Polymorphisms of surfactant protein A genes and the risk of bronchopulmonary dysplasia in preterm infants

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The pathophysiology of bronchopulmonary dysplasia (BPD) as an inflammatory disorder secondary to neonatal respitaroy distress syndrome (RDS) is not yet fully understood and still represents a major complication of prematurity. The main pathophysiologic feature of RDS is a primary surfactant deficiency in a structurally immature lung. Pulmonary surfactant contains 90 percent phospholipids and 10 percent proteins (surfactant proteins A, B, C, and D). As surfactant protein A (SP-A) has several major immunological and metabolic intrapulmonary functions, we aimed at investigating an association of polymorphisms of SP-A1 and SP-A2 encoding genes and the risk of BPD.

We performed a case-control study exclusively including Caucasian preterm infants below 32 weeks of gestation matched for the degree of immaturity and the year of birth. Venous cord blood was taken prospectively and analyzed by polymerase chain reaction (PCR), single-strand conformation polymorphism (SSCP), cloning and sequencing. BPD was defined as oxygen dependency or need for mechanical ventilation at day 28.

Twenty-three infants with BPD were enrolled (mean gestational age 26.2 weeks; mean birth weight 760.4 g) and compared with 23 infants matched on the basis of gestational age (mean gestational age 27.9 weeks; mean birthweight 1015 g). We observed a significantly increased frequency of the SP-A1 polymorphism $6A^6$ in infants with BPD compared with controls.

In addition to previously established risk factors for BPD, $6A^6$ polymorphism for SP-A1 gene is an independent co-factor. We believe treatment of neonatal RDS should also include stratification according to genetic risk factors.

Key words: surfactant protein A genes, bronchopulmonary dysplasia, preterm infants.

Bronchopulmonary dysplasia (BPD) is a chronic inflammatory pulmonary disorder[1] resulting from neonatal respiratory distress syndrome (RDS) and is observed nearly exclusively in preterm babies ventilated artificially after birth. The main pathophysiological feature of RDS is a primary pulmonary deficiency of surfactant in a structurally immature lung. The number of babies surviving without developing BPD is increasing according to different European study populations, a finding attributed to improvements in perinatal care[2-4]. Mathematical models for the prediction of BPD never reached positive predictive values beyond 70 to 75 percent using multivariate regression analyses[2,3,5]. The key variables in the afore mentioned studies were the degree of immaturity, i.e. gestational age and birth weight, severity of respiratory failure, and in one study lack of prenatal glucocorticoid administration[3]. Thus, further factors, among then those influencing inflammatory intrapulmonary activity, must be operative.

Pulmonary surfactant containing around 90 percent phospholipids and 10 percent non-serum proteins is essential for maintaining physiological surface tension at the air-water interface. Surfactant protein A (SP-A) is the most abundant surfactant protein containing both collagenous and carbohydrate-binding domains[6,7]. It was shown to exhibit major effects on both surfactant metabolism and local

pulmonary immunologic functions, among them binding to different pathogens, regulation of alveolar macrophages and enhancement of neutrophil phagocytosis[8]. The human SP-A gene locus has been assigned to chromosome 10q22-q23, consisting of two very similar genes, SP-A1 and SP-A2[9]. Polymorphisms of the SP-A genes have been described[10], however a functional effect on long-term course of neonatal RDS, i.e. risk of BPD, has not been demonstrated yet.

It thus was the goal of the present study to prove the hypothesis that polymorphisms of SP-A genes are a significant cofactor in the pathogenesis of BPD.

Material and Methods

Patients

Preterm neonates of less than 32 completed weeks gestational age were enrolled prospectively after parental informed consent, from January to July 1998 (Lübeck) and from February to December 1999 (Giessen). For evaluation of SP-A genotypes, venous cord blood samples were taken and stored at -20 °C before analysis. Infants were classified as BPD if supplemental oxygen above room air was necessary for adequate oxygenation (transcutaneous arterial oxygen saturation > 90%) at day 28 following birth or if infants were still on mechanical ventilation[11]. Further prenatal and neonatal variables were defined as follows: a complete course of prenatal corticosteroids was diagnosed if two doses of betamethasone or four doses of dexamethasone were given > 24 hours before bith, or if preeclampsia existed on the basis of the criteria proposed by the American College of Obstetrics and Gynecology (ACOG)[12]. Radiographic grading of RDS was based on the criteria proposed by Couchard and co-workers[13]. Chronic lung disease was defined as supplemental oxygen or mechanical ventilation at 36 weeks postconceptional age.

Microbiological Analyses

DNA was extracted from blood samples using the QIAquick Blood Kit (Qiagen; Hilden/Germany). Reagents for the polymerase chain reaction (PCR) were purchased from Hybaid, (Heidelberg/Germany). Oligonucleotides used as primers for the PCR and the sequencing reactions were commercially synthesized by Roth (Karlsruhe/Germany). The 6-FAM and the HEX labeled primer used for PCR single-strand conformation polymorphism (SSCP) analysis were purchased from PE Biosystems (Weiterstadt/Germany).

Amplification of SP-A1 as well as of SP-A2 was performed on a programmable thermocycler (MJ Research, Massachusetts/USA) using 100 ng DNA in a 50 ml mixture of 400 pmol of each primer, 250 pmol deoxynucleotide triphosphates (dNTP), 1.75 mmol $MgCI_2$ and 1U Proof-Mix Taq/Pwo in buffer with 10 nM Tris, 50 mM KCI.

After initial denaturing at 94°C for 2 min, 34 cycles of annealing at 58°C for 30 sec, extension at 68°C for 3.5 min and denaturing at 94°C for 30 sec were performed, followed by a final extension at 68°C for 20 min. SP-A1 was amplified by oligonucleotides 469 FW1 and 4031 RV1; for amplification of SP-A2 we used oligonucleotide 441 FW2 and 4068 RV2 (Table I).

Table I. Primers Used to Identify SP-A2 Polymorphisms

Oligonucleotide	Sequence	Orientation	Product
469 FW1	5'-CCATGACTGACCACCTTGAG-3'	up	SPA-1
4031 RV1	5'-CACATCTGAAGGCGGCTCTAG-3'	down	SPA-1
441 FW2	5'ATCACTGACTGTGAGAGGGT-3'	up	SPA-2
4068 RV2	5'-TGTCTGCAGTGGGGGGCTCTTC-3'	down	SPA-2
1077 RV1	5'-GTGGCGTCTGCAGCACAGTA-3'	down	exon 1
1511 FW2	5'-GCTGACAGATCCTACACATC-3'	up	exon 2
1729 RV2	5'-TAAGTGACTTCAGGTCGCTG-3'	down	exon 2
3088 FW4	5'GTCCCAAGGAATCCAGAGGA-3'	up	exon 4
3026 RV4	5'-AGTCGGGAGTACAGGCAGTTC-3'	down	exon 4
971 FW-cc	5'-GCAGTATACTTCCTGAGTCCTGACAGAGC-3'	up	exon 1
3430 RV-Cla	5'-GCAATCGATCTAGCATCTCACAGACCAAG-3'	down	exon 4

An aliquot of the PCR product was used to evaluate the success of the PCR reaction by agarose gel electrophoresis. These PCR products were used as templates for a nested PCR amplifying exons 1, 2, and 4 of SP-A1 and SP-A2. Exon 1 was synthesized by oligo 971 FW1 and 1077 RV14, exon 2 by 1511 FW2 and 1729 RV2, and exon 4 by 3088 FW4 and 3026 RV4 (Table I) during an extension time of 60 sec in the same conditions as described above for PCR amplifying of SP-A1 and SP-A2.

To screen for variations in the nested PCR products, nonradioactive SSCP was performed on the Capillary Genetic Analyzer 310 (PE Biosystems; Weiterstadt/Germany). The nested PCR products were diluted 10 times and 1 µl of each of the dilution was mixed with 10.5 µl deionized formamide and 0.5 µl Genescan-350 ROX internal standard (PE Biosystems; Weiterstadt/Germany), heated at 90°C for 2 min and then placed into an ice bath for 2 min and further analyzed.

Each 20 µl ligation reaction contained 100-250 ng of linearized vector, with one third to one half of purified PCR amplification product insertion in the presence of 50 mM Tris, 10 mM $MgCl_2$, 2 mM ATP and 400 U of T4 DNA ligase at 16°C for 16 h. Cloning and sequencing were carried out as published previously[16,17].

Statistics

Infants classified as BPD were matched with controls on the basis of gestational age and year of birth. Basic prenatal and neonatal variables are given as mean and standard deviation (SD) or as percentages, respectively, in Table II. Analysis of continuous variables was performed on the basis of Mann-Whitney-U tests; categorical variables were compared by chi-square tests. Differences of statistical significance were assumed if p was < 0.05 (SPSS, version 6.12 for windows; Chicago, IL).

Interim analyses were scheduled after 20 patients in each group. The study was approved by the Committee on Investigation in Human Subjects.

Table II. Prenatal and Neonatal Characteristics of Study Infants

	BPD (n=23)	Controls (n=23)	p-value
Prenatal corticosteroids (n%)	20 (87%)	18 (78%)	n.s.
Cesarean section (n%)	22 (96%)	21 (91%)	n.s.
Gestational age (weeks)	26.2±1.3	27.7±1.3	p<0.001
Preeclampsia	6 (26%)	3 (13%)	
Birth weight (g)	760.4±140	971.6±175.5	p<0.005
Male/female	13/10	8/15	n.s.
Apgar < 7 at 5 min (n%)	2 (9%)	1 (4%)	n.s.
≥ III Respiratory distress syndrome	12 (52%)	9 (39.1%)	n.s.
Mean time of mechanical ventilation (days)	24.1±10.1	2.2±1.5	p<0.0001
Mean days on oxygen	69.9±29.2	9.27±10.4	p<0.0001
Chronic lung disease (36 weeks p.c.)	9 (39%)	0 (-)	p<0.001

Data are given as mean ± SD or percentages.
BPD: bronchopulmonary dysplasia; n.s.: not significant; p.c.: postconception.

The samples showing aberrant migration on SSCP were reamplified from genomic DNA, purified using the QIAquick kit (Qiagen; Hilden/Germany) and directly sequenced. In the case of multiple polymorphisms, cloning before sequencing was necessary.

A nested PCR amplified with primer 971 FW-Acc and 3430 RV-Cla 2559 yielded such products of the gene of the gene of interest bp including exons 1, 2 and 4. They were purified using the QIAquick Kit, digested with NOT I and SAC II (NEB; Beverly/USA) and used directly in ligation reactions. The linearized pBluescrip II+ vector was dephosphorylated (3 U alkaline phosphatase/µl at 37°C for 1 h).

Results

Basic prenatal and neonatal characteristics of the exclusively Caucasian study infants are given in Table II. Although matching was performed on the basis of gestational age, infants with BPD were less mature compared to controls, thus the differences in birth weight.

The overall distribution of polymorphisms of the genes encoding the SP-A is given as an overview in Table III. In addition to the polymorphisms primarily described by Floros and co-workers[10], we found silent polymorphisms in the SP-A1 gene which were observed in exon 2, amino acid 62: $6A^5$ corresponding to $6A^4$ (G→A), $6A^6$ corresponding to $6A^3$ (A→G), $6A^7$ corresponding

to 6A (G→A) and $6A^8$ corresponding to $6A^1$ (A→G), and in exon 1, amino acid 4 (T→C).

Table III. Distribution of SP-A1 and SP-A2 Gene Polymorphisms in Study Infants

SP-A1 Allele	BPD (n=23)	Controls (n=23)	p-value
6A	2/23	4/23	n.s.
$6A^2$	9/23	12/23	n.s.
$6A^3$	12/23	11/23	n.s.
$6A^4$	4/23	3/23	n.s.
$6A^5$	3/23	1/23	n.s.
$6A^6$	6/23	0/23	0.022
$6A^7$	3/23	3/23	n.s.
$6A^8$	1/23	1/23	n.s.
SP-A2 Allele	**BPD (n=23)**	**Controls (n=23)**	**p-value**
1A	4/23	1/23	n.s.
$1A^0$	14/23	17/23	n.s.
$1A^1$	4/23	5/23	n.s.
$1A^2$	12/23	6/23	n.s.
$1A^3$	3/23	0/23	n.s.
$1A^4$	3/23	2/23	n.s.
$1A^5$	1/23	0/23	n.s.

BPD: bronchopulmonary dysplasia; n.s.: not significant.

A difference between infants with BPD and controls in the allele distribution for the SP-A1 gene was demonstrated for the $6A^6$ polymorphism, indicating the latter to be a risk factor for BPD.

Discussion

This is to our knowledge the first report to suggest an association of a polymorphism of SP-A encoding genes and the risk of BPD in preterm infants. To date only an association of combined polymorphisms of genes encoding for surfactant associated proteins A and B has been described to modify the acute course during the first week of neonatal RDS[16]. Surfactant associated qrotein B is essential for immediate maintenance of lung function postnatally, whereas a complete lack of SP-A did not affect postnatal pulmonary adaptation in knock-out mice[17]. We therefore aimed to investigate a disorder like BPD in which the function of SP-A due to its inflammation-modulating properties is thought to be more relevant than in the acute phase of RDS. The functional role of SP-A in the development of BPD may be attributed to its innate immune response to microbiological challenge and regulatory immune effects[9].

We have established silent polymorphisms in the SP-A1 gene, the functional role of which may be questioned. Consequences of single nucleotide polymorphisms have been postulated for various cardiovascular, endocrine and psychiatric disorders, among them those affecting receptor expression and tertiary protein formation[20]. For example an association of two silent polymorphisms of platelet glycoprotein receptor with cardiovascular disorders has been established recently[21].

Our findings indicate a polymorphism of SP-A encoding genes to be a significant co-factor in the pathogenesis of BPD, in addition to the well-known risk factors such as the degree of immaturity, low birth weight and absent prenatal glucocorticoid administration in preterm infants with neonatal RDS. Studies enrolling preterm neonates from other ethnic origins will be necessary to further establish the described association.

Treatment of RDS in preterm infants requires stratification of the infants according to genetic risk factors.

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