The Role of Plasminogen Activator Inhibitor-1 and Angiotensin-Converting Enzyme Gene Polymorphisms in Bronchopulmonary Dysplasia

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Background: Bronchopulmonary dysplasia (BPD) is a multifactorial disease of preterm infants that is characterized by airway injury, inflammation, and parenchymal remodeling. Activation of the coagulation cascade leads to intraalveolar fibrin deposition in many inflammatory pulmonary disorders. Increased fibrin formation or decreased fibrinolysis may cause extravascular fibrin deposition. Extravascular fibrin deposits in septae and alveoli due to the altered fibrin turnover are the pathological hallmarks of BPD, which strongly indicate the importance of the imbalance in the competing activities of coagulation and fibrinolysis. **Objective:** We investigated the predictive value of variations in plasminogen activator inhibitor-1 (PAI-1) and angiotensin-converting enzyme (ACE) genes as molecular determinants for BPD in neonates. **Methods:** The study group comprised 98 preterm infants with BPD and a control group including 94 preterm infants without BPD. Restriction fragment size analyses were performed by visualizing digested polymerase chain reaction products for ACE and PAI-1 genotypes. **Results:** No significant associations were found between ACE, PAI-1 gene polymorphisms, and BPD phenotype in our population. **Conclusions:** The two gene polymorphisms (PAI-1 and ACE) had no role in the development of BPD in our study. Further studies with other genes are required for the identification of molecular predisposing factors for BPD that may help in the development of new treatments.

Introduction

DESPITE SIGNIFICANT IMPROVEMENTS in neonatal intensive care, bronchopulmonary dysplasia (BPD) remains one of the most common long-term complications associated with preterm birth (Adcock *et al.*, 2003; Walsh *et al.*, 2006; Short *et al.*, 2007; Atac *et al.*, 2010). BPD is characterized by an initial acute inflammation followed by lung fibrosis and failure of alveolar septation, which ultimately impair the development of the immature lung (Husain *et al.*, 1998; Yanamandra *et al.*, 2005). Some preterm infants develop BPD, whereas some infants with the same gestational age and birth weight do not develop such a disease. Therefore, different individual factors may have a role in the pathogenesis of BPD. Numerous genes required for neonatal lung adaptation may have a role in the etiology of the disease (Pavlovic *et al.*, 2006; Atac *et al.*, 2010). Plasminogen activator inhibitor-1 (PAI-1) and angiotensin-converting enzyme (ACE) are two candidate genes that may have a role in the pathogenesis of the disease. Activation of the coagulation cascade leads to intraalveolar fibrin deposition in many inflammatory pulmonary disorders. Proinflammatory cytokines activate coagulation via tissue factor and attenuate fibrinolysis by increasing the level

of PAIs (Akinnusi and El Solh, 2007). Alveolar fibrin deposition is an important feature of pulmonary diseases. The plasminogen activator/plasmin system plays an important role in extracellular matrix (ECM) deposition, which leads to fibrosis. PAI-1 levels in plasma are increased in many disorders including BPD, vascular diseases, cancer, inflammation, obesity, sepsis, and fibrotic disorders (Liu, 2008). The PAI-1 gene located on chromosome 7 inhibits serin proteases, tissue type, and urokinase type plasminogen activator (tPA and uPA) (Strandberg *et al.*, 1988; Izuhara *et al.*, 2008; Yagmurdur *et al.*, 2008). PAI-1 plays an important role in the regulation of ECM degradation so that PAI-1 expression is increased in many fibrotic diseases (Akay *et al.*, 2004; Akinnusi and El Solh, 2007; Izuhara *et al.*, 2008). ACE plays a role in generating angiotensin II from angiotensin I; capillary blood vessels in the lung are one of the major sites of ACE expression. The renin angiotensin system (RAS) plays an important role in the pathogenesis of pulmonary fibrosis (Kuba *et al.*, 2006). Angiotensin II induces ECM accumulation, probably by stimulating PAI-1 expression (Akinnusi and El Solh, 2007). The human ACE gene is located on chromosome 17q23 and contains a polymorphism consisting of the presence (insertion, I) or absence (deletion, D) of a 287-bp repeat (Tiret *et al.*,

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1992). The deletion is associated with increased ACE activity (Rigat *et al.*, 1990). Angiotensin II stimulates the production of PAI-1 in cultured endothelial cells and vascular smooth muscle cells (Vaughan *et al.*, 1995). Angiotensin II induces ECM accumulation by stimulating PAI-1 expression and then inhibits ECM degradation (Oikawa *et al.*, 1997; Katoh *et al.*, 2000). The two genetic variations may have a role in the pathogenesis of BPD. In the present study, we aimed to investigate the predictive value of variations in ACE and PAI-1 genes as molecular determinants for BPD in neonates.

Materials and Methods

Ethics

The study protocol was approved by the Ethics Committee at Baskent University Faculty of Medicine in Ankara, Turkey.

Patients

The study group comprised 98 preterm infants with BPD and the control group included 94 preterm infants without BPD who were admitted to hospital from August 2006 to May 2008. BPD was defined as a state of chronic oxygen requirement at the age of 28 days or 36 weeks postmenstrual age. According to NIH criteria, the BPD patients were classified to mild, moderate, and severe BPD (Jobe and Bancalari, 2001). The neonates whose gestational ages were >37 weeks, with congenital anomalies, and who died during the first 28 days of life were excluded from the study.

After obtaining informed consent, ethylenediaminetetraacetic acid-coagulated blood samples were obtained for the analysis of PAI-1 4G/5G and ACE I/D genotypes. The isolated genomic DNA was used as a template for polymerase chain reaction (PCR) as previously described (Miller *et al.*, 1988).

Genotyping

Plasminogen activator inhibitor-1. For PAI-1 genotyping, a 99-bp PCR product was digested with *Bsi*I. The uncut product (99 bp) shows the presence of 4G allele. If the PCR

product was cut into two fragments as 77 and 22 bp, it revealed the 5G allele (Ozbek *et al.*, 2009).

Angiotensin-converting enzyme. The I/D polymorphism of ACE was also detected by electrophoretic separation of PCR products. Presence of a 190-bp product indicated the I allele, whereas presence of a 490-bp product indicated the D allele. Each DNA sample revealed one of the three possible patterns after electrophoresis: a 190-bp band (genotype DD), a 490-bp band (genotype II), or both bands (genotype ID) (Celiker *et al.*, 2009).

Statistical analysis

The distributions of PAI-1 4G/5G and ACE I/D genotypes and alleles in BPD and control groups are given in Table 2. Genotype and allele frequencies were determined by direct counting. Data were analyzed by Pearson χ^2 analysis and Fisher's exact test. Results are given as *n* (%) in Table 2. The distributions of genotypes for each polymorphism were assessed for deviation from the Hardy-Weinberg equilibrium by the χ^2 test. Distributions of the continuous variables were controlled by the Shapiro-Wilk normality test. Homogeneity of the variances was controlled by Levene's test. Parametric test assumptions were invalid so BPD and control groups were compared by the Mann-Whitney *U* test and results are given in Table 2 as mean $\pm$ standard deviation, median, and minimum-maximum values. *p* < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS software (Statistical Package for the Social Sciences, version 13.0; SSPS, Inc., Chicago, IL).

Results

A total of 192 preterm neonates were enrolled. Baseline demographic characteristics such as birth weight, gestational age, gender, and cesarean section are given in Table 1.

During the follow-up period, BPD was diagnosed in 98 neonates, including 42 (42.86%) neonates with mild/moderate disease and 56 (57.14%) with severe BPD. Ninety-four

TABLE 1. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF BRONCHOPULMONARY DYSPLASIA AND CONTROL GROUPS

	BPD (n = 98) Mean $\pm$ SD Median	Control (n = 94) Mean $\pm$ SD Median	p
Gestational age	27.97 $\pm$ 1.8 28 (24–33)	30.14 $\pm$ 1.8 30 (26–34)	<0.001
Birth weight (g)	1102.76 $\pm$ 251.83 1050 (640–2110)	1430.21 $\pm$ 351.70 1400 (830–2500)	<0.001
Mean time of mechanical ventilation (days)	14.58 $\pm$ 15.73 8.00 (0–77)	1.13 $\pm$ 2.60 0 (0–17)	<0.001
Mean time of hospitalization (days)	74.02 $\pm$ 23.81 67.5 (32–159)	30.69 $\pm$ 16.94 29.5 (5–99)	<0.001
	n (%)	n (%)	
Gender (female/male)	39/59	45/49	>0.05
Cesarean section	70 (71.4)	79 (84)	>0.05
Antenatal steroid replacement	56 (43.4)	73 (56.6)	<0.05
Surfactant replacement	96 (98)	29 (30.8)	<0.001

BPD, bronchopulmonary dysplasia; SD, standard deviation.

babies served as a control group (no BPD). The mean gestational age and birth weight of patients with BPD were 27.97 ± 1.8 weeks and 1102.76 ± 251.83 g, respectively. The same figures for control group were 30.14 ± 1.8 weeks and 1430.21 ± 351.70 g. Infants with BPD had lower birth weight and gestational age compared with the control group. Other demographic characteristics were not statistically different between the two groups; a considerable percentage of neonates was born by caesarean in each group and hence the female/male ratio in the two groups was insignificant. The mean time of ventilation and hospitalization was significantly longer in the BPD group (Table 1). Application of antenatal steroid was higher in the control group. Surfactant replacement therapy was used more frequently in the BPD babies than in the control group.

The distributions of PAI-1 4G/5G and ACE D/I genotypes in BPD and control groups are given in Table 2. There was no significant effect of PAI-1 4G/5G and ACE D/I genotypes on the development of BPD.

Discussion

BPD is a multifactorial disease of preterm infants that is characterized by airway injury, inflammation, and parenchymal remodeling (Bancalari *et al.*, 2003; Bancalari and Claure, 2006). However, the molecular basis of BPD is not clear enough. Numerous genes required for neonatal lung adaptation are involved in the etiology of this disease. There are several studies defining the candidate genes that are proposed to be in association with susceptibility to BPD (Bhandari *et al.*, 2006; Atac *et al.*, 2010). In the present study, we focused on the altered competing activities of coagulation and fibrinolysis. Therefore, the main objective of the present study was to assess the molecular risk factors for BPD development by analyzing its association with polymorphisms of PAI-1 4G/5G and ACE I/D because determination of molecular predisposing factors for BPD could help in the development of new treatments and may reduce unnecessary exposure to potentially harmful therapies.

Coagulation and anticoagulation work in balance in human body. Activation of coagulation cascade leads to intraalveolar fibrin deposition in many inflammatory pulmonary disorders. Proinflammatory cytokines activate coagulation via tissue factor and attenuate fibrinolysis by increasing the level of PAIs (Pavlovic *et al.*, 2006). Intraalveolar fibrin deposition is

an important feature of pulmonary diseases. PAI-1 regulates the plasminogen activation system through inhibition of both tPA and uPA. PAI-1 is produced in liver cells, smooth muscle cells, adipocytes, and endothelial cells, is also present in platelets, and is considered to be an important regulatory element in fibrinolysis (Strandberg *et al.*, 1988; Kohler and Grant, 2000; Pihusch *et al.*, 2005). PAI-1 expression is increased in the lung fibrotic diseases (Akinnusi and El Solh, 2007). Higher plasma PAI-1 concentrations were related with patients who are homozygous for the 4G allele than patients who are homozygous for the 5G allele (Eriksson *et al.*, 1995). In the present study, there was no significant difference between these two genotypes. In another study, *in vitro* experiments have shown that the 4G allele produces six times more PAI-1 RNA than the 5G allele in response to IL-1b (Dawson *et al.*, 1993). Among 37 preterm infants with respiratory distress syndrome (RDS) and intubated during the first 2 postnatal weeks, tracheal aspirate fluid was collected for measurement of PAI-1, tPA, and uPA levels. The ratios of PAI-1 to uPA and tPA were found to be significantly higher in this group and 15 of them developed BPD; the BPD group had higher PAI-1 levels than patients without BPD (Cederqvist *et al.*, 2006). The higher levels of PAI-1 in tracheal aspirates support intraalveolar fibrin deposition in BPD, but we showed no association with BPD and PAI-1 4G/5G gene polymorphism. Singhal *et al.* showed depressed alveolar fibrinolytic activity in the neonates with RDS and progressive BPD when alveolar fibrinolysis was greatly impaired. In that study, they thought that the degree of depression in fibrinolytic activity may predict the progression to BPD (Singhal and Parton, 1996). In the present study, on comparing the respiratory distress group, which receives surfactant, with preterms who did not receive surfactant, we showed no difference of PAI-1 4G/5G gene polymorphisms in these two groups. These results showed that PAI-1 4G/5G gene polymorphism had no role in the development of BPD. This may be due to excluding preterms who died during postnatal 28 days with severe respiratory failure.

Many genetic factors have a role in lung maturation, balance between proinflammatory and antiinflammatory mechanisms, cell damage, and tissue repairment. Inflammatory cytokines, which contribute to development of BPD, increase production of free oxygen radicals and leukocyte invasion and then prevent lung maturation (Bokodi *et al.*, 2007). Chronic lung disease in premature infants is associated with increased concentration of inflammatory cytokines in tracheal aspirates. There are several studies that investigate the relation of gene polymorphism and BPD. Adcock *et al.* (2003) investigated the role of gene polymorphisms of cytokines in the development of chronic lung disease and found that tumor necrosis factor- α -308 G/A, transforming growth factor- β 1 +915 G/C, and monocyte chemoattractant protein-1 -2518 A/G polymorphisms were not associated with chronic lung disease. Whether the increase of proinflammatory cytokines or decrease of anti-inflammatory cytokines can cause lung damage was not well understood (Gitto *et al.*, 2005). There are several studies defining candidate genes that are proposed to be in association with susceptibility to BPD. Dystroglycan plays an important role in the stability of the plasma membrane and is highly expressed within the epithelial cell layer. DAG1 N494H genotype was significantly associated with BPD (Concolino *et al.*, 2007). The

TABLE 2. THE DISTRIBUTIONS OF PLASMINOGEN ACTIVATOR INHIBITOR-1 4G/5G AND ANGIOTENSIN-CONVERTING ENZYME D/I GENOTYPES IN BRONCHOPULMONARY DYSPLASIA AND CONTROL GROUP

Genotypes	BPD (n = 98)	Control (n = 94)	p
PAI-1			
4G/4G, n (%)	43 (43.9)	40 (42.6)	>0.05
4G/5G, n (%)	27 (27.6)	27 (28.7)	>0.05
5G/5G, n (%)	28 (28.6)	27 (28.7)	>0.05
ACE			
DD, n (%)	51 (52)	60 (63.8)	>0.05
D/I, n (%)	33 (33.7)	28 (29.8)	>0.05
II, n (%)	14 (14.3)	6 (6.4)	>0.05

ACE, angiotensin-converting enzyme; D, deletion; I, insertion; PAI-1, plasminogen activator inhibitor.

expression of cathepsin K, which is responsible for ECM degradation and secretion of ECM proteins from fibroblasts, was significantly lower in the lungs of premature infants developing BPD (Knaapi *et al.*, 2006). In a study including 586 very low birth weight infants, the association of genetic polymorphisms of hemostasis genes with sepsis, BPD, intraventricular hemorrhage, and periventricular leukomalacia was investigated and the factor VII-323 del/ins polymorphism was found to be a potential protective factor against BPD (Härtel *et al.*, 2006). In our previous study, we evaluated the association between FXIII-Val34Leu, FVII-323 del/ins, and transforming growth factor-beta 1 (915G/T) gene polymorphisms in patients with BPD and also found no association between these gene polymorphisms and BPD (Adcock *et al.*, 2003).

The RAS is activated during lung injury. ACE activity is increased in bronchoalveolar lavage in acute RDS, whereas circulating ACE activity is decreased at the same time (Kuba *et al.*, 2006). The RAS plays an important role in acute and chronic lung diseases (Idell *et al.*, 1987). The increased ACE activity (DD genotype) is associated with an increased risk of developing acute RDS and other lung diseases (Morrison *et al.*, 2001; Marshall *et al.*, 2002). In another study, 245 mechanically ventilated infants were evaluated for ACE I/D polymorphism and no effect of the ACE I/D polymorphism on mortality or development of BPD was found (Yanamandra *et al.*, 2004). In the present study, ACE I/D polymorphism was not associated with the risk for the development of BPD.

Lung injury and a maladaptive repair process that leads to the development of BPD are complex, as many factors play a role in this process. Because the neonates who died during the first 28 days of life were excluded from the study, the observations of this study are limited by selection bias. If we assumed that this group had severe respiratory failure, then the impact of these polymorphisms on the development of BPD may be underestimated. Another limitation of this study is the number of patients. We believe that these polymorphisms may need to be investigated in larger study groups. Further studies conducted in different ethnic groups are needed to investigate the association between gene polymorphisms and BPD.

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Disclosure Statement

No competing financial interests exist.

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