	5 ± 2
Umbilical artery pH	7.29 ± 0.07	7.28 ± 0.12
Umbilical artery base excess	-2.1 ± 2.3	-3.1 ± 4.0
POSTNATAL		
$aAPO_2$ [2]	0.35 ± 0.24	0.27 ± 0.20
Surfactant therapy	9 (56)	22 (69)
Doses of surfactant	1 ± 1	2 ± 1
Indomethacin therapy	12 (75)	28 (88)
Dexamethasone therapy during study period [3]	0	15 (47)
Duration of mechanical ventilation (d)	19 ± 19	23 ± 18
BPD	6 (38)	18 (56)
Death	2 (13)	1 (3)

Data are presented as mean ± SD or as number with percentage in parentheses.

[1] Diagnosed on the basis of clinical signs with maternal leukocytosis (white blood cell counts > 15 x 10^9 cells/L) or increased concentration of C-reactive protein CRP (> 50 mg/L), or both.

[2] Initial arterial to alveolar oxygen tension ratio.

[3] Study period was 5 days in Study I and 2 weeks in Study II. In Study II, dexamethasone was started at a mean (± SD) age of 13 ± 7 days (range: 3-33 days).

1.2. Autopsied subjects in immunohistochemistry studies (II, III).

Immunohistochemistry studies were done with the approval of the National Authority for Medicolegal Affairs. A total of 12 fetuses aborted between 1997 and 2000, and 34 infants who died between 1991 and 1998 at the University Central Hospital, Helsinki, were included in immunohistochemistry studies. Of the fetuses, 11 were aborted because of clinical suspicion of a major extrapulmonary malformation in fetal ultrasound examination. One fetus died at 29 gestational weeks as a result of placental ablation. All fetuses had macroscopically and microscopically normal lungs.

Of the 34 infants studied, 29 were born prematurely. Of these, 9 died as a result of acute RDS (age at death 0 to 3 days), 7 of prolonged RDS (age at death 8 to 16 days), and 8 of BPD, defined as in tracheal aspirate studies I and II (Shennan *et al*. 1988). As controls, 5 newborn infants (gestational ages 22 to 33 weeks) who died within 1 day after delivery for reasons not involving lung pathologic conditions were included in Study III. Of these, 3 received no mechanical ventilation or supplemental oxygen (gestational ages 22 to 22.7 weeks). Mechanical ventilation and supplemental oxygen were administered to 2 infants (gestational ages 26.1 and 33.0 weeks) for 2 and 20 hours, respectively. In addition, 5 term infants who had normal lung histology and died on postnatal days 1 to 3 of cardiac anomalies were included in Study II. None of the fetuses and infants presented with any lung anomalies or pneumonia at the time of death. Clinical information on the fetuses and infants in Studies II and III are given in Table 2.

Autopsies were performed within 3 days after death. The lung samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and stored at room temperature. Four-micrometer sections were stained with hematoxylin-eosin. After evaluation of the whole material, one representative tissue block from each case was selected for immunohistochemical studies.

1.3. Animals (IV)

The study protocol was approved by the Institutional Review Board for Animal Studies of Helsinki University Central Hospital, Helsinki, Finland. Female Wistar rats (age 8 weeks, weight 180 g) were purchased from Harlan Nederland (Harlan Nederland, Horst, The Netherlands). After the animals were housed for 1 week, exposure to hyperoxia (>95% O_2) or normoxia was carried out at the Laboratory Animal Center of Biomedicum Helsinki, University of Helsinki.

Table 2. Characteristics of autopsied subjects (Studies II and III)

	Fetuses [1]	Newborn Controls [2]	RDS patients	Prolonged RDS	BPD patients
Study II (N=27)[3]					
Number of patients (M/F)	8 (5/2) [4]	5 (2/3)	6 (1/5)	3 (2/1)	5 (4/1)
Gestational age (wks)	19.0 (14.0-29.0)	39.7 (38.3-41.0)	24.3 (23.0-27.7)	26.4 (24.6-29.9)	26.4 (25.1-29.0)
Birth weight (g)	240 (30-1240)	3660 (2900-4270)	520 (310-840)	670 (495-810)	800 (670-1070)
Antenatal betamethasone (+/-)	-	-	3/3	1/1 [5]	2/3
Surfactant treatment (+/-)	-	-	5/1	3/0	5/5
Age at death (d)		1 (0.5-3)	2.0 (0.5-3.0)	11 (8-16)	175 (75-285)
Study III (N=36)					
Number of patients (M/F)	10 (7/2) [4]	5 (3/2)	8 (3/5)	7 (5/2)	6 (6/0)
Gestational age (wks)	18 (14-22)	22.7 (22-33)	25.4 (23.9-29.7)	26.4 (24.6-29.9)	28.9 (26.0-31.3)
Birth weight (g)	180 (30-510)	540 (340-2230)	595 (305-1525)	675 (495-925)	918 (670-1250)
Antenatal betamethasone (+/-)	-	-	5/3	3/3 [5]	2/4
Surfactant treatment (+/-)	-	-	7/1	8/0	5/1
Age at death (d)		0.08 (0.01-1)	1.5 (0.5-2.0)	11 (8-16)	148 (75-306)

Values are expressed as median (range).

[1] Diagnoses at autopsy were meningomyelocele (4), hypoplastic left heart syndrome (2), nonketotic hyperglycinemia (1), diastrophic dysplasia (1), anencephaly (1), hydrocephalus (1), and placental ablation (1).

[2] In Study II, the newborn controls were term infants who had died as a result of hypoplastic left heart syndrome (4) or Ebstein anomaly (1). In Study III, the causes of death of newborn controls were fetofetal transfusion, rupture of fetal membranes, placental ablation, spontaneous abortion, and acute asphyxia.

[3] Of the 27 subjects in Study II, 17 (6 fetuses, 5 RDS, 3 prolonged RDS, and 3 BPD) were also included in Study III.

[4] Gender was unknown in one fetus.

[5] Not known in one case.

2. METHODS

2.1. Collection and analysis for dilution of TAF samples (I, II)

In Study I, a total of 56 TAF samples were collected from 16 preterm infants during their first 5 postnatal days. In Study II, 249 samples were collected from 32 preterm infants during their first 2 postnatal weeks. The samples were collected once daily by standardized routine tracheal lavage as previously described (Varsila *et al*. 1995). Briefly, 1 mL of sterile isotonic saline was instilled into the endotracheal tube, the infant was manually ventilated for 3 breaths, and the trachea was suctioned twice, each time for 5 seconds. For analysis of tracheal aspirates, secretions were collected into a trap and transferred into tubes containing 500 I.U. of aprotinin (Trasylol ®, Bayer, Leverkusen, Germany) and 5 mg of deferoxiamine (Desferal ®, Ciba, Basel, Switzerland). Aprotinin and deferoxiamine served to minimize oxidative and proteolytic artifacts. The tubes were stored at -20 ºC until analysis.

To eliminate the effect of dilution of the TAF samples, the levels of matrix proteinases and their inhibitors in TAF were related to concentrations of the secretory component of immunoglobulin-A IgA (SC), as the reference protein, with direct ELISA. The concentration of SC in lung secretions is independent of capillary leak and not affected by gestational or postnatal age during the first month of life (Watts and Bruce 1995). Secretory IgA isolated from human colostrum served as the standard, and the results were standardized with the help of Dr. B Götze-Speer and Prof. Ch. Speer (University Children's Hospital, Würzburg, Germany). Microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at +4 ºC with 100 µL aliquots of 1:2000 diluted anti-human secretory component (Dako, Glostrup, Denmark) in 50 mM sodium bicarbonate, pH 9.5. After washing with 20 mM Tris-HCl buffer containing 500 mM NaCl (TBS), pH 7.5, the plates were blocked for unspecific protein binding by incubation with 2% bovine serum albumin (BSA) in TBS and washed with 0.05% Tween 20 in TBS (TTBS). TAF samples were diluted between 1:10 to 1:500 in diluting buffer (1% BSA in TTBS), and 100 µL aliquots were added to the wells. After incubation overnight at room temperature, the plates were washed 3 times with TTBS. 100 µL of peroxidase-conjugated rabbit anti-human SC (Dako) was added to each well, and the plates were incubated for 4 hours at room temperature. After being washed with TTBS, the plates were developed by use of 100 µL of substrate solution containing 8 mg of orthophenylenediamine (Dako) and 5 µL of 30% H_2O_2 in 12 mL water. The optical densities of the plates were read at 450 nm after 30 minutes at room temperature.

2.2. Measurement of surfactant maturity (I, II)

For determination of surfactant maturity, TAF samples were collected within 3 h after birth before the infants' treatment with surfactant. The samples were analyzed for lecithin/sphingomyelin ratio (L/S ratio) and the presence of phosphatidyl glycerol (PG) by thin-layer chromathography (Hallman *et al*. 1982).

2.3. Experimental rat model of hyperoxic lung injury (IV)

The rats were placed in a sealed Plexiglas chamber (85 x 60 x 40 cm) and exposed to >95% oxygen for 24, 48, and 60 hours by administration of pure oxygen at 5 L/min. Oxygen concentration was monitored with oxymetry. Control animals were kept in room

air. At different exposure times, animals were anesthetized by intraperitoneal injections of ketamine (Ketalar®, Parke-Davis) 50 mg/kg and xylazine (Narcoxyl®, Veterinaria AG) 10 mg/kg, and killed by cervical dislocation.

2.3.1. Collection of lung samples

From 6 rats per group, immediately after decapitation, the right lung was removed and cut into 2 pieces. The pieces were soaked in isotonic saline, snap frozen in liquid nitrogen, and stored at -80 °C until determination of myeloperoxidase activity. The left lung was removed, fixed in 10% paraformaldehyde for 24 h, embedded in paraffin, and stored at room temperature for subsequent immunohistochemistry.

2.3.2. Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed for additional 8 animals per group in order to avoid any potential artifact introduced by BAL. Immediately after the killing of each rat, the thoracic cavity was opened to expose the trachea, the trachea was cannulated, and the lungs were lavaged by flushing with 10 mL of sterile normal saline as 10 aliquots of 1 mL each. Combined aliquots of BAL fluid (BALF) were kept on ice until centrifuged at 500 x *g* at 4ºC for 10 min, and the supernatant was stored at –20 °C for subsequent analyses. Total protein in the supernatant of BALF was measured by the method of Bradford (1976), and served as an indicator of increased vascular permeability and loss of capillary endothelial membrane integrity.

2.3.3. Myeloperoxidase activity in rat lung tissue

The lung samples were thawed, homogenized in phosphate-buffered saline, and centrifuged for 5 minutes at 16 000 x *g*, + 4 °C. Myeloperoxidase (MPO) activity was determined in the pellet as previously described (Kinsella *et al*. 1997). Protein concentration was quantified by the method of Bradford (1976). The results for MPO activity were expressed as mU/mg protein.

2.4. Zymography (II, IV)

For analysis of gelatinolytic activity by zymography, the polypeptides of TAF samples (**II**) and BALF samples (**IV**) were separated by 1.5-mm-thick 8 to 10% SDS-polyacrylamide gels impregnated with 1 mg/mL gelatin (Sigma) or with gelatin labeled fluorescently with 2-methoxy-2,4-diphenyl-3(2H)-furanone (Fluka, Sigma-Aldrich, Buchs SG, Switzerland) as earlier described (Paju *et al*. 2001). In Study IV, the intensities of gelatinolytic bands were evaluated with a Bio-Rad Model GS-700 Imaging Densitometer using the Quantity-program, a new version of the Molecular Analyst/PC program (Bio-Rad Laboratories, Hercules, CA, USA) (Sorsa *et al*. 1994). Results were expressed as arbitrary units (AU).

2.5. Western blotting (I, II, IV)

The presence and molecular forms of MMP-2, -8, and -9, TIMP-2 (**I**), and trypsin (**II**) in TAF samples from preterm infants, and of these MMPs and trypsin in BALF samples from rats (**IV**) were analyzed by Western blotting. The sources of the antibodies used are listed in the original publications (**I, II, IV**). After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA, USA). The membranes were saturated with 3% gelatin or 5% milk powder (Valio, Helsinki, Finland). The membranes first reacted with specific primary antibodies

overnight at room temperature, and then with secondary antibodies conjugated with alkaline phosphatase or horseradish peroxidase for one hour. The proteins were visualized by incubation of the membranes with Nitro Blue Tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma) solution or by the enhanced chemiluminesence (ECL) detection system according to manufacturer's instructions (Amersham Pharmacia Biotech Inc, NJ, USA).

In Studies I and IV, the intensity levels of different molecular weight forms of the MMPs studied (**I, IV**) and TIMP-2 (**I**) were evaluated with the Bio-Rad Model GS-700 Imaging Densitometer using the Molecular Analyst/PC program with correction for background values (Sorsa *et al*. 1994). Results were expressed as arbitrary units (AU).

2.6. Time-resolved immunofluorometric assays (II)

The concentrations of trypsinogen-1, trypsinogen-2, and TATI in TAF were determined by specific time-resolved immunofluorometric assays as earlier described (Itkonen *et al*. 1990, Osman *et al*. 1993). The detection limit was 0.1 µg/L for trypsinogen-1, 0.3 µg/L for trypsinogen-2, and 0.2 µg/L for TATI. The inter- and intra-assay coefficients of variation were 10 to 15% for trypsinogen-1, 10 to 12% for trypsinogen-2, and 13 to 14% for TATI (Itkonen *et al*. 1990, Osman *et al*. 1993).

2.7. Immunohistochemical analyses (II, III, IV)

In autopsy lung specimens from fetuses and infants, the immunoreactivity of trypsin-2 was visualized with monoclonal anti-trypsin-2 antibody 8F7 (Itkonen *et al*. 1990) (**II, III**), and PAR_2 immunoreactivity with polyclonal anti-PAR_2 B5 antibody (Kong *et al*. 1997, Al-Ani *et al*. 1999) (**III**). In rat lung, trypsin immunoreactivity was analyzed with monoclonal anti-trypsin antibody MAB 1482 (Chemicon International, Temecula, CA, USA), and MMP-8 immunoreactivity with polyclonal anti-MMP-8 antibody (Hanemaaijer *et al*. 1997) (**IV**).

Four-micrometer sections were deparaffinized and rehydrated. To enhance antigen retrieval, the sections were pretreated with microwave heat. Endogenous peroxidase was quenched by incubating the sections in methanol and hydrogen peroxidase. After application of blocking serum, the sections were incubated overnight at room temperature with the primary antibody. Bound antibody was visualized by the avidin-biotin complex immunoperoxidase technique (ABC) (Elite ABC kit, Vectastain, Vector Laboratories, Burlingame, CA, USA) following the manufacturer's instructions. The sections were incubated with the biotinylated secondary antibody and the peroxidase-labelled avidin-biotin complex for 30 minutes each. Bound peroxidase was visualized with 3-amino-9-ethyl-carbazole (AEC) solution (A-5754, Sigma), and finally the sections were stained with hematoxylin.

For negative controls, the primary antibodies were replaced with neutral isotonic phosphate-buffered saline (**II, III, IV**). In addition, for PAR_2, negative controls were performed by preabsorbing the antibody with the non-conjugated immunogenic peptide and incubating 3 hours at room temperature before application to the tissue. In Study IV, rat pancreatic tissue served as a positive control for trypsin immunohistochemistry, and rat skin specimens served as positive controls for MMP-8 immunohistochemistry (Pirila *et al*. 2001).

2.7.1. Semiquantitative analysis

In Study III, the immunoreactivity of PAR_2, and in Study IV, the immunoreactivity of trypsin were analyzed and scored independently by two investigators in a blinded fashion. In each case, the entire section of lung tissue was evaluated. In Study III, PAR_2 level was scored in a semiquantitative manner as 5 levels (absent = 0, low = 1, moderate = 2, strong = 3, very strong = 4). Separate scores were given for bronchial and bronchiolar epithelium, alveolar epithelium, bronchial smooth muscle cells, vascular smooth muscle cells, and vascular endothelium. In Study IV, the immunoreactivity of trypsin was semiquantified according to the following method: absent = 0, low = 1, moderate = 2, or strong = 3. Bronchial and bronchiolar epithelium, alveolar epithelium, and vascular smooth muscle were scored separately.

2.8. Statistical analyses

P values < 0.05 were considered statistically significant. All calculations were done with StatView 4.1 (Abacus Concepts Inc., Berkeley, CA, USA).

Studies I and II. Patient data in both studies are given as mean ±± SD. In Study I, results are expressed as mean ±± SEM, and in Study II as medians and ranges and interquartiles. The Mann-Whitney *U*-test (**I**, **II**) or Fisher's exact test (**II**) was used to compare differences between groups. Logarithmic transformation of the data was performed for the linear regression analysis used for continuous variables (**I**, **II**).

Studies III and IV. Data are expressed as medians, interquartiles, and 10th and 90th percentiles. Comparisons between groups were performed with the nonparametric multiple comparison Kruskal-Wallis test. Dunn's test was used for the *post hoc* comparisons.

RESULTS

1. LUNG INJURY IN PRETERM INFANTS (I, II, III)

1.1. MMP-2, MMP-8, and MMP-9, and TIMP-2 in TAF (I)

In TAF samples from 16 preterm infants with respiratory distress, the presence and forms of MMP-2, -8, and -9, and TIMP-2 during the first 5 postnatal days were characterized by use of Western blotting (**I**, Figure 1). MMP-2 was detectable in TAF samples from all infants. It was detected as high molecular weight complexed forms, at the level of 70 and 60 kD corresponding to latent and active forms of MMP-2, and as lower molecular weight fragmented forms (**I**, Figure 1). Both the neutrophil-derived 70- to 80-kD isoform of MMP-8 and the mesenchymal cell-derived 40- to 60-kD MMP-8 occurred. Of the isoforms, the neutrophil-derived MMP-8 species clearly predominated, and it appeared in all infants. In 13 of them, mesenchymal cell-derived MMP-8 was also detectable. Neutrophil-derived active MMP-8 appeared in a small number of samples, and MMP-8 also in complexed and fragmented forms (**I**, Figure 1). MMP-9 was detectable in 13 infants, in complexed, 90-kD latent, and active forms (**I**, Figure 1). TIMP-2 was demonstrable as high molecular weight complexed forms, in native intact form, and 8 infants showed fragmented TIMP-2 (**I**, Figure 1). TIMP-2 could be detected in TAF samples from all but one infant who died of severe respiratory distress at the age of 12 days.

The levels of MMP-2, -8, and -9 in TAF samples were evaluated by densitometric scanning of the Western blots.

Table 3. Perinatal variables and levels of MMP-2, -8, and -9, and TIMP-2 (AU/SC unit) in TAF during the first 5 postnatal days (Study I)

Parameter	Total MMP-2	Latent MMP-8		Total MMP-9	Total TIMP-2
		PMN	Mes		
Gestational age	R = 0.37*	R = 0.03	R = 0.59**	R = 0.05	R = 0.46**
Birth weight	R = 0.16	R = -0.18	R = 0.37*	R = -0.12	R = 0.33*
PROM, present	121.9 ± 44.4	26.8 ± 6.2	4.1 ± 1.9	7.9 ± 1.9	8.6 ± 2.1
vs	vs	vs	vs	vs	vs
PROM, absent	71.1 ± 40.0*	9.8 ± 2.3***	3.4 ± 3.0	1.8 ± 0.5*	14.9 ± 9.2

PMN, neutrophil-derived; Mes, mesenchymal cell-derived; PROM, premature rupture of the membranes > 24 hours before labor

* P < 0.05, ** P < 0.001, *** P < 0.0001

MMP-2. Total MMP-2 immunoreactivity (complexed + latent + active + fragmented) in TAF during the first 5 postnatal days showed a positive correlation with gestational age, but not with birth weight (Table 3). A negative correlation existed between MMP-2 and umbilical cord artery pH and base excess (both R = -0.29, P < .05).

MMP-8. During the first 5 postnatal days, levels of mesenchymal-derived latent MMP-8 showed positive correlations with gestational age and birth weight (Table 3). In contrast, no correlations existed between levels of neutrophil-derived latent MMP-8 and gestational age or birth weight (Table 3). Levels of neutrophil-derived latent MMP-8 were strongly associated

Table 4. Parameters of respiratory distress and levels of neutrophil-derived latent MMP-8 in TAF (Study I)

Parameter	Neutrophil-derived latent MMP-8 (AU/SC unit)
L/S ratio	R = -0.39**
Phosphatidyl glycerol (+ vs -)	7.5 ± 1.2 vs 27.5 ± 6.2**
Surfactant therapy (+ vs -)	25.4 ± 6.3 vs 10.6 ± 1.5*
Doses of surfactant	R = 0.30*
Dexamethasone therapy (+ vs -)	23.0 ± 5.5 vs 16.1 ± 4.2*
Intubation >1 week (+ vs -)	22.8 ± 4.6 vs 17.2 ± 6.9*
Development of BPD (+ vs -)	27.6 ± 5.2 vs 15.1 ± 5.0***

+ vs -, levels of neutrophil-derived latent MMP-8 in infants with or without the given factor

* P < 0.05, ** P < 0.01, *** P < 0.001

with an immature surfactant profile and severe respiratory distress; an inverse correlation existed with L/S ratio, and significantly higher levels were demonstrated in infants in whom phosphatidyl glycerol was undetectable in surfactant analysis (Table 4). Levels of neutrophil-derived latent MMP-8 were significantly higher in infants who needed surfactant treatment, and these levels showed a positive correlation with number of surfactant doses given. Moreover, the levels of neutrophil-derived latent MMP-8 during the first 5 postnatal days were associated with prolonged respiratory distress, as indicated by higher levels of the enzyme in infants intubated for longer than 1 week and in those who later were treated with dexamethasone to facilitate weaning from mechanical ventilation (Table 4).

MMP-9. No correlations existed between levels of total MMP-9 immunoreactivity (complexed + latent + active) and gestational age or birth weight (Table 3). Levels of MMP-9 during the study period showed no association with surfactant treatment and no correlation with duration of mechanical ventilation.

TIMP-2. During the first 5 postnatal days, a positive correlation existed between levels of total TIMP-2 in TAF (complexed + native + fragmented) and gestational age and birth weight (Table 3). The levels showed positive correlations with levels of total MMP-2 and total MMP-9 ($R = 0.68$, $R = 0.64$, respectively; both $P < 0.0001$). In addition, a positive correlation existed between the levels of TIMP-2 and mesenchymal cell-derived latent MMP-8 ($R = 0.61$, $P = 0.003$), but no correlation existed with neutrophil-derived latent MMP-8 ($R = 0.03$, $P = 0.8$). TIMP-2 was lower in infants with more severe respiratory distress, as indicated by lower levels in infants with an initial arterial-to-alveolar oxygen tension ratio ($aAPO_2$) < 0.22 (**I**, Figure 2A), in infants who needed treatment with surfactant, and in those requiring mechanical ventilation for more than one week (**I**, Figure 2B).

Association with premature rupture of membranes (PROM). In premature infants born to mothers with PROM, TAF levels of total MMP-2 and MMP-9, as well as those of neutrophil-derived latent MMP-8, were significantly higher than in other infants. In contrast, no significant difference existed in the levels of mesenchymal cell-derived latent MMP-8 and TIMP-2 (Table 3).

1.2. Trypsin and tumor-associated trypsin inhibitor (TATI) in TAF (II)

By use of gelatin zymography and Western blotting, two major immunoreactive species of trypsin was identified: trypsin-1 and trypsin-2 with molecular weights of 25 and 28 kD. In addition, 40-kD to 100-kD relatively faint complexed forms of trypsin were present (**II**, Figure 1).

The concentrations of trypsinogen-1 and trypsinogen-2, and TATI in TAF determined by specific immunofluorometric assays were above the detection limit of the assay in 86%, 96%, and 97% of the samples, respectively.

The median (range) concentration of trypsinogen-1 was 1.6 (0.0-95) ng/SC unit during week 1, and 0.9 (0.0-17) ng/SC unit during week 2. During week 1, the median concentration of trypsinogen-2 was 14.5 (0.0-235) ng/SC unit, and during week 2 was 17.3 (0.0-267) ng/SC unit. During weeks 1 and 2, positive correlations appeared between the concentrations of trypsinogen-1 and trypsinogen-2 ($R = 0.49$ and $R = 0.45$; both $P < 0.0001$). The median concentration of TATI during week 1 was 35.1 (3.9-926) ng/SC unit, and 21.0 (2.6-342) ng/SC unit during week 2. During weeks 1 and 2, positive correlations appeared between concentrations of TATI and trypsinogen-1 ($R = 0.41$, $P < 0.001$; $R = 0.44$, $P < 0.0001$), and between those of TATI and trypsinogen-2 ($R = 0.34$, $P < 0.01$; $R = 0.50$, $P < 0.0001$).

Trypsinogen-1. During week 1, trypsinogen-1 showed a positive correlation with gestational age ($R = 0.36$, $P < 0.001$). Its concentration correlated neither with proteinuric pre-eclampsia, antenatal glucocorticoid treatment, birth weight, severity of acute respiratory distress, nor duration of mechanical ventilation.

Table 5. Parameters of respiratory distress and concentrations of trypsinogen-2 in TAF during weeks 1 and 2 (Study II)

Parameter	Trypsinogen-2 (ng/SC unit)	
	Week 1	Week 2
L/S ratio [1]	R = -0.36**	R = -0.66***
PG in TAF (+ vs -) [2]	5.5 (0.4-25) vs 19.4 (0-235)***	1.8 (0.5-10.5) vs 23.0 (0-193)***
$aAPO_2 \leq 0.22$ (+ vs -) [3]	19.1 (0.4-235) vs 11.0 (0-118)	27.2 (0.5-266) vs 9.5 (0-66)***
Surfactant therapy (+ vs -)	20.9 (0-235) vs 10.7 (0-118)*	23.0 (0-266) vs 8.0 (0-66)**
Dexamethasone (+ vs -)	19.8 (0-235) vs 8.6 (0-158)**	14.3 (0-266) vs 28.3 (0-38)
Duration of intubation (d)	R = 0.30**	R = 0.1
BPD (+ vs -)	22.0 (0.4-235) vs 10.0 (0-114)***	25.5 (0.5-266) vs 9.0 (0-66)***

Values presented as median (range).

+ vs -, concentrations of trypsinogen-2 in infants with or without the given factor

[1] Lecithin/sphingomyelin ratio and [2] phosphatidyl glycerol, measured from TAF sample obtained within 3 hours after birth; [3] Arterial to alveolar oxygen tension ratio

* P < 0.05, **P < 0.01, ***P < 0.0005

Trypsinogen-2. During week 1, the concentration of trypsinogen-2 showed a weak negative correlation with birth weight (R = -0.26, P = 0.005), but no correlation existed between the concentration of trypsinogen-2 and gestational age. No association was detectable between the concentration of trypsinogen-2 and antenatal glucocorticoid treatment. Trypsinogen-2 was significantly higher in preterm infants born to mothers with proteinuric pre-eclampsia, than in those whose mothers had chorioamnionitis or PROM, or in preterm infants without these antenatal complications (**II**, Figure 2). A negative correlation appeared between trypsinogen-2 concentration and surfactant maturity, as measured by lecithin/sphingomyelin ratio (L/S-ratio), and the presence of phosphatidyl glycerol in TAF (Table 5). Trypsinogen-2 was higher in preterm infants with a more severe acute respiratory distress, as indicated by higher concentrations of trypsinogen-2 in preterm infants with an initial $aAPO_2 \leq 0.22$, and in those who needed treatment with surfactant (Table 5). During week 1, higher concentrations of trypsinogen-2 were detectable in preterm infants who, to facilitate weaning from mechanical ventilation, received dexamethasone (age at start of treatment: mean ± SD: 13 ± 7 days; range: 3-33 days). In addition, concentration of trypsinogen-2 during week 1 showed a positive correlation with duration of mechanical ventilation (Table 5).

TATI. Positive correlations appeared between birth weight and TATI concentrations during weeks 1 and 2 (R = 0.27, P = 0.008; R= 0.38, P = 0.0002). No correlation existed between TATI and proteinuric pre-eclampsia or severity of acute respiratory distress. However, concentrations of TATI during week 1 were lower in preterm infants who needed mechanical ventilation for more than one week: median 27.2 (range 3.9 - 926) ng/SC unit vs. 61.8 (range 18.4 - 534) ng/SC-unit, P < 0.0001.

1.3. Matrix proteinases and their inhibitors in TAF, and development of BPD (I, II), MMP-2

MMP-8, MMP-9, and TIMP-2 (I). During the first 5 postnatal days, levels of neutrophil-derived latent MMP-8 were significantly higher in TAF samples from preterm infants who subsequently developed BPD than in those who survived without it (Table 4, Figure 3 in Study I). No associations existed between development of BPD and levels of mesenchymal cell-derived MMP-8, MMP-2, MMP-9, or TIMP-2. When all patients with an unfavorable respiratory outcome (6 infants who subsequently developed BPD and 2 infants who died from severe respiratory distress on days 12 and 24) were combined, in this group levels of TIMP-2 in TAF were significantly lower than in the group in which respiratory distress resolved ($P < 0.05$).

Trypsinogen-1, trypsinogen-2, and TATI (II). Those preterm infants who subsequently developed BPD had significantly higher trypsinogen-2 in TAF during days 5 to 10 than did those who survived without it (**II**, Figure 3). In addition, during weeks 1 and 2, the ratio of trypsinogen-2 to TATI was higher in preterm infants who subsequently developed BPD than in those surviving without it (**II**, Figure 4). No differences in trypsinogen-1 concentrations were detectable between the two groups.

1.4. Immunolocalization of trypsin-2 and PAR_2 in human lung (II, III)

1.4.1 Trypsin-2 and PAR_2 during the perinatal period

Trypsin-2. In lung tissue from the total 12 fetuses studied by trypsin-2 immunohistochemistry, faint positive immunostaining was detectable in bronchial and bronchiolar epithelial cells in 5, whereas samples from 7 fetuses were totally negative (**II**, Figure 5A; **III**). Similarly, bronchial and bronchiolar epithelium was weakly positive in 3 of the 5 newborn infants (gestational ages 22.4 to 33.0 weeks) who had died within 1 day after delivery without histologic signs of RDS (**III**). Faint positive staining in the bronchial and bronchiolar epithelium was also demonstrable in lung sections from all of the 5 term infants with normal histology who had died at 12 hours to 3 days. In one of them, positive staining was also detectable in alveolar type II-like pneumocytes (**II**, Figure 5F).

PAR_2. Positive immunostaining for PAR_2 was scored semiquantitatively, with separate scores given for bronchial and bronchiolar epithelium, alveolar epithelium, bronchial smooth muscle, vascular smooth muscle, and vascular endothelium (**III**, Figure 1).

In samples from all fetuses and infants, immunostaining for PAR_2 was detected in bronchial and bronchiolar epithelium as well as in alveolar epithelium. In the 10 fetuses studied, the level of PAR_2 immunoreactivity in bronchial and bronchiolar epithelium was low or moderate, although in one (gestational age 14 weeks), immunoreactivity was strong (**III**, Figures 1A and 2A). In cuboidal lining cells of the prospective pulmonary acini, immunoreactivity was moderate or strong (**III**, Figures 1B and 2B). The endothelium and the bronchial and vascular smooth muscle cells were negative, or showed only a low level of immunoreactivity (**III**, Figures 1C-E and 2A). In newborn infants who had died without lung disorders within 1 day after delivery, expression of PAR_2 in the cell types studied was predominantly low (**III**, Figures 1 and 3A).

1.4.2. Immunolocalization of trypsin-2 and PAR_2 in acute and chronic lung injury in preterm infants

Trypsin-2. In preterm infants who had died as a result of RDS at the age of 0 to 3 days, the lung sections were mainly negative in trypsin-2 immunostaining. Of the total of 9 preterm infants with RDS, bronchial and bronchiolar epithelium as well as hyaline membranes showed weak positive staining in 5 (**II**, Figure 5B; **III**). In contrast, in preterm infants who had died as a result of prolonged RDS at the age of 8 to 16 days, bronchial and bronchiolar epithelium was predominantly strongly positive. In addition, strong positive staining was detectable in type II-like pneumocytes lining walls of the airspaces, and of the 7 infants, positive staining was apparent also in vascular endothelial cells in 2 (**II**, Figure 5D; **III**, Figures 4B and 4C). In infants who had died of BPD (age at death 75 to 306 days), positive staining that ranged from low to strong was detectable in bronchial and bronchiolar epithelium, as well as in bronchial metaplastic stratified squamous epithelium, while the alveolar epithelium was mainly negative (**II**, Figure 5E; **III**, Figure 4F).

PAR_2. In preterm infants who died of acute RDS at the age of 0 to 2 days, a moderate level of PAR_2 immunoreactivity was evident in bronchial and bronchiolar epithelium, whereas of the 8 preterm infants studied, the level was low in alveolar epithelium in 4 (**III**, Figures 1A, 1B and 3B). No significant difference was detected in the level of PAR_2 immunoreactivity between preterm infants who died as a result of acute RDS and newborn infants (gestational ages 22.4 to 33.0 weeks) without histologic signs of RDS.

In preterm infants who died as a result of prolonged RDS, the level of PAR_2 expression in bronchial and bronchiolar epithelium ranged from moderate to very strong (**III**, Figures 1A and 3C), and in alveolar epithelium, the level was strong in 5 of 7 preterm infants (**III**, Figures 1A and 3D). When compared with newborn controls, the level of PAR_2 immunoreactivity in infants with prolonged RDS was significantly higher in bronchial and bronchiolar epithelium, in alveolar epithelium, and in bronchial and vascular smooth muscle (**III**, Figure 1). In addition, the level of PAR_2 in vascular endothelium tended to be higher than in newborn controls ($P = 0.07$). In all infants who died of prolonged RDS, marked expression of PAR_2 was also apparent in spindle-shaped cells of thickened alveolar walls and in fibrotic foci (**III**, Figure 3C). The cells showed strong positive staining in immunohistochemical analysis for **α**-smooth muscle actin, thus obviously representing lung myofibroblasts.

In lung specimens from preterm infants who had died of BPD, the level of PAR_2 immunoreactivity was strongest in bronchial and bronchiolar epithelium (**III**, Figure 1). In these samples, PAR_2 immunoreactivity was significantly higher in bronchial and bronchiolar epithelium and also in bronchial smooth muscle than in samples from newborn infants without lung disorders (**III**, Figure 1). As in infants who had died of prolonged RDS, in all infants with BPD, marked expression of PAR_2 was detectable in the α-smooth muscle actin-positive myofibroblasts of thickened and fibrotic alveolar walls (**III**, Figures 3E and 3F). In addition, PAR_2 expression was visible in alveolar macrophages (**III**, Figure 3E).

Co-localization of trypsin-2 with PAR_2 in lung tissue. In preterm infants who had died as a result of prolonged RDS, the expression of trypsin-2 was co-localized with PAR_2 in bronchial and bronchiolar epithelium and in type-II-like pneumocytes lining alveolar walls (**III**, Figures 4A and 4B). In addition,

in 2 of these infants (N = 7), the vascular endothelium showed strong immunoreactivity to both trypsin-2 and PAR_2 (**III**, Figures 4C and 4D). In preterm infants who had died of BPD, trypsin-2 co-localized with PAR_2 in bronchial and bronchiolar epithelium (**III**, Figures 4E and 4F).

2. EXPERIMENTAL HYPEROXIC LUNG INJURY IN THE RAT (IV)

Exposure of rats to >95% O_2 caused no evident signs of respiratory distress or macroanatomic changes until 60 hours of exposure, at which point pleural effusions were visible upon opening of the thoracic cavity, and the lungs appeared hemorrhagic and consolidated. The concentration of protein in bronchoalveolar lavage fluid (BALF) and myeloperoxidase (MPO) activity in lung tissue were not increased until at 48 hours (**IV**, Figure 1). At 60 hours, a 10-fold increase in BALF protein was detected compared with BALF from control animals exposed to room air (**IV**, Figure 1A).

2.1. Characterization of MMP-2, -8, and -9, and trypsin in BALF

At 48 hours of exposure, three gelatinolytic proteinases of molecular weight 28, 68, and 90 kD were clearly detected in gelatin zymograms, and the gelatinolytic activity was further increased at 60 hours of exposure (**IV**, Figure 2A). In contrast, almost no gelatinolytic activity was detectable in BALF samples from controls or from rats exposed to hyperoxia for 24 hours (**IV**, Figure 2A). By use of Western blotting, the 28-kD species was identified as trypsin (**IV**, Figure 2B), and the 68-kD and 90-kD species as latent forms of MMP-2 and MMP–9, respectively. At 48 hours and 60 hours of exposure, Western blot analysis of BALF samples with anti-MMP-8 antibody revealed both neutrophil-derived 80-kD and mesenchymal cell-derived 60-kD MMP-8 species, of which the latter clearly predominated. No MMP-8 immunoreactivity was visible in BALF samples from controls or from rats exposed to hyperoxia for 24 hours (**IV**, Figure 2C). Densitometric analysis showed that the levels of trypsin and MMP-2 were significantly increased by 48 hours of hyperoxia (**IV**, Figures 3A, 3B). At 60 hours, the levels of these enzymes were further increased, and significant increases were also detectable in the levels of MMP-9 (**IV**, Figure 3) as well as both neutrophil-derived and mesenchymal cell-derived MMP-8 (**IV**, Figure 4).

2.2. Immunolocalization of trypsin and MMP-8 in rat lung

Trypsin. In controls rats exposed to room air, weak expression of trypsin was detectable in the bronchial and bronchiolar epithelial cells, whereas the alveolar epithelium was predominantly negative (**IV**, Figure 5A). In rats exposed to hyperoxia for 24 hours, a tendency for increased trypsin immunoreactivity appeared in the bronchial and bronchiolar epithelium when compared with normoxia controls (**IV**, Figures 5B and 6B). At 48 and 60 hours of hyperoxia,

marked expression of trypsin appeared in the alveolar epithelium, while in the bronchial and bronchiolar epithelium the expression level remained low (**IV**, Figures 5C and 5D). In the alveolar epithelium, the level of trypsin immunoreactivity was significantly increased in rats exposed to hyperoxia for 48 and 60 hours when compared with normoxia controls ($P < 0.05$ and $P < 0.005$, respectively) (**IV**, Figure 6A). In 4 rats per exposure group ($N = 6$ in group), moderate to strong trypsin immunoreactivity was visible in vascular smooth muscle of the large arteries after exposure to hyperoxia for 48 and 60 hours. In addition, trypsin immunoreactivity appeared in the hyaline membranes and occasionally in intra-alveolar macrophages mainly seen at 60 hours of hyperoxia.

MMP-8. In sections of rat lung, almost no immunoreactivity to MMP-8 was detectable until 48 hours of hyperoxia, at which time point positive cells with a large cytoplasm, obviously representing recruited macrophages, could be demonstrable in the edematous perivascular space (**IV**, Figures 7A-7C). After 60 hours of hyperoxia, a great number of macrophages strongly positive for MMP-8 appeared in the alveoli as well as in the interstitium (**IV**, Figures 7D and 7E). Although expression of MMP-8 was predominantly localized macrophages, it was also visible in intravascular and perivascular neutrophils at 48 hours of hyperoxia, and, in addition, at 60 hours in neutrophils located in the interstitial and intra-alveolar space (**IV**, Figure 7D and 7F).

DISCUSSION

Lung ECM provides a highly structurized reservoir of various growth factors and cytokines, and it influences the proliferation, migration, differentiation, and functioning of lung epithelial and mesenchymal cells (Dunsmore and Rannels 1996). Therefore, remodeling of the ECM and BM, a process in which MMPs play key roles, is an important part of harmonious pulmonary development (Roth-Kleiner and Post 2005, Ryu *et al*. 2005). During lung inflammation, excessive production and release of matrix proteinases including MMPs and matrix serine proteinases cause dysregulated degradation of ECM and BM, which in the preterm lung may subsequently lead to abnormal repair and aberrant lung development, and eventually result in BPD (Chua *et al*. 2005).

1. MMP-2, -8, AND -9, AND TIMP-2 IN LUNG INJURY IN PRETERM INFANTS

We characterized the presence, molecular forms, and species of MMP-2 (gelatinase A), MMP-9 (gelatinase B), and MMP-8 (collagenase-2), as well as their specific inhibitor TIMP-2 in TAF samples from preterm infants with respiratory distress during the early postnatal period and showed that MMP-8 in TAF consisted of both 70-80 kD neutrophil-derived and 40-60 kD mesenchymal cell-derived isoforms of MMP-8. In line with this, both isoforms of MMP-8 also exist in BALF samples from adult patients with asthma and from those with bronchiectasis (Prikk *et al*. 2001a, 2002). Of the MMP-8 isoforms, the neutrophil-derived species predominated in preterm infants. Although neutrophil-derived MMP-8 appeared mostly in its non-converted latent 80-kD form, this form may represent an oxidatively activated form of MMP-8 *in vivo*. Activation of MMP-8 *in vitro* by reactive oxygen species does not necessarily involve any change in molecular weight (Saari *et al*. 1990), and the lungs of preterm infants with RDS and of those subsequently developing BPD show increased oxidative stress (Varsila *et al*. 1995).

We found neutrophil-derived latent MMP-8 to be strongly associated with severity of acute respiratory distress. In addition, during the first five postnatal days it was higher in those preterm infants in whom respiratory distress prolonged, i.e. who were mechanically ventilated for more than one week and who required treatment with dexamethasone. Moreover, those preterm infants who subsequently developed BPD showed higher TAF levels of neutrophil-

derived latent MMP-8 than did those who survived without it. This is consistent with the finding of Sweet *et al.* (2001) who, in BALF samples from preterm infants who later developed BPD, showed higher levels of MMP-8 during the first six postnatal days. In accordance with these findings, the concentrations and activation levels of MMP-8 are increased in BALF from adults with chronic tissue-destructive lung diseases including asthma and bronchiectasis (Prikk *et al*. 2001a, 2002).

In the preterm lung, MMP-8 can induce tissue injury through its capacity to degrade type I collagen, the major structural protein of the ECM. In addition, it can degrade and inactivate α_1-antitrypsin, thereby promoting the activities of serine proteinases trypsin and elastase (Owen *et al*. 2004). Recent studies in MMP-8 knockout animals have, however, shown that MMP-8 may also have anti-inflammatory effects, including a role in the regulation of neutrophil apoptosis during allergen-induced airway inflammation (Gueders *et al*. 2005).

In contrast to neutrophil-derived MMP-8, the levels of mesenchymal cell-derived MMP-8 during the first 5 postnatal days showed positive correlations with gestational age and birth weight. In this respect, mesenchymal cell-derived MMP-8 resembled MMP-2, which also correlated with gestational age, and appeared to be expressed constitutively. These results suggest that these two may be involved in the remodeling of ECM associated with lung growth. In experimental animals, MMP-2 is involved in late fetal lung development and postnatal lung growth (Arden *et al*. 1993, Fukuda *et al*. 2000). In general, MMP-2 is produced constitutively, and during lung development is mainly expressed by mesenchymal cells such as vascular endothelial and smooth muscle cells (Kheradmand *et al*. 2002, Masumoto *et al*. 2005). Although we found no association between levels of MMP-2 during the first five postnatal days and the subsequent development of BPD, later studies by Danan *et al.* (2002) and Ekekezie *et al.* (2004) have shown lower levels of MMP-2 early postnatally in tracheal aspirates from preterm infants subsequently developing BPD. In the study of Danan *et al*., the difference in MMP-2 levels between groups was detectable in TAF samples collected within six hours after birth, but not on day three or five. Similarly, Ekekezie *et al*. detected an association between low MMP-2 and BPD in samples collected on days zero to three.

We could show no association between levels of MMP-9 in TAF during the first 5 postnatal days and severity of respiratory distress or subsequent development of BPD. This is consistent with the results of Ekekezie *et al.* (2004) who found no difference in TAF levels of MMP-9 during the first two postnatal weeks between preterm infants developing BPD or not. They, however, detected lower levels of TIMP-1 and a higher ratio of MMP-9 to TIMP-1 during the first two postnatal weeks in preterm infants who subsequently developed BPD than in those who recovered from RDS.

Premature rupture of the membranes (PROM) is associated with intra-amnionic inflammation, a risk factor for subsequent development of BPD (Watterberg *et al*. 1996, Jobe 2003). These preterm infants may initially have no RDS or only mild respiratory disease, but after a few days or weeks show progressive deterioration of lung function and increased ventilatory and oxygen requirements (Bancalari *et al*. 2003).

Our study showed higher levels of neutrophil-derived MMP-8, but also higher levels of MMP-2 and MMP-9 in infants born to mothers with PROM than in infants born to mothers without this complication. In contrast, no difference was detectable in the levels of TIMP-2. The increase in the levels

of several pulmonary MMPs insufficiently counteracted by TIMP-2 during the early postnatal period may be involved in the increased risk for development of BPD in these infants. Our results are in agreement with those of Curley *et al*. (2003, 2004), who have demonstrated higher levels of MMP-8 and MMP-9, but not TIMP-1, in BALF from ventilated preterm infants from pregnancies complicated by chorioamnionitis.

We detected TIMP-2 predominantly as high molecular weight complexed forms reflecting TIMP-2-species bound to MMPs, but free native and fragmented lower molecular weight species also occurred. Fragmented TIMP-2 appeared in 8 of our 16 preterm infants. During the first 5 postnatal days, the levels of total TIMP-2 were lower in infants with more severe acute respiratory distress, and in those who later had a poor respiratory outcome (development of BPD or death from prolonged severe respiratory distress). It is noteworthy that these clinical conditions were also associated with higher levels of MMP-8 in TAF. These results suggest that an imbalance between MMP-8 and its endogenous inhibitor TIMP-2 in the lungs of preterm infants may play a role in the acute lung injury, and thereby may influence its outcome. Our finding of an association between low TIMP-2 and poor respiratory outcome is in agreement with that of Ekekezie *et al*. (2004) who in preterm infants subsequently developing BPD showed low TIMP-2 early postnatally (Ekekezie *et al*. 2004).

2. TRYPSIN-1, AND -2, AND TATI IN LUNG INJURY IN PRETERM INFANTS

We observed higher pulmonary concentrations of trypsinogen-2 in preterm infants with more severe acute respiratory distress. Moreover, the concentrations were higher during days 5 to 10 in those subsequently developing BPD than in those who survived without it. In addition, those infants developing BPD had a higher ratio of trypsinogen-2 to TATI during week 1 and week 2. No differences occurred in the concentrations of trypsinogen-1 between these groups.

In preterm infants who had died of acute RDS on days 0 to 3, immunohistochemical analysis for trypsin-2 showed weak positive staining in bronchial and bronchiolar epithelial cells in 5 of the 9 studied infants, whereas the other 4 showed no trypsin-2. In contrast, in those who died at age 8 to 16 days, the bronchial and bronchiolar epithelium showed intense expression of trypsin-2, and marked positive staining was also evident in type II-like pneumocytes lining the walls of the airspaces.

In the lungs of infants dying of nonpulmonary causes, trypsin-2 was expressed at low levels in bronchial and bronchiolar epithelial cells, in line with the findings of studies showing expression of trypsin in normal adult human bronchial epithelium, and this supports the hypothesis that trypsin may also participate in normal cellular functions (Koshikawa et al. 1998, Cocks et al. 1999).

Little is known about the role of trypsin in the lung. If dysregulated, trypsin may cause pulmonary tissue destruction through direct degradation of ECM and BM proteins (Koivunen et al. 1991, Koshikawa et al. 1992, Moilanen *et al*. 2003, Stenman *et al*. 2005) as well as through activation of various proMMPs, including MMP-1, -3, -8, -9, and

-13 (Sorsa *et al*. 1997, Moilanen *et al*. 2003). We observed activation of the MMP cascade in the lungs of preterm infants with respiratory distress (**I**). During the inflammatory reaction, α_1-antitrypsin is inactivated both oxidatively by reactive oxygen species released from inflammatory cells (Carp and Janoff 1980) and proteolytically by several MMPs, including MMP-8 and -9 (Liu *et al*. 2000, Owen *et al*. 2004). Thus, MMP-8 and trypsin-2 may act synergistically by potentiating each others' activity during development of lung injury in preterm infants.

The potential role of pulmonary trypsin in the development of BPD raises the possibility of preventing BPD by use of exogenous proteinase inhibitors; α_1-antitrypsin protects neonatal rats from the toxic effects of oxygen on pulmonary parenchymal and vascular development (Koppel *et al*. 1994). Furthermore, in a randomized, controlled trial, prophylactic administration of α_1-antitrypsin intravenously to preterm infants with RDS reduced the incidence of pulmonary hemorrhage and the use of inhaled steroids and bronchodilators, although it failed to reduce the risk for BPD at 36 gestational weeks (Stiskal *et al*. 1998). On the basis of our results, the favorable effects of α_1-antitrypsin may partially be explained by inhibition of pulmonary trypsin.

2.1. PAR_2 in lung injury in preterm infants

Extrapancreatic trypsin-2 potently activates PAR_2 (Alm *et al*. 2000). We observed that in preterm infants who had died of prolonged RDS, trypsin-2 was co-localized with PAR_2 in bronchial and alveolar epithelium. PAR_2 was highly expressed in the bronchial and bronchiolar epithelium of preterm infants who had died of prolonged RDS or BPD, and the expression was significantly stronger in comparison with newborn infants who had died of nonpulmonary reasons. In addition, the level of PAR_2 immunoreactivity in alveolar epithelium of preterm infants with prolonged RDS was significantly higher than in newborn infants.

Interestingly, we detected strong expression of PAR_2 in α-smooth muscle actin-positive myofibroblasts of the thickened and fibrotic alveolar walls in preterm infants who died of prolonged RDS or BPD. Trypsin is capable of inducing lung fibroblast proliferation via activation of PAR_2 (Akers *et al*. 2000). Myofibroblasts are derived from activated fibroblasts, and play an important role in tissue remodeling following acute lung injury. In preterm infants with acute lung injury, myofibroblasts increase in number and form bundles encircling terminal air spaces during the early postnatal period (Toti *et al*. 1997). Our findings of higher PAR_2 in myofibroblasts suggest that PAR_2 may be involved in the fibroproliferation associated with development of BPD.

PAR_2 was also visualized in bronchial and vascular smooth muscle, vascular endothelium, and alveolar macrophages, in accord with studies on the localization of PAR_2 in the adult human lung (D'Andrea *et al*. 1998, Akers *et al*. 2000, Ricciardolo *et al*. 2000, Knight *et al*. 2001, Asokananthan *et al*. 2002, Steinhoff *et al*. 2005).

In the respiratory epithelium, activation of PAR_2 stimulates the release of inflammatory mediators such as IL-6, IL-8, and matrix metalloproteinase-9, suggesting an important role for PAR_2 in lung inflammation and tissue remodeling (Vliagoftis *et al*. 2000, Asokananthan *et al*. 2002). In preterm infants with respiratory distress, the development of BPD is characterized by a persistent inflammatory pulmonary reaction associated with epithelial cell damage and increased alveocapillary permeability (Merritt *et al*. 1983, Groneck *et al*. 1994, Watterberg *et al*.

1996). In addition to alveolar macrophages, vascular endothelium and respiratory epithelium play an important role in the production of the pro-inflammatory cytokines (Kotecha *et al*. 1996). Up-regulated PAR_2 in the injured preterm lung may be involved in this inflammatory process.

3. MMP-2, -8, AND -9, AND TRYPSIN IN HYPEROXIC LUNG INJURY IN THE RAT

Hyperoxic lung injury is characterized by damage to the alveolar–capillary barrier with subsequent increased pulmonary vascular permeability, progressive inflammation, and pulmonary edema (Crapo *et al*. 1980). The early stages of oxygen toxicity begin with an initiation phase, occurring within the first 40 hours of hyperoxia exposure, in which few demonstrable morphologic changes exist (Crapo *et al*. 1980). We detected in the rat a rapid increase in the pulmonary expression of trypsin during the early development of hyperoxic lung injury. After 48 hours of exposure to >95% oxygen, strong expression of trypsin occurred in the alveolar epithelium, which–in contrast–showed almost no immunoreactivity for trypsin under normoxic conditions.

In addition to trypsin, zymography of BALF samples from rats exposed to 48 and 60 hours of hyperoxia demonstrated the up-regulation of two other gelatinolytic enzymes identified as MMP-2 and -9. Similarly to trypsin, MMP-2 and MMP-9 can efficiently degrade BM components (Sorsa *et al.* 1997, Chakrabarti and Patel 2005). The marked up-regulation of trypsin, MMP-2, and -9 in BALF coincided with an increase in the alveolar-capillary permeability, as indicated by an increased protein concentration in BALF, suggesting a role in the degradation of alveolar-capillary BM associated with the development of hyperoxic lung injury. In accordance with our results, increased expression of pulmonary MMP-2 and -9 has been shown in rats exposed to 100% oxygen for 60 hours (Pardo *et al.* 1996).

Although trypsin *in vitro* efficiently activates latent forms of MMPs, including MMP-8 and -9 by removing the propeptide, most of the MMPs-2, -8, and -9 detected in BALF samples were as demonstrated by Western blotting, in their non-converted proforms. These findings are in line with those of Study I, in which we found that especially MMP-8, but also MMP-9 was mainly detectable in latent form in TAF samples from preterm infants. Some of the non-converted forms may, however, represent oxidatively activated forms of these MMPs *in vivo*, since *in vitro* oxidative activation of MMP-8 or -9 does not necessarily involve changes in their molecular sizes (Westerlund *et al*. 1996).

A rat model of experimental pancreatitis-associated lung injury has shown that infusion of trypsin or trypsinogen causes acute dose-dependent pulmonary injury characterized by perivascular edema and hemorrhage (Hartwig *et al.* 1999). These authors further showed that this lung injury is neutrophil-dependent, and is possibly mediated by the ability of trypsin to up-regulate pulmonary intercellular adhesion molecule-1, a key vascular endothelial adhesion molecule necessary for transport of leukocytes from the intravascular space into inflamed tissues (Hartwig *et al.* 2004).

It is noteworthy that the increased trypsin in the alveolar epithelium may also play a

protective role in the development of lung injury. In the injured lung, active transepithelial transport of Na+ limits alveolar edema. The effect of serine proteinases, including trypsin, on ion transport has recently been studied by Swystun *et al*. (2005), who demonstrated *in vitro* that apical trypsin enhanced both ion transport across rat alveolar type II cells and paracellular resistance, indicating that trypsin may play an important role in the clearance of alveolar fluid. Moreover, in fluid-filled lungs in a rat model, inhibition of trypsin activity by intratracheal instillation of soybean trypsin inhibitor or α_1-antitrypsin decreases the amiloride-sensitive lung-fluid clearance, and this effect is partially restored by instillation of trypsin (Swystun *et al.* 2005).

We observed a marked up-regulation of MMP-8 in the rat lung after 48 hours of exposure to hyperoxia. MMP-8 has been regarded solely as a neutrophil-specific MMP or collagenase-2 stored in granules and released upon activation (Weiss 1989). However, certain activated mesenchymal cells also express MMP-8 (Hanemaaijer *et al.* 1997). In BALF, we detected both neutrophil-derived 80-kD MMP-8 and 60-kD mesenchymal cell-derived MMP-8 species, of which the latter clearly predominated. This is in contrast with our previous findings of MMP-8 in TAF samples from preterm infants, in which the neutrophil-derived MMP-8 was the main isoform of the MMP-8 detected. Immunohistochemical analysis confirmed that in the hyperoxic lung, MMP-8 was mostly expressed in recruited macrophages, which at 48 hours were detectable in the perivascular space and subsequently at 60 hours in the alveoli and interstitium.

Recently, an unexpected anti-inflammatory role was evidenced for MMP-8 in the lung (Owen *et al.* 2004, Gueders *et al.* 2005). One possible mechanism is the regulation of inflammatory cell apoptosis, as demonstrated during allergen-induced lung inflammation by reduced neutrophil apoptosis in MMP-8 -/- mice (Gueders *et al.* 2004). During the development of hyperoxic lung injury, a large number of infiltrating inflammatory cells exist in the rat lung (Barry and Crapo 1985). Whether the role of MMP-8 in hyperoxic lung injury is anti-inflammatory– possibly by regulating inflammatory cell apoptosis– remains a subject for future research.

CONCLUSIONS

1) Higher levels of MMP-8 in association with lower levels of TIMP-2 were detected in TAF from preterm infants with more severe respiratory distress. In addition, MMP-8 was higher in TAF during the early postnatal period in those preterm infants who subsequently developed BPD.

2) During the early postnatal period, higher pulmonary concentrations of trypsinogen-2 appeared in preterm infants with more severe acute respiratory distress and in those who later developed BPD. In addition, the ratio of trypsinogen-2 to its specific inhibitor TATI was higher in these infants.

3) In the injured preterm lung, trypsin-2 was expressed in the bronchial and alveolar epithelium, where it co-localized with PAR_2. The level of PAR_2 expression was higher in bronchial and alveolar epithelium in preterm infants who had died of prolonged RDS than in newborn infants without histological signs of RDS. In prolonged RDS and BPD, PAR_2 also occurred in myofibroblasts of the thickened and fibrotic alveolar walls. These findings suggest that activation of PAR_2 by high levels of trypsin-2 may participate in pulmonary inflammation and fibroproliferation associated with the development of BPD.

4) In rats exposed to hyperoxia, pulmonary levels of trypsin and MMP-8 sharply increased after 48 hours of exposure relative to levels innormoxia controls. Although alveolar epithelium was predominantly negative in controls, after 48 hours of hyperoxia it showed strong expression of trypsin. Marked up-regulation of trypsin and MMP-8 early in the course of hyperoxic lung injury suggests that they may play a role in the pathogenesis of acute lung injury.

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