LUNG DISEASE OF THE PRETERM INFANT: MEDIATORS INVOLVED IN FIBROPROLIFERATION AND FIBROGENESIS

Longziekte in het premature kind: mediatoren betrokken bij fibroproliferatie en fibrogenese

ISBN 90-73436-60-5

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PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam op gezag van de
Rector Magnificus
Prof.dr.ir. J.H. van Bemmel
en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
woensdag 22 mei 2002 om 13.45 uur

door

Willem Arnout Dik

geboren te Assen

PROMOTIECOMMISSIE

Promotor:	Prof.dr. R. Benner
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The studies described in this thesis were performed at the Department of Immunology, Erasmus University Rotterdam, The Netherlands.

The printing of this Ph.D. thesis was financially supported by: Dr.Ir. van der Laar Stichting, J.E. Jurriaanse Stichting, Biosource B.V.

Illustrations : Tar van Os
Printing : Ridderprint B.V., Ridderkerk
Cover : Tar van Os
Lay-out : Marcia Reintjes

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CONTENTS

Chapter 1

BRONCHOPULMONARY DYSPLASIA

Neonatal respiratory distress syndrome (RDS; also called hyaline membrane disease) can develop within minutes after birth in prematurely born infants and is characterized by progressive pulmonary atelectasis and respiratory failure for which the infants are mechanically ventilated (1-3).

It is already known for a long time that the development of RDS is associated with surfactant deficiency of the immature lung (4). Surfactant has important biophysical functions in lung biology. It reduces surface tension, thereby preventing the collapse of alveoli and lungs during expiration, it stabilizes and keeps the small airways open, and it prevents pulmonary edema by balancing hydrostatic filtration forces (5).

In the 1960s, with the improvement of neonatal intensive care and the use of mechanical ventilation, a steep increase in survival rate of infants with RDS was achieved. It was noted then, that some of the infants with more severe lung injury recovered spontaneously in the first week of life while others did not and developed a chronic lung disease (6).

Northway *et al.* described in 1967 a chronic lung disease that developed in prematurely born infants with RDS who where ventilated with warm, humidified 80 to 100% concentrations of oxygen. The disorder was named bronchopulmonary dysplasia (BPD), which was based on the observed progressive pathological changes in the immature lung that affected both the parenchyma and airways and interfered with normal lung development. It was speculated that RDS, ventilator-induced injury and supplemental oxygen (causing oxygen toxicity) were the most important factors contributing to BPD development (7).

In the decades following the initial description by Northway, the pathogenesis and etiology of the disease were further examined, and improvements in treatment were established. Despite this progress, BPD is still a major clinical problem and its incidence seems to increase. This increase is due to the improved survival rates of extremely premature infants with a very low birth weight (< 1000 gram), either with RDS or with initially minimal or no signs of RDS (8-15). The risk for BPD increases with decreasing gestational age and birth weight, and ranges from 50% in infants with birth weights of 700-900 g to 5% in infants with birth weights of more than 1250 g (9).

BPD is also called chronic lung disease of prematurity (CLD) and both terms are used interchangeably throughout the literature. BPD is a description of severe lung damage that is based on histological changes in the immature lungs of infants that died because of respiratory failure (7). BPD is clinically characterized by signs of respiratory distress, requirement for oxygen supplementation and chest x-ray abnormalities after 28 days of age in an infant who required mechanical ventilation in the first week of life for a minimum of three days (16). CLD can be regarded as the long-term clinical, less severe presentation of the disease and is defined as the requirement for oxygen supplementation at a postconceptional age of 36 weeks (17). In CLD there is extensive variability in disease severity, ranging from mild to severe or fatal. The milder forms often occur in very low birth weight infants with mild or no initial RDS (13, 14). In these milder forms, a patent ductus arteriosus and nosocomial infections play an important role in the development of the disease (13).

1.1 Long term sequelae of BPD

Respiratory morbidity is common in infants and young children who are born prematurely and developed BPD. Lower respiratory tract infections are a major complication in the first year of life in infants with BPD. Lung compliance has been shown to be decreased in infants with BPD, indicating decreased elastic properties of the lung, down to 80% of normal at an age of three years (18). Infants with BPD show increased airway resistance that may persist into young adulthood (18). Adolescents and young adults with former BPD may have respiratory problems such as wheezing and episodes of pneumonia (19).

Radiographic abnormalities occur in up to 90% of adolescents and children with a history of BPD and correlate with pulmonary function abnormalities (19). Airway hyperresponsiveness has been reported to occur in long term BPD survivors. It is unclear, whether this increased responsiveness is due to a genetic predisposition, neonatal lung injury, or anatomically smaller airways (19).

Barker *et al.* demonstrated that the prevalence of chronic obstructive pulmonary disease was associated both with a lower birth weight and respiratory illness during infancy (20). Therefore, it has been suggested that children with BPD are predisposed to chronic obstructive pulmonary disease later in life (18).

Other complications such as growth retardation have also been shown to occur in infants with BPD. It has been suggested that this is related to factors such as birth weight and gestational age rather than to BPD (19).

1.2. Risk factors for BPD

BPD development depends on multiple risk factors. Respiratory failure, premature birth, lung immaturity with concomitant surfactant deficiency, oxygen supplementation, and ventilator-induced injury are generally considered as the major risk factors (21). BPD may also develop as a consequence of treatment of respiratory failure due to meconium aspiration, neonatal pneumonia, congestive heart failure and congenital diaphragmatic hernia (22-25). Even three cases of BPD have been described in adults following treatment with unusually high ventilatory pressures and high oxygen concentrations (26). More recently BPD has become a frequent sequel in very low birth weight infants with mild or no RDS at birth (13, 14).

Alveolar rupture (interstitial emphysema, pneumothorax and pneumomediastinum), pulmonary edema, patent ductus arteriosus and pulmonary infection prolong the need for mechanical ventilation and supplemental oxygen, and increase the risk for BPD development (13, 27-29). However, none of these factors have been shown to be essential for BPD development. Recently, chorioamnionitis has been suggested to accelerate lung maturation but to predispose to BPD development as well (30)

Genetic factors may also predispose to RDS/BPD development. Several studies have reported an increased risk for BPD development in white preterm infants compared to black infants (9, 10, 12, 31). However, a study by Kraybill *et al.* did not confirm this (32). The prevalence of RDS is increased and the course is more severe in male than in female preterm infants (9, 10, 31, 32), resulting in a higher BPD incidence in male compared to female preterm infants (31, 32). Familial asthma has been reported to be associated with BPD development from RDS (33, 34). However, other studies did not confirm this finding (35, 36).

Carriage of HLA-A3 and HLA-B14 were found to be associated with increased risk for RDS. This made the authors to suggest that RDS development probably depends on the presence of susceptibility gene(s) in linkage equilibrium with the HLA-A3 and HLA-B14 genes (37). A polymorphism within the gene coding for surfactant protein-B, and certain surfactant protein-A variants and their corresponding haplotypes have been associated with RDS (38).

1.3. Histopathological changes in the BPD lung

The histopathology of BPD changed substantially since its recognition in 1967. Improvements in neonatal intensive care during the last decades may account largely for this change. BPD changed from a disease with marked airway abnormalities and alternating zones of severe fibrotic and emphysematous lesions to a disease with varying degrees of fibrosis and reduced numbers of alveoli. To illustrate this, the following part summarizes a number of relevant histopathological studies that have been published during the last three decades.

BPD was first described in prematurely born infants (mean gestational age (ga) 32 weeks; ranging from 23-39 weeks and mean birth weight (bw) 1893 grams; ranging from 900-3204 grams) as a sequel from RDS and its treatment (7). The development of BPD was originally divided into four stages; Stage I (2-3 days after birth), Stage II (4-10 days after birth), Stage III (10-20 days after birth) and Stage IV (beyond one month after birth) (7). Stage I reflected the histopathology of RDS, with the presence of hyaline membranes, atelectasis, lymphatic dilatation, and patchy loss of epithelial cells with metaplasia and necrosis of the bronchial mucosa. Stage II showed necrosis and repair of alveolar epithelium, persisting hyaline membranes, and emphysematous coalescence of alveoli. Additionally, focal thickening of capillary basement membranes, increased patchy bronchiolar necrosis, and increased patchy squamous metaplasia were observed. During stage III less hyaline membranes were present and there was widespread bronchial and bronchiolar mucosal metaplasia and hyperplasia. In addition, marked mucous secretion and alveolar coalescence forming groups of emphysematous alveoli surrounded by atelectatic alveoli were observed. Furthermore, focal thickening of the basement membrane and fine strands of interseptal collagen were present. Stage IV revealed hypertrophy of peribronchiolar smooth muscle cells and alternating emphysematous and atelectatic lesions. Additionally, thickened basement membranes that separated the capillaries from the alveolar septa, and fibrosis in the alveolar septal walls was

observed. Furthermore, early vascular lesions of the pulmonary hypertensive type were present (7).

In 1975, Rosan proposed another classification, also with four phases, based on characteristic histopathological features and the number of days after birth at which these features were observed (39). The first phase (acute phase; 2-4 days after birth) was, at the alveolar level, mainly characterized by hyaline membranes and epithelial necrosis. At the level of the airways, patchy bronchiolar necrosis, mucosal secretion and hyaline membranes were observed. The second phase (regenerative phase; 4-8 days after birth) was marked by foregoing injury plus attempts to cellular repair, as reflected by the presence of focal regenerating alveolar epithelium. In addition, moderate numbers of intra-alveolar macrophages were present. The airways were characterized by peribronchiolar edema and mucosal abnormalities. The third phase (transitional phase; 8-16 days after birth) was described as the transition towards chronic lung disease. At the alveolar level, fewer hyaline membranes, continuing necrosis, numerous intra-alveolar macrophages and interalveolar fibroplasia were observed. The airways were characterized by necrotizing bronchiolitis, patchy metaplasia, increased numbers of smooth muscle cells, mucosal abnormalities, and peribronchial edema. The fourth phase (chronic phase; > 16 days after birth) describes rare or absent hyaline membranes at the alveolar level, separation of epithelial and endothelial basal laminae, the presence of numerous intra-alveolar macrophages and intersaccular and intrasaccular fibrosis. At the airway level, metaplastic and hyperplastic collagenizing bronchiolitis and focal mucosal hyperplasia and necrosis were observed (39).

Bonikos *et al.* described a light and electron microscopical study that was carried out in 21 infants (ga = 33 weeks (range: 28-40 weeks); bw = 1638 gram (range: 1100-3487 gram) in whom the pathological diagnosis of BPD had been made after treatment with high oxygen concentrations and positive-pressure ventilation with positive end-expiratory pressure (40). In this study, all the examined infants survived at least one week. The histopathological changes were graded as mild, moderate, severe and very severe. The primary alterations observed in all infants were damage of the bronchial and bronchiolar ciliary system and mucosal membranes, severe necrotizing bronchiolitis, and marked bronchiolar and alveolar fibrosis. Additionally, alternating areas of alveolar fibrosis, atelectasis and emphysema were observed. The abnormalities were most pronounced in infants who survived the longest period of time and who had the longest exposure to supplemental oxygen (40).

In a histopathological study by Taghizadeh and Reynolds, high peak airway pressure during ventilation was concluded to be the major risk for BPD development from RDS. Although they identified another factor as the main risk for BPD, the pathological changes they described in infants (median ga of 30 weeks (range, 26-39 weeks); bw of 1310 gram (range, 730-2910 gram); death at a median age of 41 hours (range, 1 1/2 hour-13 months)) were in concordance with the earlier discussed studies (41).

Interstitial fibrosis and emphysema were reported to be the most pronounced complications in the longest survivors with BPD in a group of 73 infants (bw ranging from 500-7080 gram) that lived for at least two days. These complications were associated with the progres-

sion of an early reparative stage via a subacute fibroproliferative stage into a chronic fibroproliferative response (42). It was demonstrated that regenerating type-II pneumocytes contributed to the reparative and proliferative reactions by reepithelialization of damaged septal walls. Hereby, type-II pneumocytes incorporated hyaline membranes into septal walls, which seemed to stimulate interstitial fibroblast proliferation with resultant interstitial fibrosis (42).

In some of the above discussed histopathological studies also long term survivors were included (7, 40, 42). Stocker described the pathological features of long-standing "healed" BPD in infants (mean bw = 1249 gram (range, 740-1850 gram; age of death between 3 and 40 months) (43). Alveolar septal fibrosis was found to be the main residual feature in the "healed" stage of BPD in infants who had moderate to severe BPD in the neonatal period (43). Furthermore, long-standing "healed" BPD was reported to be associated with mast cell hyperplasia, particularly around small airways and in more distal alveolar regions, especially those exhibiting prominent fibrosis (44).

Erickson *et al.* reported the existence of three different histopathological patterns of BPD that occurred with increasing age and independent of birth weight in a total of 46 patients (bw ranging from 460-3000 gram) (45). Group 1 was characterized by interstitial fibrosis, group 3 by enlargement of the distal airways and little or no interstitial fibrosis, and in group 2 both lesions coexisted. They suggested that early BPD is characterized by interstitial fibrosis (group 1). With increased survival and continuing growth, the interstitial fibrosis disappeared, changing the histopathological pattern to one with enormously enlarged distal airways and reduced numbers of alveoli. Such a change in histopathological pattern suggests that normal alveolar proliferation is impaired as a consequence of the original injury and its repair (45).

Three different histopathological types of BPD were also recognized by van Lierde *et al.* in a group of infants with an interquartile range for gestational age between 25.5 and 29 weeks and for birth weight between 820 and 1165 gram (46). One histopathological type was labelled "interstitial-type" and was characterized by moderate to severe interstitial fibrosis and little or no airway abnormalities. The other type, labelled "bronchiolar-type", was characterized by marked airway lesions and alveolar emphysema and mild to moderate interstitial fibrosis. A third group of patients revealed pathological characteristics of both types of histology (46). The existence of this "mixed histology group" made the authors to speculate that the "interstitial" and "bronchiolar" types are two extreme ends of the spectrum of a single disease and that a switch from one type to the other can occur (46). The authors noted that the patients with "interstitial-type" histology suffered less barotrauma and oxygen toxicity as their bronchiolar counterparts. Therefore, they speculated that "mild pulmonary aggression" resulted in a different repair response characterized by interstitial fibrosis without significant airway abnormalities (46).

A morphometric study in infants with gestational ages between 24-30 weeks, that died between 2-28 months with BPD, revealed decreased total numbers of alveoli that had an increased diameter. In addition, mild to severe alveolar septal fibrosis was present. Furthermore, bronchial and bronchiolar smooth muscle hypertrophy and bronchial gland

hyperplasia were observed (47). Reduced alveolar development has been demonstrated to result from mechanical ventilation of the premature lung, especially when associated with RDS (48).

Surfactant deficiency of the premature lung is regarded as the primary underlying cause of RDS (4, 49, 50). Surfactant replacement therapy was generally introduced in 1989 and coincided with a decline in mortality. This decline was attributed primarily to fewer deaths from respiratory causes among preterm infants. However, the effect of surfactant treatment on BPD incidence is unclear (51). Histopathologically it has been shown that surfactant-treated RDS patients that fail to respond to therapy have continuing alveolar injury (52). Husain *et al.* assessed the effects of surfactant therapy in a group of BPD patients (mean ga of 27 weeks (range, 24-30 weeks)) on pulmonary histopathological changes (53). In the surfactant treated group, 5 out of 14 patients had mild to severe alveolar septal fibrosis, while in the non-surfactant treated group moderate to severe alveolar septal fibrosis occurred in 7 of the 8 patients. Surfactant treatment did not influence the disturbed alveolar development as observed in BPD (53).

From these studies it is evident that, due to an increased survival of less mature infants, BPD nowadays develops mostly in infants with lower gestational ages and birth weights than in the 1960s-1970s, while the infants with gestational ages and birth weights comparable to the groups described in the 1960s-1970s nowadays are less prone to develop BPD. BPD can be considered as a repair stage of RDS, with ongoing lung damage due to mechanical ventilation and oxygen toxicity during the repair attempts of the initial injury. The histopathological appearance of BPD has changed over time from a disease with alternating zones of severe fibrosis and emphysematous lesions, and marked airway abnormalities to a disease with varying degrees of fibrosis, and reduced numbers of simplified alveoli.

Recently the terms "old" BPD and "new" BPD have been introduced to distinguish the classical Northway BPD and the current milder form of the disease. The "new" BPD is common in very low birth weight infants (VLBW; birth weight of less than 1 kg). Lung pathology of the "new" BPD is characterized by mild airway injury, dilated gas exchange structures, decreased alveolization and less prominent inflammation and fibrosis than seen in the "old" BPD (54, 55).

1.4. Inflammation in the pathogenesis of bronchopulmonary dysplasia

The mechanism causing BPD is not fully understood. Numerous factors have been implicated. Studies have demonstrated that early and prolonged postnatal pulmonary inflammation occurs during BPD development. Pulmonary fibrosis results as a consequence of many types of severe lung injury and usually follows an alveolar inflammatory reaction (56). It is assumed that inflammation, as the result of lung injury due to mechanical ventilation and supplemental oxygen (i.e. oxygen toxicity), plays a critical role in tissue injury, tissue remodelling and fibrosis when BPD develops (55, 57).

1.4.1. Inflammatory cells in the pathogenesis of BPD

A variety of inflammatory cells are implicated in the pathogenesis of BPD. Hereafter the most prominent ones will be discussed in relation to this pathogenesis.

Neutrophils

Histopathologically it has been shown that infants with developing BPD have an evolving pattern of pulmonary inflammation that consists initially mainly of neutrophils (58). In the same study, neutrophils and macrophages were shown to be present in tracheobronchial washings (58). This observation was confirmed by a number of other studies, showing an early predominance of neutrophils (from a postnatal age of 4 days) in tracheal aspirates, followed by the appearance of alveolar macrophages (from a postnatal age of 8 days) later in the course of BPD development (59-61).

In 1983, Merritt *et al.* demonstrated that preterm infants with RDS had increased numbers of inflammatory cells in their airways compared to control infants who were ventilated for non-pulmonary problems. From postnatal day three, infants with subsequent BPD had increased numbers of inflammatory cells in their airways compared to infants with resolving RDS (62). These investigators showed the neutrophil to be the predominant inflammatory cell type at the early stage of inflammation (62). These findings, and observations by others, have lead to the suggestion that persistent presence of neutrophils in the lungs is associated with BPD development (63-66).

Neutrophils present in the lungs after injury originate from the peripheral blood. Adult respiratory distress syndrome (ARDS) shares many pathological features with neonatal RDS. Neutrophils are also abundant in the lungs from ARDS patients, and it has been shown that leukopenia frequently precedes the onset of ARDS (67, 68). Studies in preterm lambs with RDS demonstrated reduced numbers of circulating neutrophils within the first two hours after birth reaching normal values again within 6-8 hours. The decrease in circulating neutrophils related to the accumulation of neutrophils in the airspaces (69). Recently, a decrease in the number of circulating neutrophils within two hours after birth was found to be associated with BPD development in premature infants (70).

In the pathogenesis of BPD the neutrophil may play an important role in causing injury to the pulmonary parenchyma. Activated neutrophils release reactive oxygen radicals that cause epithelial and endothelial cell death (71, 72). In addition, neutrophil elastase is the main proteolytic enzyme for pulmonary tissue elastin and has tissue destructive properties (73). The activity of neutrophil elastase is normally controlled by naturally occurring inhibitors such as α_1-proteinase inhibitor (α_1-PI) and secretory leukocyte protease inhibitor (SLPI). An imbalance between elastase and its main inhibitor α_1-PI within the lung, as result of either an increase in elastase or a decrease in α_1-PI, has been postulated to play an important role in adult lung injury (74).

Inactivation of α_1-PI occurs by oxidation, which renders it susceptible to proteolytic digestion. Inactivated oxidized α_1-PI and a proteolytic digestion product have been found in tracheal aspirates during the early stage of BPD (62). In infants with RDS, the elastase/α_1-PI

ratio in bronchoalveolar lavage (BAL) fluid was demonstrated to be comparable to control infants. In infants that subsequently developed BPD, this ratio was elevated from one through four weeks of life, suggesting that increased elastolytic damage may occur in the pathogenesis of BPD (63). A number of studies have detected free elastase in most or all of the lung fluid samples examined (62, 63, 75-77), while other studies detected free elastase in only a few of the examined infants (78, 79). In cases that free elastase activity was detected in a sample, it was due to unavailability of α_1-PI (78, 79). In addition to α_1-PI, BPD development has shown to be associated with relatively low levels of SLPI (79, 80).

Increased urinary levels of desmosine (a elastine degradation product) were observed at the end of the first week of life in infants who subsequently developed BPD, compared to control infants, suggesting increased pulmonary elastin degradation (81). In addition, desmosine has been shown to be present in tracheal aspirate fluid (76).

Histopathological examination revealed pulmonary elastic fiber degradation in early BPD (76). Alveolar secondary septa formation is thought to be dependent on a framework of elastin fibers, around which the septa will develop (82). Increased elastolytic activity during BPD development could contribute to damaging of the elastin framework, resulting in impaired/diminished alveolization. Indeed decreased numbers of alveoli have been observed in the BPD lung (47). Therefore, the control of pulmonary elastase activity may be a potential therapeutic target for preventing BPD development. However, treatment with α_1-PI did not significantly reduce the risk of BPD development (83).

It has been shown that neutrophil culture supernatants exhibit extracellular matrix (ECM) degradative activity (84). Neutrophil collagenase (matrix metalloproteinase (MMP)-8) and gelatinase may play a role in this ECM degradation process. BAL fluid from infants who subsequently develop BPD was demonstrated to contain increased MMP-8 levels compared to infants with resolving RDS (85).

Neutrophil elastase may play an important role in the pathogenesis of pulmonary fibrosis as well. Recent evidence indicates that neutrophil elastase-deficient mice are resistant to bleomycin-induced pulmonary fibrosis (86). In addition, MMP-9 (gelatinase-B) and MMP-8 expression by neutrophils has been suggested to play a role in lung damage and fibrosis in adults (87). Furthermore, via the production and release of cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-8, macrophage inflammatory protein (MIP)-1α, and transforming growth factor (TGF)-β, neutrophils may influence the inflammatory process and the development of fibrosis (88).

Macrophages

Histopathological studies revealed increased numbers of alveolar macrophages during BPD development (7, 39-41). Murch *et al.* demonstrated a marked and rapid increase of interstitial $CD68^+$ macrophages which was already maximal by 72 hours after birth (89).

Contradictory results on alveolar macrophages have been observed in lung effluent fluids. During BPD development, decreased numbers of alveolar macrophages have been found compared with self-resolving RDS (63, 64). However, another study demonstrated that

macrophages were already increased from birth onwards in patients that subsequently develop BPD compared to those who recover from RDS (90). Others described increased numbers of alveolar macrophages from 3-5 days after birth when BPD developed (66, 91). Furthermore, it was shown that surfactant treatment may increase the number of alveolar macrophages obtained by BAL during BPD development (64).

Increased total cell numbers were found to be present in BAL fluid from BPD patients at 4 months of age compared to control infants. The cell population consisted for 93% of alveolar macrophages that produced increased amounts of hydrogen peroxide compared to controls, indicating an activated status of these cells (92). Increased numbers of HLA-DR positive macrophages, observed within pulmonary tissue from infants who died with BPD, also indicate the activated state of pulmonary macrophages in BPD (93).

Activated macrophages release a variety of products that may cause tissue injury, participate in the inflammatory process and play an important role in tissue remodelling after injury. They release oxygen radicals which may have deleterious effects on the alveolar epithelium and endothelium (72). In addition, via the release of proteolytic enzymes, alveolar macrophages are able to degrade ECM components (72). Production and secretion of proteolytic enzymes, such as MMP-2 and MMP-9, by alveolar macrophages is associated with hyperoxia induced lung injury with subsequent pulmonary fibrosis (94). Alveolar macrophages may also secrete chemoattractants for other inflammatory cells such as neutrophils (95).

Alveolar macrophages produce a variety of growth factors for epithelial cells, endothelial cells and fibroblasts, which are important in the repair process of injured lungs (96). Severity of epithelial damage, ongoing injury, and delay in epithelial repair may lead to an overstimulation of the normal repair response. An overstimulation of the repair response with exaggerated production and secretion of growth factors/cytokines such as TGF-β, platelet-derived growth factor (PDGF), insulin like growth factor-1 (IGF-1), TNF-α, and IL-1 by alveolar macrophages results in excessive fibroblast proliferation and ECM deposition (56, 97).

Eosinophils

There is only scarce evidence for the participation of eosinophils and eosinophil derived products in pulmonary inflammation and lung injury in BPD. Peripheral eosinophil cell counts are increased in the first month of life when BPD develops compared to control infants who do not develop BPD (98). Increased eosinophilic cationic protein (ECP) concentrations have been found in tracheal aspirates within the first month of life when BPD developed (98, 99).

The release of ECP by activated eosinophils can directly cause injury by altering function and integrity of resident lung cells (100). In addition, eosinophils produce reactive oxygen radicals, lipid mediators such as leukotriene C_4 (LTC_4) and platelet-activating factor (PAF) as well as cytokines and growth factors such as TNF-α, MIP-1α, TGF-β and PDGF that may contribute to tissue injury, inflammation and the development of fibrosis (100-103).

However, convincing infiltration of the lungs from BPD patients by eosinophils has not been shown.

Lymphocytes

The role of lymphocytes in the pathogenesis of BPD is also uncertain. One study describes the presence of soluble IL-2 receptor (sIL-2R; signifying T-cell activation) in plasma and tracheal aspirates from infants with or at risk for BPD and infants with resolving RDS (104). Infants with or at risk for BPD were found to have higher sIL-2R levels in plasma than infants with RDS and age matched controls. However, no differences were observed in tracheal aspirate levels, and no information about the cell population in tracheal aspirates or lung tissue was given in this study (104).

IL-2 mRNA (a cytokine produced by activated T-helper cells) was undetectable in BAL cells from infants with RDS or BPD, indicating the absence of activated T-helper cells (105, 106). Furthermore, the lack of BAL cells immunoreactive for the T-lymphocyte markers CD3, CD4, and CD8 supports the absence of T-lymphocytes (106).

1.4.2. Chemotactic factors in the pathogenesis of BPD

Tracheal aspirate fluid from infants with RDS was shown to be chemotactic for neutrophils. This chemotactic activity was increased from a postnatal age of five days in infants who subsequently developed BPD compared to those who did not (65). Increased levels of the neutrophil chemoattractants complement component C5-derived anaphylotoxin (C5a) and leukotriene-B_4 (LTB_4) were present in tracheal aspirates obtained in the first week of life in infants who later acquired BPD (65). Furthermore, the neutrophil chemoattractants LTB_4, 5-hydroxyeicosatetraenoic acid (5-HETE) and PAF were present in BAL fluid from BPD patients at postnatal ages over 30 days (107). Additionally, PAF levels in tracheal aspirates from 3 to 5 days after birth correlated with BPD severity (108).

IL-8 is considered to be the major neutrophil chemotactic factor in the lung, and increased levels have been shown to persist for a prolonged period of time in lung effluents from infants who develop BPD (65, 109-115). In addition, increased pulmonary IL-8 levels have been shown to precede neutrophil influx and to correlate with the number of neutrophils and the myeloperoxidase activity (a marker for neutrophil activation) (111, 112, 115).

Jones *et al.* demonstrated the presence of IL-8 protein in BAL fluid in early RDS, and furthermore demonstrated that the lavage cells (neutrophils and macrophages) probably add to IL-8 production as IL-8 mRNA could be detected in these cells (105). Immunohistochemically it was demonstrated that lung autopsy specimens from infants with early RDS expressed IL-8 in neutrophils and bronchial epithelial cells. In addition, if there was evidence of sepsis, IL-8 was also expressed by alveolar epithelial cells, endothelial cells, fibroblasts and smooth muscle cells of vessels as well as chondrocytes (116). The above indicates that both inflammatory cells and resident lung cells presumably contribute to IL-8 production during RDS and subsequent BPD development.

Growth-related protein-α (GRO-α) is another chemotactic factor for neutrophils and

has been shown to be present in BAL fluid obtained in the first week of life during BPD development (117).

MIP-1α, RANTES (a factor called: regulated upon activation, normal T cell expressed and secreted), and monocyte chemoattractant protein (MCP)-1 are chemotactic factors for monocytes and macrophages (118). Increased levels of MIP-1α, RANTES, and MCP-1 in BAL fluid and tracheal aspirates are associated with BPD development (90, 119). In addition, MIP-1α correlated with the later development of pulmonary fibrosis in premature infants ventilated due to RDS (90).

Granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF), are of potential importance in the recruitment and function of granulocytes and macrophages in the lung. These factors have recently been described to increase in BAL fluid during BPD development (120). Furthermore, the levels of G-CSF and GM-CSF correlated with the number of neutrophils and alveolar macrophages in BAL fluid (120). This may be related to the observation that G-CSF and GM-CSF delay apoptosis of these cells (121, 122). A summary of chemoattractants implicated in the pathogenesis of RDS/BPD is given in table 1.

Table 1. Chemoattractants implicated in the pathogenesis of RDS and BPD

chemoattractants for neutrophils	references
LTB_4	65, 107
5-HETE	107
PAF	107, 108
C5a	65
IL-8	65, 105, 110-113, 115, 119
GRO-α	117
G(M)-CSF	120
chemoattractants for monocytes/macrophages	**references**
MIP-1α	90
RANTES	90
MCP-1	117, 119
G(M)-CSF	120

1.4.3. Adhesion molecules in the pathogenesis of BPD

Adhesion of neutrophils to endothelial cells is a critical event for migration from the vasculature into the lung parenchyma. Adhesion molecules, both on neutrophils and endothe-

lial cells, play an important role in this process. Neutrophils express cell surface molecules such as β_2-integrins (lymphocyte function-associated antigen (LFA)-1, macrophage (Mac)-1) and L-selectin (123). LFA-1 binds intercellular adhesion molecule (ICAM)-1 and ICAM-2 on endothelial cells, whereas Mac-1 binds to ICAM-1 (123). In addition, endothelial cells express E-selectin and P-selectin which are adhesion molecules for neutrophils (123).

IL-8 and PAF upregulate the expression of LFA-1 and Mac-1 on neutrophils, increasing the adhesiveness of these cells (123). Neutrophil migration through the endothelial barrier results in shedding of adhesion molecules from both neutrophils and endothelial cells, resulting in soluble forms of these molecules.

Increased levels of soluble ICAM-1 have been described in BAL fluid and tracheal aspirates from infants with BPD when compared to controls (112, 124). Furthermore, increased levels of soluble ICAM-1 and soluble E-selectin in plasma and serum have been found to be associated with BPD (111, 125). However, other studies did not report differences in soluble ICAM-1 concentrations in serum from infants that did and did not develop BPD as a consequence of RDS (124).

1.4.4. Cytokines in the pathogenesis of BPD

Besides IL-8, other proinflammatory cytokines such as IL-1, IL-6 and TNF-α seem to be important mediators in the inflammatory process associated with the development of BPD. The association of these cytokines with the pathogenesis of BPD is described below.

Interleukin-1

Different isoforms of IL-1 exist and are known to be associated with inflammatory diseases (126). Two members of the IL-1 gene family, namely IL-1β and IL-1 receptor antagonist (IL-1Ra), have been associated with BPD development (90, 91).

IL-1β increases in lung effluent fluids when BPD develops and is increased compared to infants with resolving RDS (90, 91, 114, 127). IL-1Ra is known to block most IL-1β-induced inflammatory actions and may limit the inflammatory response to IL-1 (128, 129).

Rindfleish *et al.* demonstrated that the BAL fluid IL-1β concentration and IL-1 activity increased during the first week of life when BPD developed. However, IL-1Ra remained relatively unchanged during the first month of life (91). Therefore, a relative imbalance between IL-1β and IL-1Ra may contribute to prolonged inflammation in BPD (91).

Kotecha *et al.* showed increased IL-1β levels in BAL fluid from BPD patients compared to patients with resolving RDS (127). Furthermore, BAL cells obtained from both RDS and BPD infants expressed similar amounts of IL-1β mRNA. Immunocytochemistry revealed that IL-1β protein was predominantly expressed by alveolar macrophages, although IL-1β immunoreactivity was also observed in epithelial cells and neutrophils (127). This suggests that a variety of resident lung cells, which are not sampled by BAL, also synthesize and secrete IL-1β during BPD development.

IL-1β is a known inducer of IL-8 synthesis and secretion by resident lung cells such as alveolar macrophages, endothelial cells, epithelial cells and fibroblasts (109, 123, 130,

131). IL-8 expression has been shown in both resident lung cells and inflammatory cells from RDS and BPD patients (116, 127). It is therefore likely that IL-1β plays a role in the induction of IL-8 expression in the lung during the development of BPD. Indeed, it has been demonstrated that inflammatory cells in the lungs from preterm infants with RDS produce and secrete IL-8 and that IL-1β contributes to this IL-8 production (106). IL-8 expression by inflammatory cells and resident lung cells may be important in the pathogenesis of BPD, as the synthesis of IL-8 by such cells may perpetuate the recruitment of neutrophils into the site of inflammation (109).

IL-1β also induces expression of cytokines such as IL-6, TNF-α and MIP-1α and upregulates the expression of adhesion molecules such as ICAM-1 (126).

Altogether these data indicate that IL-1β may play a role in the recruitment of inflammatory cells and the maintenance of the inflammatory process in the pathogenesis of BPD.

Tumor necrosis factor

TNF-α levels were found to be increased in BAL fluids and tracheal aspirates obtained on postnatal day 3 when BPD developed compared to resolving RDS and remained increased until postnatal day 40 (114, 132). Another study demonstrated that in BAL fluid of infants who later acquired BPD, TNF-α activity was not increased compared to relevant control infants on the first postnatal days, but subsequently increased reaching peak activity on postnatal day 14 (133). This was accompanied by a significant increase of TNF-α concentrations on postnatal days 14 and 28 (133).

Immunohistochemically, Murch *et al.* have demonstrated the presence of TNF-α immunoreactive cells whose number was maximal in infants dying after 72 hours (89). TNF-α immunoreactivity was not confined to cells and could be detected through the whole interstitium (89). Increased numbers of TNF-α immunoreactive BAL cells were reported from birth to a postnatal age of 22 days when BPD developed. These TNF-α immunoreactive BAL cells in BPD were accompanied by increased concentrations of TNF-α compared to self-resolving RDS (90).

TNF-α exerts similar actions as IL-1β, such as induction of IL-8 expression (109). Therefore, TNF-α, similar to IL-1β, may play an important role in the induction of IL-8 expression during BPD development. Indeed, it has been demonstrated that TNF-α contributes to IL-8 production by lung inflammatory cells from infants with RDS (106).

Interleukin 6

IL-6 has a wide range of proinflammatory activities including the stimulation of acute phase proteins and the activation of B- and T-lymphocytes (57, 134). Bagchi *et al.* demonstrated that there were no differences in IL-6 protein concentration in BAL fluid between BPD, RDS and controls on the first day of life (133). However, increased IL-6 activity did occur on the first day of life in BPD compared to RDS and controls (133). It was suggested that the IL-6 activity in the lungs of infants with resolving RDS and control infants is inactivated or inhibited, but that such regulatory activity may be deficient in infants who develop

BPD (133). IL-6 activity remained elevated in BAL fluid from BPD infants in the first two weeks of life, and declined to low levels by 4 weeks (133). The lack of concordance between IL-6 protein concentration and functional activity of IL-6 in this study stresses the importance of measuring cytokine activity in biological fluids.

Another study demonstrated a gradual increase of IL-6 protein concentration in BAL fluid when BPD developed. In this study the IL-6 concentration peaked at ten days after birth and was significantly increased compared to RDS and control infants and declined thereafter (127). In addition, the alveolar macrophage was identified as the major source of IL-6. It was suggested that increased IL-6 levels in BPD derived from airway luminal and alveolar cells that can be recovered by BAL (127).

In tracheal aspirates, increased IL-6 concentrations were observed at two and five days after birth in BPD compared to RDS. Furthermore, IL-6 remained detectable for at least two weeks after birth in BPD samples (114, 115).

Although IL-6 is often regarded as a proinflammatory cytokine, it also exerts anti-inflammatory activities. IL-6 inhibits the production of the proinflammatory cytokines IL-1 and TNF with minimal effects on the synthesis of anti-inflammatory cytokines such as IL-10. Additionally, IL-6 induces the synthesis of glucocorticoids and promotes the synthesis of IL-1Ra and soluble TNF receptor p55 (135).

Interleukin 10

IL-10 is an anti-inflammatory cytokine that inhibits the production of cytokines such as TNF-α, IL-1α, IL-1β, IL-6 and IL-8 by macrophages and monocytes (136, 137). IL-10 mRNA was found to be absent in BAL cells from preterm infants at risk of BPD in contrast to term ventilated infants with acute respiratory failure (105). Additionally, IL-10 was undetectable in BAL fluid from infants with and at risk of BPD (105, 138).

It has been suggested that the different maturational stage between preterm and term infants accounts for the inability of the preterm infants to express IL-10. Furthermore, it was speculated that the absence of IL-10 in preterm infants with RDS may account for the inability to downregulate inflammation and predispose to BPD development (105).

Kwong *et al.* demonstrated that IL-10 inhibited IL-1β and IL-8 expression by lung inflammatory cells from preterm infants with RDS. They suggested recombinant IL-10 as a potential anti-inflammatory treatment for RDS (138). A recent study described the presence of IL-10 in BAL fluid from ventilated preterm infants without a clear association with BPD development (139).

The different time-points at which certain cytokines were found to be elevated in the studies discussed above may be due to different sample techniques used, different populations of patients examined and differences in neonatal intensive care between the different centers. Despite these differences it is clear that an early and prolonged inflammatory response occurs in the pathogenesis of BPD. A summary of the cytokines likely to be involved in the pathogenesis of BPD is given in table 2.

Table 2. Cytokines in lung effluent fluid likely involved in the pathogenesis of BPD

cytokine	level during BPD development compared to RDS or control infants	references
IL-1β	+	90, 91, 114, 127
IL-1Ra	=	91
IL-6	+	114, 115, 127, 133
TNF-α	+	90, 114, 132, 133
IL-10	-/=	105, 138, 139

+ increased
- decreased
= no difference

Cytokine mRNA expression by lung inflammatory cells in the pathogenesis of BPD

In the pathogenesis of RDS and BPD the inflammatory cells in the lungs are regarded as major sources of the inflammatory cytokines in lung effluent fluids. Besides immunocytochemical analyses of lung inflammatory cells, also mRNA analyses (reverse transcriptase polymerase chain reaction; RT-PCR) identified these cells as an important source of inflammatory cytokines. Several studies detected mRNA for IL-1α, IL-1β, TNF-α, IL-6 and IL-8 in BAL cells (105, 106, 127, 140). IL-2 mRNA was undetectable, indicating the absence of T-lymphocytes (105).

Cytokine mRNA expression profiles of cells from tracheal aspirates and BAL from the same patient have been examined and found to be different (140). This may be due to differences in the composition of the cell population in tracheal aspirate and in BAL (140). This indicates that care has to be taken when comparing data on cell differentials and cytokine expression profiles between tracheal aspirates and BAL.

A summary of cytokines likely to contribute to the pathogenesis of BPD and presumably produced by lung inflammatory cells is given in table 3.

IL-1 and TNF-α are able to induce acute lung injury and subsequent fibrosis in animal models

As stated before, the pathogenesis of BPD is characterized by initial acute lung injury that is followed by a repair process with subsequent fibrosis development. IL-1 and TNF-α contribute to, or even cause, features of acute lung injury such as neutrophil accumulation, increased pulmonary vascular permeability, edema and fibrosis. The next part describes data obtained from animal models that support a possible central role for IL-1 and TNF-α in the pathogenesis of BPD. A detailed introduction on the concept of pulmonary fibrosis and fibrogenic mediators will be given in chapter 2 of this thesis.

Table 3. Cytokines likely produced by (inflammatory) BAL cells in the pathogenesis of BPD

cytokine	immunocytochemistry	mRNA	references
IL-1α	n.d.	+	140
IL-1β	+	+	106, 127, 138, 140
IL-6	+	+	127, 140
IL-8	+	+	106, 127, 138, 140
TNF-α	+	+	90, 106, 138, 140
IL-10	n.d.	-	105, 138

+ present
- absent
n.d. not determined

Intratracheal instillation of IL-1 (IL-1α and IL-1β) in rodents induces acute lung injury characterized by neutrophil influx, generation of oxygen metabolites, increased lung lavage protein and hemoglobin concentrations, and increased lung-wet-weight to body-weight ratios (141, 142). Recent evidence suggests that PAF contributes to the lung neutrophil recruitment and lung leakage that occurs when IL-1 is administered intratracheally into rats (143).

In a rabbit model of acute ventilator-induced lung injury, administration of IL-1Ra resulted in a significant reduction of pulmonary vascular leakage as well as reduction of elastase and neutrophil numbers in lavage fluid (144). Other studies confirm that administration of IL-1Ra or transgenic expression of IL-1Ra in rat or mouse lungs inhibits the acute lung injury and neutrophil influx induced by intratracheal administration of IL-1 (141, 145, 146).

Transgenic expression of human IL-1Ra in mice resulted in decreased mRNA induction of the chemokines MIP-1α and MIP-2 after intratracheal administration of IL-1α. This suggests that the inflammatory response to intratracheal IL-1 is partly mediated by the expression of chemokines (146).

Pulmonary fibrosis can occur as the consequence of many types of severe lung injury and usually follows an alveolar inflammatory reaction (56). Piguet *et al.* demonstrated that administration of IL-1Ra via continuous abdominal perfusion was able to prevent silica or bleomycin-induced pulmonary fibrosis in mice (147). In addition, IL-1Ra was able to reverse established pulmonary fibrosis induced by either silica or bleomycin (147).

Recently, it was shown that transient overexpression of IL-1β in the epithelial cells of rat lungs, using adenovirus mediated gene transfer, induced acute inflammation (148). This inflammation was characterized by infiltration of neutrophils and macrophages, increased levels of IL-6 and TNF-α, and alveolar tissue destruction (148). The initial inflammatory response was followed by progressive interstitial fibrosis which occurred coincident with sustained induction of the profibrotic mediator TGF-β_1 (148).

Together these data demonstrate that IL-1 is able to induce acute lung injury and subsequent pulmonary fibrosis. Recently, however, also a role for IL-1β was suggested in alveolar epithelial repair (149).

Intravenous injection of TNF-α in guinea pigs caused lung inflammation and increased pulmonary vascular permeability with resultant edema (150). This could be prevented by granulocyte depletion (151). Intratracheal injection of TNF-α has been shown to increase pulmonary vascular permeability and neutrophil accumulation in the alveolar septa and to cause an acute intra-alveolar neutrophil exudate (152). Other studies in rats, however, did not show such effects of TNF-α (153). Furthermore, intratracheal instillation of TNF-α, comparable to IL-1, has been shown to induce the expression of cytokine-induced neutrophil chemoattractant (CINC), which is often considered to be the rat equivalent of human IL-8 (154).

A central role for TNF-α in the development of pulmonary fibrosis was suggested by the experiments from Piguet *et al.* They demonstrated that anti-TNF-antibodies or recombinant soluble TNF-receptor inhibited the development of bleomycin or silica-induced pulmonary fibrosis in mice (155-157).

Recently, it was demonstrated that transient overexpression of TNF-α in rat lungs, using adenovirus mediated gene transfer, induced acute inflammation. This inflammation was characterized by increased infiltration of neutrophils, macrophages and lymphocytes, and eventually resulted in pulmonary fibrosis (158). It was suggested that in this model the fibrogenesis was due to secondary upregulation of TGF-β_1 and the subsequent appearance of pulmonary myofibroblasts (158).

The use of TNF-α receptor knockout mice confirmed that TNF-α is essential for the development of pulmonary fibrosis in an asbestos model. It was postulated that TNF-α mediated its effects through the activation of other growth factors such as PDGF and TGF-α (159).

These data imply that TNF-α, like IL-1, is able to induce or participate in the pathological features that are observed in BPD development. Therefore, both IL-1 and TNF-α may be key inflammatory cytokines in the initiation and/or augmentation of the inflammatory response in the pathogenesis of BPD (Fig. 1).

1.4.5. Other factors influencing pulmonary inflammation in the pathogenesis of BPD

Adrenal insufficiency

Early adrenal insufficiency, reflected by decreased cortisol production, may also play a role in the pathogenesis of BPD (160). The normal physiological response to stress includes increased secretion of cortisol by the adrenal glands (161). Watterberg *et al.* described lower serum cortisol levels in the first week of life in infants who developed BPD compared to infants who did not (160). Infants with lower serum cortisol levels had increased lung inflammation (increased total protein, albumin, α_1-PI and IL-6 in tracheal aspirates) and increased incidence of patent ductus arteriosus (160). In addition, infants who developed BPD had lower cortisol levels after intravenous adrenal corticotropic hormone (ACTH) administration

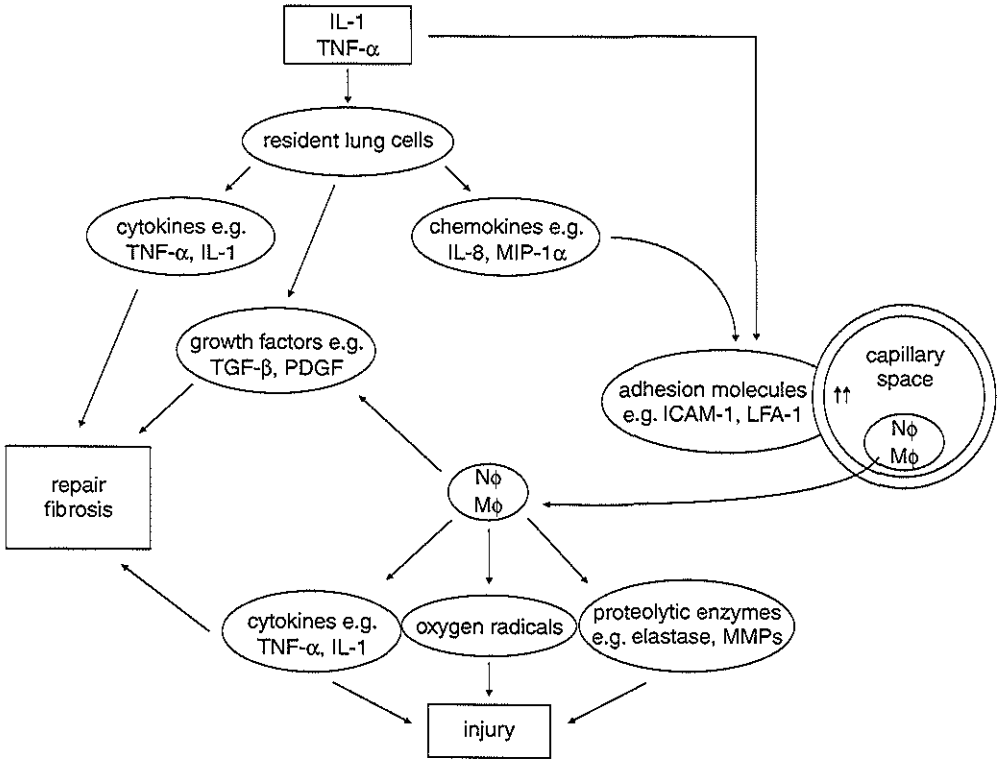

Figure 1
Processes that occur in the pathogenesis of BPD and may be induced and/or augmented by IL-1 and TNF-α. Stimulation of resident lung cells such as epithelial cells, endothelial cells, fibroblasts and alveolar macrophages by IL-1 and TNF-α may lead to the production and release of chemokines, cytokines and growth factors. Furthermore, IL-1 and TNF-α can upregulate the expression of adhesion molecules on endothelial cells and leukocytes. This facilitates the migration of inflammatory cells into the lung. These inflammatory cells produce cytokines, oxygen radicals and proteolytic enzymes that may cause pulmonary damage. Other cytokines, in concert with growth factors, participate in the repair and fibrotic response after pulmonary injury.
Nϕ = neutrophils, Mϕ = monocytes/macrophages.

than infants who did not develop BPD (162). However, another study described a weak association between plasma cortisol and BPD development, and after adjustment for gestational age and clinical risk index for babies, the predicted probability for BPD was only minimally influenced by the cortisol concentration (163).

Recent pilot studies demonstrated that early low-dose hydrocortisone treatment increased the likelihood of survival without BPD and increased the plasma cortisol levels (164, 165).

Pulmonary infection

Most individual studies and meta-analyses support an association between Ureaplasma urealyticum colonization of the respiratory tract and subsequent development of BPD in preterm infants. The suggestion that this association is independent of prematurity or low birth weight is controversial (166).

Airway colonization with U. urealyticum or bacteria at birth has been shown to be associated with an increased inflammatory response on the first day after birth in infants with RDS (167). Compared with non-colonized infants, tracheal aspirate chemotactic activity, neutrophil count, and concentrations of IL-1, LTB_4 and elastase-α_1-PI are significantly higher in the colonized group. Also concentrations of IL-8 tend to be increased in colonized infants (167). Levels of C5a and albumin in tracheal aspirates have been found comparable between colonized and non-colonized infants (167).

Increased levels of proinflammatory cytokines such as IL-1β and TNF-α in tracheal aspirates from U. urealyticum colonized preterm infants have been confirmed in other studies (168). However, U. urealyticum colonization did not influence the concentration of IL-1β, IL-8 and TNF-α in BAL fluid (169). Furthermore, nosocomial pneumonia in combination with BPD increases elastase activity in BAL fluid compared to non-infected BPD infants and may therefore result in increased elastolytic damage to the lung parenchyma (170). It has been suggested that such additional inflammatory responses in the lungs due to infection with micro-organisms results in an increased incidence of BPD in children with RDS (171)

1.4.6. Strategies to modulate pulmonary inflammation in infants with RDS/BPD

Corticosteroid treatment

Corticosteroids are effective regulators of inflammation. Corticosteroids exert their anti-inflammatory effects via glucocorticoid receptors (GR) that are present in the cytoplasm of the cell. The corticosteroid binds the GR after which the steroid/GR complex translocates from the cytoplasm to the nucleus where it binds to glucocorticoid responsive elements (GRE) in the promoter region of the target genes. The activated GR may bind to a GRE resulting in upregulation of transcription of the target gene. Alternatively, the activated GR binds to a negative GRE (nGRE) resulting in downregulation of the transcription of the corresponding gene. The susceptibility of cytokine genes to the inhibitory effects of corticosteroids is influenced by the number of nGRE's in the promoter region (172).

Corticosteroids are known to inhibit the transcription of inflammatory cytokines, including IL-1, TNF-α , IL-6, IL-8, MCP-1, MIP-1α and GM-CSF (173). These effects of corticosteroids can be mediated by interaction of GR with an nGRE in the promoter region of the genes. A number of cytokine genes, including IL-8, MCP-1 and RANTES, are negatively regulated by corticosteroids but do not have a GRE in their promoter region. IL-8 and MCP-1 are predominantly regulated via the transcription factor nuclear factor-kappa B (NF-κB). RANTES is regulated by the transcription factors activating protein (AP)-1 and NF-κB. The activated GR may directly interact with AP-1 or NF-κB and in such a way pre-

vent cytokine gene transcription. Corticosteroids may also modulate mRNA stability via increased breakdown of mRNA and thereby reduce synthesis of cytokines such as IL-1β, IL-3, IL-6 and GM-CSF (173).

Corticosteroids not only block the synthesis of cytokines but may also block their effects. Several cytokines exert their cellular effects via activation of the transcription factors AP-1 and NF-κB that subsequently activate or repress target genes and can therefore be regulated in a opposing manner by corticosteroids (172).

In addition to the downregulatory effects on inflammatory cytokines, corticosteroids may modulate inflammation by upregulating the expression and release of the IL-1 "decoy" receptor. Furthermore, corticosteroids may downregulate adhesion molecules including ICAM-1, which may be due to inhibition of cytokine synthesis or a direct effect of corticosteroids on adhesion molecule gene transcription. Additionally, corticosteroids may increase the synthesis of SLPI by increasing the transcription of the coding gene (173).

The corticosteroid dexamethasone (Dex) is commonly used in the treatment of infants with or at risk of BPD development. It has been shown that Dex treatment improves lung function and compliance and facilitates weaning from the ventilator. The long-term outcome of Dex treatment is still uncertain (174, 175). Furthermore, Dex treatment is associated with a number of adverse side effects including hyperglycemia, growth impairment, neuro-developmental impairment and gastrointestinal perforation (174, 176, 177).

The pulmonary inflammation in BPD development may be modulated by Dex treatment. It has been demonstrated that the number of inflammatory cells, especially neutrophils, significantly decreased in lung fluids from infants with or at risk of BPD after Dex treatment (178-180). The decreased neutrophil number was accompanied by a decrease in elastase level (75, 178). Additionally, a decrease in albumin levels occurred in lung fluid after Dex, indicating decreased pulmonary vascular permeability (178, 179, 181).

Inflammatory cytokines and chemokines including TNF-α, IL-1β and MIP-1α decreased in lung fluids from infants with or at risk of BPD after Dex treatment (90, 132, 179). Other markers of inflammation such as LTB_4 and 6-keto prostaglandin decreased also after Dex (179, 180). Furthermore, Dex treatment resulted in a significant reduction of the chemotactic activity of tracheal aspirates for neutrophils (179). Waisman *et al.* have demonstrated that Dex treatment of infants with or at risk of BPD caused a decreased expression of the adhesion molecule L-selectin on circulating polymorphonuclear leukocytes and monocytes. The authors speculated that downregulation of L-selectin would result in reduced migration of leukocytes into the lungs (182). Altogether these data indicate that the clinical improvement observed in infants with or at risk of BPD after Dex treatment may be mediated by suppression of the pulmonary inflammation.

Opposite to its downregulatory effect on inflammatory cytokines, Dex treatment has been shown to increase the concentration of vascular endothelial growth factor (VEGF) in BAL fluid from infants at risk of BPD. It was suggested that this Dex associated increase in VEGF may affect the disturbed pulmonary angiogenesis in infants developing BPD (183).

Besides a modulating effect on inflammation, corticosteroids are known to exert a

number of effects on lung development and maturation such as the stimulation of surfactant and antioxidant enzyme synthesis. The effects of corticosteroids on lung development have recently been reviewed (175) and will therefore not be discussed.

Influence of mechanical ventilation on inflammation in BPD

Although mechanical ventilation optimizes gas exchange, it may also induce secondary lung damage often referred to as ventilator-induced lung injury (VILI). Inflammatory cytokines such as IL-1β, IL-6, and TNF-α have been shown to influence the course of VILI (184). As it is generally assumed that pulmonary inflammation plays an important role in the pathogenesis of BPD (57), lung protective ventilation strategies that attenuate the inflammatory response may be helpful in preventing BPD development.

In an immature baboon model for BPD development it has been shown that high-frequency oscillatory ventilation (HFOV) resulted in less pulmonary inflammation than low volume positive pressure ventilation (LV-PPV) (185). After LV-PPV increased total inflammatory cell counts and increased numbers of monocytes/macrophages were found in tracheal aspirate fluids. Furthermore, increased tracheal aspirate concentrations of IL-8 were detected compared to HFOV (185). HFOV treated baboons appeared to have better inflated and less thick-walled distal lung parenchyma compared to the LV-PPV group. Additionally, a trend to improved alveolization was observed in the HFOV group (185).

A study by Thome *et al*, in preterm infants, revealed no beneficial effect of HFOV on inflammatory parameters such as albumin, IL-8 and LTB_4 in tracheal aspirates (186). Methodological differences between this study and the earlier mentioned baboon study may account for the discordant observations.

Recently, we demonstrated that applying the open lung concept to positive pressure ventilation (PPV_{OLC}) in surfactant-depleted newborn piglets resulted in a similar reduction of pulmonary inflammation as $HFOV_{OLC}$ compared to conventional PPV (PPV_{CON}) (187). BAL fluid from PPV_{CON} contained increased numbers of total inflammatory cells, neutrophils, IL-8 and thrombin activity compared to both other ventilation strategies (187). These data indicate that application of mild ventilation strategies may result in less pulmonary inflammation and therefore may be helpful in preventing BPD development.

1.5. Concluding remarks

With the improvements in neonatal intensive care, prematurely born infants with RDS were found to be at risk of BPD development, characterized by extensive remodelling of the lung parenchyma with decreased alveolar development and fibrosis as hallmarks.

During the last three decades extensive research has been performed on the pathogenesis of BPD. It has become clear that factors such as ventilator-induced lung injury and oxygen toxicity contribute to the pathogenesis of BPD by inducing and/or augmenting an excessive and prolonged pulmonary inflammation. It is generally accepted that this inflammation

contributes to the lung injury, tissue remodelling and subsequent fibrosis in the pathogenesis of BPD. Therefore, much effort is put into the development of strategies that can reduce the pulmonary inflammation and subsequent BPD development in these infants.

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Chapter

PATHOGENESIS OF PULMONARY FIBROSIS AND BRONCHOPULMONARY DYSPLASIA

Processes leading to the development of fibrosis are comparable to those in normal wound healing. Normal tissue repair follows a series of events that are tightly regulated over time. Cell recruitment and replication as well as extracellular matrix (ECM) synthesis and degradation are critical events in tissue repair and are regulated by a host of mediators derived from inflammatory and resident cells as well as from the blood (1). Fibrosis can be defined as an excessive deposition of ECM components, resulting in loss of normal tissue architecture and function.

In general, fibrosis develops after an insult followed by tissue injury, subsequent inflammation and a repair attempt (1). However, the control of cell function that occurs in proper wound healing is lost, presumably due to overproduction of mediators involved in the healing process. Additionally, the cells responding to these mediators may become more susceptible to these mediators due to an upregulation of mediator specific receptors (1).

It has been suggested that a fibrotic response is driven by three factors: 1) a continuous insult with resultant injury or a continuous stimulus; 2) excessive synthesis of ECM proteins, especially collagen; and 3) decreased degradation of deposited ECM proteins by degradative enzymes (1). Fibrosis can occur in many different organs, such as heart, liver, kidney or lung. The pathogenetic mechanisms involved appear largely the same (2-5).

The ECM is built by a large number of different components, such as collagens, elastin, laminin, entactin, fibronectin, tenascin, thrombospondins, hyaluronan and decorin (1, 6, 7). The collagen family consists of at least 19 closely related but structurally and metabolically distinct proteins. Collagens represent 60-65% of the connective tissue in adult lung (6). The interstitial fibrillar types I and III, which exist in a ratio of 2:1, comprise about 90% of the collagens in the adult lung (8). In fibrosis there is a loss of homeostasis that results in excessive accumulation of ECM components, especially collagen, elastin, fibronectin and proteoglycans (6), although the term fibrosis is generally associated with excessive collagen deposition.

2.1. Pathogenesis of pulmonary fibrosis

Pulmonary fibrosis may result from a broad range of insults such as infection, alveolar cell carcinoma, radiation, drugs, organic/inorganic dust inhalation, and hyperoxia (oxygen toxicity) (5, 9-12). Despite the wide variety of insults associated with pulmonary fibrosis, the mechanisms involved appear largely the same (13). One of the first and persistent histological features observed in the pathogenesis of pulmonary fibrosis is epithelial and endothelial cell injury leading to denuded basement membranes and the regeneration of these cells (14-18). Such injury destroys the permeability barrier with resultant proteinaceous oedema, hyaline membranes and influx of inflammatory cells. This is followed by the development of pulmonary fibrosis, characterized by excessive fibroblast proliferation and collagen deposition. Severity of epithelial damage, ongoing injury, and delay in epithelial repair appear to be key factors for overstimulation of the normal repair response leading to pulmonary fibrosis (19).

Nowadays it is generally assumed that mediators produced by pulmonary inflammatory cells, such as alveolar macrophages, and resident lung cells, such as epithelial cells, endothelial cells, and fibroblasts, and blood born mediators, induce fibroblasts to migrate, proliferate and to produce excess collagen (20-32). Figure 1 depicts the mechanisms involved in the pathogenesis of pulmonary fibrosis.

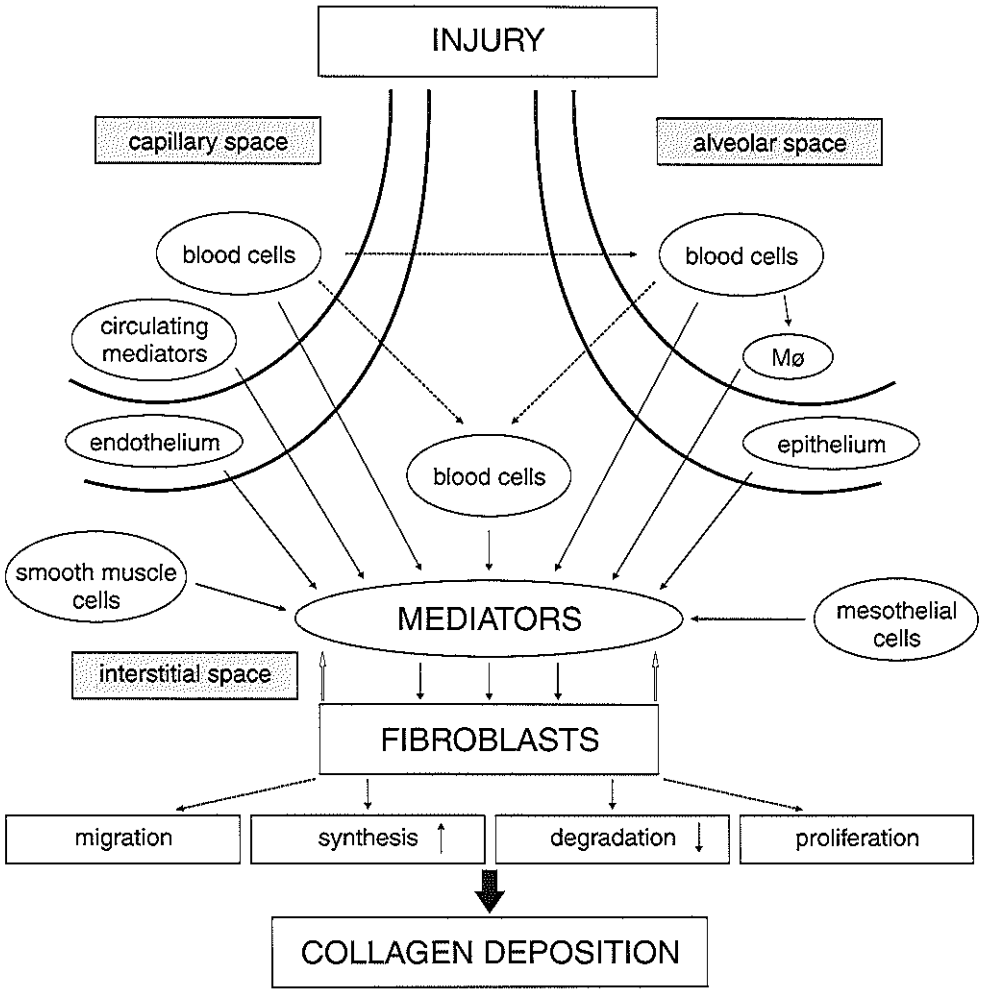

Figure 1
Mechanisms involved in the pathogenesis of pulmonary fibrosis. Epithelial and endothelial cell injury elicits the movement of inflammatory cells between capillary, alveolar, and interstitial compartments with the release of fibrogenic mediators. These mediators activate fibroblasts which migrate to the lesion, proliferate and produce and deposit increased amounts of extracellular matrix proteins, resulting in fibrosis. (Adapted with permission from: McAnulty RJ and Laurent GJ 1995, Exp Nephrol 3: 96-107).

2.1.1. Collagen in pulmonary fibrosis

Increased collagen deposition is clearly observed upon histologic examination of fibrotic lung tissue and has been confirmed by biochemical analysis (15, 16, 18, 33-40). Both biochemical and immunohistochemical studies have provided evidence that the early stages of pulmonary fibrosis are characterized by an increase of type-III collagen relative to type-I collagen while in established fibrosis an increase of type-I collagen is observed (8, 37, 41-43). Increased levels of N-terminal and C-terminal pro-peptides of collagen type-I and III, which are extracellular products of collagen synthesis, have been detected in BAL fluid and serum from adult patients with fibrosing lung disease (44-50). Broekelmann *et al.* demonstrated by *in situ* hybridization that foci of fibroblasts in organizing fibrotic lesions (early stage fibrosis) expressed type-I procollagen mRNA. In established dense fibrotic areas (late stage fibrosis), however, no type-I procollagen mRNA was expressed (51). This is in concordance with immunohistochemical analysis of type-I procollagen expression by fibroblasts in fibrosing lung disease (52). The presence of type-I procollagen in fibroblasts in fibrotic lung and its absence in fibroblasts of normal lung tissue supports the hypothesis that fibrosis is associated with an altered collagen-synthesizing phenotype of tissue fibroblasts (52, 53).

In vitro studies on collagen synthesis by fibroblasts obtained from fibrotic lung are conflicting. A number of studies did not show increased collagen synthesis by fibroblasts from fibrotic lung compared to fibroblasts from normal lung (54, 55), while others studies did demonstrate increased collagen synthesis by fibroblasts from fibrotic lungs (56, 57). Recently, Ramos *et al.* described increased α1(I) collagen mRNA expression and collagen synthesis by fibroblasts from fibrotic lung tissue (58). In these studies large variation in collagen synthesis for the different fibroblast lines was observed. On the other hand, fibroblasts from areas of active organizing fibrosis express procollagen mRNA and protein, while fibroblasts in established dense fibrotic areas express less or not at all (51, 52). Therefore, the site from which the fibroblasts are isolated from the lung tissue may influence the capacity to synthesize collagen *in vitro*.

It has been demonstrated that normal lung tissue contains different subsets of fibroblasts, based on the ability of these cells to bind C1q (the large subunit of the first component of complement). Under basal conditions, fibroblasts with high C1q binding capacity synthesize more collagen than fibroblasts with low C1q binding capacity. Therefore, fibroblasts with high C1q binding capacity may be responsible for the collagen synthesis necessary during healing. Persistence of such collagen-synthesizing cells in healing tissue may lead to the collagen accumulation characteristic for fibrotic lesions (59, 60).

In the field of pulmonary fibrosis there has been a lot of emphasis on increased collagen synthesis. However, decreased degradation of collagen by proteolytic enzymes such as matrix metalloproteinases (MMPs) may also contribute to excessive ECM accumulation. Loss of the highly regulated balance between MMPs and tissue inhibitors of metalloproteinases (TIMPs) may contribute to collagen accumulation (61-63). A detailed description of the role for MMPs and TIMPs in pulmonary fibrosis will be given in chapter 2.1.4.4.

2.1.2. The role of fibroblasts in pulmonary fibrosis

The fibroblast is the major collagen synthesizing cell-type in the lung parenchyma. Current concepts on the mechanisms leading to excessive ECM deposition in pulmonary fibrosis suggest that fibroblast proliferation may play an important role (13, 20). Histological examination of tissue taken from lungs undergoing episodes of active or end-stage pulmonary fibrosis shows evidence of increased fibroblast numbers (14-16, 34, 36, 64, 65).

In vitro studies on the proliferative characteristics of fibroblasts obtained from fibrotic lungs are inconclusive. It has been demonstrated that fibroblasts obtained from fibrotic tissues proliferate with a higher rate than fibroblast from normal lung tissue (66, 67). Raghu *et al.* demonstrated that fibroblasts obtained from early inflammatory fibrotic lesions had higher proliferation rates than fibroblasts from normal lung tissue, while fibroblasts obtained from established dense fibrotic regions proliferated with a lower rate than fibroblasts from normal lung tissue (68). Recently, a study described decreased proliferation rates in fibroblasts obtained from fibrotic lung tissue compared to fibroblasts obtained from normal lung tissue (58). In this study it was suggested that the high percentage of α-smooth muscle (α-SM) actin positive fibroblasts (myofibroblasts) in the fibrotic cell population accounted for the observed lower proliferative activity as these cell have been shown to grow slowly *in vitro* (58, 69). The slower growth rate of fibrotic fibroblasts, however, may also be explained by increased apoptosis of these cells compared to fibroblasts from normal lung tissue (58).

Mio *et al.* did not find differences in the basal growth rate of fibroblasts obtained from early fibrotic lesions, higher-intensity fibrotic lesions, and fibroblasts obtained from normal lung tissue. Fibroblasts from end-stage fibrotic lesions were not analyzed in this study (70). Furthermore, the proliferative response to the fibroblast mitogen platelet-derived growth factor (PDGF) was comparable between fibroblasts obtained from normal lung and from early and higher-intensity fibrotic lesions, which is in concordance with data described by Raghu *et al.* (68, 70).

Prostaglandin E_2 (PGE_2) is a potent inhibitor of fibroblast proliferation and collagen synthesis (71-73). TGF-β_1, which is a profibrotic mediator, has been shown to downregulate proliferation of normal lung fibroblasts at high concentrations by the induction of PGE_2 synthesis. However, TGF-β_1 was unable to downregulate the proliferation of lung fibroblasts obtained from fibrotic lung tissue (57). The reduced capacity of fibroblasts from fibrotic tissue to synthesize PGE_2, both basally and in response to TGF-β_1, was found to be due to an inability to upregulate cyclooxygenase-2 (COX-2) enzyme, which is the rate limiting enzyme in prostanoid biosynthesis (57, 74). Moreover, it was shown that the proliferation of fibroblasts from fibrotic lungs was less inhibited by PGE_2 than the proliferation of fibroblasts from normal lung tissue (70).

Suganuma *et al.* described that fibroblasts from fibrotic lung had enhanced migratory capacity, both under basal conditions and towards PDGF, compared to fibroblasts from normal lung tissue (75). Fibroblasts from tissues with dense fibrosis had a greater capacity to migrate than those from an earlier stage of fibrosis (75). Interestingly, bronchoalveolar (BAL)

fluid from patients with fibrosing lung disease is highly chemotactic for fibroblasts (76). Indeed fibroblasts have been cultured out of BAL fluid (77). *In vitro* studies have demonstrated that such migrating fibroblasts were enriched for α-SM actin (78).

Together these data suggest that different phenotypes of fibroblasts with regard to proliferative and migratory capacity occur within fibrotic lungs. These fibroblasts differ from normal lung fibroblasts. A change in phenotype of the pulmonary fibroblasts may play an important role in the pathogenesis of pulmonary fibrosis.

2.1.3. The role of myofibroblasts in pulmonary fibrosis

It has become evident over the last few years that there is phenotypic heterogeneity among fibroblasts with regard to proliferation rate, collagen synthesis rate, and surface marker expression (54, 57, 59, 66-68, 79). Myofibroblasts are a unique subpopulation of activated fibroblasts that express α-SM actin, which is a feature of smooth muscle differentiation (80).

Myofibroblasts play a central role in wound healing, presumably as an extension of their role in normal growth and differentiation. During wound healing, the myofibroblast appears to be involved in the formation and repair of the ECM, as well as the proliferation and differentiation of epithelial, vascular and neurogenic elements. Due to the expression of α-SM actin the myofibroblast is contractile and therefore can reduce the denuded surface area of wounded tissue (80).

Myofibroblast hyperplasia has been demonstrated in areas of active pulmonary fibrosis in humans and in animal models for pulmonary fibrosis. The myofibroblasts appear to be the main subset responsible for collagen accumulation (64, 81-87). Successful tissue repair after injury was characterized by a gradual disappearance of the myofibroblast as described in a rodent model of bleomycin-induced lung injury with self-limiting pulmonary fibrosis. In this model, active fibrosis was characterized by increased numbers of myofibroblasts and collagen expression. When fibrosis subsided, decreased numbers of myofibroblasts and collagen expression were observed (87-89).

Several cell types present in the lung are potential precursors of the myofibroblasts associated with pulmonary fibrosis. These include airway or vascular smooth muscle cells, pericytes, contractile interstitial cells, and septal tip cells. The fibroblast, however, is regarded as the main precursor cell for the myofibroblast (90).

Cytokines and growth factors are likely to play a role in inducing the transformation of fibroblasts into α-SM actin expressing myofibroblasts. It has been suggested that TGF-β_1 and TNF-α, which were expressed in excess by hyperplastic type-II epithelial cells in pulmonary fibrosis, stimulate the expression of α-SM actin by pulmonary myofibroblasts (85). In bleomycin-induced pulmonary fibrosis in rats, it was demonstrated that increased TGF-β_1 expression preceded the differentiation of alveolar fibroblasts into α-SM actin expressing myofibroblasts (23). Furthermore, adenoviral gene transfer of GM-CSF in the alveolus resulted in local accumulation of TGF-β_1, followed by α-SM actin and collagen synthesis by pulmonary myofibroblasts, and eventually pulmonary fibrosis (91). Among the mediators

implicated in myofibroblast modulation, TGF-β_1 can be considered as a direct inducer of the myofibroblastic phenotype as *in vitro* studies have demonstrated that TGF-β_1 induced α-SM actin expression as well as collagen synthesis by fibroblasts (92-94).

2.1.4. Mediators of pulmonary fibrosis

Pulmonary fibrosis can be the consequence of a variety of insults and is characterized by increased deposition of ECM proteins within the pulmonary interstitium. Changes in collagen deposition can result from attraction of fibroblasts to the active site of injury to increase their number, replication of fibroblasts, clonal selection of sub-populations of fibroblasts with a specific phenotype such as rapid replication or high collagen production, or changes in the collagen synthesis and degradation. Many mediators (produced by inflammatory or resident lung cells or derived from the blood) affecting these fibroblast functions have been identified and have been linked to pulmonary fibrosis both in human and in experimental animal studies. The "balance" between mediators that positively or negatively regulate fibroblast activity in the context of collagen synthesis and/or degradation and fibroblast proliferation and/or chemotaxis is thought to play a central role in the pathogenesis of pulmonary fibrosis (95). A summary of mediators having direct effect on fibroblast functions and known to be involved in pulmonary fibrosis in both humans and animal models is given in table 1.

TGF-β_1 is the most potent stimulator of collagen synthesis. Therefore its role in pulmonary fibrosis will be discussed in more detail. In addition to this, the role of PDGF, the coagulation cascade (especially thrombin) and the role of matrix metalloproteinases will be discussed as they are of special interest for this thesis.

2.1.4.1. Transforming growth factor-β

TGF-β is a dimeric protein of which three isoforms exist in mammals. These isoforms have been designated as TFG-β_1, TFG-β_2 and TFG-β_3 (165). All these isoforms are initially translated as large precursor molecules. TGF-β is secreted in a small or large latent complex that is inactive (166). The precise mechanism of TGF-β activation *in vivo* is still unknown. However, a number of candidate molecules including plasmin, MMP-2 and MMP-9, a cell surface MMP-9/CD44 complex, the ECM protein thrombospondin-1 and the $\alpha_v\beta_6$-integrin have been found to activate latent TGF-β (166-169). TGF-β_1 is the predominant and most studied isoform and often just implicated as TGF-β (165).

TGF-β_1 is a well known inducer of collagen synthesis (96, 170-172). It influences collagen production in a number of ways. It stimulates the transcription of the procollagen gene and increases the stability of procollagen mRNA (171, 173, 174).

Besides increased collagen synthesis, decreased breakdown may also result in excessive collagen accumulation. TGF-β has been demonstrated to decrease intracellular collagen degradation, to reduce the production of collagenase (MMP-1) and to increase the production of the collagenase inhibitor tissue inhibitor of metalloproteinase (TIMP) (94, 100). In such a way TGF-β can promote collagen deposition by fibroblasts.

Table 1. Mediators involved in pulmonary fibrosis that modulate fibroblast activity directly

Mediator	Effect on fibroblast	Source	Reference
TGF-β_1	Collagen ↑ Collagen degradation ↓ Proliferation ↑↓ Chemotaxis ↑ Collagenase ↓ Metalloproteinase inhibitors↑	edc, epc, eos, fib, smc, mac, meso, plat	27, 72, 94, 96-101
PDGF	Proliferation ↑ Chemotaxis ↑ Collagenase ↑↓	edc, epc, fib, smc, meso, mon, mac, plat	25, 102-107
CTGF	Collagen ↑ Proliferation ↑ Chemotaxis ↑	epc, fib	108-110
TNF-α	Collagen ↓ Proliferation ↑ Chemotaxis ↑ Collagenase ↑	edc, fib, lym, mon, mac	111-119
IGF-1	Collagen ↑ Proliferation ↑ Collagenase ↓	blood born, fib, mac, smc	120-124
ET-1	Collagen ↑ Collagen degradation ↓ Proliferation ↑ Chemotaxis ↑ Collagenase ↓	edc, epc, fib, mac	125-129
Fn	Proliferation ↑ Chemotaxis ↑	blood born, edc, epc, fib, mac	130-133
PGE_2	Collagen ↓ Proliferation ↓ Chemotaxis ↓ Collagenase ↓ / ↑ Metalloproteinase inhibitors ↓	blood born, edc, epc, fib, smc, mon, mac	57, 72, 73, 134-138
IFN-γ	Collagen ↓ Proliferation ↑ Collagenase ↓	lym, mac	139-146
IL-1	Collagen ↑ Proliferation ↑ Collagenase↑ Metalloproteinase inhibitors ↑	fib, mon, mac	119, 147-153

to be continued on the next page

Table 1. (continued)

Mediator	Effect on fibroblast	Source	Reference
MCP-1	Collagen ↑ Collagenase ↑ Metalloproteinase inhibitors ↑	edc, epc, fib, smc, mon, mac, eos	154-158
Thr	Collagen ↑ Proliferation ↑ Chemotaxis ↑	blood born	32, 159-161
Fxa	Proliferation ↑	blood born	162-164

edc = endothelial cells, eos = eosinophils, epc = epithelial cells, fib = fibroblasts, lym = lymphocytes, mac = macrophages, meso = mesothelial cells, mon = monocytes, plat = platelets, smc = smooth muscle cells

$TGF\text{-}\beta_1$ = transforming growth factor-β_1
PDGF = platelet-derived growth factor
CTGF = connective tissue growth factor
TNF-α = tumor necrosis factor-α
IGF-1 = insulin like growth factor-1
ET-1 = endothelin-1
Fn = fibronectin
PGE_2 = prostaglandin E_2
IFN-γ = interferon-γ
IL-1 = interleukin-1
MCP-1 = monocyte chemotactic protein-1
Thr = thrombin
FXa = factor Xa (activated factor X)

↑ = increased
↓ = decreased
↑↓ = biphasic
↑/↓ = increased / decreased

Furthermore, TGF-β has been shown to be chemotactic for fibroblasts, to influence fibroblast proliferation in a biphasic manner and to induce α-SM actin expression (72, 93, 99).

TGF-β in pulmonary fibrosis

In animal models of pulmonary fibrosis increased $TGF\text{-}\beta_1$ mRNA expression levels were detected before type-I and type-III procollagen mRNA expression levels increased (175, 176). Besides $TGF\text{-}\beta_1$, increased $TGF\text{-}\beta_2$ and $TGF\text{-}\beta_3$ mRNA levels have also been demonstrated in bleomycin-induced pulmonary fibrosis (177). Numerous cellular constituents of the lung, such as alveolar macrophages, bronchial and alveolar epithelial cells, endothelial cells, mesothelial cells fibroblasts and smooth muscle cells have been identified as a potential source for all three isoforms of TGF-β (177,178). A study by Coker *et al.* demonstrated that both $TGF\text{-}\beta_2$ and - β_3 share the ability of $TGF\text{-}\beta_1$ to promote collagen deposition by stimulating fibroblast procollagen synthesis *in vitro* (178). However, using *in situ* hybridization, they identified $TGF\text{-}\beta_1$ as the predominant isoform in bleomycin-induced pulmonary fibrosis, which is in concordance with earlier published PCR analysis data in bleomycin treated mice (178, 179). Increased TGF-β protein levels have been described in lung tissue after

intratracheal instillation of bleomycin and was temporally related to enhanced synthesis of collagen by pulmonary fibroblasts (23). Increased TGF-β protein expression preceded myofibroblast hyperplasia and was detected in macrophages, bronchiolar epithelium and subepithelial matrix, and ECM associated with areas of repair and fibrosis (23, 87, 101). Furthermore, after bleomycin-induced injury alveolar macrophages not only produced TGF-β but also secreted large quantities of biologically active TGF-β. Also, in BAL fluid increased levels of TGF-β have been detected after bleomycin-induced lung injury (180-183). *In vitro* studies have demonstrated that bleomycin not only stimulates TGF-β production by alveolar macrophages, but also by endothelial cells (28, 184).

The importance of TGF-β_1 in pulmonary fibrosis was nicely shown in a study in which active TGF-β_1 was transiently overexpressed in rat lung. This overexpression resulted in severe pulmonary fibrosis characterized by excessive deposition of collagen and the appearance of myofibroblast like cells (185). Furthermore, inhibition studies stress the importance of TGF-β in the pathogenesis of pulmonary fibrosis. Administration of TGF-β antibodies or recombinant soluble TGF-β receptor-II resulted in diminished collagen accumulation after intratracheal instillation of bleomycin or immune-induced (intranasal exposure to heat-killed Bacillus Calmette-Guérin) pulmonary fibrosis (186-188).

Decorin is a proteoglycan with two binding sites for all TGF-β isoforms and is an important negative regulator of this growth factor. Adenoviral transfer of decorin into the lungs has been demonstrated to inhibit bleomycin-induced pulmonary fibrosis, and this effect was probably mediated at the level of TGF-β activity (183). Furthermore, addition of taurine and niacin, which are known protectors of tissue injury, to the diet three days before intratracheal instillation of bleomycin and continuing thereafter, reduced pulmonary fibrosis at least partly via the inhibition of TGF-β_1 mRNA expression (189). IFN-γ has been shown to down-regulate bleomycin-induced pulmonary fibrosis via a reduction in the overexpression of TGF-β mRNA, and consequently a reduced production of procollagen mRNA (190).

TGF-β mediates signals from the membrane to the nucleus via the SMAD proteins. SMAD7, however, functions as an inhibitor of TGF-β signaling (191). Transient expression of SMAD7 in the lungs inhibited bleomycin-induced pulmonary fibrosis without affecting the amount of TGF-β_1 present (192).

A novel mechanism for the activation of latent TGF-β_1 has recently been suggested. In this mechanism the $\alpha_V\beta_6$ integrin expressed on epithelial cells binds latent TGF-β_1 and is involved in its activation (169). It was demonstrated that β_6 knockout mice were protected from bleomycin-induced pulmonary fibrosis, and that bleomycin induced $\alpha_V\beta_6$ expression on airway and alveolar epithelial cells throughout the lungs (169).

In patients with pulmonary fibrosis, *in situ* hybridization revealed that in areas of early active fibrosis TGF-β_1 mRNA was expressed by macrophages close to fibroblasts actively expressing type-1 procollagen mRNA (51). Additionally, immunohistochemistry revealed intense staining for TGF-β_1 around fibroblastic foci, which was co-distributed with procollagen type-1 expression by fibroblasts (51). Other immunohistochemical studies detected TGF-β_1 protein in regenerating and hyperplastic alveolar type-II epithelial cells as well as in the

fibrous tissue located beneath the hyperplastic epithelial cells (27, 85).

Recently it was shown that TGF-β_1 and TGF-β_3 mRNA localized to alveolar macrophages and bronchiolar epithelium in normal human lung. In addition to this, mesenchymal and endothelial cells only expressed TGF-β_1 mRNA (193). In fibrotic lung tissue, both isoforms of TGF were also detected in alveolar type-II epithelial cells, and it was demonstrated that TGF-β_1 mRNA expression was enhanced in fibrotic lung while TGF-β_3 was not consistently altered (193). This indicates that TGF-β_1, like in bleomycin-induced pulmonary fibrosis in mice, is the predominant TGF-β isoform in the pathogenesis of pulmonary fibrosis (193).

Increased TGF-β_1 levels have been detected in BAL fluid from patients with fibrosing lung disease. *In vitro* studies identified the alveolar macrophage as an important source of TGF-β production in the pathogenesis of pulmonary fibrosis (194-196).

Taken together, these observations suggest an important role for TGF-β in the development of pulmonary fibrosis.

2.1.4.2. Platelet-derived growth factor

PDGF is a family of growth stimulating polypeptides. The PDGFs are dimers consisting of two polypeptide chains, referred to as the A- and B-chains. These chains may assemble to form three different isoforms of PDGF, referred to as PDGF-AA, PDGF-AB, and PDGF-BB (197). The PDGF isoforms exert their effects via two receptor tyrosine kinases, denoted PDGFR-α and PDGFR-β (197). Ligand binding induces dimerization of the receptor. The PDGFR-α binds both PDGF-A and B chains while PDGFR-β only binds the PDGF-B chain (198). Therefore, dependent on the PDGF isoform, different types of receptor homo- or heterodimers (α-α, α-β or β-β) are formed, with the α-α receptor binding all three PDGF isoforms, the α-β receptor binding PDGF-AB and PDGF-BB, and the β-β receptor binding PDGF-BB only (199). Recently, two new PDGF-chains have been described and denoted PDGF-C and PDGF-D. Both chains form homodimers resulting in PDGF-CC and PDGF-DD (200-202). In addition, it was shown that PDGF-C is a ligand for PDGFR-α while PDGF-D is a ligand for PDGFR-β (200, 201). As little is know yet about PDGF-C and PDGF-D, only the "classic" PDGF isoforms will be discussed here.

Among the different fibroblast mitogens, PDGF is regarded as the most potent one. *In vitro* studies have demonstrated that all three isoforms of PDGF stimulate lung fibroblast proliferation. However, fibroblasts from different species may differ in their responsiveness to PDGF isoforms (102, 103). In addition to its effect on replication, *in vitro* studies have identified PDGF as a chemoattractant for fibroblasts (104, 203).

Several *in vitro* studies on the effect of PDGF on collagen mRNA expression and collagen synthesis by fibroblasts are inconclusive. It has been described that the different PDGF isoforms did not affect type-I and -III procollagen mRNA expression and collagen synthesis by fibroblasts (103, 105). However, if fibroblasts were cultured on dishes coated with 2-hydroxyethyl methacrylate (poly (HEMA)) or in three dimensional collagen gels, PDGF-BB increased collagen synthesis with or without mediating type-I procollagen

mRNA, respectively (204). Furthermore, PDGF may influence the accumulation of collagen as it has been demonstrated that PDGF-BB influences collagenase expression by fibroblasts in a biphasic manner (106). Additionally, PDGF-BB may upregulate TIMP expression by fibroblasts in a synergistic manner with other factors (105, 106).

PDGF in pulmonary fibrosis

A potential role for PDGF in pulmonary fibrosis has become evident from a number of animal studies. In rats, intratracheal instillation of an expression vector containing the human PDGF-B gene resulted in pulmonary overexpression of this gene and caused significant fibroproliferation and collagen synthesis (205). Overexpression of the PDGF-B gene under control of the surfactant protein-C promoter in mice resulted in alternating areas of emphysema and fibrosis (206). Intratracheal injection of PDGF-BB in rats resulted in increased incorporation of bromodeoxyuridine (BrdU) into peribronchial and perivascular stromal cells and spindle-shaped mesenchymal cells in the alveolar parenchyma and increased collagen deposition (207).

Asbestos exposure may also lead to pulmonary fibrosis. It has been demonstrated that in lungs from rats exposed to chryostile asbestos, mRNA encoding PDGF-A and -B increased as soon as 5 hours after exposure and remained elevated for at least 30 days (26). Additionally, it was shown that PDGF-A and -B chains were produced by bronchiolar-alveolar epithelial cells, macrophages and interstitial cells (26). Increased levels of PDGF-A and -B mRNA and protein have also been demonstrated in BAL cells and fluid, and lung tissue from animals with bleomycin-induced pulmonary fibrosis, and have been shown to contribute to BAL fluid-induced fibroblast proliferation (107, 124, 181, 208).

The tyrphostin AG1296 is a tyrosine kinase inhibitor that specifically inhibits autophosphorylation of the PDGFR. AG1296 has been shown to inhibit vanadium pentoxide (V_2O_5) induced pulmonary mesenchymal cell proliferation and collagen deposition (209). Yoshida *et al.* demonstrated that transgenic overexpression of the extracellular domain of the PDGFR-β in the lung inhibited bleomycin-induced fibrosis (210). Pirefenidone (PD) is a drug that inhibits bleomycin-induced lung injury in hamsters when added to the diet (211). Additionally, it has been demonstrated that PD decreased the levels of PDGF isoforms in BAL fluid after bleomycin and reduced BAL fluid mitogenicity for fibroblasts (208).

In human patients with pulmonary fibrosis, increased expression of PDGF-A and - B mRNA and protein has been demonstrated in alveolar macrophages, and PDGF-B appeared to be the most abundantly expressed (25, 212, 213). Alveolar macrophages from adult patients with pulmonary fibrosis were found to secrete increased amounts of PDGF, and increased concentrations of PDGF have been described in BAL fluid (21, 194, 195, 213, 214). Culture medium, conditioned by alveolar macrophages from patients with pulmonary fibrosis, was found to be mitogenic for fibroblasts, and PDGF largely accounted for this mitogenic activity (21, 213).

Increased PDGF-B mRNA and protein were also detected in fibrotic lung tissue, and in situ hybridization revealed localization to alveolar epithelial cells, capillary endothelial

cells, alveolar and interstitial macrophages and pleural mesothelial cells (24, 215). Increased protein expression of PDGF-A, PDGF-B, PDGFR-α and PDGFR-β in endothelial cells, vascular smooth muscle cells and fibroblasts was demonstrated to occur only in early fibrotic lesions. Macrophages, however, expressed these proteins in both early and established fibrosis (25). Furthermore, mRNA expression for PDGF-B and PDGFR-β was present in alveolar type-II cells of lung tissues from early fibrotic lesions and not established fibrosis, while mRNAs for PDGF-A and PDGFR-α were never detected (25).

Altogether these data suggest an important role for PDGF in the pathogenesis of pulmonary fibrosis.

2.1.4.3. Coagulation cascade

Tissue injury associated with vascular damage results in activation of the coagulation cascade (216). The main function of the coagulation cascade is preventing the loss of blood, and it does so via the formation of a provisional clot (consisting of platelets and fibrin). Stepwise activation of coagulation serine proteases present in the plasma results in the conversion of prothrombin into active thrombin. Thrombin plays a central role in the coagulation cascade by converting soluble plasma fibrinogen into an insoluble fibrin clot and stimulates aggregation and degranulation of platelets (216, 217). Two pathways, referred to as the extrinsic and intrinsic pathways, are involved in the generation of active thrombin. It is thought that the extrinsic pathway is critical in the initiation of fibrin formation, while the intrinsic pathway plays an important role in the maintenance of fibrin formation (216).

Dramatic activation of the coagulation cascade has been documented in cases of acute lung injury and pulmonary fibrosis (164, 218-221). In pulmonary fibrosis and diffuse alveolar damage increased coagulant activity has been shown to be associated with increased pulmonary fibrin deposition (164, 219).

The next part will shortly discuss the coagulation pathways resulting in thrombin activation. Thereafter, cellular effects elicited by thrombin and evidence for thrombin as an active mediator in pulmonary fibrosis will be discussed. Although not discussed, one should realize that thrombin can also influence lung pathology via coagulation (fibrin deposition and fibrinogen derivatives) itself (29-31, 222-224).

The extrinsic pathway

The initiation of the coagulation cascade occurs following vascular injury and the exposure of tissue factor (TF; expressed on the surface of several different cell types) to the blood. TF forms a complex with factor VII which circulates in the blood, and activates this factor VII (leading to factor VIIa). The complex VIIa-TF converts factor X towards factor Xa. The newly generated factor Xa forms a complex with factor Va (prothrombinase) which is capable of converting prothrombin to thrombin, which then acts directly on fibrinogen (216).

The intrinsic pathway

The intrinsic pathway is an alternative coagulation pathway which is triggered by the contact system of coagulation when blood comes in contact with negatively charged components of the subendothelial connective tissue, such as collagen (217, 225). The contact system involves factor XII, prekallikrein, high-molecular-weight kininogen and factor XI (225). It results in the activation of factor XI which then activates factor IX of the intrinsic pathway (216, 225). Factor IXa associates with factor VIIIa and then activates factor X at the point where the intrinsic and extrinsic pathways of coagulation converge (216).

Thrombin not only converts fibrinogen into fibrin, but also amplifies the coagulation cascade by converting factor V into its active form. In addition thrombin activates factor XIII. Activated factor XIII stabilizes the fibrin clot by covalent cross-linking of fibrin. Additionally, thrombin activates factor XI, which results in amplification of the intrinsic pathway (216, 225). A simplified scheme of both coagulation pathways is depicted in figure 2.

Under normal conditions the coagulation cascade is tightly controlled by anticoagulant pathways. The main inhibitor of the extrinsic pathway of coagulation is tissue-factor-pathway-inhibitor (TFPI). TFPI forms an inactive complex with factor VIIa/TF/factor Xa thereby inhibiting fibrin formation (216). Another important inhibitor of coagulation is antithrombin III (ATIII). ATIII is capable of inhibiting thrombin as well as the factors IXa, Xa, and XIa (216). The complex formation of ATIII with different coagulation factors is greatly enhanced by heparin. Additionally, heparin cofactor II, α_2-macroglobulin, activated protein C inhibitor and α_1-PI may also play an important role in the regulation of the coagulation cascade (216). Blood coagulation may also be regulated by protein-C. Thrombin can bind to thrombomodulin on endothelial cells. Thrombomodulin stimulates the activation of

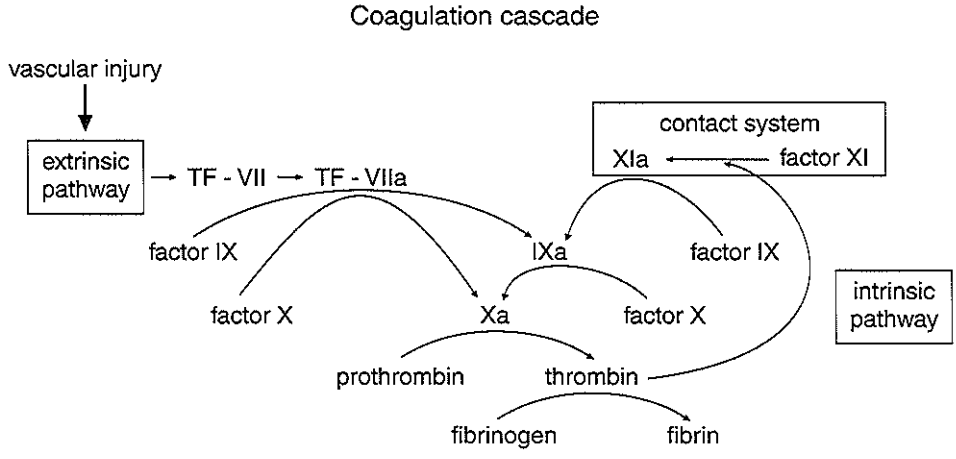

Figure 2
Simplified scheme of the coagulation cascade, involving the stepwise activation of serine proteases by the extrinsic and the intrinsic pathways. At the point of convergence of these two pathways, factor Xa converts prothrombin into active thrombin. Thrombin converts soluble fibrinogen into insoluble fibrin.

protein-C by thrombin. In this case thrombin loses its activity toward its substrates while activated protein-C inactivates factor Va and factor VIIIa (216).

Thrombin

Besides its central role in blood coagulation, thrombin mediates other cellular effects that may play an important role in inflammatory, tissue repair, and fibrotic responses. Thrombin has been shown to directly increase endothelial permeability, in addition to its effects on permeability secondary to intravascular coagulation (226). Furthermore, thrombin acts as a chemoattractant for inflammatory cells and fibroblasts and as a mitogen for fibroblasts and vascular smooth muscle cells (160, 227-229). The mitogenic effect is likely to be due to autocrine release of PDGF-AA and an upregulation of the PDGFR-α (162, 229, 230). Thrombin has also been shown to increase procollagen type-1 mRNA expression, as well as procollagen production by fibroblasts (159). Additionally, thrombin has been demonstrated to induce connective tissue growth factor (CTGF) mRNA expression by fibroblasts. CTGF is known to be a potent mitogen and chemoattractant for fibroblasts, as well as a stimulator of procollagen production by these cells (231). Furthermore, thrombin upregulates the production of inflammation, repair and fibrosis associated mediators, such as IL-6, IL-8, IL-1β, fibronectin, endothelin-1 and -2, and PDGF-AA by fibroblasts, epithelial and endothelial cells, smooth muscle cells and alveolar macrophages (162, 232-239).

Thrombin in pulmonary fibrosis

Increased levels of active thrombin have shown to be present in BAL fluid from rats with bleomycin-induced acute lung injury with subsequent pulmonary fibrosis. Thrombin activity contributed significantly to BAL fluid-induced fibroblast proliferation (161, 240). Immunohistochemical staining for thrombin was found to be associated with alveolar macrophages within inflammatory and fibroproliferative foci, and fibroblast-like interstitial cells (161).

Studies using inhibitors of the coagulation cascade have clearly demonstrated a role for coagulation and thrombin in acute lung injury. Exogenous delivery of hirudin and ATIII have shown to be protective and to result in decreased pulmonary fibrinogen deposition and BAL fluid procoagulant activity in animal models of acute lung injury (241, 242). Additionally, it has been shown that heparin improved gas exchange in a model of acute lung injury in newborn piglets (243). Heparin has also been shown to inhibit bleomycin-induced pulmonary fibrosis in mice, without reducing the number of inflammatory cells (244). BAL fluid from mice treated with bleomycin contained decreased levels of activated protein-C (245). It was demonstrated that intratracheal administration of activated protein-C to bleomycin-treated mice reduced thrombin activity in BAL fluid as well as collagen deposition in the lung (245). Direct inhibition of thrombin activity (using the compound UK-156406) reduced collagen accumulation and type-I procollagen and CTGF mRNA expression levels in the lungs from bleomycin-treated rats without modulation of the inflammatory cell influx (161).

In adult patients with pulmonary fibrosis increased levels of active thrombin have

been detected in BAL fluid and contributed significantly to BAL fluid-induced fibroblast proliferation (32, 230). Increased levels of the thrombin-ATIII complex (TATIII), a sensitive marker of thrombin generation, and decreased pulmonary protein-C activation have also been demonstrated in BAL fluid from patients with pulmonary fibrosis (246, 247).

Altogether, these data suggest an important role for thrombin in the pathogenesis of pulmonary fibrosis.

2.1.4.4. Matrix metalloproteinases and their involvement in pulmonary fibrosis

MMPs play a role in many normal biological processes such as development, bone remodelling, wound healing, angiogenesis and apoptosis (248). Inadequate MMP production or regulation of activity has been implicated in a variety of pathological processes such as tumour invasion, Sjögren's syndrome, rheumatoid arthritis, lung disease and organ fibrosis (62, 249-256).

MMPs comprise a large family of zinc endoproteases that share structural domains and are collectively capable of degrading all ECM components. The enzymes have both a descriptive name and an MMP number. MMPs can be divided into different families based on substrate specificity, which is summarized in table 2 (248, 249, 257, 258). All MMPs are synthesized as prepro-enzymes. The "pre"region targets for secretion and the "pro"region contains a sequence which ligates the catalytic zinc-domain to preserve the latency of the pro-MMP (258). Additionally, MMPs contain domains such as a hemopexin region or a fibronectin region that are essential for substrate recognition (258). A subset of MMPs are the membrane-type MMPs (MT-MMPs), which are not secreted but remain attached to the cell surface via a transmembrane domain (258).

MMP activity is controlled at multiple levels. MMP expression is regulated by a wide variety of cytokines and growth factors, such as TNF-α, IL-1, IL-6, TGF-β and PDGF (259). MMP expression may also be mediated by components of the ECM (260-262). MMP proteins are secreted or membrane-bound as latent enzymes that require proteolytic processing to release the catalytically active enzyme (258). This processing can be achieved by other MMPs (e.g., MMP-3 can activate proMMP-1, -2, and -9) or by proteases like plasmin (263-267). MT1-MMP has been demonstrated to be an activator of proMMP-2 and proMMP-13. Neutrophil proteinases may participate in this process (266, 268, 269).

The major endogenous regulators of MMP activity are the tissue inhibitors of metalloproteinases (TIMPs). At present four different TIMPs have been described (TIMP-1, 2, 3, and 4) (270). TIMPs inhibit MMP activity via non-covalent binding of the active MMP forms at a molecular equivalence (270). TIMP-1 and -2 can also bind proMMP-9 and proMMP-2, respectively (270). In addition to inhibition by TIMPs, MMP activity may also be inhibited by α_2-macroglobulin (257).

Besides inhibition of MMP activity, TIMPs may also exert a number of other effects. For example, TIMP-2 may be involved in the activation of MMP-2 by MT1-MMP (271). TIMPs may also modulate cell proliferation, influence cell morphology, inhibit angiogenesis and promote apoptosis (270, 272).

Table 2. Matrix metalloproteinases according to substrate specificity

Family	Substrates	MMP	Descriptive Name(s)
Collagenases	fibrillar collagens, type-I, II, III non-fibrillar collagens, type-VII, X, gelatins, proteoglycan core protein	MMP-1	interstitial collagenase collagenase-1
		MMP-8	neutrophil collagenase, collagenase-2
		MMP-13	collagenase-3
		MMP-18	Xenopus collagenase, collagenase-4
Gelatinases	fibrillar collagen type-V non-fibrillar collagens, type-IV, VII, X, XII, gelatin, fibronectin, elastin, proteoglycan core protein	MMP-2	gelatinase-A, 72kDa gelatinase, 72kDa type-IV collagenase
		MMP-9	gelatinase-B, 92kDa gelatinase, 92kDa type-IV collagenase
Stromelysins	non-fibrillar collagen type-IV, laminin, gelatin, elastin, proteoglycans, fibronectin, entactin/nidogen, proteoglycan core protein	MMP-3	stromelysin-1, transin, proteoglycanase, procollagenase activator
		MMP-10	stromelysin-2, transin-2
		MMP-7	matrilysin, matrin, PUMP-1, uterine-metalloproteinase
		MMP-11	stromelysin-3
Elastase	elastin, non-fibrillar collagen (IV, VI, VII, VIII, IX, X, XII, XIV)	MMP-12	metalloelastase, macrophage elastase
Membrane type-MMP	pro-MMP-9, undefined		
MT1-MMP		MMP-14	
MT2-MMP		MMP-15	
MT3-MMP		MMP-16	
MT4-MMP		MMP-17	
MT5-MMP		MMP-24	
MT6-MMP		MMP-25	
Unclassified	undefined	MMP-19	
		MMP-20	enamelysin
		MMP-21	
		MMP-22	
		MMP-23	

MMPs in pulmonary fibrosis

A role for MMPs in the pathogenesis of acute lung injury and pulmonary fibrosis has become clear from animal studies. Increased gelatinolytic activity has been described in lung tissue from rabbits after intratracheal instillation of bleomycin (273). In the early stages after bleomycin, gelatinolytic activity due to MMP-9 increased, while in the later stages of disease MMP-2 was the predominant form (273). MMP-2 and 9 were found to be expressed by infiltrating macrophages, bronchial and bronchiolar epithelial cells, and intra-alveolar fibroblasts (273). Denholm *et al.* demonstrated that alveolar macrophages indeed secreted increased amounts of MMP-9 after bleomycin stimulation (274). After bleomycin, MMP-2 was found to be expressed by regenerating type-II pneumocytes and MMP-9 by infiltrating neutrophils (273). Therefore, MMP-9 was suggested to contribute to the injury of the alveolar basement membrane in the early stages of bleomycin-induced pulmonary fibrosis, while a role for MMP-2 was suggested in the reepithelialization of the alveoli in the later stages of disease (273). Increased immunolocalization of MMP-1 has been described in bronchial and bronchiolar epithelial cells, type-II pneumocytes and alveolar macrophages. Increased TIMP-2 expression was found to colocalize with MMP-1. TIMP-2 was also expressed by fibroblasts in fibrotic lesions, suggesting that the TIMP-2 counteractivity to MMP-1 in fibroblasts may promote excessive collagen deposition in pulmonary fibrosis (273).

In BAL fluid, increased collagenase activity against type-I and -IV collagen and collagen degradation products occurred as early as 3 days post-bleomycin in rats. Peak levels of MMP-2 and -9 were demonstrated in BAL fluid in the first week post-bleomycin after which MMP-9 almost completely disappeared while MMP-2 gradually declined (275). Maximum basement membrane degradative activity coincided with the onset of alveolar epithelial cell proliferation one week after bleomycin. Therefore, it was suggested that basement membrane-bound growth factors may be released by MMPs and then stimulate epithelial cell regeneration (275). Other studies have confirmed that both MMP-2 and MMP-9 may play an important role in the reepithelialization of the injured alveoli after bleomycin (276, 277). Different MMPs may regulate the epithelial repair at different levels of the airways (276).

Several *in vitro* studies support a role for different MMPs in the reepithelialization process after injury. *In vitro* wound repair has been shown to require MMP-9 to facilitate bronchial epithelial cell migration (278, 279). In addition to MMP-2 and MMP-9, MMP-7 has also been implicated in the repair of airway epithelium after injury. It was demonstrated that human tracheal epithelium required MMP activity, likely due to MMP-7, to migrate and reepithelialize wounds in tracheal explants. Furthermore, wounds in tracheas from MMP-7 knock-out mice showed no evidence of epithelial migration, and wound opening did not change during the study period (280). MMP-1 is known to be required for keratinocyte migration during epidermal repair and has also been shown to facilitate *in vitro* alveolar epithelial repair by facilitating cell migration on type-1 collagen (281, 282).

In mice it has been demonstrated that intraperitoneal injection of bleomycin induced MMP-2 and metalloelastase (MMP-12) gene expression and that this remained elevated for

a long period (283). In this study, no effect of bleomycin was found on MMP-13, MMP-3 and MMP-9 gene expression. However, others did find increased MMP-9 gene expression after bleomycin (276, 283). Bleomycin had the strongest effect on MMP-12 gene expression, which was paralleled by a marked elevation of mRNA encoding type-1 procollagen and TIMP-1 (283). Increased TIMP-1 mRNA expression in lung tissue after bleomycin has been confirmed by others and was demonstrated to be restricted to areas of lung injury (276, 284). Additionally, lung extracts of bleomycin-treated mice contained increased metalloproteinase inhibitory activity and BAL fluid of bleomycin treated mice showed increased TIMP-1 and -2 protein levels (284).

Recently, it was demonstrated that administration of the synthetic MMP inhibitor batimastat reduced bleomycin-induced pulmonary fibrosis in mice and limited the increase in MMP-2 and MMP-9 activity as well as TIMP-1 in BAL fluid (285). Batimastat did not reduce the bleomycin-induced increase in TGF-β but did inhibit the influx of inflammatory cells (285). These data indicate that MMPs are involved in the modulation of tissue inflammation and remodeling. However, in MMP-9 knockout mice, the bleomycin-induced pulmonary fibrosis and inflammatory cell influx were not inhibited. Thus MMP-9 does not seem to play an essential role in the initiation or evolution of the inflammation and fibrotic changes after bleomycin (276). In a model of immune complex-mediated acute alveolitis in wild-type and MMP-2 and MMP-3 knock-out mice it was demonstrated that both MMP-2 and MMP-3 were involved in the pathogenesis of the acute lung injury. Only MMP-3 appeared to be involved in the recruitment of neutrophils into the lung (286).

Other animal models of acute lung injury and fibrosis, such as hyperoxia-induced lung injury and experimental silicosis have also been shown to be associated with an upregulation of MMP-2, MMP-9, MMP-13 and TIMPs. It was suggested that an imbalance in the expression of MMPs and TIMPs contributed to the lung injury and fibrosis (287, 288). Indeed, decreased collagenolytic activity in lung tissue has been found to be associated with the phase of active fibrogenesis (i.e., collagen accumulation) in animal models of lung fibrosis (289).

In adult patients with fibrosing lung disease increased collagenase activity has been detected in BAL fluid, and was associated with the alveolitis (290, 291). Studies by Selman *et al.* confirm that increased BAL collagenase or collagenolytic activity indeed might reflect the presence of activated inflammatory cells in the lavage fluid or the inflammatory stage of the disease. Increased BAL collagenase or collagenolytic activity not necessarily reflects the collagen turnover that occurs in the parenchymal ECM during active fibrogenesis. In contrast to BAL fluid, collagenolytic activity in lung tissue from patients was decreased when there was a predominance of fibrosis over inflammation when compared to control lung (289). Furthermore, it was demonstrated that the collagenolytic activity decreased in patients with hypersensitivity pneumonitis who deteriorated (with risk of pulmonary fibrosis), compared to patients with hypersensitivity pneumonitis who improved or healed (289). Although the identity of the MMPs contributing to the determined activity remained unclear in these studies, the higher rates of MMP activity in control tissue suggest that stromal cells rather than the inflammatory cells are the source of the MMP activity. These findings support the hypothe-

sis that decreased local collagenolysis may be a crucial event in the development of pulmonary fibrosis. Fibrotic lung tissue has been demonstrated to contain increased collagenase inhibitory activity and fibroblasts isolated from fibrotic lungs have a diminished capacity to synthesize MMP-1 resulting in an increased TIMP/MMP-1 ratio with decreased collagenolytic activity as a consequence (289, 292).

Fukuda *et al.* demonstrated immunolocalization of MMP-1 in interstitial cells present in fibrotic foci. They also demonstrated that interstitial cells (predominantly myofibroblasts) stained intensely for TIMP-2 in irreversible lung fibrosis (idiopathic pulmonary fibrosis), while interstitial cells of reversible lung fibrosis (bronchiolitis obliterans organizing pneumonia) stained predominantly for MMP-1 (62). These data have been confirmed by others and suggest that decreased collagenolytic activity, due to increased TIMP expression, contributes to ECM deposition in progressive pulmonary fibrosis (62, 63, 293). Furthermore, increased expression of MMP-1, MMP-2, MMP-8, MMP-9, MT1-MMP, TIMP-1, TIMP-2, TIMP-3 and TIMP-4 has been demonstrated in regenerating alveolar epithelial cells, myofibroblasts, alveolar macrophages, inflammatory cells, interstitial cells, endothelial cells, and smooth muscle cells. MMPs and TIMPs have also been detected in association with the ECM (61-63).

BAL fluid from patients with fibrosing lung disease has been demonstrated to contain increased levels of MMP-2 and MMP-9 (63, 252, 253, 293, 294). These MMP-2 and MMP-9 levels correlated with the presence of basement membrane degradation products. Furthermore, MMP-2 levels correlated with the number of lymphocytes and MMP-9 levels with the number of neutrophils in BAL fluid (252, 253, 293).

In vitro studies have suggested a role for MMPs in the migration of T lymphocytes across the basement membrane (295). Another *in vitro* study demonstrated that activated neutrophils secrete MMP-9 and elastase, and use these enzymes to migrate across the basement membrane (296). This suggests that neutrophils can be considered as an important source of MMP-9 in fibrosing lung disease. Alveolar macrophages may also be an important source of MMP-9 as these cells were found to secrete increased amounts of MMP-9 in pulmonary fibrosis (294). In addition to MMPs, increased levels of TIMP-1 have been detected in BAL fluid from patients at risk of developing pulmonary fibrosis (252, 253, 294).

Altogether these data suggest an important role for MMPs in basement membrane degradation, inflammatory cell infiltration, epithelial repair processes and disordered ECM remodeling in pulmonary fibrosis.

2.2. Pulmonary fibrosis in bronchopulmonary dysplasia

Besides a large number of studies that have demonstrated histological evidence of collagen deposition in the lungs of infants who died because of BPD (discussed in chapter 1.3.) several studies have examined the collagen content of the lungs from these infants. These studies will be discussed hereafter. In addition, the little information available on fibrogenic

mediators in the pathogenesis of BPD will be discussed.

2.2.1. Collagen in the lungs of infants with BPD

Increased hydroxyproline levels, implicating increased collagen levels, have been reported in the lungs from infants with RDS and BPD compared to age-matched controls (297, 298). Cherukupalli *et al.* found a decrease in type-I/type-III collagen ratios in the lungs from infants with BPD (298). This decrease implies a relatively increased synthesis of type III collagen, which is in concordance with biochemical and immunohistochemical studies of early fibrosis in the adult human lung (8, 41, 42). In addition to this, the N-terminal propeptide of type-III collagen has been detected in tracheal aspirates and serum from infants at risk for the development of BPD. Tracheal aspirate levels of the N-terminal propeptide of type-III collagen did not correlate with BPD development, while increased serum N-terminal propeptide of type-III collagen levels were detected when infants developed BPD (299). Shoemaker *et al.*, however, described elevated type-I/type-III collagen ratios in the lungs from infants ventilated because of respiratory insufficiency due to various causes (300). The discrepancy between the studies by Shoemaker *et al.* and Cherukupalli *et al.* may be due to differences in the control and experimental groups. Increased urinary excretion of hydroxyproline has been observed in infants with BPD and may also indicate increased pulmonary collagen synthesis (301). Altogether, these data implicate that there is an altered collagen metabolism in infants with BPD with a resultant increase in total lung collagen.

2.2.2. Fibrogenic mediators in the pathogenesis of BPD

Only few and largely descriptive data are available on fibrogenic mediators in BPD. Nothing is known yet about the contribution of fibrogenic mediators to the fibroproliferation and fibrogenesis in the pathogenesis of BPD.

Fibronectin (Fn) is known to stimulate fibroblast proliferation and chemotaxis and excessive amounts of Fn have been associated with pulmonary fibrosis (see table 1). Decreased levels of Fn in plasma have been shown to be associated with early RDS (302). It was demonstrated that Fn was higher in tracheal aspirates at weeks 3 and 4 of life in infants with BPD compared to infants with RDS at 1 week of life (302). It was suggested that low plasma Fn concentrations in early RDS contributed to the pulmonary capillary leak, and that high tracheal lavage Fn contributed to the development of pulmonary fibrosis in BPD (302).

Watts *et al.* distinguished between plasma Fn (pFn; which is synthesized by the liver) and cellular Fn (cFn; which, in the lung, is synthesized by alveolar macrophages, epithelial cells, fibroblasts, smooth muscle cells and endothelial cells) in tracheal aspirates (303, 304). Despite the structural differences between these two forms of Fn, their biological and immunological functions are remarkably similar (304). Watts *et al.* described increased levels of pFn in tracheal aspirates during the first two weeks of life from infants who developed BPD compared to those who recovered from RDS, suggesting a greater pulmonary capillary permeability in infants developing BPD (303). cFn levels in aspirates from infants with BPD were also higher in the first two weeks of life in comparison with infants who recovered from RDS. This suggested that increased synthesis of cFn occurs in the lungs dur-

ing BPD development and that this excessive Fn could contribute to the development of the pulmonary fibrosis in BPD (303).

Immunohistochemical findings are also suggestive for a role of cFn in the development of pulmonary fibrosis in BPD, as it has been demonstrated that the highest cFn expression in the BPD lung coexisted with excessive fibroblast proliferation (305).

TGF-β. Kotecha *et al.* found increased levels of both active and total (active + latent) TGF-β_1 in BAL fluid in the first week of life in infants who developed BPD compared to infants who recovered from the initial RDS and infants who required ventilation for non-pulmonary reasons (306). Findings for TGF-β_1 similar to those described by Kotecha *et al.* have been observed in tracheal aspirate fluids (307). Additionally, it has been demonstrated that tracheal aspirates from infants who were oxygen dependent at the time of discharge from the hospital contained higher levels of active TGF-β within the first 24 h of life than infants who were discharged without oxygen supplementation (308).

Immunocytochemical analysis of BAL cells with a pan-TGF-β antibody revealed alveolar macrophages and to a lesser extent neutrophils and epithelial cells as potential sources for TGF-β in the pathogenesis of BPD (306). Intense immunoreactivity for TGF-β has been found in alveolar macrophages and myofibroblasts in lung tissue from infants who died because of BPD, and myofibroblasts were found to infiltrate alveoli at sites rich in TGF-β (309). In addition to TGF-β, increased expression of TGF-α and its receptor (epidermal growth factor receptor) has been demonstrated in alveolar epithelium, vascular smooth muscle cells and alveolar macrophages in lung tissue from late stage BPD (310).

Endothelin-1 is a mediator with both chemoattractant and mitogenic properties as well as a stimulant of fibroblast collagen synthesis and has been associated with pulmonary fibrosis (125-128). A role for endothelin-1 has been implicated in BPD development, as increased levels were present in tracheal aspirates during the first week of life from infants who subsequently developed BPD (311).

Inflammatory cytokines such as IL-1 and TNF-α have clearly been shown to be associated with pulmonary fibrosis and have been found to be increased in lung effluent fluids from infants who develop BPD compared to resolving RDS. Data on IL-1 and TNF-α in BPD and their contribution to acute lung injury and fibrosis have been discussed in the section "Inflammation in the pathogenesis of BPD" (chapter 1.4.4.) of this thesis.

So far only limited data is available on the presence of "classical" fibrogenic mediators in the pathogenesis of BPD. Furthermore, no data is available on the mechanisms via which fibrogenic mediators could be involved in the pathogenesis of BPD.

2.2.3. Coagulation cascade in the pathogenesis of BPD

Intravascular and intra-alveolar deposition of fibrin has been described in preterm infants with RDS (312, 313). These fibrin deposits are the result of activation of the coagulation system. In concordance with this, it has been demonstrated that levels of TATIII (a sensitive and specific marker of thrombin generation) were increased in the plasma from infants with severe RDS compared to infants with mild to moderate RDS (314-316). Viscardi *et al.*

demonstrated that procoagulant activity, mainly related to tissue factor associated with factor VII, was detectable in BAL fluid from infants with both RDS and BPD and did not differ from control infants (317). Furthermore, it has been demonstrated in a premature baboon model for BPD development that procoagulant activity, mainly due to tissue factor associated with factor VII, increased in BAL fluid after 6 and 21 days of hyperoxia compared to the first 24 hours of RDS (318). Although there is indirect evidence of thrombin activation in the alveolar compartment during the development of BPD, to date no reports have been published on thrombin activity or TATIII in the alveolar compartment of these infants.

2.2.4. Matrix metalloproteinases in the pathogenesis of BPD

Increased levels of the collagenase MMP-8 in tracheal aspirate and BAL fluid within the first week of life have been found to be associated with BPD development from RDS (319, 320). As opposed to MMP-8, the collagenase MMP-1 was undetectable in BAL fluid, obtained during the first 6 postnatal days, from infants with resolving RDS and infants who developed BPD (319). Recently, it was demonstrated that oxidative stress increased the expression of the gelatinase MMP-9 and the inhibitor TIMP-1 in BAL fluid obtained within the first 6 postnatal days of ventilated premature infants. The ratio of MMP-9 to TIMP-1 levels, however, did not alter with increasing oxygen toxicity. But the methods used in this study did not enable the determination of changes in net enzyme activity (321). It has been described that tracheal aspirate levels of both MMP-2 and MMP-9 within the first 5 postnatal days were not associated with BPD development from RDS, while low levels of TIMP-2 were found to be associated with an unfavorable respiratory outcome in ventilated premature infants with RDS (320).

This data suggests the possible involvement of different MMPs and TIMPs in the pathogenesis of BPD. Data on MMP expression in BAL fluid during RDS and BPD development is still scarce. Also, nothing is known yet about the long-term expression and cellular source of different MMPs and TIMPs in the pathogenesis of BPD.

2.3. Effect of corticosteroid treatment on collagen and fibrogenic mediators in pulmonary fibrosis and BPD

Corticosteroids have the capability to directly regulate collagen synthesis by fibroblasts via different mechanisms. It has been demonstrated that dexamethasone (Dex) reduced collagen synthesis by fibroblasts *in vitro* and *in vivo* (322, 323). Such an effect of Dex on collagen synthesis by fibroblasts might be species or organ specific, as recent evidence indicated that Dex increased collagen production by human fetal lung fibroblasts (324). Furthermore, Dex may decrease the collagenase expression by fibroblasts or influence fibroblast proliferation (324-326).

Several studies have demonstrated that steroids such as methylprednisolone and Dex inhibit lung inflammation and pulmonary fibrosis in different animal models (327-330).

Corticosteroid treatment is the mainstay of therapy in pulmonary fibrosis, however, the response of such patients to corticosteroids is minimal (331). Although alveolar macrophages from patients with pulmonary fibrosis have been shown to contain glucocorticoid receptors, corticosteroid treatment did not alter the secretion of fibroblast mitogens by these cells (332). Therefore, it has been suggested that the inability of steroids to suppress the release of fibroblast mitogens by alveolar macrophages may account in part for the limited effect of steroids in pulmonary fibrosis (332). In patients who did respond to corticosteroid treatment a decrease in serum procollagen type-III peptide has been observed (333). This implicates that a good response to treatment does interfere with collagen synthesis.

It has been suggested that corticosteroids may only be effective when there is a major inflammatory component associated with the disease state (334). Acute respiratory distress syndrome (ARDS) is a rapid fibrosing lung disease characterized by an early inflammatory and fibroproliferative phase. The inflammatory phase is increased and persists in non-survivors with ARDS (335). It has been reported that unresolving ARDS patients with histological evidence of dense acellular fibrosis at open lung biopsy do not improve with methylprednisolone treatment. Such treatment was found to be more effective when given early in the fibroproliferative phase, before the occurrence of dense acellular fibrosis. In this early phase the treatment led to a decrease in plasma and BAL fluid levels of the N-terminal propeptides of type-I and -III collagen (336, 337). This antifibrotic action of methylprednisolone was associated with improvement of pulmonary function (337). From this it becomes clear that the initiation time of corticosteroid treatment may be crucial for its effect on the development of pulmonary fibrosis.

Dex treatment has been clearly shown to result in a clinical improvement and a reduced inflammatory response in the lungs of infants with or at risk of BPD, as has been discussed in chapter 1.5. of this thesis. However, little is known about the effect of Dex treatment on fibrogenic mediators and fibrosis in the lungs from infants with or at risk of BPD. Gerdes *et al.* demonstrated that Dex treatment did not influence Fn concentrations in BAL fluid from infants who had developed or were developing BPD (338). From this study it was concluded that Dex treatment may not inhibit the development of pulmonary fibrosis in infants with BPD.

Watts *et al.* demonstrated that Dex did decrease total Fn, cFn and pFn in tracheal aspirates from infants with BPD who clinically improved and survived (339). Additionally, in infants without clinical improvement who died after Dex, Fn levels were three to four times greater at start of the Dxm therapy than in the surviving infants. Although the levels of Fn decreased in this group after Dex, they remained increased compared to levels in surviving infants throughout therapy. A third group of infants initially showed clinical improvement, but died. This group had significantly lower levels of Fn at the start of the therapy than the survivors had, however, their levels remained unchanged after Dex (339). From this study it was suggested that Dex has the potential to decrease the lung production of Fn and thereby may limit the ongoing fibrosis in BPD.

Treatment with Dex or hydrocortisone has been shown to have no effect on the levels

of total TGF-β_1 and biologically active TGF-β in lung fluids from infants with or at risk of BPD (307, 308). This is in concordance with a study by Khalil *et al.* demonstrating that Dex did not alter the increased TGF-β secretion by alveolar macrophages that were activated *in vivo* by bleomycin-induced injury (180).

Co *et al.* demonstrated that Dex treatment of infants with BPD resulted in decreased urinary hydroxyproline excretion, indicating decreased collagen synthesis (301). Although the assay used in the study by Co *et al.* was not specific for lung collagen, the authors speculated that Dex therapy resulted in an overall suppression of collagen synthesis including a reduction in pulmonary fibrosis (301). No data are available yet on the effect of Dex treatment on fibroblast activity and on the collagen synthesis and accumulation in the lungs from infants with or at risk of BPD. Therefore, the effect of Dex treatment on pulmonary fibrosis in BPD remains unclear.

2.4. Concluding remarks

Pulmonary fibrosis develops after a pulmonary insult followed by tissue injury, subsequent inflammation and a repair attempt. In this process the control of fibroblast function is lost, presumably due to an overproduction and sustained presence of fibrogenic mediators that may be derived from inflammatory cells, resident lung cells or the blood. Eventually this results in excessive fibroblast proliferation and collagen deposition which may lead to BPD. So far only limited data is available on the production of fibrogenic mediators and their contribution to fibroblast activation in BPD. In addition, little insight is available on the effect of the commonly used Dex treatment on the pulmonary expression levels of fibrogenic mediators and progression of fibrosis in BPD.

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Chapter

AIMS OF THE STUDIES

The overall aim of the studies was to increase the insight into the process of fibroproliferation and fibrogenesis during the development of BPD. The studies described in this thesis are based on the analysis of BAL fluid from RDS infants with and without subsequent BPD development and on immunohistochemical analysis of lung tissue from infants who died at different phases of BPD development. In addition, studies were performed in which pulmonary fibrosis was induced in rats via intratracheal instillation of the antitumor agent bleomycin.

Fibroblast proliferation is a central process in the development of pulmonary fibrosis. In adult patients with fibrosing lung disease it has been demonstrated that BAL fluid contained increased mitogenic activity for fibroblasts as well as increased concentrations of fibrogenic mediators.

Increased numbers of fibroblasts and excessive collagen deposition are hallmarks in lung tissue from infants that died with BPD. Increased serum levels of the N-terminal propeptide of type-III collagen have been observed in the serum of infants who develop BPD. In addition to this, fibrogenic mediators such as fibronectin and TGF-β have been found increased already within the first week of life in the lungs of infants with subsequent BPD. These data suggest that increased fibroproliferation and fibrogenesis may already occur within the first week of life in infants who develop BPD.

In chapter 4 the mitogenic potential of BAL fluid for human fetal lung fibroblasts is described. The analyzed BAL fluids were obtained at different postnatal ages from premature ventilated infants with resolving RDS and from infants that acquired BPD. Thrombin, which exerts a central role in blood coagulation, is a fibroblast mitogen that is present in increased levels in the lungs of adult patients with pulmonary fibrosis and in animal models of pulmonary fibrosis. The presence of fibrin-rich hyaline membranes in the lungs during BPD development suggests that extravascular coagulation occurs in the lungs when BPD develops. Indeed, procoagulant activity has been demonstrated in BAL fluid from infants with RDS and subsequent BPD. However, thrombin activity has never been described in the lungs from premature infants with RDS and subsequent BPD. Therefore, the presence of active thrombin in BAL fluid and its contribution to BAL fluid-induced fibroblast proliferation was determined. In addition to thrombin activity in BAL fluid, the thrombin-antithrombin III complex (TATIII) was determined as a marker of thrombin generation.

Dexamethasone (Dex) treatment is commonly used in infants with or at risk of BPD. Dex clearly improves pulmonary function in such infants and the effect is probably partly established via downregulation of pulmonary inflammation. The effect of Dex treatment on fibroblast activity and fibrogenic mediators in the lungs of infants at risk of BPD is unclear yet. In chapter 5 the effect of Dex treatment on the BAL fluid mitogenic potential and the fibrogenic mediator PDGF-BB are described. As a marker of fibrogenesis in the lungs, the N-terminal propeptide of type-III collagen was determined in BAL fluid. To address the anti-inflammatory effect of Dex, the cytokine IL-1β and the inflammatory cell population were examined in BAL fluid.

MMP-1 (interstitial collagenase) is an enzyme capable of degrading the fibrillar col-

lagens (type-I and -III) that are deposited in the lungs in the case of fibrosis. Studies have implicated that dysregulation of MMP-1 activity by its inhibitors TIMP-1 and TIMP-2 is likely to contribute to excessive collagen accumulation in the lungs. Furthermore, MMP-1 has been implicated in reepithelialization after injury. As the pathology of BPD is characterized by excessive pulmonary injury followed by reepithelialization of the injured areas and the development of fibrosis, MMP-1 may play an important role in BPD development. In chapter 6 the immunolocalization and staining intensity of MMP-1, TIMP-1 and TIMP-2 are described in lung tissue in different phases of BPD development.

MMP-2 (gelatinase A) and MMP-9 (gelatinase B) have been described to participate in pulmonary fibrotic disorders in both adult patients and animal models. Gelatinases are suggested to play a role in the acute injury phase of lung disease as well as in the regeneration of the epithelium after lung injury. It has been described that oxidative stress in newborn babies increases both MMP-9 and its inhibitor TIMP-1. The presence and kinetics of expression of gelatinases in BAL fluid and lung tissue from infants with RDS and subsequent BPD are unclear yet. In chapter 7 these aspects were investigated during the first 10 days of life in BAL fluid from infants who did and did not develop BPD. Additionally, the immunolocalization of MMP-9 is described in lung tissue from infants who died at different phases of BPD development.

Bleomycin-induced pulmonary injury with subsequent pulmonary fibrosis is a well-characterized animal model to study the pathogenesis of the fibrotic response in the lungs. This model is characterized by recruitment and activation of inflammatory cells and increased proliferation and collagen synthesis by fibroblasts. A variety of cytokines and growth factors such as IL-1, PDGF, TGF-β_1 and thrombin are implicated in the modulation of the inflammatory response and in regulating the pathogenesis of the developing pulmonary fibrosis. Corticosteroids are the mainstay of treatment in patients with pulmonary fibrosis and have been shown to reduce pulmonary fibrosis in the bleomycin model. Short-course Dex treatment has been demonstrated to increase survival without BPD in prematurely born infants. So far the effect of a short-course Dex treatment on the fibrotic reponse in the lungs is unclear. Therefore, we studied whether a three-day course of Dex, initiated three days after the induction of lung injury by bleomycin, was able to reduce the developing pulmonary fibrosis. In addition, we studied whether the effect of short-course Dex treatment could be related to cell proliferation and to the levels of the fibrogenic mediators TGF-β_1, PDGF-AB and thrombin in BAL fluid. The results of this study are described in chapter 8.

In chapter 9, the findings presented in chapters 4 through 8 are discussed in the context of current literature data. In this chapter also suggestions are done for further studies in this field.

Chapter

BAL FLUID FROM PRETERM INFANTS IS MITOGENIC FOR LUNG FIBROBLASTS

submitted

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This work was supported by a grant from: Sophia Foundation For Medical Research

Abstract

Aims: To determine whether bronchoalveolar lavage (BAL) fluid from preterm infants with resolving respiratory distress syndrome (RDS) and those developing bronchopulmonary dysplasia (BPD) differ in their mitogenic activity for fibroblasts, and if thrombin contributes to the mitogenic activity and is detectable in BAL fluid. **Methods:** Sequential BAL (postnatal days 2-14) was obtained from 37 ventilated infants with RDS. Twenty six infants developed BPD whereas eleven resolved. BAL fluid mitogenic activity was determined in a proliferation assay using human fetal lung fibroblasts. The contribution of thrombin to the mitogenic activity was determined using the thrombin inhibitor PPACK. Furthermore, thrombin levels in BAL fluids were measured using a specific substrate to detect thrombin activity and by measuring thrombin-antithrombin III complex (TATIII). **Results:** BAL fluid mitogenic activity was comparable between BPD and RDS (BPD: 33% proliferation on d2 to 41% on d14; RDS: 21% on d2 to 54% on d7). Thrombin inactivation by PPACK completely inhibited mitogenic activity in BAL samples obtained from d2 and d4 (BPD: $p < 0.001$ on d2 and d4; RDS: $p < 0.05$ on d4). From d7 onwards, inhibition of thrombin only partly reduced ($p < 0.05$) BPD BAL fluid mitogenic activity, indicating that other mitogenic factors contribute as well. Surprisingly, thrombin activity and TATIII were decreased in BAL fluid from BPD compared with RDS patients at d2 and d4. **Conclusions:** Our study shows that BAL fluid from infants with and without BPD development is equally mitogenic for lung fibroblants and that thrombin is a major mitogen in these samples. This study suggests that fibroproliferation occurs early in the lungs of preterm infants with both BPD and RDS and that thrombin may play an important role in these disorders.

Introduction

Neonatal respiratory distress syndrome (RDS) is mainly caused by lung immaturity with concomitant surfactant deficiency. RDS may resolve (uncomplicated RDS), or progress towards bronchopulmonary dysplasia (BPD) also named chronic lung disease of prematurity (1).

Histologically, RDS is characterized by the presence of alveolar and bronchiolar fibrin-rich hyaline membranes (2). During the progression towards BPD, excessive fibroblast proliferation occurs and hyaline membranes are replaced by fibrous tissue (3). In infants who developed BPD, increased levels of fibrogenic growth factors such as transforming growth factor-β_1 (TGF-β_1) and fibronectin have been described in BAL fluid during the first week of life (4, 5). Furthermore, N-terminal propeptide of collagen type-III, an indicator of collagen type-III synthesis, is present in lung effluent fluids and increased in serum from infants at risk for BPD (6). These data suggest that increased fibroproliferation and fibrogenesis may already occur during the first week of life, and play a role in the pathogenesis of BPD.

However, still little is known about the nature of mediators involved in these processes.

The serine protease thrombin plays a central role in the coagulation cascade by converting fibrinogen to insoluble fibrin (7). In addition to its role in haemostasis, thrombin may also act as a profibrotic mediator as it has been shown to stimulate fibroblast chemotaxis, proliferation and synthesis of extracellular matrix components as well as the production of fibrogenic mediators such as platelet-derived growth factor (PDGF) and TGF-β_1 (8-11). Thrombin activity has shown to be present in BAL fluid from experimental animals and adult patients with fibrosing lung disease and to contribute to BAL fluid-induced fibroblast proliferation (12, 13).

It has been demonstrated that vascular blood clotting is activated in infants with RDS and that the degree of activation correlated with disease severity (14). In addition, the appearance of intra-alveolar fibrin-rich hyaline membranes during the pathogenesis of RDS/BPD implies that extravascular coagulation occurs in the injured lung. Procoagulant activity has been shown to be present in BAL fluid when BPD develops (15). However, the presence of thrombin activity in BAL fluid during BPD development has never been shown.

Given the increased presence of markers for fibroproliferation and fibrogenesis in the first week of life when BPD develops, we hypothesized that BAL fluid from infants who develop BPD contains increased mitogenic activity for lung fibroblasts compared to BAL fluid from infants with an uncomplicated course of RDS. In addition, we expected thrombin levels to be increased and to contribute to BAL fluid mitogenicity when BPD develops. Therefore, in this study we included patients who did and did not develop BPD from RDS. BAL fluid, obtained during the first 14 days of life, was examined for mitogenic activity for human fetal lung fibroblasts. We determined the presence of thrombin activity in BAL fluid and assessed whether thrombin contributed to this mitogenic activity. In addition, as a marker for thrombin generation, we determined the thrombin-antithrombin III complex (TATIII) in BAL fluid.

Materials and methods

Patients

Thirty seven prematurely born infants, admitted to the neonatal intensive care unit, were included in this study. Inclusion criteria were: (1) gestational age $\leq$ 30 weeks and (2) requirement for mechanical ventilation on the first day of life because of RDS. Informed consent was obtained and the study was approved by the local medical ethics committees. Serial BAL was performed in a standardized way on postnatal days 2, 4, 7, 10, and 14, as long as the infant remained intubated. BPD was defined as having an abnormal chest radiograph and requirement for supplemental oxygen at 28 days of life (16).

Bronchoalveolar lavage

BAL was performed as follows, the infant was placed in supine position with its head

turned left. A 5' French straight suction catheter was inserted via the sideway of a 3-way endotracheal tube connector. The catheter was placed into wedge position and two aliquots of 1ml/kg of saline were instilled. BAL samples were aspirated into a suction trap using 60 cm H_2O negative pressure. The total lavage time was less than 1 min. Samples were put on ice immediately and processed within one hour. Samples were centrifuged (10 min, 1500 rpm, 4°C). Thereafter, the fluid fraction was centrifuged for 10 min at 10000 rpm at 4°C to remove debris and stored in aliquots at -80°C. Cell numbers were determined using a haemocytometer. May Grünwald Giemsa staining was performed and cell differentials were determined by counting 300 cells per cytospin.

Fibroblast culture and proliferation assay

The human fetal lung fibroblast cell line HFL-1 (American Type Culture Collection, Maryland, U.S.A) was cultured in Dulbecco's modified Eagle's medium (DMEM; Bio Whittaker Europe, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS; Bio Whittaker Europe), 4 mM ultraglutamine-I, antibiotics (penicillin, 100 U/ml and streptomycin, 100 µg/ml), and 15 mM Hepes buffer (Bio Whittaker Europe). Cells were grown in a humidified atmosphere of 5% CO_2 in air at 37°C. Proliferation was assessed by seeding fibroblasts (6 x 10^3 cells per well in 50 µl DMEM containing 0.4% FCS) into 96-well microtitre plates and allowing them to adhere for 24 h. BAL fluid was diluted 8 times in DMEM containing 0.4% FCS and in DMEM/0.4% FCS containing 2.3 x 10^{-6} M of the thrombin inhibitor PPACK (D-Phenylalanine-Proline-Arginine-Chloromethyl Ketone, Dihydrochloride; Calbiochem, Darmstadt, Germany). 50 µl of the 1/8 dilutions of BAL fluid was added in triplicate to the fibroblasts (yielding a final 1/16 dilution of BAL fluid as preliminary experiments showed this to be optimal). As control, fibroblasts were stimulated with thrombin (1U/ml DMEM/0.4% FCS; ICN Biomedicals Inc, Oh) and thrombin plus PPACK. After 72 h, the proliferative response was determined using a colorimetric assay based on the uptake and subsequent release of methylene blue dye (17). Briefly, medium was aspirated and cells were washed with phosphate buffered saline. Cells were fixed with 10% formol saline for 1/2 h and then stained with 1% methylene blue in 0.01 M borate buffer, pH 8.5. Excess dye was removed from the plate and bound dye was eluted from the cell layer by the addition of 100 µl of acidified alcohol (0.01 M HCl-ethanol absolute, 1:1). Absorbance was measured at a wavelength of 650 nm on a microplate spectrophotometer. Fibroblast proliferation was expressed as a percentage change in mean absorbance above that of cells exposed to medium containing 0.4% FCS alone. DMEM supplemented with 5% FCS was used as a positive control for fibroblast proliferation. Fibroblast proliferation assessed by this assay was validated against proliferation assessed by direct cell counting and tritium thymidine incorporation and revealed comparable results.

Analyses of BAL fluid

Thrombin activity was determined using the thrombin specific chromogenic substrate Tos-Gly-Pro-Arg-pNA (Sigma, St Louis, MO) (18). Briefly, 25 µl of BAL fluid was diluted

in 25 µl Tris-buffered saline (TBS; pH 8.3) and 25 µl BAL fluid was diluted in TBS containing 4 x 10^{-6} M of the thrombin inhibitor PPACK. These mixtures were added to a 96 well microtitre plate and incubated for 20 min at 37 °C to allow thrombin-PPACK complexes to form. Thereafter, 50 µl of 1mM Tos-Gly-Pro-Arg-pNA (in 1.5 mM HCL) was added to the BAL fluid dilutions and incubated at 37 °C. The optical density (OD) was measured at 405 nm for a period of 48 h. During this time period a linear increase in optical density of the BAL samples was found (data not shown). Thrombin activity in every BAL sample is expressed as an OD value at 405 nm, which was determined as the difference in OD, measured at 48 h, between the BAL sample without and with PPACK. As a control for the assay 1 x 10^{-6} M of thrombin (Sigma, St Louis, MO) was also added to the substrate. This resulted in an optical density which was never reached by one of the BAL fluid samples. Preincubation of thrombin with PPACK resulted in complete inhibition of thrombin activity.

TATIII levels were determined using a commercially available enzyme immunosorbent assay (Kordia Laboratory Supplies, Leiden, The Netherlands). Results were expressed as picomolar (pM) TATIII in BAL fluid.

Active TGF-β_1 levels were determined using a commercially available enzyme-linked immunosorbent assay (Promega Corp., Madison, Wis.). Results were expressed as pg/ml BAL fluid.

Statistical Analysis

Results for BAL fluid-induced fibroblast proliferation within the patient groups are presented as median values. Because BAL was performed in a standardized way, we did not use a marker of dilution, but expressed measurements per volume unit of BAL fluid as recommended by the ERS task force on BAL in children (19). Data for measurements in BAL fluid are presented as mean values (± SEM) (4). The Mann-Whitney U test was used to compare between groups. The effect of thrombin inhibition by PPACK on BAL fluid-induced fibroblast proliferation was analyzed using the paired students t-test. Pearson's correlation was used to compare the relationship between different parameters determined in BAL fluid and parameters in BAL fluid with patient characteristics. A p value < 0.05 was considered significant.

Results

Patient characteristics

Seventy BAL procedures were performed in 37 preterm infants. Twenty six infants subsequently developed BPD and 11 recovered from the initial RDS. Infants who developed BPD were more immature and had a lower birthweight than those that recovered from RDS (table 1).

Table 1. Patient characteristics

	BPD	RDS
Number	26	11
Gestation* (weeks)	27.4 (25.6-29.4)	28.3§ (26.7-30)
Birth weight* (grams)	930 (760-1525)	1125§ (780-1650)
Surfactant$	24 (92.3%)	9 (81.8%)
Antenatal steroids$	22 (84.6%)	8 (72.7%)

* median value (range)
$ number of patients treated (percentage treated)
§ $p < 0.05$ vs BPD

Bronchoalveolar lavage

For all performed BAL procedures the recovery was 50 ± 2% (mean ± SEM) from the initial lavage volume used. No difference in recovery was observed between the different groups at the different postnatal ages or within a patient group during the study period. Due to extubation, BAL samples from RDS patients were only obtained until day 7. The BAL cell population consisted mainly of neutrophils and macrophages of which neutrophils were predominant (Fig. 1). No significant difference in the number of neutrophils and macrophages per ml BAL fluid was observed between RDS and BPD, and the number of neutrophils remained relatively constant during the study period (Fig. 1A). In contrast, an increase in the number of macrophages per ml BAL fluid was observed in the BPD group, but not in the RDS group during the study period (Fig. 1B).

BAL fluid-induced fibroblast proliferation

All BAL fluids, apart from one (BPD day 2), stimulated fibroblast proliferation above media control levels. In the BPD group the median BAL fluid-induced fibroblast proliferation ranged from 33% on day 2 to 41 % on day 14. In the RDS group median BAL fluid-induced fibroblast proliferation ranged from 21% on day 2 to 54 % on day 7 (Fig. 2). Although the mitogenic activity of BAL fluid from the BPD group tended to be greater than that of the RDS group at postnatal day 2, no statistical significant difference was observed at any time-point between the BPD and RDS group.

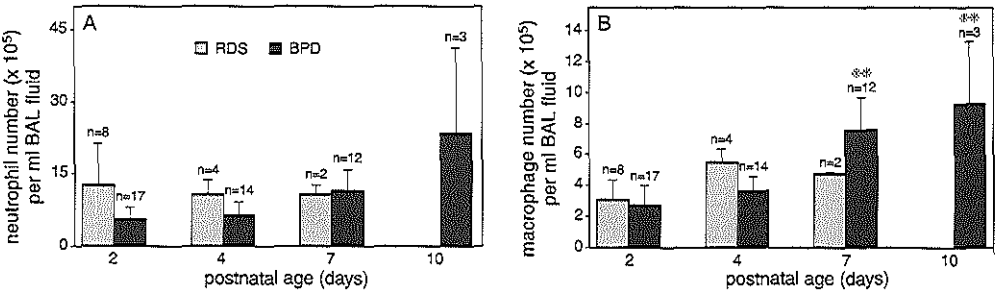

Figure 1
The number (mean ± SEM) of neutrophils (A) and macrophages (B) per ml BAL fluid at different postnatal ages in RDS and BPD group. ** $p < 0.05$ compared to postnatal day 2. n represents the number of patients in each group at the different postnatal ages. Due to unavailability of cell differentials from some samples, no value for BPD at a postnatal age of 14 days is shown.

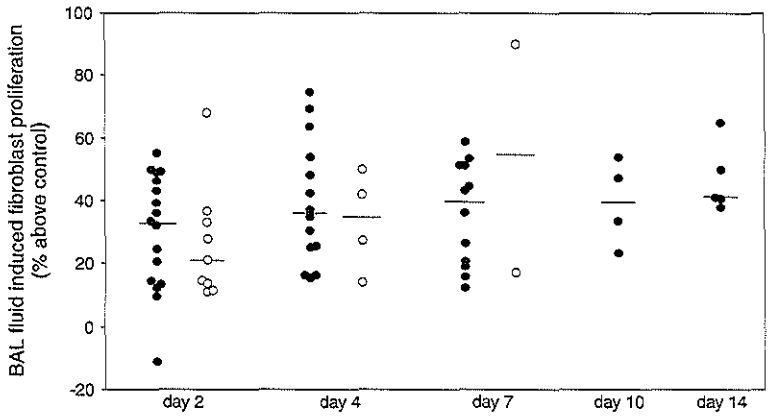

Figure 2
The effect of a 1/16 dilution of BAL fluid on fibroblast proliferation per individual at postnatal ages of 2, 4, 7, 10 and 14 days. Bars represent median values within each group (o = RDS, • = BPD).

Inhibition of BAL fluid-induced fibroblast proliferation by PPACK. Thrombin (1 U/ml) induced fibroblast proliferation was completely blocked by PPACK (data not shown). PPACK inhibited the fibroblast mitogenic activity of BAL fluid in 16 of the 17 BPD samples on day 2. This resulted in significant inhibition ($p < 0.001$) of fibroblast proliferation to media control levels. PPACK inhibited day 4 BAL fluid-induced fibroblast proliferation in all 14 BPD samples. For the whole group this resulted in significant inhibition ($p < 0.001$) down to control levels. Comparable to day 2 some BAL fluid samples retained mitogenic activity after addition of PPACK. PPACK inhibited day 7 BAL fluid-induced fibroblast proliferation in 8

out of 12 BPD samples, resulting in a significant inhibition (from 40% to 23 % proliferation; $p < 0.05$) for the whole group (Fig. 3). PPACK inhibited day 10 BPD BAL fluid-induced proliferation in 2 out of 4 samples without reaching statistical significance for the whole group (from 40% proliferation without PPACK to 30% with PPACK). In contrast, PPACK inhibited proliferation from all 5 day 14 BPD samples, resulting in a reduction of proliferation from 41% to 31% ($p < 0.05$) for the whole group.

In the RDS group, PPACK inhibited day 2 BAL fluid-induced fibroblast proliferation in 5 out of 9 samples, resulting in a reduction of proliferation from 21% to 17% for the whole group (not significant). PPACK blocked BAL fluid-induced fibroblast proliferation in all day 4 RDS samples, resulting in inhibition of proliferation ($p < 0.05$) down to media control levels for this group. Inhibition of BAL fluid-induced fibroblast proliferation with PPACK was not attempted for the day 7 RDS samples because there were only 2 samples available.

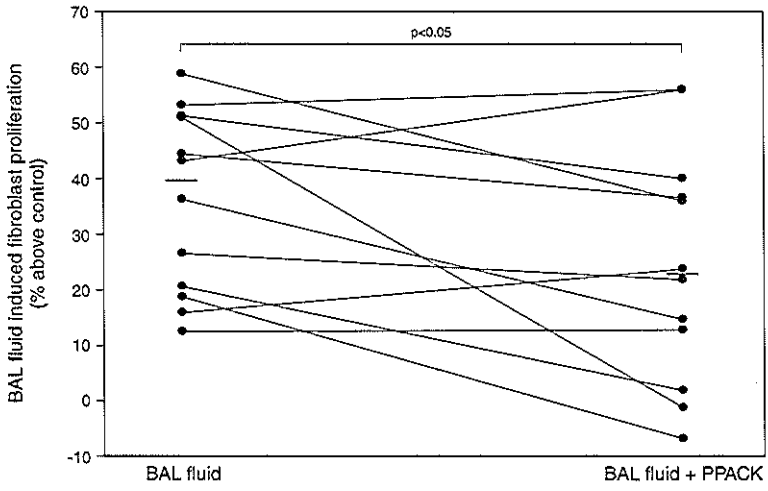

Figure 3
The effect of the thrombin inhibitor PPACK on BAL fluid-induced fibroblast proliferation from BPD patients with a postnatal age of 7 days. Lines represent individual BAL fluid samples with and without PPACK. Bars represent median values.

Analyses of BAL fluid

Thrombin activity. Day 2 RDS samples contained increased thrombin activity compared to BPD ($p = 0.06$). Thrombin activity in day 4 samples from infants with resolving RDS was significantly ($p < 0.05$) increased compared to the BPD group (Fig. 4). Thrombin activity in BAL fluid significantly increased ($p < 0.05$) in the BPD group with increasing age, to a level comparable with the RDS group at all examined time-points (Fig. 4).

To determine whether the lower thrombin activity in the BPD group was related to relative immaturity of these infants compared to infants with resolving RDS, we determined the

correlation between thrombin activity in day 2 BAL samples with the gestational age. Thrombin activity in day 2 samples showed a significant positive correlation with gestational age ($r = 0.463$; $p < 0.05$).

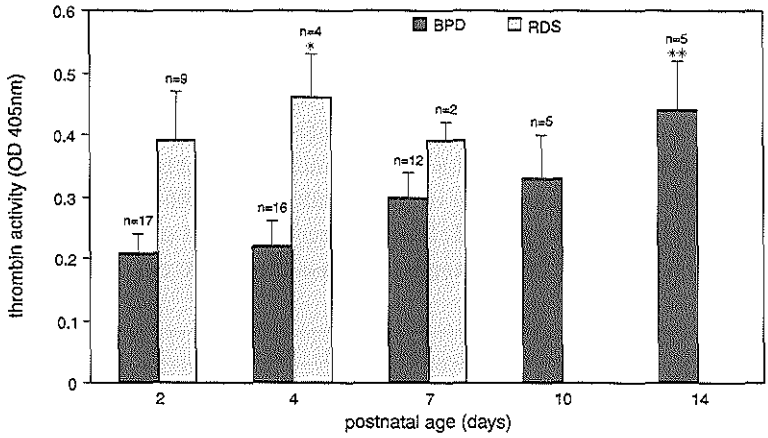

Figure 4
Thrombin activity (mean value ± SEM) in BAL fluid at different postnatal ages in RDS and BPD group. * $p < 0.05$ compared to BPD; ** $p < 0.05$ compared to postnatal day 2. n represents the number of patients in each group at the different postnatal ages.

TATIII. Antithrombin III (ATIII) is a naturally occurring inhibitor of thrombin which inactivates thrombin by forming a thrombin-antithrombin III complex (TATIII). TATIII has been used as a biochemical marker of thrombin generation (20). BAL fluid from infants with resolving RDS at postnatal days 2 and 4 revealed increased levels of TATIII compared to the BPD group at these ages. However, the difference in TATIII was not statistically significant (day 2: $p = 0.16$ and day 4: $p = 0.06$; Fig. 5.). As for thrombin activity, TATIII in BAL fluid increased significantly during BPD development reaching a maximum at day 10 (Fig. 5.) No correlation was found between TATIII levels in day 2 BAL fluid and gestational age. TATIII was determined in a total of 64 BAL samples. In these samples TATIII levels correlated positively with thrombin activity ($r = 0.616$; $p < 0.001$; Fig. 6).

Active TGF-β_1. Active TGF-β_1 has been described to be increased in BPD BAL fluid obtained during the first week of life compared to RDS BAL fluid, and to gradually decline with increasing age (4). To compare the samples in our patient groups with this previous study we determined active TGF-β_1 in a total of 54 BAL fluid samples. Active TGF-β_1 was elevated in BPD BAL fluid at postnatal day 4 compared to the RDS group at this age but declined thereafter (Fig. 7). No correlation was observed between active TGF-β_1 levels and thrombin activity or TATIII levels. Furthermore, no correlation was observed between the concentration of active TGF-β_1 in BAL fluid from postnatal day 2 with gestational age.

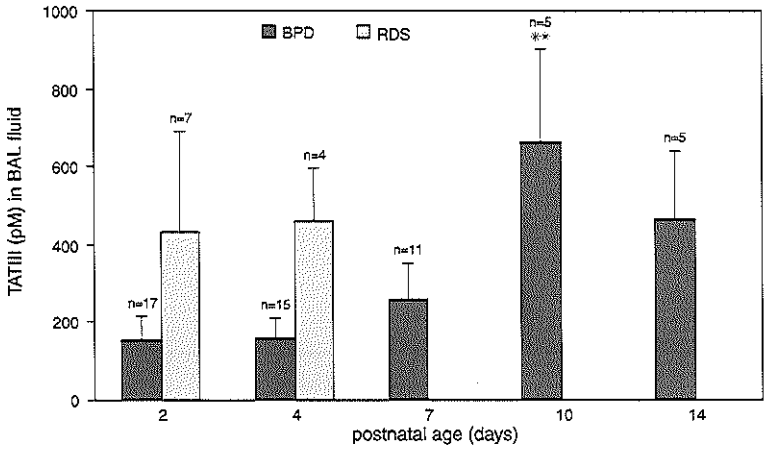

Figure 5
TATIII level (mean value ± SEM) in BAL fluid at different postnatal ages in the RDS and BPD group. ** $p < 0.05$ compared to postnatal day 2. n represents the number of patients in each group at the different postnatal ages. Due to shortage of BAL fluid TATIII was not determined in day 7 RDS samples.

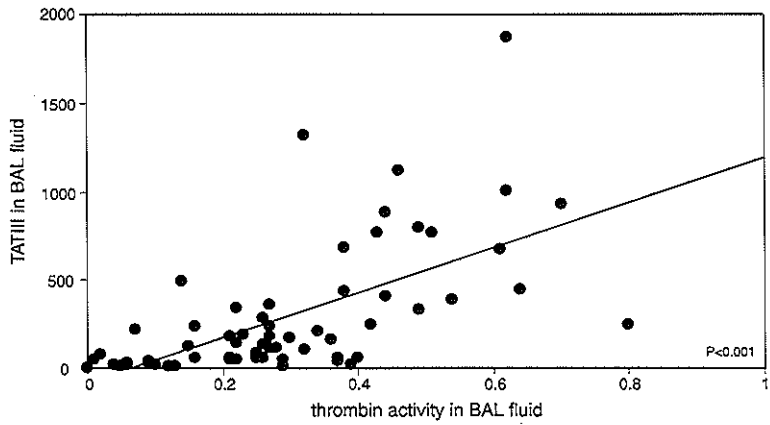

Figure 6
Correlation between BAL fluid TATIII levels and thrombin activity in 64 BAL fluid samples.

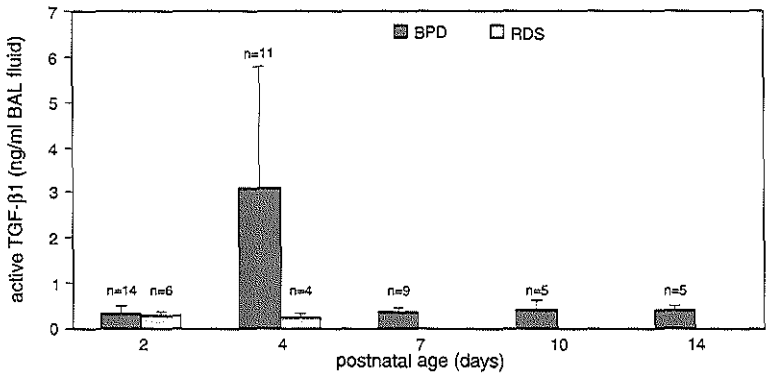

Figure 7
Active TGF-β_1 levels (mean value ± SEM) in BAL fluid at different postnatal ages in RDS and BPD group. n represents the number of patients in each group at the different postnatal ages. Due to shortage of BAL fluid TATI-II was not determined in day 7 RDS samples.

Discussion

In our study we found no difference in mitogenic activity of BAL fluid between the BPD and RDS groups in the first week of life. Acute lung injury begins with an inflammatory phase that is followed by a repair phase. It is generally accepted that orderly repair involves the migration and proliferation of endothelial cells, epithelial cells and fibroblasts (21, 22). Therefore, it is likely that the mitogenic activity in BAL fluid from infants with resolving RDS is a normal physiological response to the initial lung injury and presumably contributes to repair. We were not able to obtain samples from resolving RDS after 7 days of age for ethical reasons. Therefore, it is not possible to draw definite conclusions regarding the likelihood that prolonged mitogenic activity in the lungs from patients with BPD plays an important role in driving the fibrotic response in these patients. Nevertheless, the observation that BAL fluid from patients with BPD contained significant mitogenic activity to a postnatal age of 14 days makes it tempting to speculate that prolonged mitogenic activity in the lungs may result in chronic fibroblast activation leading to excess fibroblast proliferation, thereby contributing to the development of fibrosis in BPD.

PPACK inhibited BAL fluid-induced fibroblast proliferation in only 5 out of 9 day 2 RDS samples, indicating that factors other than thrombin are present and contribute to proliferation. PPACK inhibited BAL fluid-induced fibroblast proliferation in all (except one) BAL samples from the BPD group at postnatal ages of 2 and 4 days and the RDS group at day 4. In the majority of the samples PPACK completely inhibited mitogenic activity, indi-

cating that thrombin is a major fibroblast mitogen present in these samples. Although PPACK attenuated BAL fluid-induced fibroblast proliferation in BPD patients from 1 week of age, remarkable fibroblast mitogenicity remained present in these samples. This indicates that mitogenic factors other than thrombin become important from 1 week of age when BPD develops. Alveolar macrophages play an important role in pulmonary fibrosis as producers of fibroblast mitogens such as PDGF, insulin like growth factor (IGF) and tumor necrosis factor (TNF)-α (21). We observed an increase in alveolar macrophages in BAL samples from 1 week of age in the BPD group. Therefore, it is likely that these alveolar macrophages produce and secrete fibroblast mitogens which contribute to BAL fluid-induced fibroblast proliferation from postnatal day 7 onwards.

Although thrombin clearly contributed to BAL fluid mitogenicity at postnatal ages of 2 and 4 days in the BPD group, we surprisingly found that these samples contained lower thrombin activity than the RDS samples. There are four possible explanations for these findings. First, BAL fluid at postnatal days 2 and 4 from BPD patients may contain increased amounts of factors that synergistically increase the proliferative effect of thrombin present in BAL fluid compared to the RDS group. Indeed it has been demonstrated that the presence of basic fibroblast growth factor (bFGF) is essential for thrombin to exert its full mitogenic effect (23). It is therefore possible that factors like bFGF are present in the BPD samples to enhance the proliferative effects of the relative low thrombin levels. Second, thrombin present in BAL fluid, may upregulate the autocrine production of factors by fibroblasts that synergistically increase fibroblast proliferation induced by a factor present in BPD BAL fluid only. Third, BAL fluid from BPD patients may and from RDS patients may not upregulate the number of thrombin receptors on fibroblasts and in such a way increase the mitogenic effect of thrombin. TGF-β_1, TNF-α and interleukin-1β, which are present in elevated levels in BAL fluid when BPD develops, have been shown to upregulate thrombin receptor expression (24). Fourth, it has been described that thrombin is able to release growth factors from the peri-cellular matrix (25). Therefore, thrombin may release factors from the peri-cellular matrix of the fibroblasts that influence the proliferative activity of these cells.

TATIII levels in BAL fluid during BPD development revealed comparable kinetics as thrombin activity in BAL fluid, and a positive correlation between TATIII and thrombin activity was observed. TGF-β_1 levels in BAL fluid revealed the opposite of thrombin activity and TATIII as it tended to be increased in the BPD group at day 4 and decreased thereafter towards postnatal day 14. Our results for TGF-β_1 are comparable to those described by Kotecha *et al.* (4) although they failed to reach statistical significance at day 4 between BPD and RDS patients, possibly due to the small patient number in our study. Importantly, no correlation was observed between TGF-β_1 with either thrombin activity or TATIII levels. Therefore, it is unlikely that the differences in thrombin activity and TATIII between BPD and RDS are due to dilutional differences of the BAL samples.

We found a positive correlation between BAL fluid thrombin activity and gestational age. This is consistent with the notion that the most premature infants maybe less effective in generating thrombin in the bronchoalveolar compartment than older patients. It is known that

term neonates have a mild coagulation deficiency at birth and that premature neonates have a more definite deficiency which becomes more severe with increasing prematurity (26). The generation of active thrombin requires the conversion of the zymogen prothrombin into active thrombin via the activation of the coagulation cascade. It has been described that prothrombin mRNA in the liver and prothrombin plasma levels rise with increasing gestation (27-29). Conversion of prothrombin to thrombin in the lung is primarily generated via the extrinsic pathway of coagulation (30). Parameters of the extrinsic pathway of coagulation, such as factor VII and X increase in plasma with increasing gestational age (31-33). In the extrinsic pathway a complex of tissue factor-factor VII activates factor X. Activated factor X is involved in the conversion of prothrombin to active thrombin. Viscardi *et al.* described the presence of procoagulant activity due to activation of factor X by tissue factor-factorVII in BAL fluid from both RDS and BPD patients. But they did not find a difference in BAL fluid factor X activating activity between RDS and BPD (15). However, the patients used were less premature (gestational age ≤ 34 weeks) than the patients used in our study (gestational age ≤ 30 weeks) and there was no difference in gestational age between RDS and BPD groups. In our BPD group the gestational age was significantly lower compared to the RDS group. Therefore, we speculate that the lower thrombin activity in the BPD group compared to the RDS group at the postnatal ages of 2 and 4 days may be explained by a more immature coagulation system in the BPD group. The increase in thrombin activity during BPD development may then be explained by disease associated persistent pulmonary vascular leak and gradual development of the coagulation system as the infants become older. Alternatively, Brus *et al.* demonstrated activation of the coagulation system in plasma of RDS patients and additionally demonstrated that the degree of activation correlated with RDS severity (14). Therefore, it is likely that prothrombin consumption is increased in the vascular system from infants with subsequent BPD compared to self-resolving RDS. Such increased prothrombin consumption in the vasculature would leave smaller amounts of prothrombin available to be converted to thrombin in the alveolar compartment from infants with subsequent BPD.

Increased thrombin activity in BAL fluid from the resolving RDS group compared to the BPD group in our study may indicate a role for thrombin in normal repair after acute lung injury. Interestingly, Schmidt *et al.* have shown that treatment with antithrombin in preterm infants who suffer from RDS significantly prolonged the duration of mechanical ventilation and supplemental oxygen (34). This indirectly also suggests a role for thrombin in the restoration of normal lung function in premature infants after acute lung injury.

In conclusion, our study shows that BAL fluid from infants with developing BPD and uncomplicated RDS contains mitogenic activity for human fetal lung fibroblasts, and that thrombin is a major mitogen present in these samples. This suggests that fibroproliferation occurs early in the lungs of preterm infants with both BPD and RDS and furthermore that thrombin may play an important role in these disorders.

Acknowledgements

We gratefully acknowledge Dr. R.C. Chambers (Centre for Cardiopulmonary Biochemistry and Respiratory Medicine, University College London Medical School, Rayne Institute, London, UK) for critical reading of the manuscript.

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Chapter

DEXAMETHASONE TREATMENT INCREASES MITOGENIC ACTIVITY IN HUMAN NEONATAL LUNG LAVAGE FLUID

Submitted

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This work was supported by a grant from: Sophia Foundation For Medical Research

Abstract

Pulmonary fibrosis results from excessive fibroblast proliferation and increased collagen deposition and occurs in bronchopulmonary dysplasia (BPD). Platelet-derived growth factor (PDGF)-BB is mitogenic for fibroblasts and is increased in fibrotic lung disorders. Systemic dexamethasone (Dex) treatment improves pulmonary function and reduces inflammation in infants with or at risk of BPD. However, the effect of Dex treatment on fibroblast activity, PDGF-BB and collagen synthesis in lungs from BPD patients is uncertain. We analyzed BAL fluids obtained before and after Dex treatment from 15 infants at risk of BPD, for fibroblast mitogenicity, PDGF-BB, N-terminal-propeptide of collagen type-III (PIIINP), IL-1β and inflammatory cells. BAL fluid-induced fibroblast proliferation significantly increased after Dex treatment. The change in mitogenicity correlated with a change in BAL fluid PDGF-BB levels. Furthermore, BAL fluid-induced fibroblast proliferation was blocked using a specific inhibitor of the PDGF-receptor. Dex treatment did not influence the levels of PIIINP, but did reduce IL-1β levels and inflammatory cell numbers in BAL fluid. This study implies that Dex treatment might promote fibroproliferation despite an apparent downregulation of inflammation. Therefore, Dex treatment may not inhibit the development of pulmonary fibrosis and contribute to persistent BPD.

Introduction

Neonatal respiratory distress syndrome (RDS) is characterized by pulmonary inflammation with neutrophils and macrophages as the main cell types (1, 2). RDS can progress towards bronchopulmonary dysplasia (BPD), also known as chronic lung disease of prematurity. Risk factors for the development of BPD include premature birth with concomitant lung immaturity, mechanical ventilator-induced lung injury, oxygen toxicity, and pulmonary inflammation (3).

Pulmonary fibrosis is characterized by excessive fibroblast proliferation and increased collagen deposition (4) and is a common feature in infants dying because of BPD (5). Increased pulmonary levels of fibrogenic mediators such as fibronectin and transforming growth factor-β_1, which increase proliferation and collagen synthesis by fibroblasts have been associated with BPD development (6, 7).

Treatment with systemic dexamethasone (Dex) is commonly used in infants with or at risk of BPD and improves pulmonary function, facilitates weaning from the ventilator, and reduces pulmonary inflammation (8). However, it has been suggested that Dex treatment may not inhibit the development of pulmonary fibrosis in BPD, as it did not decrease fibronectin concentrations in bronchoalveolar lavage (BAL) fluid (6). On the other hand, Dex treatment of BPD infants resulted in decreased urinary excretion of hydroxyproline, indicating suppressed collagen synthesis in these infants (9). However, the specific effect of Dex treatment

on lung collagen synthesis during BPD development remains unclear, as urinary excretion of hydroxyproline reflects total body collagen synthesis.

BAL fluid from adults with acute respiratory distress syndrome, who are at risk of pulmonary fibrosis, is mitogenic for lung fibroblasts *in vitro*, indicating the presence of soluble mitogens (10). Platelet-derived growth factor (PDGF)-BB is a potent mitogen for fibroblasts, and increased pulmonary levels are associated with pulmonary fibrosis (4). Additionally, PDGF-BB has been shown to contribute to BAL fluid-induced fibroblast proliferation in an animal model of bleomycin-induced acute lung injury (11). It has been reported that expression of PDGF-B mRNA by alveolar macrophages, which are considered as an important source of PDGF in the development of pulmonary fibrosis, is upregulated by Dex (12).

Because excessive fibroblast proliferation is a central event in the pathogenesis of pulmonary fibrosis and may be driven by PDGF, we studied if Dex treatment influenced the fibroblast mitogenic activity of BAL fluid from infants at risk of BPD. Additionally, the effect of Dex treatment on PDGF-BB levels in BAL fluid was examined. To analyze the effect of Dex treatment on pulmonary collagen synthesis we determined the N-terminal-propeptide of type-III collagen (PIIINP) as a marker for collagen type-III synthesis and pulmonary fibrosis (13). To determine the anti-inflammatory effect of Dex, interleukin (IL)-1β and BAL cells were examined.

Methods

Patients and Dex regimen

Included were fifteen ventilated preterm infants with a gestational age < 30 weeks. Criteria for inclusion were: 1) ventilator dependency because of RDS, and 2) treatment with Dex because of risk of BPD development. Informed consent from the parents and approval by the local medical ethics committees were obtained. All infants received 0.5 mg/kg per day of Dex sodium-phosphate, administered intravenously in two doses for three days. This was followed by 0.3 mg/kg per day in two divided doses for an additional three days, after which the Dex dose was weaned over the next 2 to 4 weeks. BPD was defined as having an abnormal chest radiograph and oxygen dependency at 28 days of age (14).

Bronchoalveolar lavage

BAL was performed as described by Grigg, using two aliquots of 1 ml saline per kg bodyweight (15) within 24 hours prior to initiating Dex treatment and between 24-72 hours after initiation of treatment, or just before weaning from the ventilator whichever occurred first. The recovered volume was determined and BAL fluid was stored at -80°C until analysis. Cell numbers were determined using a haemocytometer. May Grünwald Giemsa staining was performed on cytospin preparations and cell differentials were determined by counting 300 cells per cytospin.

Fibroblast proliferation assay

The human fetal lung fibroblast cell line HFL-1 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 4 mM ultraglutamine-I, antibiotics (penicillin, 100 U/ml and streptomycin, 100 µg/ml), and 15 mM Hepes buffer. BAL fluid-induced fibroblast proliferation was determined using a colorimetric assay based on the uptake and subsequent release of methylene blue dye (16). Briefly, fibroblasts were seeded (6 x 10^3 cells per well) into 96-well microtitre plates in DMEM/0.4% FCS and allowed to adhere for 24 h. A 1/16 dilution of BAL fluid in DMEM/0.4% FCS was used to determine the effect on fibroblast proliferation, as dilution experiments revealed this to be the optimal dilution to stimulate proliferation. Proliferation was determined after 72 h and expressed as percentage change in mean absorbance above that of cells exposed to DMEM/0.4% FCS only. DMEM/5% FCS was used as a positive control. The assay was validated by direct cell counting and tritium thymidine incorporation.

Analyses of BAL fluid

PDGF-BB was determined with an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, UK). PIIINP was determined using a radioimmunoassay (Orion diagnostica, Espoo, Finland). IL-1β was determined by ELISA (Human Il-1β cytoset™, Biosource International, Camarillo, CA). Assays were performed according to the methodology of the manufactors. The detection limits were 4.6 pg/ml, 0.2 µg/L and 1.6 pg/ml for PDGF-BB, PIIINP and IL-1β, respectively. Concentrations below the detection limit were interpreted as < detection limit. No marker for dilution was used, and PDGF-BB, PIIINP and IL-1β levels in BAL fluid are presented as volume concentration, according to the most recent ERS task force guidelines on BAL in children (17).

Inhibition of BAL fluid-induced fibroblast proliferation

Tyrphostin AG1296 is a compound that specifically inhibits the platelet-derived growth factor receptor tyrosine kinase, thereby inhibiting PDGF induced proliferation (18). After adherence of the cells for 24 h, the medium was changed to DMEM/0% FCS for 6 h. Thereafter, fresh DMEM/0% FCS containing 30µM of the tyrphostin AG1296 (Calbiochem, Darmstadt, Germany) in vehicle (dimethylsulfoxide (DMSO)) or vehicle alone was added for another 18 h. Medium was replaced by BAL fluid (known to contain PDGF-BB) in DMEM/0.4% FCS for 48 h (three replicates per BAL fluid sample for AG1296 and vehicle alone). As control, HFL-1 pre-incubated with AG1296 or vehicle were stimulated with 50 ng/ml PDGF-BB (R&D Systems, UK).

Statistical analysis

Patient characteristics and BAL cell-values are presented as mean values ± standard error of the mean (SEM). Results for fibroblast proliferation, PDGF-BB, PIIINP and IL1β are presented as median values and range. The paired students t-test was used for comparison between values obtained before and after initiation of Dex treatment. Pearson's correlation

was used to compare the relationship between BAL fluid mitogenic activity and PDGF-BB concentrations. A p value < 0.05 was considered to indicate statistical significance.

Results

Patients and Dex regimen

The patients (n=15) had a gestational age of 27.2 ± 0.3 weeks (ranging from 26-30 weeks) and a birthweight of 972.6 ± 66.2 gram (ranging from 650-1529 gram). Dex treatment was initiated at an age of 18.3 ± 2 days after birth (ranging from 7-33 days). All patients responded to the Dex therapy as they were extubated within days after initiation of treatment. However, despite treatment, all patients developed BPD.

Bronchoalveolar lavage

BAL was performed at 2.3 ± 0.2 days after initiation of Dex treatment (ranging from 1-3 days). BAL-recovery was determined before and after initiation of treatment. No difference was observed in BAL-recovery before and after initiation of Dex (40.1 ± 5.2 and 33.9 ± 4.2% of the initial lavage volume used, respectively). Table 1 shows the results of cell numbers and composition of the cell population obtained by BAL. The total number of cells as well as the number of cells per ml BAL fluid was significantly decreased after Dex treatment. Percentages of neutrophils and macrophages in the cell population were not influenced by Dex treatment. However, absolute numbers of macrophages per ml BAL fluid decreased significantly after Dex. The number of neutrophils per ml BAL fluid also declined after Dex, however, without reaching significance.

Table 1. Effect of Dex treatment on cell population in neonatal lung lavage

patients (n=15)	before initiation of Dex*	after initiation of Dex*
total # cells obtained by BAL ($x10^6$)	1.39 ± 0.31	0.51 ± 0.07$
total # cells/ml BAL fluid ($x10^6$)	1.74 ± 0.39	0.9 ± 0.26$
% macrophages in BAL fluid	43.7 ± 5.8	39.8 ± 6.4
% neutrophils in BAL fluid	48.6 ± 6.0	47.3 ± 7.0
total # macrophages/ml BAL fluid ($x10^6$)	0.7 ± 0.16	0.28 ± 0.06$
total # neutrophils/ml BAL fluid ($x10^6$)	0.93 ± 0.27	0.54 ± 0.23

* = mean ± standard error of the mean
$ = $p < 0.05$

BAL fluid-induced fibroblast proliferation

For the whole group of patients, BAL fluid-induced fibroblast proliferation increased from 34.7% (7.7-75.3% proliferation above medium control) before Dex to 51.2% (17.8-115.1%) after initiation of Dex treatment ($p < 0.05$). From the fifteen patients, twelve showed an increase and three a decrease in BAL fluid-induced fibroblast proliferation after the initiation of Dex treatment (Fig. 1A). Interestingly, the three patients with decreasing BAL fluid mitogenicity contained the highest mitogenic activity before initiation of Dex. When fibroblast proliferation was expressed relative to the 5% FCS similar results were obtained (data not shown).

Analyses of BAL fluid

A summary of the analyses per individual BAL sample is presented in table 2. Dex treatment did not influence the PDGF-BB concentration in BAL fluid from the fifteen patients before and after Dex (11.8 (< 4.6-92.9 pg/ml BAL fluid) compared to 27 (< 4.6-80.4 pg/ml BAL fluid, respectively). However, when the mitogenic activity of BAL fluid from patients (n=3) decreased after Dex, it was accompanied by a decrease in PDGF-BB levels. From the twelve patients with increased mitogenic activity after Dex, ten showed an increase in PDGF-BB levels after Dex, one patient showed a minimal decrease and one had undetectable PDGF-BB levels before and after Dex (Fig. 1B). A statistically significant positive correlation was observed between the change in PDGF-BB levels in BAL fluid and the change in BAL fluid-induced fibroblast proliferation after initiation of Dex treatment ($r = 0.7$; $p < 0.01$).

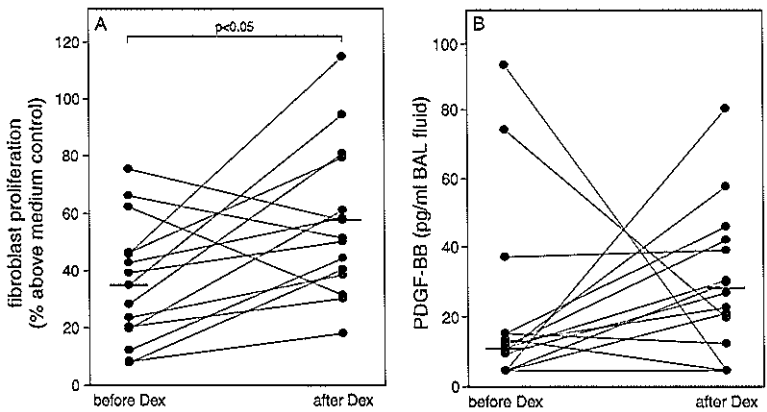

Figure 1

The effect of BAL fluid (1/16 diluted) on fibroblast proliferation for each individual (1A), twelve patients show an increase in BAL fluid mitogenic activity after Dex, and three patients a decrease. Horizontal bars represent median values. 1B depicts PDGF-BB concentrations in BAL fluid before and after Dex treatment. Horizontal bars represent median values. The three patients with a decrease in BAL fluid mitogenic activity after Dex (1A) also show a decrease in PDGF-BB levels after Dex (1B). From the twelve patients with increasing mitogenic activity (1A) ten have increased PDGF-BB levels after Dex, one has a minimal decline in PDGF-BB after Dex, and in one patient PDGF-BB was undetectable before and after Dex treatment (1B). When PDGF-BB was undetectable in a BAL sample it is depicted as 4.6 pg/ml BAL fluid, which is the detection limit of the used assay.

Table 2. Analyses of BAL fluid per individual patient

	Fibroblast proliferation (% above ctrl)		PDGF-BB (pg/ml BAL fluid)		PIIINP (µg/L BAL fluid)		IL-1β (pg/ml BAL fluid)	
patient	before Dex	after Dex	before Dex	after Dex	before Dex	after Dex	before Dex	after Dex
1	38.9	50.0	15.2	46.1	n.d.	n.d.	52.3	26.1
2	23.3	38.3	15.1	12.3	n.d.	n.d.	n.d.	n.d.
3	42.6	58.1	11.8	42.1	7.7	16.2	n.d.	n.d.
4	45.9	115.1	< 4.6	29.7	n.d.	n.d.	n.d.	n.d.
5	7.7	40.3	< 4.6	80.4	7.6	215	78.8	68.2
6	19.5	60.8	< 4.6	20.9	51.2	18.2	< 1.6	< 1.6
7	20.5	30.2	37.1	39.1	43.1	25.9	23.2	5.3
8	34.7	94.4	11	30.4	12.3	23.2	n.d.	n.d.
9	46.0	79.2	12.6	22.8	9.5	6.6	30.7	< 1.6
10	28.3	80.8	9.3	27.0	n.d.	n.d.	48.9	< 1.6
11	8.3	17.8	< 4.6	< 4.6	9.5	6.6	n.d.	n.d.
12	12.1	44.2	10.5	57.5	n.d.	n.d.	n.d.	n.d.
13	66.2	51.2	74.0	19.7	9.5	6.8	31.8	< 1.6
14	75.3	57.6	13.6	< 4.6	24.5	6.0	30.7	< 1.6
15	62.2	31.3	92.8	< 4.6	11.4	6.3	n.d.	n.d.
median	34.7	51.2$	11.8	27	10.5	11.5	31.3	< 1.6$
range	7.7-75.3	17.8-115.1	< 4.6-92.8	< 4.6-80.4	7.6-51.2	6.0-215	< 1.6-78.8	< 1.6-68.2

$ = $p < 0.05$ compared to before Dex
n.d. = not determined

PIIINP levels were determined in BAL fluid from ten patients (based on sample availability). Dex treatment did not significantly change the levels of PIIINP in these ten patients (10.5 (7.6-51.2 μg/L BAL fluid) before Dex compared to 11.5 (6-215 μg/L BAL fluid) after Dex).

Due to limited amounts of BAL fluid, IL-1β levels were investigated for eight patients. A significant decrease of IL-1β levels in BAL fluid was observed after Dex treatment (31.3 (< 1.6-78.8 pg/ml BAL fluid) before Dex compared to (< 1.6 (< 1.6-68.2 pg/ml BAL fluid) after Dex; $p < 0.05$). Seven patients showed decreased IL-1β levels after compared to before Dex treatment, in one patient IL-1β was undetectable before and after Dex treatment.

Inhibition of BAL fluid-induced fibroblast proliferation

To determine whether PDGF-BB contributed to BAL fluid-mitogenicity, the PDGF receptor system in fibroblasts was blocked by using the specific inhibitor AG1296. AG1296 was able to inhibit BAL fluid-induced fibroblast proliferation and to block PDGF-BB induced proliferation (Fig. 2). AG1296 had no effect on HFL-1 proliferation after 48 h in DMEM containing 0.4% FCS compared to pre-incubation with vehicle alone.

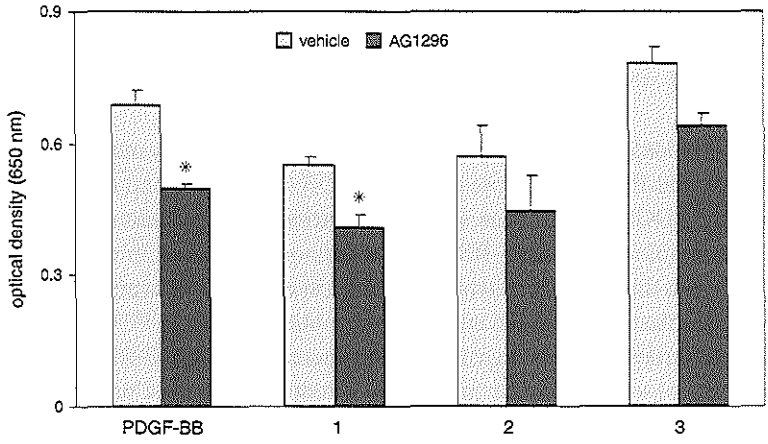

Figure 2
Tyrphostin AG1296 blocks PDGF-BB (50 ng/ml) and BAL fluid-induced fibroblast proliferation as indicated by a reduction in optical density after methylene blue elution. Results for three BAL samples from three individual patients (denoted 1-3) are depicted in this graph. * = $p < 0.05$

Discussion

Pulmonary fibrosis is a feature of BPD (5), and is a consequence of excessive fibroblast proliferation and increased collagen synthesis (4). PDGF, is a potent proliferative stimulus for mesenchymal cells such as fibroblasts and known to be involved in pulmonary fibrosis (4). PDGF is composed of two polypeptide chains, termed A and B, and may exist as either one of the homodimers or as a heterodimer (4). Although it is known that Dex treatment of infants with or at risk of BPD improves pulmonary function, and decreases pulmonary inflammation (8) and total body collagen synthesis (9, 19), the effect on lung fibroblast proliferation, pulmonary collagen synthesis and PDGF levels in infants at risk of BPD is uncertain.

As reported previously (8, 20), our study demonstrates an anti-inflammatory effect of Dex, reflected by a reduction in IL-1β levels and inflammatory cells in BAL fluid. A novel finding, however, is that the mitogenic activity of BAL fluid from premature infants at risk for pulmonary fibrosis, increases after treatment with systemic Dex and that this increase is associated with an increase of PDGF-BB levels in these patients. In our study three patients, with a relatively high BAL fluid mitogenicity before initiation of Dex treatment, showed a decrease in BAL fluid mitogenicity after Dex. In contrast, twelve with a relatively low BAL fluid mitogenicity before Dex showed an increase after Dex. The observed change in BAL fluid mitogenicity and PDGF-BB levels after Dex treatment did not correlate with surfactant treatment, antenatal steroids, type of ventilation, birth weight or gestational age (data not shown).

In animal models of acute lung injury and pulmonary fibrosis increased expression of PDGF-B mRNA and PDGF-BB protein preceded DNA synthesis and tissue repair (11, 21, 22). Additionally, PDGF-BB has been shown to contribute to fibroblast mitogenic activity of BAL fluid, and inhibition of PDGF-BB reduces pulmonary fibrosis in experimental models (11, 23). In line with this is the correlation we found in our study between the change in BAL fluid-induced fibroblast proliferation and the concomitant change in PDGF-BB levels before and after Dex treatment. This suggests that the increase in PDGF-BB is, at least partly, responsible for the observed increase in BAL fluid-induced fibroblast proliferation after Dex treatment. Furthermore, we demonstrated that PDGF indeed contributed to BAL fluid-induced fibroblast proliferation as blocking the PDGF-receptor system in fibroblasts reduced BAL fluid-induced proliferation. Therefore, PDGF may be an important fibroblast mitogen in the pathophysiology of BPD. However, other mitogens are likely to contribute as well since blocking the PDGF receptor system did not completely inhibit the BAL fluid mitogenic activity.

Alveolar macrophages are considered as a main source of PDGF in pulmonary fibrosis and increased numbers are present in the pathogenesis of BPD (2, 4). In our study, the BAL cell population obtained from infants prior to Dex treatment contained considerable numbers of alveolar macrophages that can be considered as a potential source of the

detected PDGF-BB. It has been demonstrated that macrophages stimulated with Dex express increased amounts of PDGF-B mRNA, secrete increased amounts of PDGF protein, and stimulate fibroblast proliferation and collagen synthesis (12, 24). Although we found that the number of alveolar macrophages decreased after Dex treatment, it may well be that this treatment increases the net production of PDGF-BB per cell. This could result in increased total PDGF-BB levels in the bronchoalveolar compartment, resulting in increased fibroblast proliferation. Activation of such a pathway may result in a profibrotic environment in the lungs from infants with or at risk of BPD and therefore Dex may not inhibit the development of pulmonary fibrosis in these infants. However, Dex itself may also influence fibroblast activation, as it has been reported that Dex stimulates the proliferation of rat lung fibroblasts *in vitro*, presumably by increasing the expression of the PDGF-α receptor (25). *In vitro* studies with Dex in primary human fetal lung fibroblasts, however, revealed reduced proliferation and increased collagen synthesis (26). Increased pulmonary vascular leak is a pathogenetic hallmark of BPD development (3). It is therefore possible that, in our study, leakage of Dex from the vascular compartment into the alveolar space has occurred. As a consequence of such leakage, Dex may have been present in the BAL samples obtained after Dex treatment and may have influenced BAL fluid-induced fibroblast proliferation. However, we found that Dex itself was unable to stimulate fibroblast proliferation in our culture system. Furthermore, the addition of the glucocorticoid antagonist RU 38486 to BAL fluid obtained after Dex treatment did not influence BAL fluid-induced fibroblast proliferation and neither did the addition of Dex to BAL samples obtained before Dex treatment (data not shown). Therefore, it is unlikely that if Dex was present in BAL fluid that it influenced the observed BAL fluid-induced fibroproliferation.

In vitro experiments have shown that Dex reduces collagen synthesis by fibroblasts (27, 28). However, recent evidence indicates that Dex increases collagen synthesis by human fetal lung fibroblasts (26). Interestingly, Chen *et al.* demonstrated that prenatal Dex administration with prolonged exposure of preterm rats to hyperoxia resulted in a pulmonary pathologic picture similar to BPD, and with even greater severity of septal fibrosis compared to hyperoxia exposed control rats (29). As it is likely that postnatal Dex administration may exert comparable effects these data make the possible effect of Dex on pulmonary collagen synthesis in BPD uncertain. We observed no difference in PIIINP levels before and after Dex treatment, indicating that Dex treatment does not influence collagen synthesis in the lungs from infants at risk of BPD. However, the fact that no difference was observed may be due to the limited number of patients and great variability in PIIINP levels, which has also been shown to exist for PIIINP levels in BAL fluid from adult patients with acute respiratory distress syndrome (10). Alternatively, an effect of Dex on PIIINP levels may be obscured due to drainage of PIIINP from the lung by lymph vessels (30).

We suggest that Dex treatment of infants at risk of BPD does not decrease the development of pulmonary fibrosis, presumably due to increased PDGF-BB driven pulmonary fibroblast proliferation with increased collagen synthesis as a consequence. We can not exclude that the observed differences of the determined parameters in BAL fluid before

and after Dex treatment are just the natural course of the disease, as no control patients were included in this study. Because of the variation in initiation of Dex treatment in our patient population (7-33 days after birth) and the fact that both centers find it unethical to withdraw patients who are at risk of BPD from Dex treatment we did not include a placebo control group. However, regarding the expected and observed anti-inflammatory effects of Dex treatment and the heterogeneity of initiation of Dex treatment, the effects on BAL fluid mitogenicity and PDGF-BB levels are likely to be due to Dex treatment and not to reflect the natural disease course.

In conclusion, our study implies that Dex treatment might promote fibroproliferation despite an apparent downregulation of inflammation, and therefore may not inhibit the development of pulmonary fibrosis and contribute to persistent BPD.

Acknowledgements

We gratefully acknowledge Prof. Dr. R. Benner and Dr. V.H.J. van der Velden for critical reading of the manuscript, and M. Huysman for assistance with bronchoalveolar lavage.

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Chapter

LOCALIZATION AND POTENTIAL ROLE OF MATRIX METALLOPROTEINASE-1 AND TISSUE INHIBITORS OF METALLOPROTEINASE-1 AND -2 IN DIFFERENT PHASES OF BRONCHOPULMONARY DYSPLASIA

Pediatr Res 2001; 50: 761-766

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This work was supported by a grant from: Sophia Foundation For Medical Research

Abstract

Bronchopulmonary dysplasia (BPD) can evolve in prematurely born infants who require mechanical ventilation because of hyaline membrane disease (HMD). The development of BPD can be divided in an acute, a regenerative, a transitional, and a chronic phase. During these different phases, extensive remodelling of the lung parenchyma, with reepithelialization of the alveoli and formation of fibrosis occurs. Matrix metalloproteinase-1 (MMP-1) is an enzyme that is involved in reepithelialization processes, and dysregulation of MMP-1 activity contributes to fibrosis. Localization of MMP-1 and its inhibitors, tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2, were investigated in lung tissue obtained from infants who died during different phases of BPD development. In all studied cases (n=50) type-II pneumocytes were found to be immunoreactive for MMP-1, TIMP-1, and TIMP-2. During the acute and regenerative phase of BPD, type-II pneumocytes reepithelialize the injured alveoli. This may suggest that MMP-1 and its inhibitors, expressed by type-II pneumocytes, play a role in the reepithelialization process after acute lung injury. Although MMP-1 staining intensity remained constant in type-II pneumocytes during BPD development, TIMP-1 increased during the chronic fibrotic phase. This relative elevation of TIMP-1 compared with MMP-1 is indicative for reduced collagenolytic activity by type-II pneumocytes in chronic BPD and may contribute to fibrosis. Fibrotic foci in chronic BPD contained fibroblasts immunoreactive for MMP-1 and TIMP-1 and -2. This may indicate that decreased collagen turnover by fibroblasts contributes to fibrosis in BPD development.

Introduction

Respiratory distress syndrome develops within minutes after birth in premature infants and is associated with surfactant deficiency of the immature lung (1, 2). Pathologically, it is called hyaline membrane disease (HMD), with the formation of hyaline membranes in the terminal airways being one of the most striking pathologic features (3).

Hyaline membrane formation is caused by necrosis and desquamation of epithelial cells lining the alveolar basement membrane and the occurrence of pulmonary edema (3-5). To sustain life, infants are treated with mechanical ventilation and supplemental oxygen, which are both risk factors for the progression of HMD toward bronchopulmonary dysplasia (BPD) (6).

BPD, also called chronic lung disease of prematurity, can be regarded as the end stage of HMD treatment and is associated with mortality and long term pulmonary morbidity (6, 7). The development from HMD to BPD is characterized by extensive tissue remodelling and can be divided into four phases based on days after birth: acute (2-4 days), regenerative (4-8 days), transitional (8-16 days), and chronic (> 16 days) (8). A main feature of the acute and regenerative phase is reepithelialization of the denuded alveoli. The transitional and chronic

phases are characterized by alveoli primarily lined with type-II pneumocytes and the occurrence of fibrotic areas and increased numbers of fibroblasts (3, 9, 10).

During the reepithelialization process, hyaline membranes form a matrix on which type-II pneumocytes adhere and regenerate. This process incorporates hyaline membranes into the alveolar septal wall. Incorporated hyaline membranes, which contain fibronectin, are associated with areas of fibrosis during BPD development (9, 11, 12).

Matrix metalloproteinases (MMPs) are a group of enzymes capable of degrading extracellular matrix proteins. MMPs play a critical role in normal physiological processes like development, tissue remodelling, inflammation, angiogenesis, wound healing, and cell migration (13). The expression of MMPs is regulated at the transcriptional level by cytokines, growth factors, and extracellular matrix components (14, 15, 16). MMPs are secreted as latent pro-enzymes and require proteolytic cleavage for activation. MMP-1 is capable of degrading the fibrillar collagens- type-I, -II, and -III, collagen type-X, gelatin and proteoglycans (17).

In vitro studies have shown that type-II pneumocytes, the cells that initiate alveolar reepithelialization, are able to produce MMP-1 and thereby promote their own migration (18, 19). Therefore, MMP-1 may play a role in the reepithelialization process of the alveoli during the acute and regenerative phase of BPD development, comparable with the role for MMP-1 in reepithelialization of the skin after injury (20, 21). Tissue inhibitors of matrix metalloproteinase (TIMP)-1 and TIMP-2 inhibit active MMP-1 (22). It was shown that disturbed MMP-1, TIMP-1, and TIMP-2 regulation plays an important role in pathologic processes like fibrotic liver disease and pulmonary fibrosis (23-26, 27).

Therefore, we expect MMP-1, TIMP-1, and TIMP-2 to contribute to the remodelling process of lung tissue when HMD progresses toward BPD. This urged us to study the immunohistochemical localization of MMP-1, TIMP-1, and TIMP-2 in lung tissue obtained from infants who died during different phases of BPD development.

Materials and methods

Patients

Autopsy lung specimens were selected in the files of the department of Pathology from the years 1988-1998, based on premature infants who were histologically and clinically diagnosed as HMD/BPD. HMD was clinically defined as respiratory distress for which ventilation was necessary and a pulmonary x-ray pattern compatible with HMD (28). All patients were still on oxygen when they died. Therefore, per definition all patients that died after 28 days fulfilled the clinical BPD definition according to Bancalari (29). Staging of the histologic samples was performed according to Rosan (8). In total, 50 prematurely born infants with pathologic findings of BPD or HMD, who died of respiratory insufficiency were selected (all patients before 1990 (n=12) died because of respiratory insufficiency alone; after 1990, 26 infants died because of respiratory insufficiency alone; in the other 12 patients respiratory insufficiency coexisted with heart failure or intraventricular hemorrhage).

As controls, specimens were taken from three term infants who died of nonpulmonary causes and lived for 9, 14, and 30 d, respectively.

Immunohistochemistry

Paraffin embedded lung tissue was cut into 4 μm sections, incubated overnight at 37°C, deparaffinized, and rehydrated. Afterward, the slides were washed in phosphate-buffered saline (PBS, pH 7.8) for 5 min and incubated with 0.1% pepsin A (Sigma Chemical Company, St Louis, MO, U.S.A.) in 0.01M HCl for 30 min at 37°C for antigen retrieval. The slides were subsequently rinsed in PBS of 4°C for 5 min, followed by blocking of endogenous biotin activity with an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA, U.S.A.). Afterward, the slides were rinsed for 15 min in PBS followed by 10 min in PBS containing 0.2% Tween 20. The slides were then incubated for 5 min with PBS containing 1% bovine serum albumin (BSA) followed by a 1 h incubation with PBS containing 1% BSA and 10% normal human serum (NHS). This was followed by rinsing the slides for two times 5 min in washbuffer (PBS containing 0.1%BSA and 0.2% Tween 20). Subsequently, the slides were incubated for 1 h with 10% normal goat serum diluted in PBS containing 1% BSA for MMP-1 and TIMP-2 staining or 10% normal rabbit serum diluted in PBS containing 1% BSA for TIMP-1 staining. Then the slides were incubated with the primary antibodies diluted in PBS containing 1% BSA and 10% normal goat or rabbit serum (mouse anti-human MMP-1; 1:200 dilution, mouse anti-human TIMP-2; 1:20 dilution, ICN Biomedicals, Aurora, Ohio, and goat anti-human TIMP-1; 1:40 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) overnight at 4°C. Afterward, the slides were rinsed for 5 min in washbuffer, which was followed by two wash steps of 15 min. Subsequently, the slides were incubated for 30 min with biotin-labeled goat-anti-mouse (Biogenex, San Ramon, CA, U.S.A.) or rabbit-anti-goat (DAKO, Glostrup, Denmark) at a 1:50 and 1:20 dilution in PBS containing 1% BSA and 10% NHS, respectively. Then the slides were rinsed for 5 min followed by two times 15 min in washbuffer. This was followed by incubating the slides for 30 min with a 1:50 dilution of Streptavidin-alkaline-phosphatase (Biogenex) in PBS containing 1% BSA. Afterward, the slides were rinsed once 5 and once 15 min with washbuffer and rinsed for 5 min in 0.2M Tris-HCl (pH 8.0). Subsequently, the slides were incubated for 30 min with New Fuchsin substrate (Chroma, Stuttgart, Germany). Finally the slides were rinsed with PBS, counterstained with Mayers hematoxylin (Merck, Darmstadt, Germany) and mounted in Kaiser's glycerol (Merck). Control staining was performed by substitution of the primary antibody with PBS. R.R.d.K. and L.B. performed the evaluation of the stained sections in a blinded fashion. The cells and structures examined in this study were the alveolar macrophage, the alveolar type-II pneumocyte, the alveolar basement membrane, and fibroblasts in fibrotic foci. The staining intensity of the sections was scored in a semiquantitave manner according to the following method: no staining = 0, diffuse very faint staining = 1, diffuse faint staining = 2, diffuse moderate staining = 3, and diffuse strong staining = 4.

Statistical analysis

Data on staining intensity are presented as median and percentiles (10 to 90%) for the different phases of BPD development. For evaluation of differences in staining intensity between different phases of BPD development the Mann-Whitney U test was used. A p value less than 0.05 was considered significant.

Results

Patients

BPD is considered as a gradually developing sequel of the original diagnosed HMD (acute phase of BPD development) and its treatment (8). Therefore, we applied the time phases for BPD development, according to Rosan, to the patient group (8). Twenty seven patients were included in the acute phase (0-4 d), seven in the regenerative phase (5-8 d), nine in the transitional phase (9-16 d), and seven in the chronic phase (>16 d). The control patients were regarded as one group, independent of their age at death. Clinical data of the different phases of BPD development and of control patients are shown in table 1.

Table 1. Clinical data of BPD and control patients

BPD stage	gestational age (weeks)*	age at death (days)*	weight at death (kilograms)*	male : female	number of patients
Acute phase	28.9 (24.9-36)	2 (0.08-4)	1.1 (0.58-3.0)	2:1	27
Regenerative phase	25.6 (25-30)	6 (5-8)	1.1 (0.56-1.4)	4:3	7
Transitional phase	27.9 (26.3-30)	13 (9-16)	1.1 (0.77-1.7)	7:2	9
Chronic phase	28 (25-31.7)	35 (21-304)	1.1 (0.85-5.0)	6:1	7
Controls	term	14 (9-30)	2.9 (2.9-3.7)	1:2	3

* Median value (range)

MMP-1, TIMP-1 and TIMP-2 expression.

An overview of the immunohistochemical detection for MMP-1, TIMP-1, and TIMP-2, presented as positive or negative immunoreactivity, is shown in table 2.

Table 2. Localization of MMP-1, TIMP-1, and TIMP-2 during different phases of BPD and in control tissue

	Acute phase			Regenerative phase			Transitional phase			Chronic phase			Control tissue		
	MMP-1	TIMP-1	TIMP-2	MMP-1	TIMP-1	TIMP-2	MMP-1	TIMP-1	TIMP-2	MMP-1	TIMP-1	TIMP-2	MMP-1	TIMP-1	TIMP-2
Type-II pneumocytes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Alveolar basement membrane	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-
Alveolar macrophages	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fibroblasts in fibrotic area	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-

+ = immunoreactivity
− = no immunoreactivity

Control lung tissue

Control lung tissue revealed scattered MMP-1, TIMP-1 and -2 positive type-II pneumocytes and alveolar macrophages. TIMP-1 reactivity was also detected in the alveolar basement membrane (Fig. 1).

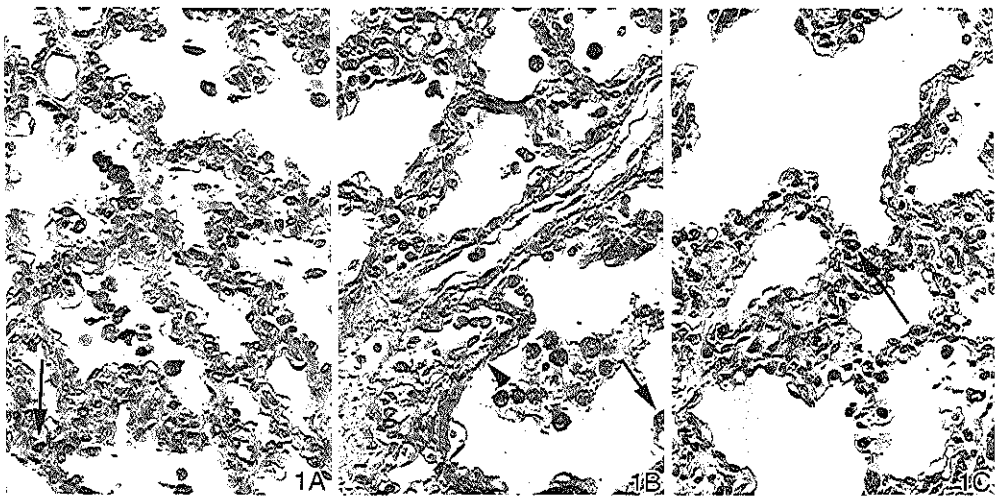

Figure 1
Immunohistochemical staining of control lung tissue. MMP-1 (A), TIMP-1 (B), and TIMP-2 (C) immunoreactivity is detected in type-II pneumocytes (arrow) and alveolar macrophages. TIMP-1 (B) immunoreactivity is detected in the alveolar basement membrane (arrowhead).

Acute phase (0-4 d)

Lung tissue of the patients who died early in the acute phase (< 1 d) was characterized by extensive loss of alveolar epithelial cells and the formation of hyaline membranes. Because of extensive loss of alveolar epithelial cells, virtually no reactivity with MMP-1, TIMP-1, and TIMP-2 was observed in the alveolar epithelium. However, if cuboidal type-II-like pneumocytes were present, they stained positive for MMP-1, TIMP-1, and TIMP-2. During progression toward day 4 in the acute phase, the lung tissue showed hyaline membranes covering cells positive for MMP-1, TIMP-1, and TIMP-2. Furthermore, regenerating epithelial cells positive for MMP-1, TIMP-1, and TIMP-2 epithelialized the hyaline membranes, incorporating them into the alveolar septum. In most cases a clear positive staining for TIMP-1 was localized to the alveolar basement membrane, which was observed underneath epithelial cells as well as underneath hyaline membranes. Alveolar macrophages showed no difference in immunostaining for MMP-1, TIMP-1, and TIMP-2 compared with control lung tissue.

Regenerative phase (5-8 d)

During the regenerative phase hyaline membranes were still present in the tissue and clearly became incorporated into the alveolar wall. The incorporated membranes were covered by cuboidal type-II-like pneumocytes that stained positive for MMP-1, TIMP-1, and TIMP-2 (Fig. 2). In most cases TIMP-1 positivity was also observed in parts of the alveolar basement membrane, similar to the situation in the acute phase. Alveolar macrophages present in this phase showed a similar positivity for MMP-1, TIMP-1, and TIMP-2 as detected in the control lung tissue.

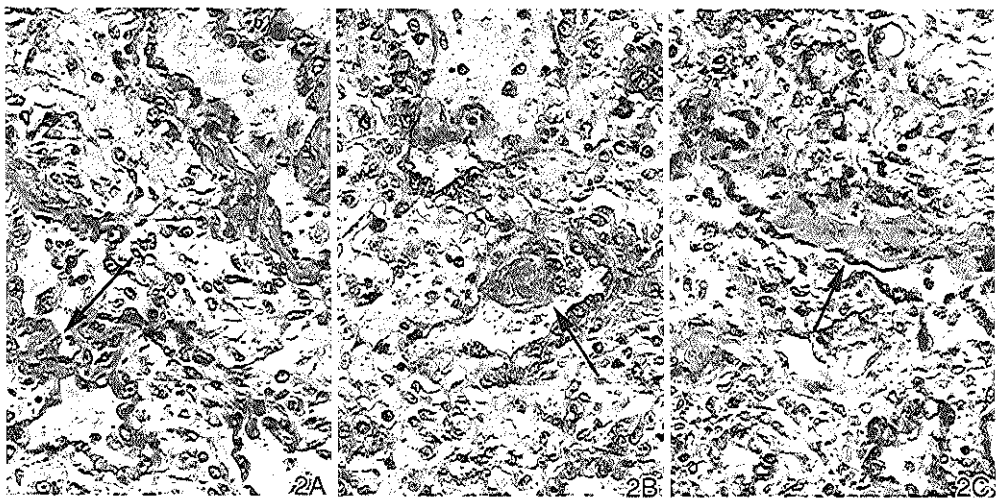

Figure 2
Immunohistochemical staining of lung tissue in the regenerative phase of BPD development. MMP-1 (A), TIMP-1 (B), and TIMP-2 (C) immunoreactivity is detected in type-II pneumocytes reepithelializing the alveoli and incorporating hyaline membranes (arrow).

Transitional phase (9-16 d)

During the transitional phase less hyaline membranes were present. If present, they were covered with cuboidal type-II-like pneumocytes, which stained positive for MMP-1, TIMP-1, and TIMP-2. Furthermore, the alveolar surface appeared to be covered exclusively with type-II-like pneumocytes, which were MMP-1, TIMP-1, and TIMP-2 positive. Alveolar macrophages, which were more frequently present than in the previous stages, also stained positive for MMP-1, TIMP-1, and TIMP-2 (Fig. 3). TIMP-1 immunoreactivity in the alveolar basement membrane differed from barely detectable in some patients to prominent in others.

Chronic phase (>16 d)

During the chronic phase hyaline membranes were virtually absent. The alveoli appeared to be covered exclusively with type-II-like pneumocytes immunoreactive for

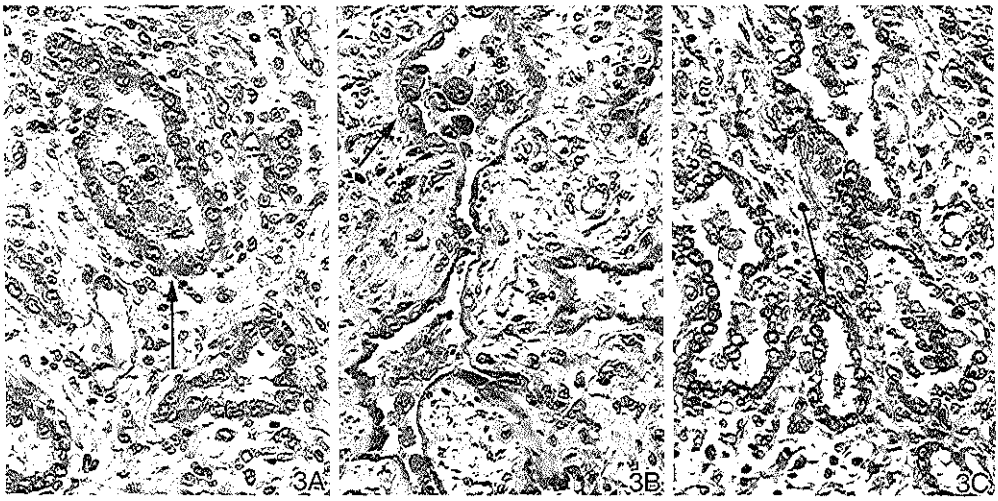

Figure 3
Immunohistochemical staining of lung tissue in the transitional phase of BPD development. MMP-1 (A), TIMP-1 (B), and TIMP-2 (C) immunoreactivity is detected in type-II pneumocytes covering the entire alveolar surface (arrow) and in alveolar macrophages.

MMP-1, TIMP-1, and TIMP-2. In some cases the alveoli appeared emphysematous. Alveolar macrophages also revealed positive staining for MMP-1, TIMP-1, and TIMP-2. During this chronic phase, interstitial fibrosis was a prominent feature. Fibrotic areas contained fibroblasts positive for MMP-1, TIMP-1, and TIMP-2 (Fig. 4). Immunostaining for TIMP-1 in the alveolar basement membrane varied from barely detectable in some patients to prominent in others. If the primary antibody was omitted no immunoreactivity was detected (Fig. 4D).

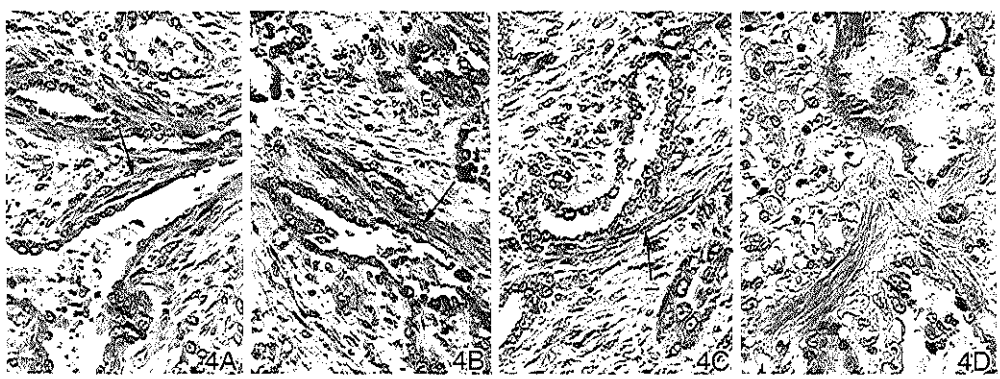

Figure 4
Immunohistochemical staining of lung tissue in the chronic phase of BPD development. MMP-1 (A), TIMP-1 (B), and TIMP-2 (C) immunoreactivity is detected in fibroblasts present in fibrotic foci (arrow) and in type-II pneumocytes covering the entire alveolar surface. 4D is negative control.

Semiquantitative analysis of immunoreactivity during BPD development

Semi-quantitative analysis of immunoreactivity revealed no difference in MMP-1 and TIMP-2 staining intensity between the four phases of BPD development. However, TIMP-1 showed a marked increase in staining intensity in the type-II pneumocytes that formed the alveolar lining in the chronic phase of BPD compared with the earlier phases (Fig. 5).

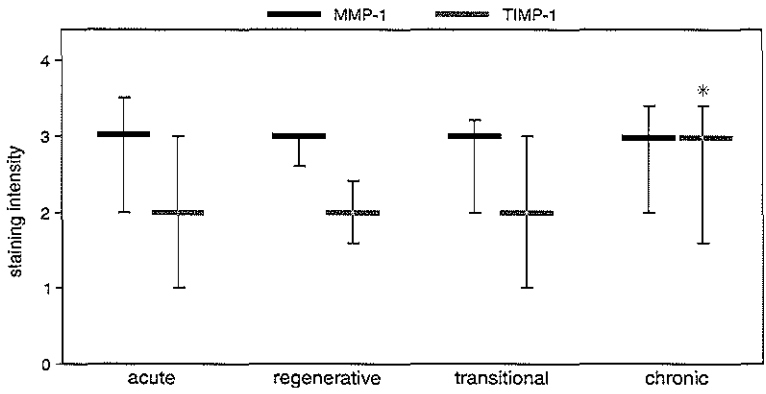

Figure 5

Semiquantitative analysis of MMP-1 and TIMP-1 in type-II pneumocytes during different developmental phases of BPD. Results for staining intensity are expressed as median and percentiles (10 to 90%) per group of patients in the four different phases of BPD development. Semiquantitative scoring was assessed as follows: no staining = 0, diffuse very faint staining = 1, diffuse faint staining = 2, diffuse moderate staining = 3, and diffuse strong staining = 4. * $p < 0.05$ compared to acute phase.

Discussion

In the present study we investigated the expression pattern of MMP-1 and its inhibitors, TIMP-1 and TIMP-2, in the lung during the development of BPD. MMP-1 colocalized with TIMP-1 and TIMP-2 in type-II pneumocytes and alveolar macrophages. TIMP-1 also localized to the alveolar basement membrane.

The expression of MMP-1, TIMP-1, and TIMP-2 by type-II pneumocytes and alveolar macrophages in the healthy lung may play a role in the extracellular matrix turnover, which is a constant feature in lung tissue (30, 31). The presence of TIMP-1 in the basement membrane may prevent this structure from degradation by MMPs or may provide a barrier function for active MMPs to reach the lung interstitium to control extracellular matrix turnover.

After lung injury and during lung development, the type-II pneumocyte is the progen-

itor cell for the formation of a functional alveolar epithelium (32, 33). Migration of type-II pneumocytes over hyaline membrane-like matrices has been suggested to play an important role during reepithelialization after acute injury. The $\alpha_2\beta_1$-integrin on type-II pneumocytes mediates the migration on collagen type-I, a substrate for and inducer of MMP-1 production (17, 34, 35). Recently, an *in vitro* study described that MMP-1 decreased the type-II pneumocyte cytoskeleton stiffness, the adhesion to collagen type-I, and increased cell migration across collagen type-I (19). Regarding these observations and the expression of MMP-1, TIMP-1, and TIMP-2 by type-II pneumocytes on hyaline membranes in the acute and regenerative phases of BPD development, we speculate that MMP-1 is involved in reepithelialization of the alveolar surface during the acute and regenerative phase of BPD development. The colocalization of TIMP-1 and TIMP-2 with MMP-1 indicates that these inhibitors regulate the activity of MMP-1 during this process. Analysis of lung homogenates could reveal information about *in vivo* MMP-1 activity and the ratio of MMP-1/TIMPs. Furthermore, the relative amounts of active and latent MMP-1 could be determined, because both forms are recognized with the antibody used in this study.

The saccular stage of fetal lung development is characterized by alveoli mainly lined by type-II pneumocytes. During this stage, thinning of the interstitial matrix between alveoli occurs. It has been suggested that epithelial cells over-expressing MMP-1 relative to TIMP-1 contribute to the net degradation of interstitial collagens during this process (36-39). During the transitional and chronic phase of BPD we observed virtually no hyaline membranes and the alveoli were almost exclusively lined by hyperplastic MMP-1, TIMP-1, and -2 positive type-II pneumocytes. Furthermore, fibrotic areas in the alveolar septa were clearly present in chronic BPD. Fibrotic areas are characterized by increased numbers of fibroblasts and accumulation of fibrillar collagens, mainly collagens type-I and type-III (3, 40-43). MMP-1 exerts collagenolytic activity against fibrillar collagen. We observed increased staining intensity for TIMP-1 in type-II pneumocytes in the chronic phase of BPD compared with the earlier phases, whereas no difference in MMP-1 staining intensity in type-II pneumocytes was observed. This might result in an increased TIMP-1/MMP-1 ratio leading to decreased collagenolytic activity by type-II pneumocytes, thereby favoring interstitial collagen accumulation (fibrosis) as is observed in chronic BPD and is opposed to normal lung development.

Fibroblasts are the key cells in a fibrotic response, contributing to collagen deposition via increased proliferation, increased collagen synthesis, or decreased collagen breakdown (44). Lung tissue from patients with idiopathic pulmonary fibrosis has been shown to contain decreased collagenolytic activity and lung fibroblasts from these patients show an increased ratio of TIMP/MMP-1 compared with normal lung fibroblasts (25, 45). It has been suggested that co-expression of MMP-1 and TIMP-2 in fibroblasts in fibrotic foci from patients with idiopathic pulmonary fibrosis contributes to progressive collagen deposition caused by decreased collagenolytic activity. On the other hand, a predominance of MMP-1 in fibroblasts in fibrotic foci in bronchiolitis obliterans organizing pneumonia could explain the reversibility of fibrotic changes in that disease (27). Our study revealed that during the chron-

ic phase of BPD, fibroblasts in fibrotic areas were associated with the expression of MMP-1, TIMP-1, and TIMP-2. Inhibition of collagen degradation by decreasing the production of MMP-1 and increasing the production of TIMPs by fibroblasts has been reported for transforming growth factor-β (TGF-β_1) (46). Interestingly, intense immunoreactivity for TGF-β_1 has been shown in alveolar macrophages and fibroblasts in lung tissue during the regenerative and transitional stages of BPD (37). Therefore, during BPD development TGF-β_1 could increase the TIMP/MMP-1 ratio in fibroblasts. This would lead to a decreased collagenolytic activity and favors net deposition of collagen by fibroblasts, thereby contributing to fibrosis of the lung tissue.

In the present study we observed MMP-1 expression in type-II epithelial cells reepithelializing the injured alveoli. From the literature there is abundant evidence that MMP-1 is involved in reepithelialization after injury. Therefore, this may suggest a role for MMP-1 expressed by type-II pneumocytes in the reepithelialization process after acute injury during the acute and regenerative phase of BPD development. The increase in intensity of TIMP-1 relative to MMP-1 in type-II pneumocytes during the chronic phase of BPD might result in decreased interstitial collagen breakdown, thereby contributing to fibrosis as observed in chronic BPD. Furthermore, fibroblasts co-expressing MMP-1 and TIMPs in fibrotic areas may contribute to fibrosis in chronic BPD via a decreased collagenolytic activity, due to an increased TIMP/MMP-1 ratio.

Acknowledgements

We gratefully acknowledge Prof. Dr. R. Benner and Dr. V.H.J. van der Velden for critical reading of the manuscript.

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Chapter

EXPRESSION OF MATRIX METALLOPROTEINASE-9 AND -2 IN PRETERM INFANTS AT RISK FOR BRONCHOPULMONARY DYSPLASIA

submitted

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This work was supported by a grant from: Sophia Foundation For Medical Research

Abstract

Neonatal respiratory distress syndrome (RDS) in ventilated preterm neonates can either resolve or develop into bronchopulmonary dysplasia (BPD). Pulmonary inflammation, injury, diminished alveolization and fibrosis characterize the disturbed lung development in BPD. We analyzed sequential bronchoalveolar lavage (BAL) fluids from infants with resolving disease and from those developing BPD for matrix metalloproteinase-2 (MMP-2) and MMP-9. These MMPs degrade native type-IV collagen, fibronectin and elastin, and thus are likely to play a prominent role in the disturbed lung development in BPD. Unexpectedly, MMP-9 levels were increased at postnatal day 2 in BAL fluid from patients with resolving RDS compared to patients developing BPD ($p < 0.05$). After day 4, however, MMP-9 levels increased in BAL fluid from BPD patients and exceeded levels as observed in RDS at day 2. MMP-2 levels were equal in RDS and BPD and remained constant or showed a small increase after day 4 for BPD patients. Immunostaining revealed that the increased levels of MMP-9 in RDS and BPD appear to originate from neutrophils and macrophages, and for BPD also alveolar type-II epithelial cells. These findings indicate that the difference between resolving RDS or developing BPD may be related to the ability to raise an early adequate response to the initial injury.

Introduction

To save life, preterm infants with neonatal respiratory distress syndrome (RDS) are artificially ventilated. RDS can resolve within days after birth (uncomplicated RDS) or progress towards bronchopulmonary dysplasia (BPD) (1). BPD is characterized by interstitial fibroplasia, fibrosis and diminished alveolization. The exact mechanism of progression from RDS towards BPD is unknown, but involves artificial ventilation, oxygen toxicity and inflammation (1-3).

It has been proposed that proteases degrading components of the extracellular matrix (ECM), such as matrix metalloproteinases (MMPs) and neutrophil elastase, may lead to the impaired alveolar septation and fibrosis in BPD (2). In line with this proposal, neutrophil elastase and matrix metalloproteinase (MMP)-8 (neutrophil collagenase) have both been found to be increased in lungs from infants who develop BPD (2, 4). Furthermore, we demonstrated that an imbalance between MMP-1 and tissue inhibitors of metalloproteinases (TIMPs) correlated with excessive collagen accumulation in the lungs from premature infants who died of BPD (5).

MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B) degrade native type-IV collagen, fibronectin, elastin and denatured collagens (gelatin) (6-9). Both MMPs are secreted as latent proteases that require proteolytic processing to yield active enzyme (10). Analyses of bronchoalveolar lavage (BAL) fluid from patients with acute respiratory distress syndrome

(ARDS), which has a pathophysiology comparable to RDS, revealed increased levels of MMP-2 and MMP-9 (11, 12). Oxygen toxicity, one of the risk factors for the development of BPD, resulted in increased MMP-2 and MMP-9 levels in an animal model of hyperoxia (9). Also, oxidative stress in lungs from newborns was associated with increased MMP-9 levels (13). Finally, increased levels of MMP-2 and MMP-9 were detected in the lungs from patients with idiopathic pulmonary fibrosis (14). A causal relationship between fibrosis and MMP-2 and MMP-9 is suggested from studies in which mice received an intranasal injection of bleomycin together with the MMP inhibitor batimastat. This led to inhibition of MMP-2 and MMP-9 activity in BAL fluid and prevented the bleomycin induced pulmonary fibrosis (15). Therefore, both MMP-2 and MMP-9 are likely to play a role in the pathophysiology of BPD, although opposing findings have been reported (16, 17).

To determine whether MMP-2 and MMP-9 may play a role in the development of BPD we examined whether sequential BAL fluids from infants with uncomplicated RDS and from infants with developing BPD differed in the expression of MMP-2 and MMP-9. We found marked differences for MMP-9 but not for MMP-2. To reveal the possible sources of MMP-9 in BAL fluid, BAL cells from uncomplicated RDS and BPD patients and lung tissue from BPD patients were analyzed for antigenic MMP-9.

Materials and methods

Patients

Thirty two prematurely born infants, admitted to the neonatal intensive care unit, were included in this study. Inclusion criteria were: (1) gestational age ≤ 30 weeks and (2) requirement for mechanical ventilation on the first day of life because of RDS. BAL was performed on postnatal days 2, 4, 7 and 10, as long as the infant remained intubated. BPD was defined as having an abnormal chest radiograph and requirement for supplemental oxygen at a postnatal age of 28 days (18). Informed consent from parents was obtained. The study was approved by the local medical ethics committees.

Bronchoalveolar lavage

BAL was performed in a standardized way using 2 times 1 ml saline per kg body weight as described before (19). No marker for dilution was used, in accordance with the most recent guidelines from the ERS task force on BAL in children (20). The fluid fraction was separated from the cellular fraction by centrifugation and stored in aliquots at -80° C until analysis. Cell numbers were determined using a haemocytometer. May Grünwald Giemsa staining was performed and cell differentials were determined on 300 cells per patient per time-point.

Zymography

SDS-polyacrylamide (10%; w/v) gels containing 0.2% (w/v) gelatin (Sigma, St Louis, MO) were used to identify proteins with gelatinase activity in BAL fluid. With this method even latent MMP-9 and -2 bands show gelatinase activity. After electrophoresis, the gels were washed extensively in a solution of 2.5% (v/v) Triton X-100 to remove SDS, and incubated overnight at 37°C in Tris-HCl pH 7.5 containing 0.5M $CaCl_2$, 0.02% (w/v) NaN_3 and 1% (v/v) Triton X-100. The gels were stained with PhastGel™ Blue R (Amersham Pharmacia Biotech AB; Upsala, Sweden) and destained in a solution of 7% (v/v) acetic-acid and 20% (v/v) methanol. Gelatinase activity appeared as a clear band against a blue background. Purified MMP-2 and MMP-9 (Roche; Mannheim, Germany) and prestained molecular weight marker (Bio-Rad, Richmond, CA) were used to identify the different gelatinase bands. The gelatinase activities were quantified using densitometry. Values (arbitrary units (A.U.)) were related to a control collagenase (Clostridium histolyticum type 1A; Sigma) which was run in parallel on every zymogram. Representative zymograms are shown.

Immunocytochemistry

Cytospins were fixed with 4% (w/v) paraformaldehyde in PBS. Endogenous peroxidase was blocked by 3% (v/v) hydrogen peroxide in PBS/0.1% (w/v) saponin. Thereafter cytospins were blocked with 10% (v/v) normal rabbit serum in PBS/0.1% saponin, followed by 1 h incubation at 37 °C with 1.5 μg/ml (in PBS/0.1% saponin) goat polyclonal anti-human MMP-9 antibody (Santa Cruz Biotechnology, Santa Cruz, Ca), or goat IgG (Zymed, San Francisco, Ca) as negative control. Then cytospins were incubated for 30 min at room temperature with a biotin-labeled rabbit-anti-goat antibody (1.6 μg/ml in PBS/0.1% saponin; Dako, Glostrup, Denmark), followed by incubation (30 min at room temperature) with a horse-radish peroxidase-labeled streptavidin (Dako; 1/50 dilution in PBS/0.1% saponin). Hereafter, an amplification step was performed using the TSA™ Biotin System (NEN™ Life Science Products, Inc., Boston, MA). Subsequently, cytospins were incubated for 3 min with diaminobenzidine (Sigma Fast™ DAB, Sigma), counterstained with hematoxylin (Merck Diagnostica, Darmstadt, Germany) and embedded in Kaiser's gelatin glycerine (Merck Diagnostica).

Immunohistochemistry

Immunohistochemical staining for MMP-9 was performed on 4 μm lung tissue sections from infants who died during different phases of BPD development. The same antibody and concentration as mentioned in the section immunocytochemistry was used. The method used and clinical data from these infants were described before (5). Twenty five infants died in the acute phase (0-4 days after birth), seven in the regenerative phase (5-8 d), nine in the transitional phase (9-16 d) and seven in the chronic phase (> 16 d).

Statistical analyses

Results are presented as mean ± standard error of the mean (SEM) and were analyzed using the Mann Whitney U test. Pearsons correlation was used to examine relations between gelatinase bands with BAL cells, gestational age, birth weight and BAL fluid recovery. A p value < 0.05 was considered to indicate statistical significance.

Results

Patients

Sixty eight lavage procedures were performed in 32 preterm infants who were initially ventilated because of RDS. All patients were included before postnatal day 7. Twenty two infants subsequently developed BPD and 10 recovered from the initial RDS. Infants who developed BPD had a shorter period of gestation and tended to have lower birth weight than infants who recovered from RDS (table 1).

Table 1. Patient characteristics

	BPD (n=22)	RDS (n=10)	p value$
Gestational age* (weeks)	27.1 (25.7-29.4)	28.3 (26.7-30)	0.02
Birth weight* (g)	958 (720-1525)	1110 (780-1650)	0.1

* median (range)
$ Mann Whitney U test

Bronchoalveolar lavage

Recovery of lavage fluid was 49 ± 2% (mean ± SEM) of the used lavage volume. Recoveries did not significantly differ between patient groups and between postnatal ages. Due to extubation, BAL samples from RDS patients were obtained only until day 4. The number of BAL samples obtained during the different postnatal ages were: day 2: RDS = 9, BPD = 13; day 4: RDS = 4, BPD = 18; day 7: BPD = 15; day 10: BPD = 9. The BAL cell population consisted mainly of neutrophils and macrophages, of which neutrophils were more numerous (Fig. 1). No significant differences in total leukocytes, neutrophil and macrophage cell numbers per ml of BAL fluid were observed between RDS and BPD for days 2 and 4. Within the BPD group, there was a significant increase in the number of macrophages per ml BAL fluid on postnatal days 4, 7, and 10 as compared to postnatal day 2. Neutrophils were significantly increased only on postnatal day 10, compared to postnatal day 2 (Fig. 1).

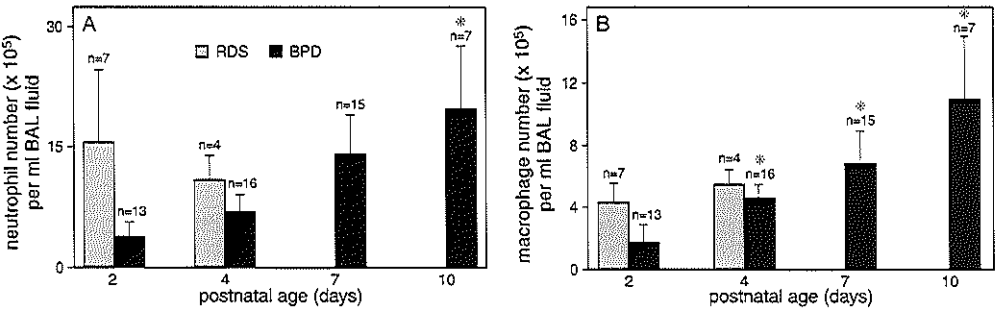

Figure 1
Number of neutrophils (A) and macrophages (B) per ml BAL fluid from RDS and BPD patients during the study period. * $p < 0.05$ (Mann Whitney U test) compared with day 2.

Zymography

Analyses of BAL fluid from RDS and BPD patients revealed several bands with gelatin zymography. Most markedly, a 92 and a 135 kDa band were found, representing latent MMP-9 and presumably neutrophil-gelatinase B-associated lipocalin (NGAL) associated with MMP-9 (NGAL associated with MMP-9, will from here be further indicated as NGAL), respectively. Occasionally a 86 kDa and a high molecular weight (~200 kDa) band were observed, likely to represent activated MMP-9 and a homodimer of MMP-9, which is resistant to dissociation by SDS. The identity of the MMP-9 bands was confirmed by immunoprecipitation, using protein A-Sepharose beads, removing MMP-9-anti-MMP-9 complexes from the BAL fluid and by Western blotting (data not shown). Finally, a 72 kDa and sometimes a weak 68 kDa band were found, typical of latent MMP-2 and activated MMP-2, respectively.

Comparison of gelatinases in RDS and BPD BAL fluids revealed marked differences at postnatal day 2 (Fig. 2) and, to a lesser extent, at day 4 (data not shown). By densitometric analyses, latent MMP-9 and NGAL were shown to be significantly increased at postnatal day 2 in BAL fluid from patients who recovered from RDS (Fig. 3). In contrast, latent MMP-2 was detected equally in BAL fluid from both RDS and BPD patients at postnatal days 2 and 4. The amount of NGAL showed a weak but significant correlation with the number of neutrophils per ml BAL fluid ($r = 0.448$; $p = 0.04$), but did not correlate with macrophage or total white blood cell numbers per ml BAL fluid. The amount of latent MMP-9 showed no significant correlation with the total number of white blood cells or the number of neutrophils and macrophages per ml BAL fluid. There was a significant correlation between the amount of NGAL and latent MMP-9 ($r = 0.931$; $p < 0.001$). No statistically significant correlation was observed between latent MMP-9 and NGAL with gestational age, birth weight or BAL recovery. After day 4, particularly amounts of latent MMP-9 and NGAL increased substantially in BAL fluid from BPD patients to levels higher than seen for MMP-9 and NGAL in RDS patients at day 2 (compare Figure 4A with Figure 2A), while

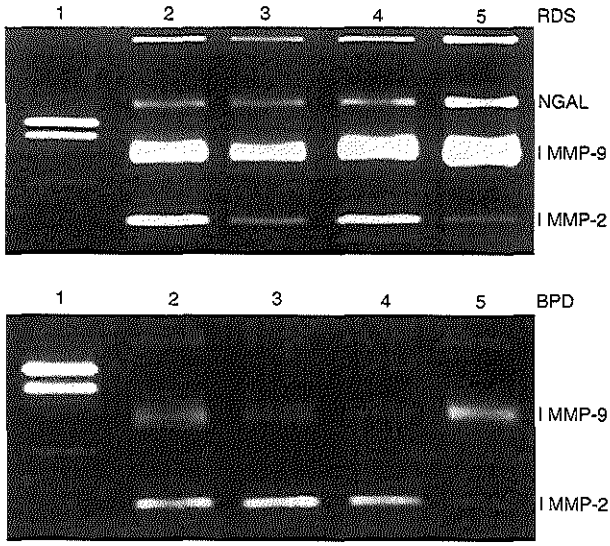

Figure 2
Zymograms of RDS and BPD BAL fluid samples from postnatal day 2. Lane 1 is collagenase control. Lanes 2-5 are BAL samples from individual patients. I MMP-2 is latent MMP-2.
I MMP-9 is latent MMP-9. NGAL is neutrophil gelatinase B-associated lipocalin associated with MMP-9.

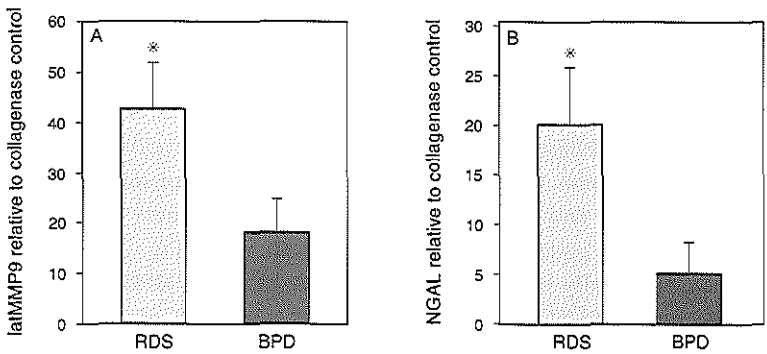

Figure 3
Densitometric analyses of latent MMP-9 (A) and NGAL (B) in postnatal day 2 BAL fluid samples from RDS (n = 9) and BPD (n = 13) patients. Densitometric value is expressed relative to a control collagenase (A.U.) that was run parallel with the samples on every zymogram. * $p < 0.05$ (Mann Whitney U test) compared with BPD.

latent MMP-2 remained relatively constant or, at most, showed a slight increase over the study period (Fig. 4).

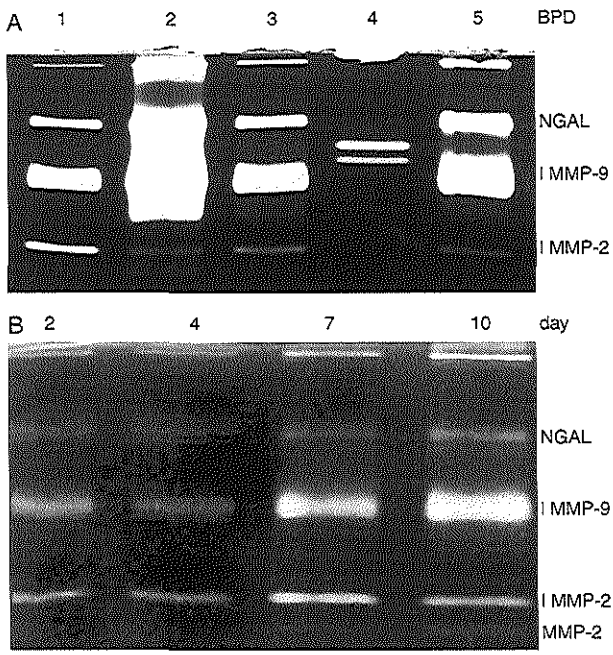

Figure 4
(A). Zymogram of BAL fluid samples obtained at postnatal day 10 from BPD patients. Lanes 1-3 and lane 5 are BAL samples from individual patients, lane 4 is a collagenase control. (B). Sequential BAL samples obtained at postnatal days 2, 4, 7 and 10 from an infant developing BPD. MMP-2 is active MMP-2, l MMP-2 is latent MMP-2, l MMP-9 is latent MMP-9, NGAL is neutrophil gelatinase B-associated lipocalin associated with MMP-9.

Immunocytochemistry

Immunocytochemical analyses of BAL cells revealed immunoreactivity for MMP-9 in neutrophils and macrophages from both RDS and BPD patients. However, also numerous MMP-9 negative neutrophils and macrophages were observed (Fig. 5).

Immunohistochemistry

Lung tissue from infants who died in the acute phase (0-4 days) was characterized by absence or sparse scattered immunostaining for MMP-9 (Fig. 6A). In the regenerative phase of the disease (5-8 days), clusters of MMP-9 immunoreactive cells (comprising inflammatory cells such as monocytes/macrophages and neutrophils) became evident (Fig. 6B). In the transitional phase (9-16 days), numerous clusters of immunoreactive cells were present and stained more intensely compared to the previous phase (Fig 6C). In the chronic phase (>16 days), MMP-9 expressing inflammatory cells were still present (data not shown). Additionally, from the regenerative phase to the chronic phase onwards, weak MMP-9

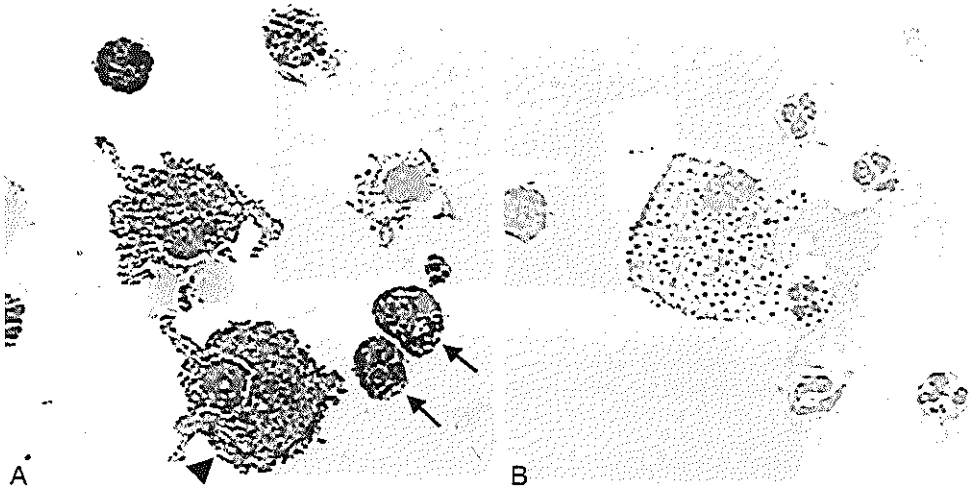

Figure 5
Immunocytochemical staining with anti-MMP-9 antibody (A) and control IgG (B) on BAL cells from an RDS patient at postnatal day 2. MMP-9 immunoreactivity was observed in neutrophils (arrow) and macrophages (arrowhead).

immunoreactivity was observed in regenerating alveolar type-II epithelial cells and persisted into the chronic phase (Fig 6C). In the chronic phase, fibroblasts in fibrotic foci were immunoreactive for MMP-9 (data not shown).

Discussion

Here we show that MMP-9 levels in BAL fluid from premature infants (gestational age ≤ 30 weeks) who develop BPD as compared to infants with resolving RDS are significantly reduced at postnatal day 2. After postnatal day 4, however, MMP-9 levels in BAL fluid from BPD patients increased markedly and remained high during the study period. Levels of MMP-2 were comparable between RDS and BPD at day 2 and remained constant or showed a small increase after day 4 for BPD patients.

The higher MMP-9 levels in BAL fluid at day 2 from patients who recover from RDS is a novel finding. The neutrophil is considered an important source of MMP-9 and is known to secrete different molecular weight forms of MMP-9 (21). In the zymograms of BAL fluid collected at postnatal day 2 we observed MMP-9 bands which, based on molecular weight represent NGAL, latent MMP-9 and the homodimer of latent MMP-9. The presence of NGAL indicates that neutrophils contributed to the MMP-9 detected in BAL fluid, which is supported further by the significant correlation between absolute numbers of neutrophils per ml of BAL fluid and the levels of NGAL. Furthermore, MMP-9 immunoreactive neutrophils were present in the BAL fluid from RDS and BPD patients at postnatal day 2. No such cor-

Figure 6
Immunohistochemical staining for MMP-9 on lung tissue from patients at different phases of BPD development. Sparse MMP-9 immunoreactivity was observed during the acute phase (0-4 d after birth) of BPD (A). In the regenerative phase (5-8 d) clusters of MMP-9 immunoreactive inflammatory cells became evident (B). In the transitional phase (9-16 d) of BPD (C) strong MMP-9 immunoreactivity was still observed in inflammatory cells and weak MMP-9 immunoreactivity was present in regenerating type-II epithelial cells (arrow).

relation existed for latent MMP-9, indicating that other cellular sources such as monocytes, macrophages and alveolar type-II epithelial cells may also contribute to the increased levels of MMP-9 in RDS during the initial stage of the disease. Indeed, MMP-9 immunoreactivity was observed in macrophages, monocytes and alveolar type-II epithelial cells, in line with previous studies (22-25).

In the present study we determined MMP-9 levels which do not necessarily reflect MMP-9 activity in BAL fluid, particularly as proteolytic activation and natural inhibitors (TIMPs) modulate MMP-9 activity (24). Nevertheless, the presence of MMP-9 on day 2 in BAL fluid from patients with resolving RDS and its relative absence in patients with developing BPD suggests that early activation and/or recruitment of cells, among which neutrophils, is an important mechanism leading to the clearance of RDS. Given that activation and recruitment of cells require initial triggers it is likely that these initial responses are adequate in patients with resolving RDS and inadequate (too low and/or too slow) in patients who develop BPD. Such an inadequate response in BPD patients may result in delayed reepithelialization of the injured alveoli, a process in which MMP-9 is implicated (26).

After day 4, MMP-9 levels increase in BAL fluid from BPD patients and actually increase to much higher levels than seen for MMP-9 in RDS patients on day 2. Here too, neutrophils appear an important source of MMP-9, but the involvement of alveolar type-II epithelial cells and fibroblasts is likely too. This prolonged and extensive increase of MMP-9 might be implicated in lung damage in BPD, as it has been demonstrated that increased MMP-9 levels in BAL fluid from ARDS patients coexisted with the 7S portion of type-IV collagen, which is considered as a marker of basement membrane disruption (12).

The latter data on MMP-9 are in line with those reported in a preliminary report by Sweet and co-workers (16), but are conflicting with those from Cederqvist *et al* (17) who reported no difference in the levels of MMP-9 between RDS and BPD. In their study, however, tracheal aspirates were analyzed instead of BAL fluid, thus sampling another compartment of the airways. Furthermore, the data for the initial five postnatal days were pooled and presented as one value, thereby possibly overlooking the temporal differences in MMP-9 levels as described here. Taken together it is likely that these methodological differences may account for the observed discrepancy.

In conclusion, our study provides evidence that the difference in resolving RDS or developing BPD may be related to the ability to raise an early response to the initial changes in the lung. The low MMP-9 levels, particularly those of NGAL at postnatal day 2, may help to identify RDS patients who are at risk of developing BPD.

Acknowledgements

We gratefully acknowledge Prof. Dr. G. Opdenakker (Rega Institute, University of Leuven, Belgium) and Dr. V. Everts (AMC, University of Amsterdam, The Netherlands) for helpful discussions, and Prof. Dr. R. Benner and Dr. V.H.J. van der Velden for critical reading of the manuscript.

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Chapter

POST-INJURY SHORT-COURSE DEXAMETHASONE TREATMENT INHIBITS COLLAGEN DEPOSITION IN BLEOMYCIN-INDUCED FIBROSIS IN RATS

submitted

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This work was supported by a grant from: Royal Dutch Academy of Sciences

Abstract

Bronchopulmonary dysplasia (BPD) is a neonatal chronic lung disease with varying degrees of fibrosis that can develop over days to weeks following mechanical ventilation for neonatal respiratory distress syndrome. Recently, it was demonstrated that a short-course of dexamethasone (Dex), initiated 12-48 hours postnatally, increased survival without developing chronic lung disease in these infants. However, whether or not this treatment regimen inhibits fibrosis, is not known. In this study, we examined the effect of treating rat lungs exposed to bleomycin, with a 3-day course of Dex (0.5 mg/kg/body weight, comparable to the dose in infants at risk of BPD), initiated three days after injury, on cell proliferation and collagen production. We clearly demonstrated that treating the animals with Dex for three days, inhibited collagen accumulation in their lungs compared with bleomycin exposed untreated animals despite initiating treatment three days after initiation of lung injury. Dex treatment reduced the number of proliferating cells in the lung parenchyma of bleomycin exposed rats but did not influence BAL fluid mitogenic activity for lung fibroblasts or alter the BAL fluid levels of the fibrogenic mediators TGF-β_1, PDGF-AB, or thrombin. We conclude that a 3-day course of Dex treatment initiated after induction of fibrosis by bleomycin, reduces lung collagen accumulation but this occurs by mechanisms other than through reduction of TGF-β_1, PDGF-AB or thrombin levels in BAL fluid. We propose that the beneficial effects of short-course Dex treatment of infants likely to develop BPD, may be, at least partly, due to antifibrotic effects.

Introduction

Pulmonary fibrosis is the end stage of a heterogeneous group of disorders of known and unknown etiology. Despite the wide variety of insults associated with this condition, such as bacterial infection, inhalation of organic and inorganic dusts, radiation, drugs, and trauma the mechanisms involved appear largely the same (1). It is assumed that in response to injury, inflammatory cells enter the lung and, together with resident lung cells, release mediators that stimulate fibroblast proliferation and collagen deposition within the lung interstitium (1). A host of mediators have been implicated in the pathogenesis of pulmonary fibrosis because they fit three basic criteria: 1) As mentioned, they stimulate fibroblast replication or procollagen synthesis, 2) The gene expression and protein production of the mediator is increased in the lungs of patients with pulmonary fibrosis and 3) Inhibitors of its function attenuates fibrosis in animal models of the disease (2). Mediators which fit these criteria include: platelet-derived growth factor (PDGF), transforming growth factor-β_1 (TGF-β_1), insulin-like growth factor-1, endothelin-1, fibronectin and thrombin (1, 3-9).

Bronchopulmonary dysplasia (BPD; also named chronic lung disease of prematurity) is a neonatal chronic lung disease associated with varying degrees of fibrosis, that develops

within days to weeks after mechanical ventilation treatment for neonatal respiratory distress syndrome (RDS) (10, 11). Early and prolonged pulmonary inflammation is thought to play a critical role in tissue injury, tissue remodelling and fibrosis in the pathogenesis of BPD (12). Infants with, or at risk of developing BPD, are routinely treated with a systemic course of the corticosteroid dexamethasone (Dex) for a prolonged period of time. The Dex regimen used in our neonatal intensive care unit at Erasmus MC, University Medical Center Rotterdam, is generally initiated after the first week of life. It comprises 0.5 mg/kg per day of Dex sodium-phosphate for three days, followed by 0.3 mg/kg per day for an additional three days, after which the Dex dose is weaned over the next 2 to 4 weeks. This treatment has proven to be beneficial as it facilitates weaning from the mechanical ventilator and reduces pulmonary inflammation (13). However, the effect of this treatment on lung fibrosis is uncertain as it does not downregulate the pulmonary expression levels of fibrogenic mediators such as fibronectin and TGF-β_1 in these infants (14, 15). Recently, it was found that a short-course of Dex (using a dose comparable to what we use to initiate treatment in our hospital) starting at 12-48 hours postnatally, increased survival without BPD and reduced the requirement for subsequent late Dex therapy (16, 17). It was stated that the pulmonary benefits of early short-course Dex in these infants should be weighed against the side-effects such as gastrointestinal perforation. However, it is not known if a short-course of corticosteroid treatment initiated early after injury is able to prevent lung fibrosis.

Intratracheal instillation of the antitumor agent bleomycin is the most commonly used animal model for pulmonary fibrosis. This model is characterized by an early, predominantly neutrophilic inflammatory response, increased fibroblast proliferation and enhanced collagen deposition due to increased collagen synthesis and decreased collagen degradation (18, 19). Prolonged administration of corticosteroids such as methylprednisolone or Dex, initiated prior to, or simultaneously with bleomycin, reduces pulmonary inflammation, lung injury and collagen deposition in this model (20-22). However, as treatment for BPD commences after lung injury in infants, the clinical relevance of experimentally treating animals with anti-inflammatory therapy prior to or at the time of injury is questionable.

The current study was undertaken to determine if a 3-day course of Dex treatment (0.5 mg/kg body weight which is comparable with the initial three-day dose of the Dex regimen used in our neonatal intensive care unit), initiated after the induction of lung injury with bleomycin, affected collagen deposition and cell proliferation and modulated the expression profile of the profibrotic mediators TGF-β_1, PDGF-AB, and thrombin in BAL fluid.

Methods

Male Lewis rats weighing 140-210 g were anesthetized by intramuscular injection of 0.75-1.0 ml/kg body weight Hypnorm (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml; Janssen Pharmaceutical, High Wycombe, UK). Bleomycin disulphate (Kyowa Hakko, Slough, UK) was administered by a single intratracheal injection (1.5 mg/kg body

weight in 0.3 ml of sterile saline) as described previously (6). Control animals received 0.3 ml of saline alone.

Corticosteroid treatment

Three days after administering bleomycin, animals receiving corticosteroid treatment were given a daily intraperitoneal (i.p.) injection of Dex sodium-phosphate (0.5 mg/kg body weight in 0.5 ml of sterile saline; Sigma, St Louis, MO) from days three to five. Control animals received an i.p. injection of sterile saline. Four different experimental treatment groups were included in this study: 1) bleomycin receiving saline i.p. (BLM); 2) bleomycin receiving Dex i.p. (BLMdex); 3) control receiving saline i.p. (CTRL), and 4) control receiving Dex i.p. (CTRLdex). Groups of 6 rats were killed 3, 7 and 14 days after bleomycin or saline instillation by an overdose of pentobarbitone. Lungs were lavaged 3 times with 4 ml of sterile saline and total and differential cell counts were performed. BAL fluid was aliquoted and stored at -80°C for further analyses. The lungs were removed, blotted dry and immediately snap-frozen in liquid N_2 after removing the trachea and major airways. Lung collagen content and BAL fluid mitogenic activity and growth factor levels were measured in 6 animals for all groups except CTRL on day 7 (n = 3), and CTRLdex at day 7 (n = 5)

Groups of 3 animals were also killed 3 and 5 days after bleomycin or saline injection with and without Dex treatment and proliferating cells were identified by bromodeoxyuridine (BrdU) immunoreactivity. Briefly, animals were injected i.p. with BrdU (15 μg/ g body weight in 0.3 ml sterile saline; Sigma) 1 h prior to killing and the lungs fixed by intratracheal instillation of freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) at a pressure of 25 cm H_2O. The trachea was ligated and the thoracic contents removed *en bloc*. After overnight immersion in fixative (4 °C), tissues were transferred to 15% sucrose in PBS (overnight, 4 °C), dehydrated and embedded in paraffin wax.

Collagen measurement

Lung collagen was assessed by measuring hydroxyproline levels in proteins by high-pressure liquid chromatography (HPLC) as previously described (6). Briefly, approximately 100 mg of powdered lung tissue (obtained by crushing tissue while frozen at -196 °C) was weighed and hydrolyzed in 2 ml of 6 M HCl at 110°C for 16 h. Hydrolysates were mixed with activated charcoal and filtered (Milipore, type DA, pore size 0.65 μm). A 200 μl aliquot of a 1-in-10 dilution of filtered hydrolysate was dried using a centrifugal vacuum concentrator. Hydroxyproline was isolated and measured by reverse-phase-HPLC after derivatization with 7-chloro-4-nitrobenz-2-oxa-1,2,-diazole (NBD; Sigma) (6). The hydroxyproline content in each sample was determined by comparing peak areas of samples from the chromatogram with those generated from standard solutions. The amount of collagen in total lung tissue was calculated assuming that lung collagen contains 12.2% w/w hydroxyproline (23), and expressed as mg collagen/lung.

Fibroblast proliferation assay

Fibroblast proliferation was assessed using a colorimetric assay based on the uptake and subsequent elution of the dye methylene blue as previously described (24). Briefly, cells (human fetal lung fibroblasts; HFL-1) were seeded at 6 x 10^3 cells/well into 96-well plates in 50 µl Dulbecco's modified Eagle's medium (DMEM; Gibco, Renfrewshire, UK) supplemented with 0.4% normal calf serum (NCS), L-glutamine and antibiotics and allowed to adhere for 24 h. Thereafter, 50 µl of a 1/4 dilution of BAL fluid in DMEM/0.4% NCS was added to the fibroblasts cultures (six replicates per BAL fluid sample; yielding a 1/8 dilution of BAL fluid) and proliferation assessed after 48 h. The medium was removed and the plates immersed in PBS and blotted on absorbent paper. Cells were fixed in 10% formol saline and then stained for 30 min with 1% methylene blue (Sigma) in 0.01 M borate buffer, pH 8.5. Excess dye was removed with 0.01 M borate buffer using an automatic plate washer. Excess moisture was removed from the plate by blotting on absorbent paper and the dye eluted from the cells by the addition of 100 µl of acidified alcohol (0.01 M HCL-ethanol, 1:1). Absorbance was measured at a wavelength of 650 nm on a microplate spectrophotometer. Fibroblast proliferation was expressed as a percentage change in mean absorbance above that for cells exposed to DMEM/0.4% NCS alone.

Measurement of growth factors in BAL fluid

Levels of total protein in BAL fluid were measured using a Bradford-based reagent (Bio-Rad; München, Germany). Briefly, the Bradford-based reagent was diluted 5 times with milli Q H_2O. Thereafter, 5 µl of BAL sample was added to 100 µl of reagent. Bovine serum albumin (BSA) was used to generate a standard curve. Absorbance was measured at a wavelength of 620 nm and protein contents of the BAL samples were calculated from the standard curve.

TGF-β_1 was measured by enzyme-linked immunosorbent assay (ELISA; Promega; Madison WI) after acid activation of the BAL fluid samples to determine total TGF-β_1 (both latent and active TGF-β_1).

PDGF-AB levels were determined using a human PDGF-AB ELISA (R&D systems; Abingdon, UK) which has previously been shown to be cross-reactive with rat (25).

The ELISA assays were performed according to the methods outlined by the manufacturer. All BAL fluid samples were analyzed undiluted in duplicate. Results were expressed as pg/ml BAL fluid. The sensitivity of the assays was 25 pg/ml and 8.4 pg/ml for TGF-β_1 and PDGF-AB, respectively.

Thrombin activity was determined using the thrombin specific chromogenic substrate Tos-Gly-Pro-Arg-pNA (Sigma) as described previously (26). Briefly, 25 µl of BAL fluid was diluted in 25 µl Tris-buffered saline (TBS; pH 8.3) and 25 µl BAL fluid was diluted in TBS containing 4 x 10^{-6} M of the thrombin inhibitor PPACK (Bachem; Bubendorf, Switzerland). These solutions were added to a 96 well microtitre plate and incubated for 20 min at 37 °C to allow thrombin-PPACK complexes to form. Thereafter, 50 µl of 1mM Tos-Gly-Pro-Arg-pNA (in 1.5 mM HCL) was added to the diluted BAL fluid and incubated at 37 °C. The opti-

cal density (OD) was measured at 405 nm for 24 h. During this time, a linear increase in optical density of the BAL samples was found (data not shown). Thrombin activity in each BAL sample is expressed as an OD value at 405 nm, which was determined as the difference in OD, measured at 24 h, between the BAL sample with and without PPACK. As a positive control, 1 x 10^{-6} M thrombin (Sigma) was added to the substrate. Preincubation of thrombin with PPACK resulted in complete inhibition of thrombin activity (data not shown).

Cell proliferation by BrdU incorporation

Paraffin sections (5 μm) were dewaxed, rehydrated and boiled for 15 min in citric acid (pH 6.0). BrdU staining was performed using a BrdU Staining Kit (ZYMED Laboratories INC.; South San Francisco, CA) according to the manufacturer's instructions. Slides were examined by light microscopy (Zeiss; Axiolab) and the number of BrdU positive cells determined in 10 high power fields (400x magnification) per section of lung tissue in groups of 3 bleomycin exposed and unexposed animals. Data are presented as the mean number of positive cells per high power field.

Statistical analyses

Results are presented as mean ± standard error of the mean (SEM). Data between groups were compared using the unpaired Students t-test. A p value < 0.05 was considered to be statistically significant.

Results

Animal Weights

Rats injected with bleomycin lost weight during the first 3 days after treatment but gained weight thereafter. Rats treated with bleomycin and receiving Dex continued to lose weight during the three days of Dex administration and started to gain weight again when Dex administration was stopped. Control animals receiving Dex also lost weight during the Dex treatment and started to gain weight again when Dex administration was stopped.

BAL fluid cell count and differential

Table 1 shows the total leukocyte counts and composition of the cell population in BAL fluid from the different groups at the different time-points examined. Three days after bleomycin the total number of cells in BAL fluid was significantly increased compared with control. Furthermore, the percentage of neutrophils was significantly increased and the percentage of macrophages significantly decreased compared with control.

	day 3		day 7				day 14			
	CTRL	BLM	CTRL	CTRLdex	BLM	BLMdex	CTRL	CTRLdex	BLM	BLMdex
# cells/ml BAL fluid (x 10^4)	5.2±1.0	26.8±5.7*	10.0±3.0	5.5±1.3	10.0±3.8	7.3±2.8	5.8±1.3	4.8±0.6	10.4±2.0*	7.2±1.4
% neutrophils	4.1±2.9	47.0±5.2*	1.6±0.7	1.0±0.4	24.4±8.6	15.7±7.4	0.7±0.3	1.0±0.2	2.0±1.0	5.6±2.1
% macrophages	95.1±2.9	46.9±6.8*	98.3±0.6	98.3±0.5	73.2±9.3	81.1±8.3*	99.0±0.4	98.9±0.3	97.1±1.1	93.6±2.1*
% lymphocytes	0.9±0.3	6.1±1.9	0.1±0.1	0.7±0.2	2.4±0.9	3.1±1.1	0.3±0.1	0.1±0.1	1.0±0.5	0.9±0.3*

* $p < 0.05$ (Mann Whitney U test) compared to appropriate control group

At day 14, the total number of cells was still elevated in bleomycin treated animals, however, the proportions of cell types comprising the BAL cell population was comparable with controls. There was a trend towards decreased lavage cell numbers in Dex treated animals compared with their respective controls at days 7 and 14 but these changes were not statistically significant. However, there was a significantly decreased percentage of macrophages at day 7 and day 14 compared with control animals, while the percentage of lymphocytes was increased at day 14 compared with control.

Collagen measurement

Figure 1 shows the changes in total lung collagen 3, 7 and 14 days after instillation of bleomycin with and without Dex treatment. Bleomycin did not change lung collagen content at day 3. At day 7, the collagen content was significantly increased in BLM compared with BLMdex (14.2 ± 1.6 mg collagen/lung compared with 9.7 ± 0.7 mg collagen/lung; $p < 0.05$). The collagen content continued to increase in the BLM group, and by day14 was double that of controls (22.5 ± 2.1 mg compared with 11.2 ± 1.1 mg for controls; $p < 0.05$). Lung collagen content in bleomycin exposed Dex treated animals did not increase compared with Dex treated or untreated controls at either 7 or 14 days. Bleomycin exposed animals treated with Dex had significantly reduced lung collagen at day 14 compared with bleomycin alone (22.5 ± 2.1 mg for BLM compared with 15.2 ± 2.2 for BLMdex; $p < 0.05$). There was no significant change in total lung collagen for controls at any of the times examined. Dex treatment of control animals did not influence total lung collagen content at any of the times examined.

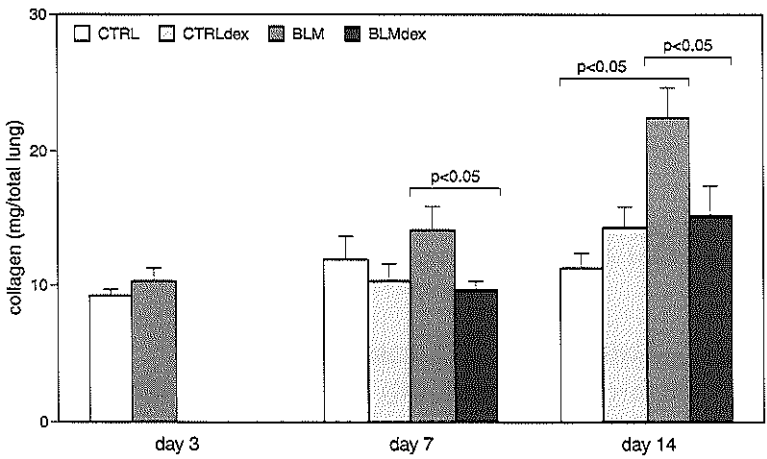

Figure 1
Change in lung collagen content at various times following bleomycin instillation. Collagen content was measured 3-14 days after intratracheal instillation of bleomycin or saline with and without a three-day course of Dex treatment. Each value represents the mean ± SEM.

BAL fluid-induced fibroblast proliferation

Figure 2 shows the mitogenic effect of the BAL fluid from animals in each group on fibroblast proliferation over 48 h. There was a significant increase in BAL fluid-induced fibroblast proliferation at day 3 in bleomycin compared with saline treated animals (28.4 ± 1.8% proliferation above medium control compared with 5.7 ± 1.4% for control; $p < 0.05$). No differences were observed between bleomycin treated and control animals at days 7 and 14. Dex treatment did not significantly influence the mitogenic activity of BAL fluid from bleomycin treated animals at days 7 and 14. At day 7, BAL fluid from control animals exposed to Dex revealed significantly decreased mitogenic activity compared with bleomycin exposed animals treated with Dex (20.2 ± 4.9% proliferation for BLMdex compared with 0.3 ± 4.6% for CTRLdex; $p < 0.05$). No significant change in BAL fluid mitogenic activity was observed for Dex treated and untreated controls at any of the times examined.

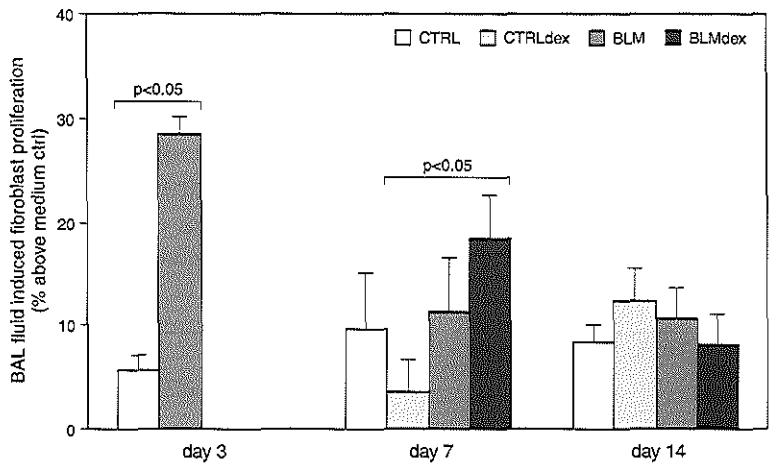

Figure 2
The effect of a 1/8 dilution of BAL fluid on fibroblast proliferation 3-14 days after intratracheal instillation of bleomycin or saline with and without a three-day course of Dex treatment. Each value represents the mean ± SEM.

Measurement of growth factors in BAL fluid

Total protein levels were significantly increased 3 days after bleomycin treatment (631.1 ± 56.4 μg/ml BAL fluid compared with 118.8 ± 47.6 μg/ml for controls; $p < 0.05$). Thereafter, total protein levels declined in bleomycin treated animals but were still significantly increased compared with control at day 14 (158.5 ± 26.7 μg/ml BAL fluid compared with 78.1 ± 12.3 for controls; $p < 0.05$). Treatment with Dex did not influence BAL fluid total protein levels in bleomycin treated or control animals. There was no significant change in BAL fluid total protein levels for controls at any of the times examined (Fig. 3A).

TGF-β_1 levels in BAL fluid were significantly increased at day 3 after bleomycin

treatment (616.4 ± 165.9 pg/ml BAL fluid compared with 98.9 ± 27.9 pg/ml BAL fluid for controls; $p < 0.05$). TGF-β_1 levels declined at 7 days after bleomycin, but were still significantly increased compared to control (194.6 ± 48.0 pg/ml BAL fluid compared with 46.1 ± 12 pg/ml BAL fluid for controls; $p < 0.05$). At day 14 after bleomycin, TGF-β_1 in BAL fluid was still significantly increased (230.6 ± 177.7 pg/ml BAL fluid compared with 24.9 ± 7.6 pg/ml BAL fluid for controls; $p < 0.05$). Dex treatment of bleomycin exposed and control animals did not influence TGF-β_1 in BAL fluid at any of the times examined. There was no significant change in BAL fluid TGF-β_1 levels for controls at any of the times examined (Fig. 3B).

PDGF-AB levels in BAL fluid were significantly increased at day 3 after bleomycin (209.9 ± 25.8 pg/ml BAL fluid compared with 90 ± 6.2 pg/ml BAL fluid for controls; $p < 0.05$) and remained elevated at relative constant levels during the study period. Dex treatment of bleomycin exposed and control animals did not influence PDGF-AB in BAL fluid at any of the times examined. There was no significant change in BAL fluid PDGF-AB levels for controls at any of the times examined (Fig. 3C).

Thrombin activity in BAL fluid was significantly increased at day 3 after bleomycin exposure (0.6 ± 0.07 OD units at 405nm compared with 0.1 ± 0.03 OD units at 405nm for

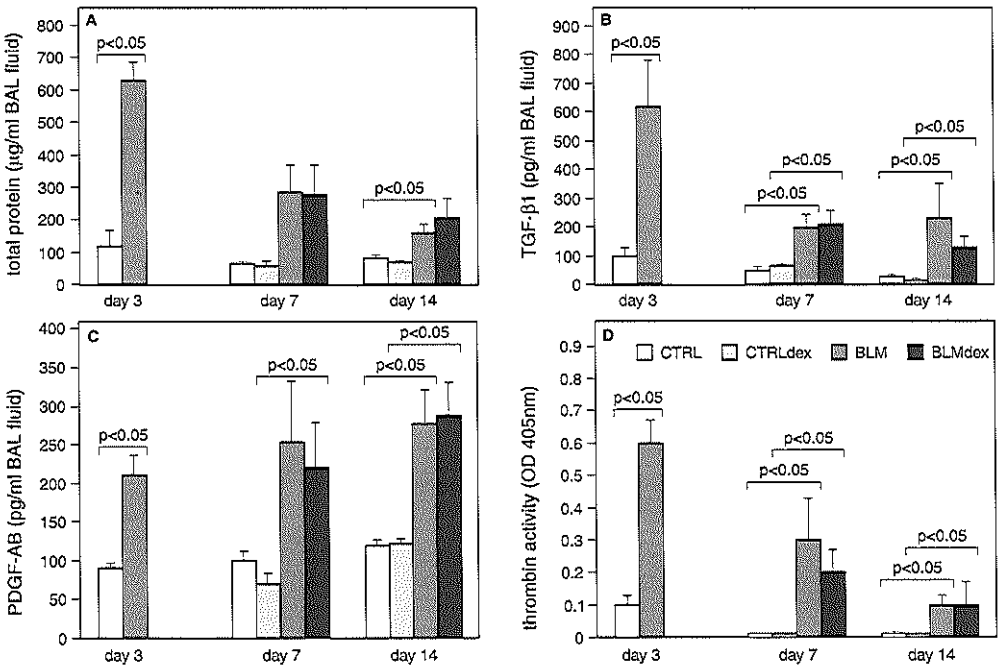

Figure 3
Changes in BAL fluid total protein (A), total TGF-β_1 (B), PDGF-AB (C), and thrombin activity (D) 3-14 days after bleomycin or saline instillation with and without a three-day course of Dex treatment. Each value represents the mean ± SEM.

controls; $p < 0.05$).

Thereafter thrombin activity gradually declined in BAL fluid of bleomycin treated animals but remained significantly elevated at all times examined compared with controls (Fig. 3D). Dex treatment of bleomycin exposed and control animals did not influence thrombin activity in BAL fluid at any of the times examined. In control animals, BAL fluid thrombin activity was elevated at day 3 compared to controls at day 7 and 14 (Fig. 3D).

Cell proliferation by BrdU incorporation

Very few cells were proliferating in control lung tissue as assessed by BrdU immunoreactivity with staining limited to a few alveolar type-II epithelial cells (Fig. 4A). In animals exposed to bleomycin, increased numbers of BrdU positive alveolar type-II epithelial cells and interstitial cells were clearly evident both in fibrotic (Fig 4B-D) and normal areas of lung parenchyma at all times examined. Treatment of bleomycin exposed animals with Dex significantly reduced the number of BrdU positive cells both in areas of fibrosis and normal lung parenchyma (Fig. 5).

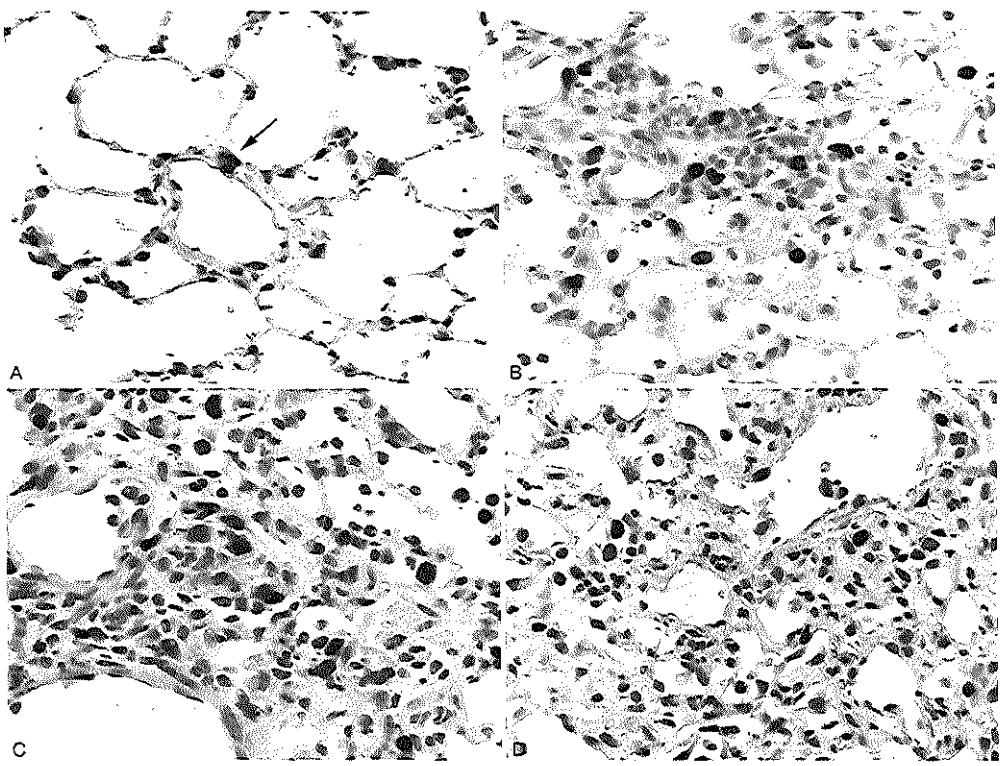

Figure 4
BrdU immunoreactive cells (arrow) in control lung tissue 3 days after saline instillation (A), 3 days after bleomycin instillation (B), 5 days after bleomycin instillation without Dex treatment (C) and 5 days after bleomycin instillation with Dex treatment (D). Magnification is 400x.

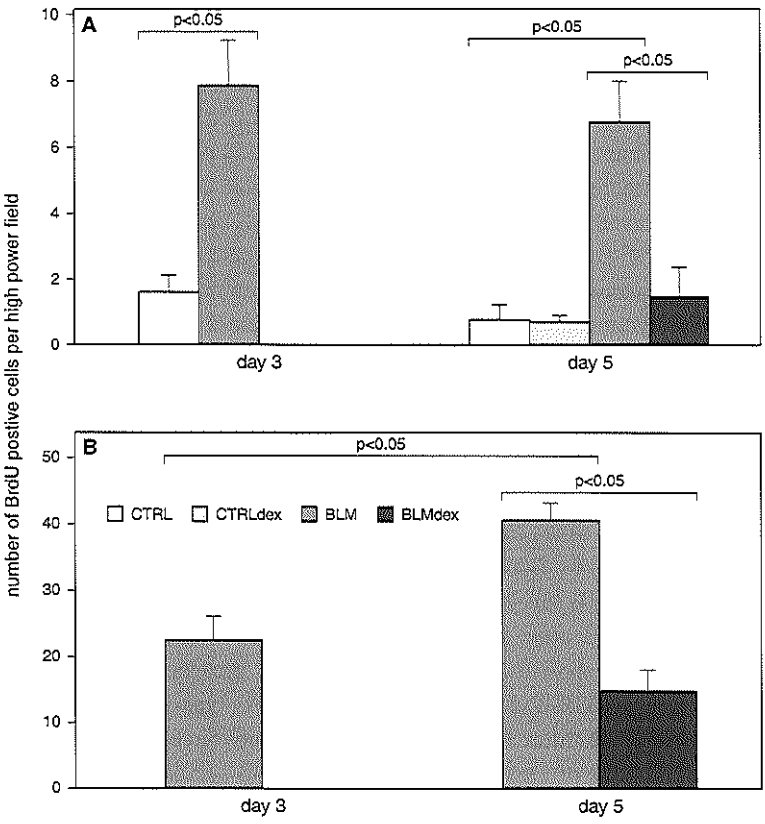

Figure 5
The number of BrdU immunoreactive cells per high power field at 400 times magnification. (A) shows the number of BrdU positive cells in areas of normal appearing lung tissue at 3 and 5 days after bleomycin or saline instillation with and without Dex treatment. (B) shows the number of BrdU positive cells in fibrotic lung tissue 3 days after bleomycin instillation and 5 days after bleomycin instillation with and without Dex treatment.

Discussion

Previous studies have shown that prolonged administration of corticosteroids, initiated prior to or together with bleomycin-administration inhibited the development of lung fibrosis in rats (20-22). Although an important finding, its relevance clinically is questionable as anti-inflammatory therapy is always given after lung damage has occurred. In this study we clearly show that a 3-day course of Dex treatment, initiated three days after bleomycin-induced lung injury, inhibits excessive collagen deposition in the lungs of rats, suggesting that this treatment regimen, comparable to the Dex dose initially used for three days in our neonatal intensive care unit to treat infants at risk for BPD, may be useful in

reducing fibrosis in these infants.

The current theory on the pathogenesis of pulmonary fibrosis is based on the hypothesis that mediators released by inflammatory cells and resident lung cells as well as blood born mediators induce fibroblasts to proliferate and/or to produce excess collagen (1). We observed no significant difference in the inflammatory cell population between Dex treated and untreated bleomycin exposed animals. This may have accounted for the fact that no differences were observed in BAL fluid levels of TGF-β_1 and PDGF-AB between these groups of animals. TGF-β_1 is a potent stimulator of collagen synthesis by fibroblasts (27) and TGF-β_1 mRNA expression is elevated prior to increases in type-I and type-III procollagen mRNAs in bleomycin-induced lung fibrosis (28). Different approaches to inhibit TGF-β have been successfully used to prevent bleomycin-induced pulmonary fibrosis. Neutralizing TGF-β antibodies, soluble TGF-β type II receptors, and decorin are all able to inhibit TGF-β_1-activity and reduce collagen accumulation after bleomycin treatment (29-31). In our study we detected increased levels of TGF-β_1 in BAL fluid after bleomycin, comparable with levels reported previously (31, 32). We found that Dex did not downregulate TGF-β_1 levels in BAL fluid at any of the times examined after bleomycin treatment. Although we did not examine protein expression by BAL cells, it is likely that alveolar macrophages substantially contributed to the TGF-β_1 levels in BAL fluid as these cells have been demonstrated to express and secrete TGF-β after bleomycin exposure (33, 34). Additionally, it has been described that Dex application to alveolar macrophages previously activated *in vivo* by bleomycin did not reduce TGF-β secretion by these cells (34). Our observation that Dex treatment did not influence BAL fluid TGF-β_1 levels after bleomycin exposure may therefore be a reflection of the inability of Dex to downregulate TGF-β secretion by activated alveolar macrophages.

BAL fluid from bleomycin treated animals has previously been demonstrated to contain mitogenic activity for lung fibroblasts, to which both PDGF and thrombin contributed (35, 36). Additionally, *in vivo* inhibition of thrombin and PDGF activity has been shown to reduce bleomycin-induced pulmonary fibrosis in rats (3, 9). We found the mitogenic activity of BAL fluid to be significantly increased three days after bleomycin and comparable with control animals at day 7 and 14, which supports previously reported findings (35). Dex treatment had no effect on the BAL fluid mitogenic activity in bleomycin exposed animals. Both PDGF-AB levels and thrombin activity were significantly increased at day 3 after bleomycin, and presumably contribute to BAL fluid mitogenicity at that time-point. Thrombin activity decreased gradually thereafter but remained significantly increased compared with controls. The PDGF-AB levels remained increased at a relative constant level during the whole study period. Thrombin activity and PDGF-AB levels in BAL fluid were not modulated by Dex treatment of bleomycin exposed animals. These data suggest that although a 3-day course of Dex is able to inhibit bleomycin induced pulmonary fibrosis in rats, its actions are not through reducing levels of the fibrogenic mediators TGF-β_1, PDGF-AB or thrombin in BAL fluid.

However, we can not rule out the possibility that Dex treatment affects parenchymal

levels or activity of these growth factors. For instance fibroblasts have also been identified as a source of TGF-β_1 in pulmonary fibrosis (37), and it has been demonstrated that Dex reduces TGF-β_1 mRNA and TGF-β secretion by lung fibroblasts (38). In addition, Dex is known to reduce collagen synthesis and steady state levels of procollagen mRNA by lung fibroblasts (39). Therefore, the Dex regimen used in our study may have reduced parenchymal TGF-β_1 expression by fibroblasts with subsequent decreased autocrine stimulation of collagen synthesis. Alternatively, stimulation of fibroblasts with Dex stimulates the production of the proteoglycan decorin that binds and inactivates TGF-β (40). Transient expression of decorin in the lungs from bleomycin treated animals has been demonstrated to reduce pulmonary fibrosis without reducing total TGF-β_1 levels in BAL fluid (31). Therefore, it is possible that the Dex treatment in our study reduced parenchymal TGF-β_1 activity by increasing the decorin levels in the lung with a resultant decrease of collagen deposition.

We found that intratracheal instillation of bleomycin resulted in BrdU incorporation into alveolar type-II epithelial cells in areas of normal lung architecture and fibrotic foci indicating an increase in the number of proliferating cells. This finding supports previous studies and reflects active repair of the injured epithelial surface (41). Dex treatment reduced the number of BrdU stained cells in both fibrotic and normal areas of the lung parenchyma in bleomycin treated animals. This suggests that Dex either prevents cell proliferation or reduces the amount of epithelial damage and apoptosis. Excessive apoptosis of bronchiolar and alveolar epithelial cells has shown to be associated with bleomycin-induced lung fibrosis as well as with the development of BPD (42, 43). Dex has indeed been demonstrated to inhibit Fas-induced apoptosis of alveolar epithelial cells *in vitro* (44). Furthermore, a previous study demonstrated that prolonged administration of methylprednisolone suppressed the expression of Fas and Fas ligand mRNA, bronchial and alveolar epithelial apoptosis, as well as evidence of histological fibrosis induced by bleomycin (45). Therefore, the Dex regimen used in our study may have reduced epithelial cell apoptosis and limited fibrogenesis.

In conclusion, this study demonstrates that a 3-day course of Dex treatment, started after initiation of bleomycin-induced fibrosis, is able to reduce excessive pulmonary collagen accumulation in rats. The observed effect of Dex is established without a reduction of the fibrogenic mediators TGF-β_1, PDGF-AB, or thrombin in the alveolar space. We speculate that the anti-fibrotic effect of Dex is mediated via other pathways, which may include a reduction in cell proliferation, decreased epithelial apoptosis or inhibition of tissue profibrotic mediator activity. Our findings are of clinical relevance as we show that a 3-day course of Dex, in a comparable dose with that used to treat premature infants who are at risk of developing BPD, is able to inhibit the development of pulmonary fibrosis in an animal model. The beneficial effects of short-term early treatment with Dex in infants at risk of BPD may be due, at least in part, to antifibrotic effects.

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Chapter

GENERAL DISCUSSION

Bronchopulmonary dysplasia (BPD) is a neonatal chronic lung disease associated with varying degrees of fibrosis that can develop over days to weeks after treatment for neonatal respiratory distress syndrome (RDS). Pulmonary fibrosis generally follows an alveolar inflammatory reaction, and the pathogenesis of pulmonary fibrosis is characterized by excessive fibroblast proliferation (fibroproliferation) and collagen deposition (fibrogenesis). These processes are thought to be driven by mediators derived from the blood or produced by resident lung cells and/or inflammatory cells.

Corticoisteroids are widely used in the treatment of pulmonary fibrosis as they have potent anti-inflammatory effects. The effects on pulmonary fibrosis and the mechanisms modulated by corticosteroids are largely unknown yet.

This chapter discusses the results of the studies described in this thesis in relation to the present understanding of the pathogenesis of pulmonary fibrosis and BPD as well as the effect of corticosteroid treatment on the development of pulmonary fibrosis.

9.1. BPD: an inadequate early but prolonged fibroproliferative response that may be modulated by dexamethasone treatment

RDS is a form of acute lung injury characterized by bronchiolar and alveolar epithelial damage with denudation of the basement membrane as well as endothelial damage (1-3). It is generally accepted that with the resolution of RDS the initial lung injury is repaired in an orderly manner. However, when this repair response is disordered BPD can develop (2). Orderly repair after lung injury involves the migration and proliferation of epithelial cells, endothelial cells and fibroblasts and is driven by a host of mediators (4, 5).

Bronchoalveolar lavage (BAL) provides information on the cellular and noncellular components present in the alveolar space and can be used to evaluate disease activity. In active disease, BAL fluid from patients may contain biological/immunological markers of inflammation and/or active progression to fibrosis (6).

In this thesis studies were performed in which BAL fluids, obtained at different postnatal ages, from infants who did and did not develop BPD from RDS, were analyzed. The BAL procedure was performed in a standardized way as described by Grigg and previously used in our hospital (7, 8). The BAL fluids were analyzed for their mitogenic activity for human fetal lung fibroblasts as well as for the presence of mediators known to be involved in repair and fibrosis development. Measurements for mitogenic activity were performed at least in triplicate per BAL fluid sample. Mediator levels were determined in duplicate in all the examined BAL fluid samples. Previously it was demonstrated in our hospital that there was a strong correlation between the concentration of proteins expressed per ml BAL fluid and the corrected concentration of these parameters (i.e. expressed per ml epithelial lining fluid) (8). Therefore, the parameters determined in the BAL fluid samples in our studies were expressed as concentration per ml BAL fluid, which is in accordance with the most recent ERS task force guidelines on BAL in children (6).

In chapter 4, it was demonstrated that there was no difference in BAL fluid mitogenic activity for fibroblasts in the first week of life between the BPD and resolving RDS patients. It is therefore likely that the mitogenic activity in BAL fluid from resolving RDS is a normal physiological response to the initial lung injury that is needed for the restoration of the injured structures. Unfortunately, we have been unable to include patients ventilated because of non-pulmonary reasons. Inclusion of such a group of patients would have learned if the mitogenic activity in BAL fluid was due to the disease or just a sequel of mechanical ventilation. However, such a group of patients would always consist of infants of longer gestation as almost all infants with a gestational age of less than 30 weeks have to be ventilated for respiratory problems.

Acute lung injury in general is characterized by destruction of both the epithelial and endothelial sides of the alveolar-capillary barrier. This results in extravasation of fluid and cells into the interstitium and alveolar space. Once the insult has taken place, inflammatory cells appear in the injured site. Alveolar exudates containing fibrin fragments, fibronectin and other adhesive and chemotactic substances will provide a three dimensional matrix (provisional matrix) for the migration of inflammatory cells, epithelial cells, endothelial cells and fibroblasts (9, 10). Epithelial damage and successive denudation of the basement membrane may promote fibroblast growth and fibroblast infiltration into the alveolar space. Under normal conditions these processes are inhibited by alveolar type-II epithelial cells. Therefore, a rapid restoration of the injured alveolar epithelial barrier is thought to be crucial to prevent the development of pulmonary fibrosis (4, 10, 11). This view is supported by the observation that keratinocyte growth factor (KGF; a factor important in maintaining the epithelial integrity) treatment prevents the development of bleomycin-induced pulmonary fibrosis in rats (12).

As a consequence of immoderate and ongoing injury, fibroblast proliferation and migration into the alveolar space occurs in the pathogenesis of BPD and pulmonary fibrosis in general (3, 4, 13). Rapid alveolar epithelial proliferation can restore the surface layer, prevent fibroblasts from entering the alveolar lumen and control their activity. The type-II alveolar epithelial cell is considered as the cell regenerating the alveolar epithelium after injury (14). Alveolar type-II as well as bronchiolar epithelial cell migration and proliferation are prominent features after lung injury (15-17). Type-II alveolar epithelial cell proliferation is evident during the early reparative stages of RDS/BPD (3, 13).

Although we did not examine it, it is likely that the BAL fluid examined in chapter 4 exerts mitogenic activity for epithelial cells and that (mitogenic) factors present in the BAL fluid contribute to epithelial repair. If such is the case, it can be speculated that the mitogenic activity of the BAL fluid from the RDS group may be appropriate to restore the epithelial layer within days after injury, thereby preventing fibroblasts from entering the alveolar space and controlling their activity.

In our study, the infants who developed BPD had more severe RDS (based on a clinical score) than patients with resolving RDS. Therefore, it is likely that the infants who developed BPD had more severe lung injury. The mitogenic activity in the BAL fluid in combination with severe ongoing lung damage in the BPD group may be inappropriate to

restore the epithelial barrier rapidly enough to prevent alveolar fibroblast infiltration and fibroproliferative progression of the disease. The view of delayed epithelial repair in the pathogenesis of BPD is supported by recent observations by Currie and colleagues. They demonstrated decreased levels of epidermal growth factor (EGF; a factor promoting mitogenesis of all epithelial cells and known to stimulate epithelial repair) on postnatal day 1 in patients developing BPD compared to patients with resolving RDS. It was concluded that this decreased EGF levels reflected the immaturity of the infants and predisposed to BPD (18).

KGF is another epithelial mitogen that is upregulated after lung injury and implicated in epithelial repair (17). Preliminary data from our laboratory indicate that KGF concentrations in BAL fluid on postnatal day 2 are comparable between RDS and BPD in contrast to the expectation that the levels of KGF would correlate with the severity of the initial damage. Therefore, the KGF levels in the BPD patients may be too low to contribute to rapid restoration of the epithelial integrity in these patients.

BAL fluid from adults with fibrosing lung disease has been demonstrated to contain increased and prolonged mitogenic activity for lung fibroblasts compared with BAL fluid from controls (19, 20). Thrombin is a blood-born mediator that stimulates fibroblast proliferation and collagen synthesis (21, 22), and contributes to bleomycin-induced pulmonary fibrosis in animals (23). Additionally, thrombin has been shown to contribute to the increased mitogenic activiy of BAL fluid from fibrosing lung disease (19, 24).

In chapter 4 we demonstrated that thrombin contributed to BAL fluid mitogenic activity for fibroblasts in both RDS and BPD and that the mitogenic activity of BPD BAL fluid remained stable during the first 14 postnatal days. Due to extubation of patients, we obtained BAL fluid samples from just two patients with resolving RDS on postnatal day 7 and no BAL fluid samples from resolving RDS patients after postnatal day 7. Therefore, no conclusions can be drawn on differences in BAL fluid mitogenic activity from postnatal day 7 onwards between resolving RDS and BPD. Furthermore, due to the small groups of resolving RDS patients on postnatal days 4 and 7 and the small groups of BPD patients on postnatal days 10 and 14 the data on BAL fluid-induced fibroblast proliferation may have been biased. Nevertheless, the observation that BAL fluid from patients with BPD contained significant mitogenic activity to a postnatal age of 14 days makes it tempting to speculate about prolonged mitogenic activity in the lungs when BPD develops.

Minimal mitogenic activity has been demonstrated in BAL fluid from adults and animals without pulmonary disease (19, 24, 25). Therefore, it is reasonable to assume that the mitogenic activity of BAL fluid decreases in the RDS patients with resolution of the initial injury. The prolonged BAL fluid mitogenicity in the BPD group could well indicate that persistent fibroproliferation may occur in the lungs from infants developing BPD and that thrombin contributes to this process. Infiltration of (myo)fibroblasts into the alveolar exudate (hyaline membranes) has been described to occur within the first week after birth during BPD development (3, 13). A coincident infiltration of fibroblasts with the observed persistent mitogenic activity in the lungs may account for the observed excessive fibroproliferation and fibrosis in BPD.

When we determined thrombin activity and TATIII levels (as a marker for thrombin generation) we surprisingly found them decreased in BAL fluid from BPD compared to RDS patients on postnatal days 2 and 4. Recently, we demonstrated that thrombin activity in BAL fluid from newborn surfactant depleted piglets was influenced by the type of mechanical ventilation used (26). However, no correlation between the mode of mechanical ventilation and thrombin activity in BAL fluid was found in the infants younger than 4 days described in chapter 4. In a later stage, the thrombin activity increased in BAL fluid during BPD progression, reaching peak activity on day 14 comparable to that observed at postnatal days 2 and 4 in resolving RDS. As for the data on BAL fluid-induced fibroblast proliferation the thrombin data may be biased due to the small groups of resolving RDS and BPD patients at later timepoints.

Thrombin exerts multiple cellular effects important for wound healing. It promotes platelet aggregation, migration of inflammatory cells and the proliferation of mesenchymal cells. It has been demonstrated that application of thrombin and the thrombin receptor-activating peptide (TRAP) promoted wound healing. It was suggested that thrombin or TRAP accelerated wound healing partly via an earlier development of the proliferative phase (27). It may well be that alveolar thrombin generation is necessary to initiate a normal proliferative repair response for the resolution of RDS. When BPD develops, this initial response may be too low due to decreased alveolar thrombin generation, resulting in inadequate (too low and/or too slow) repair during the first postnatal days. A role for thrombin in the repair process in RDS is supported by the observation that treatment of infants suffering from RDS with antithrombin significantly prolonged the duration of mechanical ventilation and supplemental oxygen (28). However, when pulmonary thrombin activity is present for a prolonged time coinciding with ongoing lung injury as observed in BPD, it possibly contributes to the persistent fibroproliferation and excessive collagen deposition.

In our study, the group of infants that developed BPD was of significantly shorter gestation than the one with resolving RDS. This is in concordance with previously reported data, demonstrating that the most immature infants are the most likely to develop BPD (18, 29). We found a significant positive correlation between thrombin activity in BAL fluids at postnatal day 2 with the gestational age of the infants. This suggests that the ability to generate alveolar thrombin activity is related to the relative maturity of the infant. It is indeed known that the coagulation system matures with increasing gestation (30-36). Recently thrombin activity has been described in BAL fluid from preterm lambs (gestational age: 132 d; term is 145 d) ventilated because of RDS (37). It would be of interest to determine in such a model whether lambs with gestation of less than 132 d are indeed impaired in alveolar thrombin generation and whether they benefit from local thrombin or TRAP therapy.

An alternative explanation for the decreased alveolar thrombin levels in the BPD group can be derived from data on coagulation activation as described by Brus *et al.* They demonstrated that preterm infants with RDS had increased plasma concentrations of TATIII on the first day after birth compared to healthy preterm infants and that these levels decreased within three days (38). Additionally, they demonstrated that infants with severe RDS had

increased plasma levels of TATIII compared to infants with mild/moderate RDS (39). This indicates that there is a positive correlation between vascular thrombin generation and the RDS severity. In our study, the infants who developed BPD had the most severe symptoms of RDS and therefore might have had increased intravascular thrombin generation (prothrombin consumption). Consequently this may have resulted in a decreased availability of prothrombin to be converted into thrombin in the alveolar space. Examination of TATIII levels in both plasma and BAL fluid and thrombin activity in BAL fluid could reveal if there is an inverse correlation between vascular and pulmonary thrombin generation when BPD develops.

Treatment with systemic dexamethasone (Dex) is commonly used in infants with or at risk of BPD. It is known to improve pulmonary function, to reduce pulmonary inflammation and to facilitate weaning from the ventilator (40). The effect of Dex treatment on pulmonary fibrosis, however, is uncertain as it does not downregulate the concentrations of fibronectin and TGF-β_1 in BAL fluid from preterm ventilated infants with BPD (41, 42).

We were the first to demonstrate that BAL fluid from infants who develop BPD is mitogenic for human fetal lung fibroblasts (chapter 4). Increased fibroblast proliferation occurs in BPD and pulmonary fibrosis in general (13, 43). The data in chapter 5 are therefore of special interest as they show that Dex treatment of infants at risk of BPD increases the BAL fluid mitogenic activity for fibroblasts.

In the study described in chapter 5 no placebo control group was included. Therefore, it can not be excluded that the observed increase in BAL fluid mitogenic activity is due to the natural course of the disease. However, comparison between data on BAL fluid mitogenic activity from chapter 4 with chapter 5 supports the finding that Dex treatment is responsible for the increase in mitogenic activity of BAL fluid. In chapter 4, BAL fluid from infants who acquired BPD stimulated fibroblast proliferation with 33-41% at postnatal ages of 2, 4, 7, 10 and 14 days. In chapter 5, BAL fluid obtained from patients before Dex treatment (7-33 days of age) stimulated fibroblast proliferation with 35 %, thus comparable with the increased proliferation described in chapter 4. After Dex treatment, BAL fluid-induced fibroblast proliferation increased to 51%, exceeding all previous values as described in chapter 4. This observation makes it likely that it is indeed the Dex treatment that caused the increase in BAL fluid mitogenic activity. This is further supported by the observation that Dex treatment did reveal the expected anti-inflammatory effects despite the observed increase in BAL fluid mitogenic activity.

Interestingly, we noticed that from the 15 patients included in this study the 3 with decreasing mitogenic activity after Dex had a higher mitogenic activity before treatment than the ones with increasing mitogenic activity after Dex treatment. Although clinically all patients improved during Dex treatment and the ventilator settings were weaned, the data on mitogenic activity might suggest that possibly two types of responses to Dex treatment exist in infants at risk of BPD. Further studies should be performed in which more patients receiving Dex treatment are included. In addition to examination of the BAL fluid mitogenic activity, lung function tests and chest radiographs should be included at fixed time-points in

these studies. That could reveal whether the change in BAL fluid mitogenic activity after Dex treatment correlates with pulmonary function. Furthermore, although lung pathology is difficult to score from chest radiographs, especially in the case of minor changes, a correlation between the change in BAL fluid mitogenic activity and lung pathology (e.g. fibrosis/interstitial abnormalities, emphysema) might become evident. Analyses like these could elucidate if there are indeed two different groups of patients at risk of BPD that respond differently to Dex treatment.

In vitro studies have demonstrated that macrophages stimulated with Dex express increased amounts of PDGF-B mRNA, secrete increased amounts of PDGF protein, and stimulate fibroblast proliferation and collagen synthesis (44, 45). In concordance with this, it was demonstrated in chapter 5 that the increase in BAL fluid mitogenicity after Dex treatment was accompanied by increased PDGF-BB concentrations in BAL fluid. Furthermore, by blocking the PDGF receptor system, it was demonstrated that PDGF indeed contributed to the BAL fluid mitogenic activity. In preliminary experiments, we found with RT-PCR that BAL cells expressed mRNA for both the PDGF-A and PDGF-B chain. By immunocytochemical analysis we identified the alveolar macrophage as the major cell type expressing PDGF. It is possible that Dex treatment stimulated the alveolar macrophages to increased PDGF-B expression and secretion, resulting in the increased concentrations of PDGF-BB that we detected in BAL fluid after this treatment. *In vitro* experiments, using BAL cells from infants at risk of BPD development, should be performed to elucidate if these cells indeed upregulate PDGF-B synthesis and PDGF-BB secretion upon Dex stimulation. Additionally, BAL cells obtained from patients before and after Dex treatment could be analyzed for PDGF-B mRNA expression and PDGF-BB secretion.

Dex is known to reduce collagen synthesis by fibroblasts both *in vivo* and *in vitro* (46, 47), and has been demonstrated to decrease total body collagen synthesis in infants with or at risk of BPD (48, 49). However, recent *in vitro* studies indicate that Dex stimulates collagen synthesis by human fetal primary lung fibroblasts (50). This makes the effect of Dex treatment on lung collagen synthesis in infants at risk of BPD highly uncertain. In adults with ARDS it was demonstrated that methylprednisolone treatment decreased the concentrations of the type-I and type-III procollagen aminoterminal propeptides (PINP and PIIINP, respectively) in BAL fluid (51). We observed no differences in BAL fluid PIIINP levels before and after Dex. This indicates that, opposite to the reported effect on total body collagen synthesis, Dex treatment does not influence lung collagen synthesis during BPD development.

Possible effects of Dex on pulmonary collagen synthesis in our study could be obscured by the large variations in PIIINP levels, the small number of patients and drainage of PIIINP from the lung by lymph vessels. On the other hand, Meduri *et al.* only observed a significant reduction of PIIINP between 8 to 15 days after initiation of methylprednisolone in ARDS patients (51). We can not exclude that Dex treatment did influence pulmonary collagen synthesis at later time-points after treatment in our study as the infants were extubated within days after treatment initiation. Due to this, BAL could not be performed anymore for ethical reasons. It is known, however, that Dex treatment reduces total body collagen syn-

thesis already within three days after initiation of treatment in infants with or at risk of BPD (48, 49). Therefore, it is reasonable to assume that if Dex treatment did influence pulmonary collagen synthesis, this should have been evident within three days of treatment.

We suggest that the Dex regimen used in our study enhances the pulmonary fibroproliferation. Such increased fibroproliferation could contribute to the finding that Dex fails to downregulate pulmonary collagen synthesis in the lungs from infants at risk of BPD.

Lung biopsy specimens obtained before and after initiation of Dex could reveal insight into the histopathological response (cell proliferation and collagen deposition) to Dex treatment in infants at risk of BPD. However, studies like these are hard to perform due to ethical considerations. An alternative approach would be to examine lung tissue from Dex treated and untreated infants who died during BPD development and to determine the effect of the treatment on fibroblast numbers and collagen deposition.

From the data in chapter 4 and chapter 5, it can be speculated that BPD development is characterized by an early inappropriate (too low and/or too slow) proliferative response to the initial pulmonary injury. Prolongation of this proliferative phase may contribute to the development of pulmonary fibrosis in BPD. This prolonged fibroproliferative response increases when Dex treatment is initiated after the first week of life. Therefore, initiation of Dex treatment after the first week of life may be no longer able to reduce the development of pulmonary fibrosis in the pathogenesis of BPD. Highly speculative is to suggest that initiation of Dex treatment within 1-2 days after birth boosts the proliferative response in infants at risk of BPD, which could lead to enhanced repair, thereby preventing progression towards BPD.

9.2. Matrix metalloproteinases and their inhibtors in the pathogenesis of BPD

MMP-1, TIMP-1, and TIMP-2

Besides mechanisms resulting in increased collagen synthesis, decreased collagen degradation (due to decreased collagenolytic activity) is another mechanism that may contribute to excessive collagen deposition. Decreased collagenolytic activity has been demonstrated in lung tissue from adults with pulmonary fibrosis (52, 53). The fibrillar collagens type-I and type-III are the main types of collagen deposited in the pathogenesis of pulmonary fibrosis (43). MMP-1, also named (interstitial) collagenase, is able to degrade these collagens. Therefore, MMP-1 expression and regulation of MMP-1 activity are of potential importance to the pathogenesis of pulmonary fibrosis. Several studies in adult human pulmonary fibrosis support the view that increased expression of TIMPs compared with MMP-1 results in a non-collagen degrading microenvironment (54-56). Evidence is accumulating indicating that MMP-1 may also be important in the regeneration of the injured alveolar epithelial barrier after injury, comparable with the role of MMP-1 in reepithelialization after skin injury (57, 58).

Chapter 6 describes the immunolocalization of MMP-1, TIMP-1 and -2 during different developmental phases of BPD. MMP-1 localized to regenerating type-II alveolar epithelial cells, which is in concordance with observations made by others in human adult pulmonary fibrosis (55). This observation suggests that MMP-1 expressed by these cells contributes to the restoration of the alveolar epithelial barrier. This is supported by recent *in vitro* experiments demonstrating that MMP-1 contributes to alveolar type-II epithelial cell migration (57).

In the chronic phase of BPD, alveolar type-II epithelial cells revealed increased staining for TIMP-1 while MMP-1 remained comparable to the previous phases. This suggests that an increase in TIMP-1 compared with MMP-1 results in reduced collagenolytic activity by type-II pneumocytes in the chronic phase of BPD. Additionally, fibroblasts in fibrotic foci during the chronic phase of BPD coexpressed MMP-1, TIMP-1, and TIMP-2, which may indicate decreased collagen degradation by fibroblasts. This finding is in concordance with a previous report on patients with idiopathic pulmonary fibrosis that suggested that the co-expression of MMP-1 and TIMP-2 in fibroblasts from fibrotic foci contributed to progressive collagen deposition due to a decreased collagenolytic activity by these cells (55).

From these data we conclude that decreased pulmonary collagenolytic activity may occur and contribute to the fibrotic changes seen in BPD. Examination of fresh lung tissue could reveal if there is indeed a decrease in pulmonary collagenolytic activity when BPD develops. In addition to this, fibroblasts could be cultured from lung tissue of BPD patients and analyzed for their capacity to synthesize MMP-1, TIMP-1 and TIMP-2 compared to control lung fibroblasts. Furthermore, fibroblast culture supernatants could be analyzed for collagenolytic activity.

MMP-2 and MMP-9

Collectively, MMPs are able to degrade all ECM components and play key roles in normal physiological processes that involve ECM remodeling, such as wound healing, angiogenesis, and development (59). The gelatinases MMP-2 and MMP-9 are secreted as latent (pro)enzymes that require processing to become active and are able to degrade several components of the basement membrane (60).

Increased levels of MMP-2 and MMP-9 have been demonstrated in BAL fluid and lung tissue from adult patients with pulmonary fibrosis (55, 56, 61, 62). Furthermore, a causal relationship between pulmonary fibrosis and MMP-2 and MMP-9 is suggested from studies in mice where the administration of an MMP inhibitor together with bleomycin prevented the development of pulmonary fibrosis (63). Oxygen toxicity, generally regarded as one of the major risk factors for BPD, resulted in increased MMP-2 and MMP-9 levels in an animal model of hyperoxia (64), and also oxidative stress in newborn humans was found to be associated with increased MMP-9 levels (65).

Most studies on MMP-2 and MMP-9 suggest that these enzymes contribute to lung damage and subsequent fibrosis. Recent evidence, however, also indicates a role for MMP-9 in epithelial repair after injury (66-68). MMP-9 is expressed by regenerating alveolar type-II

epithelial cells in human pulmonary fibrosis and hyperoxia-induced lung injury in rats (55, 64).

Chapter 7 describes the presence of MMP-2 and MMP-9 in sequential BAL fluids from patients with resolving RDS and BPD development. We found that MMP-2 levels were comparable in BAL fluid from resolving RDS and BPD patients. Contradictory to this, MMP-9 levels were decreased during the first four postnatal days in BAL fluid from infants with subsequent BPD. In our study we determined the amount of MMP-9 in BAL fluid by zymography and desitometric analysis. Values for MMP-9 were related to a control collagenase and expressed as arbitrary units. A number of BAL fluid samples were reanalyzed by zymography and revealed comparable results to the first measurements. In addition to this, we analyzed 12 BAL fluid samples from postnatal day 2 in a commercially available MMP-9 ELISA that detects latent MMP-9 and latent MMP-9/TIMP-1 complex. A statistically significant positive correlation ($r = 0.906$, $p < 0.0001$) was observed between the values for latent MMP-9 as determined by zymography and the values determined by ELISA. This indicates that zymography is a reliable way to determine MMP-9 levels in BAL fluid.

When BPD developed, the MMP-9 levels increased over time and from postnatal day 7 onwards exceeded the levels observed in resolving RDS. In accordance with the increase of MMP-9 in BAL fluid during BPD progression we also found that MMP-9 immunoreactivity increased in lung tissue with the progression of BPD.

BAL fluid samples from both infants with resolving RDS and infants that developed BPD revealed a gelatinolytic band representing a heterodimer of MMP-9 with neutrophil-gelatinase-B-associated lipocalin (NGAL). The presence of this MMP-9/NGAL complex indicates that neutrophils are a source of the detected MMP-9 (69, 70). At postnatal day 2, particularly the NGAL/MMP-9 levels were increased when patients resolved from the initial RDS compared to infants that developed BPD. Measurement of this complex in BAL fluid may therefore be helpful to identify RDS patients who are at risk of BPD development. This identification can be of importance with respect to therapeutic intervention. The difference in NGAL/MMP-9 between resolving RDS and BPD is an interesting finding as NGAL/MMP-9 exists exclusively in the specific granules of the neutrophil (71).

Degranulation of specific granules in our study seems, therefore, to be associated with resolving RDS. It can be that the trigger required for the release of specific granules from neutrophils is initially present when RDS resolves but less or absent when BPD develops. Although we only demonstrated NGAL in association with MMP-9 in our assay, it is tempting to speculate that also free NGAL is released from the specific granules and present in increased levels in BAL fluid when RDS resolves. Free NGAL has been demonstrated to bind inflammatory mediators such as LTB_4 and PAF, and it was speculated that NGAL may exert a negative feedback on the inflammatory response by the binding of such inflammatory mediators (71, 72). Increased levels of LTB_4 and PAF in lung effluent fluids within the first week of life have been shown to be associated with BPD development (73, 74). Therefore, the control of LTB_4 and PAF activity by NGAL may control the pulmonary inflammatory response and contribute to RDS recovery. Decreased levels of NGAL could lead to impaired

regulation of the pulmonary inflammatory response and predispose to BPD.

We observed no correlation between latent MMP-9 and the number of neutrophils in BAL fluid. This indicates that cell types, other than neutrophils, are likely to contribute the MMP-9 levels as well. We found that, besides neutrophils, also monocytes/macrophages and regenerating alveolar type-II epithelial cells expressed MMP-9, supporting the view that these cells may contribute to the MMP-9 detected in BAL fluid.

A dramatic increase in latent MMP-9 expression has been identified within the first 24 hours of normal healing wounds, followed by a decline at 48 hours and reaching baseline levels between 72 and 96 hours. Contradictory to this, it was found that in chronic wounds MMP-9 remained elevated (75). Initiation of normal healing follows sequential completion of coagulation, inflammation, removal of damaged matrix components, cellular proliferation and migration (fibroblasts, endothelial and epithelial cells), angiogenesis, matrix synthesis, epithelialization and remodeling (4, 5, 76). During normal healing MMP-9 may be involved in the removal of the damaged ECM as well as contributing to cellular migration and angiogenesis. The increased levels of MMP-9 in resolving RDS are likely to reflect a normal response to lung injury as these infants recover without pulmonary problems. Therefore, it can be suggested that MMP-9 is involved in the repair mechanisms in the injured lung when RDS resolves.

Migration and proliferation of epithelial progenitor cells are critical events in the regeneration of an epithelial barrier. During recovery from hyperoxia-induced lung injury the alveolar type-II epithelial cells have been demonstrated to secrete increased amounts of MMP-9 which exactly parallels peak alveolar epithelial repair *in vivo* and facilitates migration through gelatin (68, 77). An important role for MMP-9 in reepithelialization after injury has also been suggested from studies using bleomycin-induced lung injury and *in vitro* and *ex vivo* wound repair of human bronchial epithelial cells (67, 78, 79). Regarding these data it can be suggested that MMP-9 produced by alveolar epithelial cells and possibly other cell types contributes to the restoration of the alveolar epithelial barrier when RDS resolves. The increased MMP-9 levels in resolving RDS may, therefore, be a reflection of active epithelial regeneration by alveolar type-II epithelial cells that produce MMP-9 for this process. When BPD develops, this epithelial restoration may be delayed due to decreased MMP-9 production resulting in impaired repair with subsequent pulmonary fibrosis.

In addition to the effects of MMP-9 on reepithelialization after injury, MMP-9 has also been associated with angiogenesis and MMP-9 levels have been demonstrated to correlate with increases in vascularity during wound healing (80, 81). BPD is characterized by decreased pulmonary vascular development (82). Therefore, it is possible that the decreased MMP-9 levels we detected in BPD within the first four postnatal days contribute to or reflect the aberrant pulmonary vascular development in BPD, comparable to that suggested for decreased pulmonary VEGF and VEGF receptor expression in the pathogenesis of BPD (82). *In vitro* and *ex vivo* wound repair models could reveal if there is a difference in BAL fluid samples from resolving RDS and BPD to restore an injured epithelial barrier and to stimulate angiogenesis. Furthermore, such analyses could reveal if the MMP-9 present in the BAL fluid

contributes to these processes.

EGF is a mitogen for epithelial cells that also upregulates MMP-9 secretion and migration of keratinocytes (83). It is possible that EGF exerts similar effects on epithelial cells in the lungs from preterm infants. The decreased latent MMP-9 levels in BPD during the first four postnatal days may therefore be due to decreased pulmonary EGF levels which have been shown to be associated with BPD development (18).

Alternatively, the decreased MMP-9 levels in BPD patients within the first four postnatal days may be due to differences in lung maturity between patients with resolving RDS and patients developing BPD. During lung development specific MMPs are expressed in a temporal manner. MMP-9 was found to be mainly expressed in the later stages of lung development (84). In our study, the patients developing BPD were of significantly shorter gestation than infants with resolving RDS. Although we observed no correlation between MMP-9 levels and gestational age, it is possible that pulmonary maturational differences contributed to the differences in MMP-9 in BAL fluid from the RDS and BPD group.

When BPD developed, the MMP-9 levels in BAL fluid increased and finally exceeded the levels in resolving RDS. This increase in MMP-9 may contribute to the ongoing lung damage in the pathogenesis of BPD. In patients with ARDS it has been demonstrated that increased MMP-9 levels in BAL fluid coexisted with the 7S region of type-IV collagen, which is considered as a marker of basement membrane disruption (61). Increased levels of the 7S region of type-IV collagen have also been demonstrated in BAL fluid from infants who developed BPD compared to resolving RDS. The authors suggested that oxygen metabolites were involved in basement membrane degradation in the pathogenesis of BPD (85). However, taking our data on MMP-9 into account, one can speculate that, at least from postnatal day 7, MMP-9 also contributes to the excessive basement membrane degradation in the pathogenesis of BPD. Studies should be undertaken to determine whether a correlation exists between basement membrane degradation products and the levels of MMP-9 in BAL fluid when BPD develops. Increased basement membrane disruption in the pathogenesis of BPD would allow fibroblasts to enter the alveolar lumen. This, together with the prolonged presence of pulmonary fibroblast mitogens as described in chapter 4, could result in persistent fibroblast activation with fibrosis as a consequence.

Altogether, these findings suggest that the difference between resolving RDS and developing BPD may be related to the ability to raise an early adequate MMP-9 response to the initial pulmonary injury. Such an early MMP-9 response would be required for normal pulmonary restoration. However, when this MMP-9 response is delayed and finally excessive and prolonged it may contribute to disordered repair and extensive ECM injury, thereby contributing to the lung pathology as observed in BPD.

9.3. Post-injury short-course dexamethasone treatment inhibits pulmonary fibrosis: a possible therapy for preventing the development of fibrosis in BPD

Infants who are at risk of BPD are generally treated with a systemic course of Dex. Numerous different Dex regimens have been examined in preterm infants at risk for BPD. The outcomes of these studies on BPD incidence are inconclusive due to different patient populations and the different Dex regimens and concentrations used (86-91). Dex treatment has been shown to improve the pulmonary function and to facilitate weaning from the mechanical ventilator. These beneficial effects may be partly explained by the anti-inflammatory effects that Dex exerts in the lungs of these infants (40, 41, 92-95). In addition, decreased urinary excretion of hydroxyproline and decreased plasma levels of the C-terminal propeptide of type-I collagen and the N-terminal propeptide of type-III collagen have been detected after Dex treatment (48, 49). These measurements are a reflection of the whole body collagen synthesis and do not necessarily reflect the collagen metabolism in the lung. It has been demonstrated that despite an apparent anti-inflammatory effect, Dex may not influence pulmonary levels of fibrogenic mediators such as TGF-β_1 and fibronectin in BPD (41, 42), suggesting that Dex may not prevent fibrosis development in BPD. In line with this it was reported in chapter 5 that Dex treatment, initiated between postnatal days 7 and 33, did not reduce the concentration PIIINP in BAL fluid, suggesting that Dex treatment started at this time-point does not interfere with pulmonary collagen synthesis in infants at risk of BPD.

Recently, it was demonstrated that when Dex treatment was initiated early after birth in infants at risk of BPD and given for a short period, it increased the survival without BPD (96, 97).

Whether such a short-course of Dex is able to interfere with pulmonary collagen metabolism in fibrotic lung disorders was never examined. It is, however, known that steroids are able to inhibit bleomycin-induced pulmonary fibrosis in animals when given prior to or together with bleomycin and for a prolonged period of time (98, 99). Because the bleomycin model has been proven to be sensitive to corticosteroids we used this model to examine if a short-course of Dex, initiated after fibrosis induction, was able to prevent the development of pulmonary fibrosis in this model as well.

In chapter 8 we describe that a three-day course of Dex (0.5 mg/kg bw; comparable to what is used in infants at risk of BPD), initiated after bleomycin-induced injury/fibrosis in rats, inhibited excessive pulmonary collagen accumulation. Furthermore, we demonstrated that this inhibition of collagen accumulation was not accompanied by a decrease in BAL fluid levels of the profibrotic mediators TGF-β_1, PDGF-AB and thrombin. This indicates that the BAL fluid not necessarily reflects what is going on in the lung parenchyma. However, we can not exclude that Dex treatment reduced the expression levels of other fibrogenic mediators in the BAL fluid. For instance, TNF-α and IL-1 expression levels are known to be downregulated by corticosteroids and these cytokines are involved in the development of pulmonary

fibrosis after bleomycin (100-103). Up till now we have been unable to detect TNF-α and IL-1β in our BAL fluids. The total lavage volume we used (12 ml) may have resulted in a dilution of TNF-α and IL-1β in the BAL fluid to concentrations too low to detect by the assays we used. Concentrating the BAL fluids could resolve this problem.

We noticed that Dex treatment reduced the number of proliferating cells in both fibrotic and normal areas of the lung parenchyma of bleomycin treated animals. We therefore speculate that the anti-fibrotic effect of the Dex regimen used in our study is mediated via pathways that may include a reduction in parenchymal cell proliferation, decreased epithelial apoptosis or inhibition of profibrotic mediator activity rather than reducing the levels of profibrotic mediators in the bronchoalveolar compartment.

The classical model for the pathogenesis of ARDS suggested that epithelial and endothelial damage result in an inflammatory phase that is followed by a fibroproliferative phase which, if excessive, results in established fibrosis. The current hypothesis, however, is that fibroproliferation occurs in parallel with inflammation. It has been suggested that therapies preventing the progression to established fibrosis will need to affect both proinflammatory and profibrotic mechanisms (104).

Corticosteroid treatment of ARDS has been reported to be more effective when administered early in the fibroproliferative phase, thus before dense acellular fibrosis with deranged alveolar architecture occurs. The response to therapy was found to be associated with pulmonary improvement and a decrease of PINP and PIIINP levels in plasma and BAL fluid (51, 105). From this it seems that the time at which corticosteroid therapy is initiated is crucial for its outcome effect to inhibit the development of fibrosis. Also for patients with idiopathic pulmonary fibrosis it has been suggested that corticosteroids may only be effective when there is a major inflammatory component associated with the disease state (106). However, it is not known whether corticosteroids establish these effects by interfering with inflammatory or fibrotic pathways or the cross-talk between inflammatory and fibrotic pathways or other mechanisms.

The data in chapter 8 suggest that also in the bleomycin model of pulmonary fibrosis the fibroproliferative phase coexists with the inflammatory phase. This implicates that when Dex treatment is initiated early in the fibroproliferative/inflammatory phase, thus before the establishment of dense acellular fibrosis, it is indeed able to inhibit the development of pulmonary fibrosis. It would be of interest to examine whether Dex treatment initiated between 7 and 10 days after bleomycin exposure prevents fibrosis development in this model as well. This could testify the hypothesis that corticosteroid treatment is indeed more effective when administered early in the fibroproliferative response.

In chapter 8 we show in a rat model of pulmonary fibrosis that a three-day course of Dex (0.5 mg/kg bw/day), initiated at an early time-point after injury at which inflammation and fibroproliferation are likely to occur in parallel, inhibited collagen accumulation in the lungs after bleomycin exposure. The data from chapter 4 suggest that fibroproliferation is likely to occur early in the lungs from infants that develop BPD, and that this fibroproliferation coincides with a major inflammatory component in these infants. Early initiation of a

short-course of Dex treatment has been demonstrated to increase survival without BPD in preterm infants (96, 97). However, whether such a course of Dex can inhibit pulmonary collagen deposition in these infants or pulmonary fibrosis in general was not known so far. The data from chapter 8 suggest that the pulmonary beneficial effects of short-course early treatment with Dex in infants at risk of BPD may be partly due to antifibrotic effects. An early short-course Dex treatment of infants at risk of developing BPD, and possibly also patients with other fibrosing lung diseases may therefore be useful to prevent the development of lung fibrosis.

Studies could be undertaken in which infants at risk of BPD are treated with the early three-day course of Dex and compared with a placebo control group. BAL fluid obtained before and during the treatment could be analyzed for the presence of N-terminal propeptides of collagen molecules and the mitogenic activity for fibroblasts as well fibrogenic and inflammatory mediator profile. This would reveal whether this treatment is efficient in controlling collagen synthesis in the lungs and whether it correlates with pulmonary improvement. Additionally, it could reveal whether the time-point at which Dex treatment is initiated is crucial in the control of pulmonary collagen metabolism, since we described that Dex initiated between postnatal days 7 and 33 did not influence collagen type-III synthesis in infants at risk of BPD. Alternatively, an animal model for BPD development, such as the baboon model described by Coalson and colleagues, can be used (107). Such an animal model raises the possibility to perform biochemical and histological analyses of the lung tissue as well. A major point of concern, however, is that early and short-course Dex treatment in prematurely born infants is associated with serious side effects such as gastrointestinal perforation and growth retardation (91, 97). Therefore, the potential benefits on pulmonary outcome should be weighed against the serious potential side effects of such treatment. Studies to determine the minimal dose of Dex needed to inhibit the development of pulmonary fibrosis and to improve pulmonary function with the least side effects for infants at risk of BPD are therefore of prime importance.

Concluding remarks

1. The pathogenesis of BPD is characterized by an inadequate repair response (too low and/or too slow) to the initial lung injury. This is reflected by inappropriate fibroproliferation and decreased levels of thrombin and MMP-9 in BAL fluid at a postnatal age of 2 to 4 days. Subsequently, due to ongoing ECM damage to which excessive amounts of MMP-9 contribute, a prolonged and disordered repair response occurs. This results in persistent fibroblast proliferation to which thrombin as well as other factors contribute. In addition, from a postnatal age of 16 days, decreased collagenolytic activity of alveolar type-II epithelial cells and fibroblasts, due to a decreased MMP-1/TIMP ratio in these cells, is likely to contribute to excessive collagen accumulation in chronic BPD. The inability to raise an adequate

response may be related to the relative immaturity of infants that develop BPD compared to infants with resolving RDS. Figure 1 schematically depicts the time-points of BPD development at which fibroproliferation, thrombin, MMP-9, MMP-1 and TIMPs are present in the lungs and explains the possible roles of these factors in the pathogenesis of BPD.

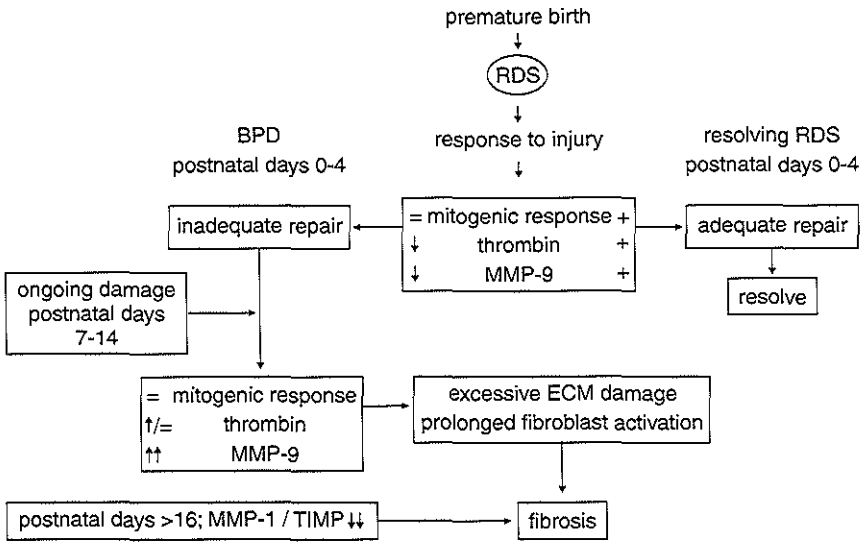

Figure 1

+ is adequate response to injury, = is comparable to response elicited in resolving RDS, ↓ is decreased compared to resolving RDS, ↑ /= is increased compared with postnatal days 2-4 and comparable with resolving RDS, ↑↑ is excessive increase compared to postnatal days 2-4 and exceeding levels in resolving RDS, ↓↓ is decreased compared with earlier stages of BPD.

RDS can either resolve or progress towards BPD. Resolving RDS is characterized by an adequate repair response. This consists of at least a sufficient mitogenic response that elicits cellular proliferation (epithelial and endothelial cells, fibroblasts), the generation of sufficient amounts of thrombin that contributes to the mitogenic response, and appropriate MMP-9 production/secretion that contributes to removal of damaged extracellular matrix components, cellular migration and angiogenesis. BPD development from RDS is characterized by an inadequate repair response to the initial injury. Within the first week of life this is due to an insufficient mitogenic response (comparable to the one elicited in resolving RDS in contrast to the expectation that the mitogenic response would correlate with the severity of the initial damage), decreased thrombin generation and MMP-9 production and secretion. During BPD progression there is ongoing injury, prolonged pulmonary mitogenic activity, an increase in pulmonary thrombin levels and an excessive increase in pulmonary MMP-9 levels. The increased MMP-9 levels contribute to lung damage, especially to the basement membrane. Persistent mitogenic activity, due to thrombin as well as other mediators, results in chronic fibroblast activation with sustained proliferation and collagen deposition within the lung parenchyma as a consequence. From postnatal day 16 onwards, MMP-1 (collagenolytic) activity is depressed in the lungs due to increased TIMP expression. Together these processes contribute to fibrosis development as observed in the lungs from infants with BPD.

2. Dexamethasone treatment, initiated between postnatal days 7 and 33, reduces pulmonary inflammation in infants at risk of BPD. However, dexamethasone treatment initiated beyond the age of 1 week is unable to reduce pulmonary collagen synthesis within 3 days. This is possibly due to increased fibroproliferation driven by increased pulmonary PDGF-BB levels after dexamethasone treatment.

3. A three-day course of dexamethasone, initiated three days after the induction of bleomycin-induced pulmonary fibrosis in rats, inhibits excessive collagen accumulation in the lungs. Such a treatment course may be of potential therapeutic interest in preventing the development of pulmonary fibrosis in infants at risk of BPD.

9.5. References

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107

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Abbreviations

α_1-PI	:	alpha$_1$-proteinase inhibitor
α-SM	:	alpha-smooth muscle
ACTH	:	adrenalcorticotropic hormone
AP-1	:	activating protein-1
ARDS	:	adult respiratory distress syndrome
ATIII	:	antithrombinIII
BAL	:	bronchoalveolar lavage
BPD	:	bronchopulmonary dysplasia
BrdU	:	bromodeoxyuridine
BSA	:	bovine serum albumin
bw	:	birth weight
C5a	:	C5-derived anaphylotoxin
cFn	:	cellular fibronectin
CINC	:	cytokine-induced neutrophil chemoattractant
CLD	:	chronic lung disease of prematurity
COX-2	:	cyclooxygenase-2
CTGF	:	connective tissue growth factor
Dex	:	dexamethasone
DMEM	:	Dulbecco's Modified Eagle's Medium
ECM	:	extracellular matrix
ECP	:	eosinophilic cationic protein
EGF	:	epidermal growth factor
ERS	:	European Respiratory Society
ET-1	:	endothelin-1
FCS	:	fetal calf serum
Fn	:	fibronectin
FXa	:	factor Xa
ga	:	gestational age
G-CSF	:	granulocyte colony-stimulating factor
GM-CSF	:	granulocyte macrophage colony-stimulating factor
GR	:	glucocorticoid receptor
GRE	:	glucocorticoid responsive element
GRO-α	:	growth-related protein-α
5-HETE	:	5-hydroxyeicostatetranoic acid
HFOV	:	high frequency oscillatory ventilation
$HFOV_{OLC}$	:	high frequency oscillatory ventilation applying the open lung concept
HLA	:	human leukocyte antigen
HMD	:	hyaline membrane disease
ICAM	:	intercellular adhesion molecule
IFN-γ	:	interferon-gamma
IGF-1	:	insulin-like growth factor-1
IL	:	interleukin
IL-1Ra	:	interleukin-1 receptor antagonist
KGF	:	keratinocyte growth factor
LFA-1	:	lymphocyte function associated protein-1
LTB_4	:	leukotriene-B_4
LV-PPV	:	low volume positive pressure ventilation
MAC-1	:	macrophage-1
MCP-1	:	monocyte chemoattractant protein-1
MIP	:	macrophage inflammatory protein
MMP	:	matrix metalloproteinase
MT-MMP	:	membrane type-matrix metalloproteinase
NCS	:	normal calf serum
NF-κB	:	nuclear factor-kappa B
NGAL	:	neutrophil-gelatinase B-associated lipocalin
nGRE	:	negative glucocorticoid responsive element
NHS	:	normal human serum
OD	:	optical density
PAF	:	platelet-activating factor
PBS	:	phosphate-buffered saline
PD	:	pirefenidone
PDGF	:	platelet-derived growth factor
PDGFR	:	platelet-derived growth factor receptor
pFn	:	plasma fibronectin
PGE_2	:	prostaglandin E_2
PPACK	:	D-Phenylalanine-Proline-Arginine-Choromethyl Ketone, Dihydrochloride
PPV_{CON}	:	conventional positive pressure ventilation
PPV_{OLC}	:	positive pressure ventilation applying the open lung concept
PINP	:	N-terminal-propeptide of collagen type-I
PIIINP	:	N-terminal-propeptide of collagen type III
RANTES	:	regulated upon secretion, normal T cell expressed and secreted
RDS	:	respiratory distress syndrome
RT-PCR	:	reverse transcriptase polymerase chain reaction
SEM	:	standard error of the mean
sIL-2R	:	soluble interleukin-2 receptor
SLPI	:	secretory leukocyte protease inhibitor
TATIII	:	thrombin-antithrombinIII complex
TF	:	tissue factor
TFPI	:	tissue-factor-pathway-inhibitor
TGF	:	transforming growth factor
Thr	:	thrombin
TIMP	:	tissue inhibitor of metalloproteinase
TNF	:	tumor necrosis factor
TRAP	:	thrombin receptor-activating peptide
VEGF	:	vascular endothelial growth factor
VILI	:	ventilator-induced lung injury

Summary

To save life, prematurely born infants with neonatal respiratory distress syndrome (RDS) are artificially ventilated. RDS can resolve within days after birth (uncomplicated RDS) or progress towards a chronic lung disease called bronchopulmonary dysplasia (BPD). The lung pathology of BPD is associated with varying degrees of fibrosis that develops within days to weeks after mechanical ventilation treatment for RDS. The development of pulmonary fibrosis generally follows an inflammatory phase and is characterized by an increase in pulmonary fibroblast numbers and increased deposition of extracellular matrix components (especially collagen) in the lung interstitium. Numerous studies have focussed on inflammatory mediators in the pathogenesis of BPD, but only a few reports are available on the presence of fibrogenic mediators in the lungs during BPD development.

The studies described in this thesis focussed mainly on mediators known to be involved in fibroproliferation (fibroblast proliferation) and fibrogenesis (collagen deposition) and are, therefore, of interest in understanding the pathogenesis of BPD.

When sequential BAL fluids were examined from infants with resolving RDS and from infants developing BPD for their mitogenic activity for human fetal lung fibroblasts (HFL-1) no differences were observed. This suggests that the mitogenic activity in the lungs of prematurely born infants is associated with RDS resolution and contributes to (or reflects) repair of the injured lung structures. The infants that developed BPD, experienced more severe RDS than the resolving infants. This suggests that the infants with BPD development had more severe lung tissue damage than infants with resolving RDS. At postnatal days 2 to 7, the mitogenic activity of BAL fluids was comparable between RDS and BPD, in contrast to the expectation that the BAL fluid mitogenic activity would correlate with the severity of the initial damage. Therefore, the mitogenic activity of BAL fluid from postnatal days 2 to 7 from infants developing BPD may be inadequate to initiate a proper repair response.

When infants progressed to BPD, the mitogenic activity of BAL fluid remained at the same level for at least 2 weeks after birth. This suggests that prolonged fibroproliferation occurs in the lungs of infants developing BPD. It can be postulated that an initially inadequate repair response followed by prolonged pulmonary fibroproliferation contributes to the development of the fibrosis in the lungs of these infants. The coagulation cascade serine protease thrombin was identified as an important fibroblast mitogen in the BAL fluids, especially during the first four postnatal days in both resolving RDS and BPD. Furthermore, it became evident that from postnatal day 7 on factors other than thrombin also contributed to the mitogenic activity of the BAL fluid.

Although the effect of dexamethasone (Dex) on inflammatory mediators in the lungs during BPD development has been intensively studied, the effect on fibrogenic mediators has received little attention yet. From the studies in this thesis it became evident that the currently used Dex regimen to treat infants at risk of BPD increased the BAL fluid mitogenic activity for lung fibroblasts. This increased BAL fluid mitogenic activity was found to be

associated with an increase in the level of the fibrogenic mediator platelet-derived growth factor (PDGF)-BB. Additionally, Dex treatment did not reduce the BAL fluid concentrations of the N-terminal propeptide of type-III collagen (PIIINP) but did reduce the interleukin (IL)-1β and inflammatory cell numbers in BAL fluid. This suggests that the currently used Dex treatment, which is initiated after the first week of life, might promote fibroblast proliferation despite an apparent downregulation of inflammation. Therefore, this treatment may not inhibit the development of pulmonary fibrosis in infants at risk of BPD.

Although analysis of BAL fluid has been proven to be useful in the examination of fibrotic lung disorders, additional information can be obtained from examination of pulmonary tissue. This is especially true since BAL fluid not always reflects what is actually happening within the lung parenchyma. Decreased parenchymal collagen degrading capacity has been demonstrated to be associated with pulmonary fibrosis in adult patients. This can be related to increased parenchymal expression of tissue inhibitors of metalloproteinases (TIMPs) compared with matrix metalloproteinases (MMPs). Examination of lung tissue from infants that died during different phases of BDP development revealed that the chronic (fibrotic) phase of BPD was characterized by an increased expression of TIMP-1 in type-II epithelial cells compared with the MMP-1 expression by these cells. In addition to this, fibroblasts in fibrotic foci expressed MMP-1 as well as TIMP-1 and TIMP-2. These data suggest that decreased collagenolytic activity occurs in the lung parenchyma in the chronic phase of BPD. Such a decrease in parenchymal collagenolytic activity could contribute to excessive collagen accumulation as observed in BPD.

MMP-2 and MMP-9 have also been implicated with fibrosing lung disease in both adult humans and experimental animal models of the disease. Examination of sequential BAL fluids revealed comparable MMP-2 levels at postnatal days 2 and 4 in RDS and BPD. MMP-9 levels, however, were decreased at these ages in BAL fluid from BPD patients compared to resolving RDS. This suggests that early MMP-9 expression in BAL fluid is correlated with resolving RDS. Especially the heterodimer consisting of NGAL and latent MMP-9 was decreased in postnatal day 2 BAL fluids from infants that developed BPD. Therefore, the low MMP-9 levels, particularly those of NGAL/MMP-9 at postnatal day 2, may help to identify RDS patients who are at risk of developing BPD.

With the progression towards BPD a dramatic increase in MMP-9 BAL fluid level was observed, while MMP-2 remained relatively stable. It is suggested that this extensive increase of MMP-9, which is evident from postnatal day 7 onwards, contributes to the ongoing lung damage, especially to the basement membrane, during BPD development. Neutrophils, monocytes/macrophages, type-II alveolar epithelial cells and fibroblasts were identified as sources of MMP-9 in both resolving RDS and during BPD development.

It has been demonstrated that short-course Dex treatment initiated between 12 and 48 hours after birth in infants with RDS reduces the incidence of BPD. However, whether these beneficial effects can be attributed to an anti-fibrotic action of this therapy was not known. In this thesis we demonstrated that a short-course (3-days) Dex treatment, initiated three days after lung injury induction in rats by the anti-neoplastic agent bleomycin, was clearly able to

prevent excessive pulmonary collagen deposition. The observed Dex effect was established without a reduction in inflammatory cell numbers or levels of the fibrogenic mediators TGF-β1, PDGF-AB or thrombin in the BAL fluid. Short-course Dex treatment did, however, reduce the number of proliferating cells in normal and fibrotic lung tissue after bleomycin exposure. From these data we conclude that the anti-fibrotic effect of Dex is mediated via pathways other than reducing the concentrations of the fibrogenic mediators TGF-β1, PDGF-AB and thrombin in the bronchoalveolar compartment, for instance a reduction of cell proliferation, decreased epithelial apoptosis or inhibition of tissue profibrotic mediator activity.

The studies in this thesis suggest that the pathogenesis of BPD is characterized by an inadequate response (too low/or too slow) to the initial lung injury. This inadequate response leads to ongoing lung damage and persistent fibroproliferation. MMP-9, thrombin as well as other fibroblast mitogens are likely to contribute to these processes. In addition, increased expression of TIMP-1 by type-II alveolar epithelial cells relative to MMP-1 expression by these cells as well as co-expression of MMP-1, TIMP-1 and TIMP-2 by fibroblasts in the chronic (fibrotic) phase of BPD may result in decreased parenchymal collagenolytic activity. This decreased collagenolytic activity favours excessive pulmonary collagen accumulation in the BPD lung.

The currently used Dex treatment in infants at risk of BPD that is started after the first week of life may not be able to reduce the development of pulmonary fibrosis in these infants, possibly due to increased pulmonary fibroblast proliferation driven by increased pulmonary levels of PDGF-BB. In contrast, an early 3-day course of Dex may be able to inhibit the development of pulmonary fibrosis in BPD as this treatment regimen was proven to inhibit the development of bleomycin-induced lung fibrosis in rats.

Samenvatting voor niet-ingewijden

Kinderen die geboren worden na een zwangerschapsduur van minder dan dertig weken (40 weken is normaal), zogenaamde prematuren, ontwikkelen binnen enkele minuten na de geboorte ademhalingsproblemen. Dit ziektebeeld wordt het neonataal respiratoir distress syndroom genoemd (RDS). Om deze patiënten in leven te houden, moeten ze beademd worden met vaak hoge concentraties zuurstof. Een aantal van deze patiënten herstelt in de eerste levensweek, anderen ontwikkelen een ernstige longziekte die bronchopulmonale dysplasie (BPD) of chronische longziekte van de prematuriteit (CLD) genoemd wordt. De ziekteverschijnselen van BPD zijn onder andere aanhoudende ademhalingsproblemen en het achterblijven van de lichaamsgroei. Het merendeel van de kinderen moet langdurig (weken tot maanden) beademd worden en heeft nog vele maanden extra zuurstof nodig. Hoewel de ziekteverschijnselen verminderen gedurende de kinderjaren, blijken jong volwassenen, die BPD hebben gehad, bij onderzoek van het functioneren van de longen nog steeds afwijkingen te hebben. Deze afwijkingen kunnen een voorteken zijn van chronisch longlijden op latere leeftijd.

Ondanks de verbeteringen in de neonatale zorg, overlijden er nog steeds kinderen aan de gevolgen van BPD. Microscopisch onderzoek van de longen van overleden patiënten met BPD laten een ontstekingsreactie zien, die gevolgd wordt door overmatige bindweefselvorming in de longen. Deze zogenaamde longfibrose heeft tot gevolg dat het functioneren van de longen ernstig wordt verstoord. Centraal in het proces dat leidt tot fibrose staat de bindweefselcel, ook wel fibroblast genoemd. Tijdens het ontstaan van longfibrose worden er bepaalde stoffen (fibrogene mediatoren) geproduceerd door ontstekingscellen of andere cellen in de longen. Deze fibrogene mediatoren hebben tot gevolg dat de fibroblasten zich gaan vermeerderen (prolifereren) en teveel collageen gaan produceren.

In overlevende BPD patiënten kan de ontstekingsreactie, en het ziekteproces dat leidt tot de bindweefselvorming, onderzocht worden door het afnemen van een longspoelsel ofwel bronchoalveolaire lavage (BAL). Het afnemen van een BAL is een eenvoudige procedure bij beademde prematuren. De BAL vloeistof kan dan onderzocht worden op de aanwezigheid van mediatoren die bijdragen aan de ontwikkeling van de longfibrose bij BPD.

In deze studie is onderzocht of BAL vloeistof van patiënten, die BPD ontwikkelen, al gedurende de eerste levensweek afwijkt ten opzichte van BAL vloeistof van patiënten die herstellen van RDS. Uit deze analyses bleek dat de BAL vloeistof van BPD en RDS patiënten niet verschilt in het vermogen om fibroblasten te laten prolifereren. Hoewel we het niet hebben kunnen analyseren, is het aannemelijk dat mét het herstel van de longen van RDS patiënten, de activiteit in de BAL vloeistof om fibroblasten te laten prolifereren, afneemt. Als een patiënt BPD ontwikkelt, blijft deze activiteit gedurende de eerste twee levensweken echter aanwezig. Verder is aangetoond dat het enzym thrombine voor een belangrijk deel bijdraagt aan de het vermogen van BAL vloeistof om fibroblasten te laten prolifereren. Hieruit concluderen wij dat, in RDS patiënten die herstellen, het vermogen van BAL vloeistof om

fibroblasten te laten prolifereren een normale reaktie is op de schade in de long en dat deze capaciteit mogelijk bijdraagt aan het genezingsproces. In geval van BPD ontwikkeling is deze reaktie in eerste instantie zwak, waardoor het herstel van de longschade niet snel genoeg gaat. Echter, de activiteit in de BAL vloeistof om fibroblasten te laten prolifereren, is bij BPD patiënten gedurende langere tijd aanwezig. Dit draagt bij aan de langdurige fibroblast-proliferatie, en uiteindelijk tot de ontwikkeling van fibrose in de longen van deze kinderen.

Kinderen die het risico lopen BPD te ontwikkelen worden na de eerste levensweek behandeld met een langdurige kuur met het corticosteroid dexamethason (Dex). Het is bekend dat Dex de ontsteking in de longen van deze patiënten vermindert. Dit draagt waarschijnlijk in grote mate bij aan het feit dat de patiënten met deze behandeling na enige dagen geen beademing meer nodig hebben. Of deze behandeling de vorming van fibrose in de longen van deze kinderen remt, is onbekend. Het bleek dat het vermogen van BAL vloeistof van patiënten met het risico op BPD ontwikkeling om fibroblasten te laten prolifereren, toenam binnen 1 tot 3 dagen na de start van de Dex behandeling. Dit ging gepaard met een verhoging van de concentratie van de fibrogene mediator platelet-derived growth factor (PDGF) in de BAL vloeistof. Verder bleek dat de collageen produktie in de longen niet beïnvloed werd binnen 1 tot 3 dagen na de start van de Dex behandeling. Hieruit concluderen wij dat de huidige Dex behandeling, die gestart wordt ná de eerste levensweek, de vorming van fibrose in de longen van kinderen, die BPD ontwikkelen, mogelijk niet remt. Dit kan het gevolg zijn van de verhoogde fibroblastproliferatie stimulerende activiteit in de longen na Dex behandeling.

Overmatige collageen vorming in de longen hoeft niet alleen het resultaat te zijn van een verhoogde produktie van collageen. Normaal gesproken is er in het lichaam een balans tussen collageen produktie en afbraak. Als de collageen afbraak vermindert, kan dit ook overmatige bindweefselvorming in een orgaan, zoals de long, tot gevolg hebben.

Matrixmetalloproteinase-1 (MMP-1) is een enzym dat collageen afbreekt. De activiteit van MMP-1 wordt echter geremd door TIMP-1 en TIMP-2. In de longen van kinderen, die overleden zijn aan BPD, bleek er een sterke toename te zijn in de hoeveelheid TIMP-1 ten opzichte van MMP-1. Hieruit concluderen wij dat de activiteit van MMP-1 verminderd is in de longen van patiënten met BPD. Het gevolg hiervan is dat nieuwgevormd collageen niet voldoende wordt afgebroken, met een ophoping van collageen in de longen als resultaat.

Matrixmetalloproteinase-9 (MMP-9) is een enzym dat betrokken is bij wondgenezing, maar dat, als het in te grote hoeveelheden voorkomt, kan leiden tot weefselschade. Patiënten, die BPD ontwikkelden, bleken tijdens de eerste vier levensdagen minder MMP-9 in de longen te hebben dan patiënten die herstelden van RDS. Dit suggereert dat MMP-9 een rol speelt in het herstelproces van RDS. In geval van BPD ontwikkeling is dit herstelproces mogelijk verstoord door te lage concentraties MMP-9 in de longen. Het bepalen van MMP-9 in de BAL vloeistof twee dagen na de geboorte kan mogelijk helpen bij het onderscheid maken tussen patiënten met RDS die wel of geen BPD gaan ontwikkelen.

Na de eerste levensweek was er een sterke toename in de concentratie van MMP-9 in

de longen van kinderen die BPD ontwikkelden. Deze concentratie was aanmerkelijk groter dan die tijdens de eerste vier levensdagen in patiënten die herstelden van RDS. Dit suggereert dat vanaf de eerste levensweek MMP-9 weefselschade veroorzaakt in de longen van kinderen die BPD ontwikkelen. Dit, in samenhang met de aanwezigheid van fibrogene mediatoren, kan bijdragen aan de fibrosevorming in de longen van kinderen met BPD.

Recent heeft men aangetoond dat als Dex behandeling binnen twee dagen na de geboorte, en slechts voor een korte periode, gegeven wordt, de kans op BPD ontwikkeling in te vroeg geboren kinderen vermindert. Hoewel deze behandeling duidelijk gunstige effecten heeft op de longfunctie, gaat het wel gepaard met ernstige bijeffecten, zoals een vertraagde lichaamsgroei en darmproblemen. Het is niet bekend of deze behandeling de vorming van longfibrose kan remmen.

Om te onderzoeken of een korte en vroeg gestarte Dex behandeling de vorming van longfibrose kan remmen, hebben wij gebruik gemaakt van een diermodel voor longfibrose. Het bleek dat kort (drie dagen) toedienen van Dex, gestart snel na de inductie van longschade (drie dagen), de vorming van longfibrose volledig remde in dit diermodel. Hieruit concluderen wij dat de beschreven gunstige effecten van kortdurende, en vroege, Dex behandeling op de longfunctie van kinderen met risico op BPD mogelijk deels veroorzaakt wordt door een anti-fibrotisch effect van deze behandeling. Een dergelijke kortdurende en vroege Dex behandeling van kinderen met risico op BPD ontwikkeling zou daarom bruikbaar kunnen zijn voor het verminderen van de kans op longfibrose. Er dient echter altijd een afweging gemaakt te worden tussen de wellicht gunstige effecten op de longen en de bijeffecten van deze behandeling.

Uit het onderzoek concluderen wij dat BPD ontwikkeling bij te vroeg geboren kinderen het gevolg is van een inadequate reaktie van het lichaam op de oorspronkelijke schade in de longen. Deze inadequate reaktie leidt uiteindelijk tot overmatige longschade en fibroblast activatie, met als gevolg verbindweefseling. De huidige toegepaste Dex behandeling, die start na de eerste levensweek, heeft wel een gunstig effect op de ontsteking in de longen, maar mogelijk geen remmend effect op de longfibrose in kinderen met risico op BPD ontwikkeling. Een vroege en korte behandeling met Dex, zoals door ons getest in een diermodel voor longfibrose, zou mogelijk de verbindweefseling van de longen in kinderen met risico op BPD kunnen remmen.

Dankwoord

Nu het boekje bijna af is, besef ik me eens temeer dat zonder de hulp van vele mensen dit proefschrift nooit tot stand had kunnen komen. Dit proefschrift is het resultaat van een samenwerking tussen vele verschillende groepen, zowel nationaal als internationaal. Het begon als een samenwerking tussen de afdelingen Immunologie en Kindergeneeskunde/Neonatologie van het Erasmus MC in Rotterdam. Snel volgde er samenwerking met de afdelingen Pathologie van het Erasmus MC, Longziekten/Experimentele Immunologie en Neonatologie van het AMC in Amsterdam en het Centre for Cardiopulmonary Biochemistry and Respiratory Medicine, van het University College London, UK. Bij deze wil ik een ieder uit deze groepen die heeft bijgedragen aan dit proefschrift van harte bedanken.

De afgelopen jaren heb ik met zeer veel plezier gewerkt. Dit plezier kwam niet alleen voort uit het onderzoek doen, maar ook door de collega's op het lab. Daarom bedank ik al mijn huidige en voormalig labgenoten voor al het plezier en de (wetenschappelijke) discussies van de afgelopen jaren. Eén persoon wil ik in het bijzonder noemen. Gita, jij hebt een zeer grote bijdrage geleverd aan het tot stand komen van dit proefschrift. Je hebt altijd met zeer veel enthousiasme aan dit onderwerp gewerkt. Een kritische noot van jou bij de door mij bedachte ultieme experimenten heb ik menigmaal gehoord en bijzonder kunnen waarderen. Een betere analist om mee samen te werken had ik mij niet kunnen wensen.

Tot slot wil ik mijn ouders, zus en vrienden bedanken voor hun belangstelling voor mijn werkzaamheden en de steun en hulp gedurende de afgelopen periode.

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