Am. J. Hum. Genet. 66:1569–1579, 2000

Association between the Surfactant Protein A (SP-A) Gene Locus and Respiratory-Distress Syndrome in the Finnish Population

Mika Rämet,[1] Ritva Haataja,[1] Riitta Marttila,[2] Joanna Floros,[3] and Mikko Hallman[1]

[1]Department of Pediatrics and Biocenter Oulu, University of Oulu, Oulu, Finland; [2]Central Hospital of Southern Ostrobotnia, Seinäjoki, Finland; and [3]Department of Cellular and Molecular Physiology and Pediatrics, Pennsylvania State University College of Medicine, Hershey, PA

Respiratory-distress syndrome (RDS) in the newborn is a major cause of neonatal mortality and morbidity. Although prematurity is the most-important risk factor for RDS, the syndrome does not develop in many premature infants. The main cause of RDS is a deficiency of pulmonary surfactant, which consists of phospholipids and specific proteins. The genes underlying susceptibility to RDS are insufficiently known. The candidate-gene approach was used to study the association between the surfactant protein A (SP-A) gene locus and RDS in the genetically homogeneous Finnish population. In the present study, 88 infants with RDS and 88 control infants that were matched for degree of prematurity, prenatal glucocorticoid therapy, and sex were analyzed for SP-A genotypes. We show that certain SP-A1 alleles ($6A^2$ and $6A^3$) and an SP-A1/SP-A2 haplotype ($6A^2/1A^0$) were associated with RDS. The $6A^2$ allele was overrepresented and the $6A^3$ allele was underrepresented in infants with RDS. These associations were particularly strong among small premature infants born at gestational age <32 wk. In infants protected from RDS (those that had no RDS, despite extreme prematurity and lack of glucocorticoid therapy), compared with infants that had RDS develop despite having received glucocorticoid therapy, the frequencies of $6A^2$ (.22 vs. .71), $6A^3$ (.72 vs. .17), $6A^2/1A^0$ (.17 vs. .68), $6A^3/1A^1$ (.39 vs. .10), and $6A^3/1A^2$ (.28 vs. .06) in the two groups, respectively, were strikingly different. According to the results of conditional logistic-regression analysis, diseases associated with premature birth did not explain the association between the odds of a particular homozygous SP-A1 genotype ($6A^2/6A^2$ and $6A^3/6A^3$) and RDS. In the population evaluated in the present study, SP-B intron 4 variant frequencies were low and had no detectable association with RDS. We conclude that the SP-A gene locus is an important determinant for predisposition to RDS in premature infants.

Introduction

Respiratory-distress syndrome (RDS [MIM 267450]) in the newborn is the main cause of mortality and morbidity in prematurely born infants. RDS is characterized by deficient gas exchange that is caused by diffuse atelectasis and high-permeability lung edema and that results in fibrin-rich alveolar deposits called "hyaline membranes" (hyaline-membrane disease). Deficiency in lung surfactant is the main cause of RDS (Avery and Mead 1959; for a review, see Merritt et al. 1993). Surfactant consists of lipids and specific surfactant proteins A–C (SP-A–SP-C). Its main function is to lower surface tension at the air-liquid interface of the alveolus, thus preventing diffuse atelectasis (Hawgood and Clements 1990). Lung effluent in RDS is deficient in surfactant components (Hallman et al. 1991), and the lungs of infants that have died of RDS have low levels of surfactant proteins (deMello et al. 1989, 1993). Surfactant components in the amniotic fluid can predict the risk of RDS (Gluck et al. 1971; Hallman 1992). Moreover, levels of SP-A have been associated with severity of RDS (Moya et al. 1994).

Although prematurity is the most-important risk factor for RDS, the syndrome does not develop in many premature infants. Race, sex, and maternal diseases are among the risk factors affecting the incidence of RDS (Farrell and Wood 1976; Richardson and Torday 1994). Antenatal glucocorticoid therapy (Crowley 1995) and surfactant supplementation at birth (Merritt et al. 1993) reduce the incidence of RDS. According to the results of a twin study, genetic factors contribute to the etiology of RDS (Myrianthopoulos et al. 1971). Although the findings of other studies point to hereditary factors having a role in RDS as well (Graven and Mesenheimer 1965; Lankenau 1976; Olowe and Akinkugbe 1978; Hafez et al. 1989), the specific genes underlying susceptibility to RDS have not been identified. Because multiple factors can contribute to the pathogenesis of RDS, the etiology of RDS is thought to be multifactorial and/or multigenic (Floros and Kala 1998).

Received December 17, 1999; accepted for publication March 2, 2000; electronically published April 4, 2000.

Address for correspondence and reprints: Dr. Mikko Hallman, Department of Pediatrics and Biocenter Oulu, University of Oulu, P.O. Box 5000, FIN-90014, Oulu, Finland. E-mail: mhallman@cc.oulu.fi

© 2000 by The American Society of Human Genetics. All rights reserved.
0002-9297/2000/6605-0012$02.00

Deficiency in the synthesis of either phospholipids or surfactant proteins could be responsible for compromised surface activity. Congenital defects in phospholipid metabolism would be likely to cause multiple disorders. On the other hand, variability in the genes encoding surfactant proteins could explain some of the genetic predisposition to RDS. The results of American population-based studies that include both white and black subjects and that examine SP-A and SP-B as candidate genes (Floros et al. 1995; Kala et al. 1998) show significant associations between certain subgroups of RDS and SP-A and/or SP-B alleles.

The human SP-A gene locus, which consists of two highly homologous functional SP-A genes (SP-A1 and SP-A2) and a pseudogene, has been assigned to chromosome 10q22-q23 (Bruns et al. 1987; Fisher et al. 1987). Both SP-A genes consist of four coding exons. Although both gene products may be needed for fully functional mature human SP-A protein (Voss et al. 1991), individually expressed SP-A genes are also bioactive (Wang et al., in press). SP-A improves both the surface activity of surfactant and the resistance against surfactant inhibitors that accumulate in alveolar space in high-permeability lung edema (Hallman et al. 1991; Jobe 1994); however, SP-A deficiency does not result in respiratory failure in mice born at term (Ikegami et al. 1998). As a member of the mammalian C-type lectin family, SP-A also participates in host defense (for a review, see Crouch 1998). Several alleles that differ by a single amino acid have been identified for each SP-A gene (Floros et al. 1996; DiAngelo et al., in press). The alleles of the SP-A1 gene are denoted as "$6A^n$," and those of the SP-A2 gene as "$1A^n$" (Karinch and Floros 1995; Floros and Hoover 1998). On the basis of its high-heterozygosity index and polymorphism-information-content values, the SP-A locus has the characteristics of a good genetic marker (Floros et al. 1996).

There is conflicting evidence regarding the role of SP-A in surfactant function. According to the hypothesis examined in the present study, deficiency in SP-A contributes to susceptibility to RDS. We have evaluated the association between the SP-A gene locus and the risk of RDS in the Finnish population, which is characterized by homogeneity (de la Chapelle 1993, Peltonen et al. 1995). The data presented here indicate that the human SP-A locus affects predisposition to RDS.

Subjects and Methods

Protocol and Study Population

The present study was performed at the Children's Hospital, Oulu University Central Hospital, Oulu, Finland; the Central Hospital of Southern Ostrobotnia, Seinäjoki, Finland; and the Children's Hospital, University Central Hospital, Tampere, Finland. The ethics committees of these centers approved the study protocol. The parents of the neonates gave written consent for their infants' blood samples to be used in this study. Samples were collected from infants born in 1996–99.

Clinical data on sex, gestational age, and maternal and neonatal clinical histories were obtained from the medical records. The diagnosis of RDS was made on the basis of the published clinical (grunting, retraction, flaring, need for supplemental O_2 for $\geqslant$48 h, or need for exogenous surfactant therapy), radiographic (diffuse reticulogranular pattern and air bronchograms), and/or pathologic (diffuse atelectasis and hyaline membranes) criteria. None of the newborns had been treated with surfactant prophylactically.

The infants with RDS were matched pairwise to those without RDS, on the basis of the following criteria: (1) sex, (2) length of gestation (within one week), and (3) prenatal glucocorticoid treatment for prevention of RDS. Twin pairs were not matched with each other. Infants were excluded from the study either if intrauterine blood transfusion was given or if one or both parents were not of Finnish origin. Altogether, 88 pairs could be matched. These 176 infants were further studied. Table 1 lists the clinical data for the patients. Three infants with RDS were born at term (i.e., at gestation $\geqslant$37.0 wk). Cord-blood specimens were obtained from all infants born at term during one month ($n = 225$) at Oulu University Central Hospital. These specimens were genotyped. In this general population, none of the infants had RDS, which is a rare disease in infants born at term.

Table 1

Characteristics of the Infants with RDS and the Controls

Characteristic	Infants with RDS (n = 88)	Controls (n = 88)
Gestational age (wk)[a]	31.6 ± 2.6	31.7 ± 2.6
Birth weight (g)[b]	1,788 ± 646	1784 ± 604
Male sex (n)	56	56
Female sex (n)	32	32
Prenatal glucocorticoid (n)	50	50
Premature birth (n):[c]		
Spontaneous vaginal	12	24
PROM	16	22
Twin pregnancy	25	17
Preeclampsia	15	10
Placental ablation	7	2
Other	10	10
Term birth (n)[d]	3[e]	3

[a] Mean ± SD (23.6–37.4 wk [for infants with RDS] vs. 23.0–37.4 wk [for controls]).

[b] Mean ± SD (570–3,280 g [for infants with RDS] vs. 630–3,860 g [for controls]).

[c] Gestation <37.0 wk.

[d] Gestation $\geqslant$37.0 wk.

[e] All three infants recovered from RDS and did not have an SP-B exon 4 insertion mutation (121ins2).

DNA Samples

Whole-blood samples (0.5–3 ml) from the umbilical cord were collected into plastic EDTA tubes and were stored at −70°C until they were sent to the laboratory of the Department of Pediatrics, Oulu University Central Hospital, for genetic analysis. Genomic DNA was isolated from the whole-blood specimens by use of the Puregene DNA Isolation Kit (Gentra Systems). An aliquot of the DNA solution was diluted to 50 ng/μl, for use in the PCR amplification. When whole-blood samples were not available (for 55 subjects), genotypes were determined by use of a blood spot dried on a filter paper. A 3-mm disk (corresponding to ~12,000 white blood cells) was punched from the blood spot on the paper, by use of a handheld paper punch (Wallac). To decontaminate the punch between samples, multiple punches were done on clean filter paper. DNA was bound to the disk, and cellular contaminants were released by three successive 15-min incubations with 50 μl DNA purification solution (Gentra Systems), followed by three washes with 100% ethanol. After drying at either 55°C or room temperature, the purified paper disk was directly used as a template for PCR amplification. A blank paper disk treated in a similar manner was included in each series, as a control for DNA cross-contamination.

Genotyping of SP-A Genes

Genotyping of both SP-A genes was performed as described elsewhere (DiAngelo et al. 1999). In brief, SP-A genes were amplified with the use of gene-specific primers, under conditions described elsewhere (Floros et al. 1996). Genomic SP-A1 and SP-A2 clones were used as controls of gene specificity in each set of PCR reactions. PCR-cRFLP–based methodology was used to detect single-nucleotide polymorphisms at codons 19, 50, 62, 133, and 219 (for the SP-A1 gene) and at codons 9, 91, 140, and 223 (for the SP-A2 gene). Codon 85 was analyzed for both genes, to further ensure the gene specificity of the PCR amplifications. Various combinations of polymorphisms at these sites distinguish different alleles. At present, 19 alleles have been described for the SP-A1 gene $6A^n$ (DiAngelo et al. 1999; R. Rämet, R. Haataja, R. Marttila, A.-M. Hämäläinen, M. Knip, and M. Hallman, unpublished data). For 22 whole-blood specimens, genotyping was done by use of allele-specific oligonucleotide probes, as described elsewhere (Floros et al. 1996). A total of 100 specimens were analyzed with the use of both methods. The results were identical in each case.

Genotyping of the SP-B Intron 4 Length Variation (SP-B Δi4)

Genotyping of SP-B Δi4 polymorphism (Floros et al. 1995) was performed with the use of a PCR fragment amplified with the use of forward primer SPBi4F 5′ CTGGTCATCGACTACTTCCA 3′ and reverse primer SPBi4R 5′ TGAAGGGCACGTAGTTTCCTA 3′. PCR was conducted in 15 μl reaction mixture containing 50 ng genomic DNA, 0.2 μM each dNTP, 0.6 μM each primer, 1 × GeneAmp PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% w/v gelatin), and 0.75 U Ampli*Taq* Gold DNA polymerase (PE Biosystems). Samples were initially heated at 94°C for 10 min. Thereafter, they went through 5 cycles at 94°C for 2 min, 55°C for 30 s, and 72°C for 2 min 30 s; then 30 cycles at 94°C for 1 min, 55°C for 30 s, and 72°C for 2 min 30 s; and then a final extension at 72°C for 8 min (Thermal cycler PTC-200; MJ Research). The resulting 240–590-bp PCR fragments were analyzed in ethidium bromide–stained LE (BIOzym) or on NuSieve GTG (FMC BioProducts) agarose gels. The blood samples on paper were amplified as described above, with the exception that the reaction volume was 20 μl. The most-common SP-B Δi4 fragment (510 bp) was denoted as the "invariant allele." All deletion variants (five different sizes) were pooled together as the deletion alleles. The most-common deletion variant (366 bp) accounted for ~80% of all deletion alleles; others (240, 330, 400, and 480 bp) were less frequent. In this population sample, only one insertion-variant allele (590 bp) was detected at a frequency of .013.

Statistical Analysis

Allele-frequency comparisons were performed with the use of χ^2 analysis. Allele distributions in infants with RDS and controls were compared with the use of 2 × *k* tables. Frequencies of individual alleles were compared by use of 2 × 2 tables. Fisher's exact test was used when an expected value was <5. The odds ratios (ORs) and 95% confidence intervals (95% CIs) of RDS were calculated by means of the Woolf (logit) method. The observed genotype frequencies were compared with expected Hardy-Weinberg distributions, by use of χ^2 analysis. Logistic-regression analysis was used to investigate whether the diseases resulting in premature birth were associated with the odds of RDS and whether the association between the SP-A1 allele frequencies and RDS was independent of diseases causing premature birth. The ORs of allelic variables and potential confounders (spontaneous premature vaginal birth, premature rupture of fetal membranes [PROM], preeclampsia, and twin pregnancy) for RDS were estimated by use of conditional regression models. Two sets of independent variables were included. Set 1 included four di-

chotomous covariates for the etiology of premature birth, and set 2 included two variables for alleles $6A^2$ and $6A^3$ classified into three categories. The software used for computations included SPSS for Windows (for basic statistical calculations), Arcus Quickstat (for χ^2 analysis and Fisher's exact test), and Egret for Windows (for conditional logistic-regression analysis).

Results

Association between SP-A Alleles and RDS

DNA samples from 88 infants with RDS and from control infants matched for the degree of prematurity, prenatal glucocorticoid therapy, and sex were analyzed. There was a significant difference in SP-A1 ($P = .017$)—but not SP-A2 ($P = .23$)—allele distributions in infants with RDS, compared with controls. The SP-A1 allele $6A^2$ was overrepresented in infants with RDS, compared with that in controls (frequency .65 vs. .52, respectively; $P = .017$). Likewise, the SP-A1 allele $6A^3$ was underrepresented in infants with RDS, compared with that in controls (frequency .24 vs. .34, respectively; $P = .034$) (fig. 1*A*). Infants with RDS showed a trend toward overrepresentation of the SP-A2 allele $1A^0$ (frequency .63 [in those with RDS] vs. .54 [in controls]; $P = .11$), which is in linkage disequilibrium with $6A^2$ (Floros et al. 1996, R. Rämet, R. Haataja, R. Marttila, A.-M. Hämäläinen, M. Knip, and M. Hallman, unpublished data) (fig. 1*B*). The frequency of the SP-A1/SP-A2 haplotype $6A^2/1A^0$ was significantly higher in infants with RDS than in controls (.62 vs. .50, respectively; $P = .018$) (fig. 1*C*). In term infants ($n = 225$), the frequencies of the $6A^2$, $6A^3$, and $1A^0$ alleles and of the $6A^2/1A^0$ haplotype were .60, .26, .59, and .57, respectively.

The frequency of the SP-A1 $6A^2/6A^2$ genotype tended to be different in infants with RDS and controls (.43 vs. .31, respectively; $P = .086$), whereas the frequency of the heterozygous $6A^2/*$ genotype was similar in the two groups (.43 vs. .43; $P = 1.0$). Similarly, the frequency of the $6A^3/6A^3$ genotype differed in infants with RDS and controls (.034 vs. .15, respectively; $P = .009$, OR .20, 95% CI .06–.74), whereas the frequency of the heterozygous $6A^3/*$ genotype did not differ in the two groups (.41 vs. .39, respectively; $P = .76$). The frequency of the homozygous SP-A2 genotype $1A^0/1A^0$ tended to be different in infants with RDS and controls (.40 vs. .28, respectively; $P = .11$), whereas the frequency of the heterozygous $1A^0/*$ genotype was similar (.45 vs. .51, respectively; $P = .45$). The somewhat similar results for the SP-A1 allele $6A^2$ and the SP-A2 allele $1A^0$ were consistent with the fact that these two genes are in strong linkage disequilibrium, which is also evident in this study population. The observed frequency distributions did not

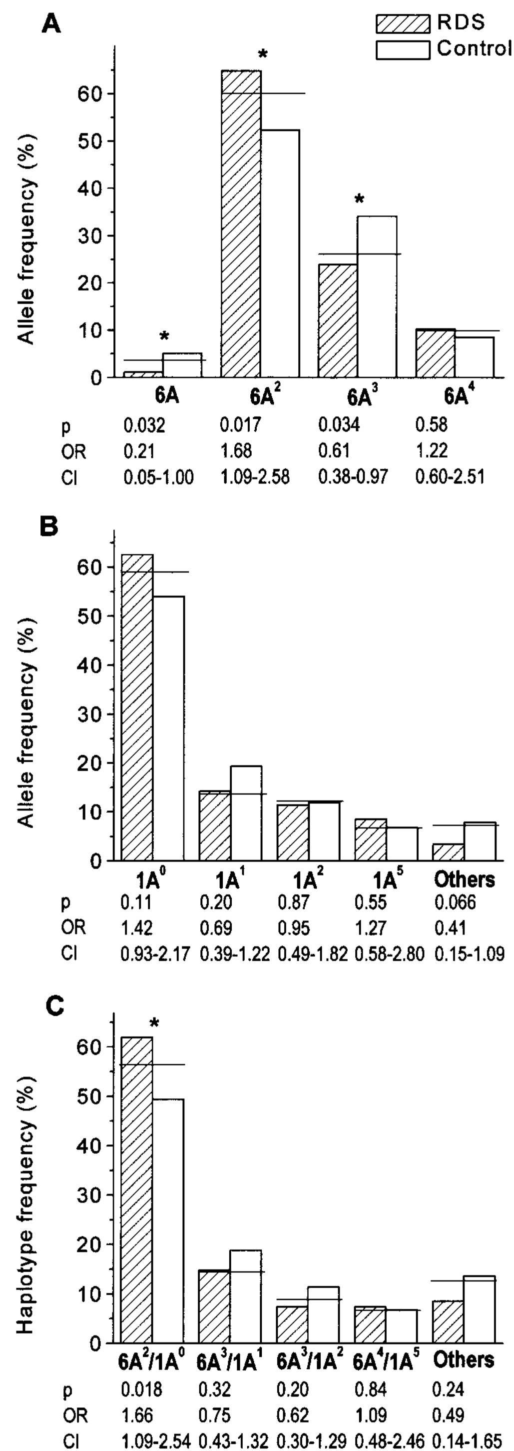

Figure 1 *A,* Distribution of SP-A1 alleles. *B,* Distribution of SP-A2 alleles. *C,* Distribution of the major SP-A1/SP-A2 haplotypes. Altogether, 88 infants with RDS and 88 controls matched for the degree of prematurity, prenatal glucocorticoid therapy, and sex were analyzed. Horizontal lines represent allele frequencies of term infants. The *P* values, ORs, and 95% CIs, which illustrate the association of individual alleles with RDS, are shown below the histograms. An asterisk (*) above the histogram indicates $P < .05$.

deviate from Hardy-Weinberg equilibrium (data not shown).

Influence of Risk Factors on Association between RDS and Either SP-A Alleles or Haplotypes

Prematurity is the most-important risk factor for RDS. The allele frequencies of both SP-A genes in infants with RDS and controls were determined separately for infants born at gestation <32 wk and those born at gestation >32 wk. The median gestational age in the present study was 32 wk. The association between RDS and the SP-A gene locus tended to be dependent on the degree of prematurity (fig. 2*A* and *B*). Infants born at gestation ⩾32.0 wk showed no significant differences in the distribution of the SP-A1 ($P = .56$) or SP-A2 ($P = .53$) alleles in infants with RDS, compared with controls. In contrast, among infants born at gestation <32 wk, there was a difference in SP-A1 ($P = .036$) allele distribution but no difference in SP-A2 ($P = .26$) allele distribution, in a comparison of infants with RDS and controls. The frequency of the SP-A1 allele $6A^2$ was .66 in infants with RDS, compared with .49 in controls ($P = .018$; fig. 2*A*). Similarly, the frequency of the SP-A1 allele $6A^3$ was lower in infants with RDS than it was in controls (.21 vs. .37, respectively; $P = .015$). In very premature infants, the frequency of the homozygous SP-A1 genotype $6A^2/6A^2$ tended to differ in infants with RDS, compared with controls (.48 vs. .30, respectively; $P = .090$). In contrast, the frequency of the heterozygous $6A^2/*$ genotype did not differ in the two groups (.37 vs. .37; $P = .98$). The frequency of the homozygous $6A^3/6A^3$ genotype differed in infants with RDS and controls (.022 vs. .21, respectively; $P = .006$), whereas the frequency of the heterozygous $6A^3/*$ genotype did not differ in the two groups (.37 vs. .30, respectively; $P = .50$). A similar trend was observed for the frequency of the homozygous SP-A2 genotype $1A^0/1A^0$ (.44 vs. .28 in infants with RDS and controls, respectively; $P = .13$) and for the heterozygous SP-A2 genotype $1A^0/*$ (.43 and .47 in infants with RDS and controls, respectively; $P = .77$).

The incidence of RDS is higher in males than in females (Farrell and Wood 1976). Taking this into account, we separately determined, for males (56 pairs) and females (32 pairs), the differences in the frequencies of the SP-A alleles in infants with RDS and controls. In infants with RDS, the frequency distribution of SP-A1 was similar in males and females ($P = .40$). In both males and females, the frequency of $6A^3$ tended to be lower in infants with RDS, compared with controls, whereas the frequency of $6A^2$ tended to be higher in infants with RDS, compared with controls (data not shown).

Glucocorticoid therapy in threatened premature birth reduces the risk of RDS. The least-susceptible infants are those that, despite very premature birth and lack of antenatal corticosteroid therapy, do not have RDS develop. Among infants born at gestation <32 wk, there was a striking difference between those that had received steroid therapy and had RDS develop ($n = 36$) and those that had not received steroid therapy and did not have RDS develop ($n = 9$; fig. 3*A*). In the group that did not receive steroid therapy and did not have RDS develop, the frequency of SP-A1 allele $6A^2$ was .22, compared with a frequency of .71 in the group that had received steroid therapy and had RDS develop ($P = .0002$). Correspondingly, the frequency of the SP-A1 allele $6A^3$ dif-

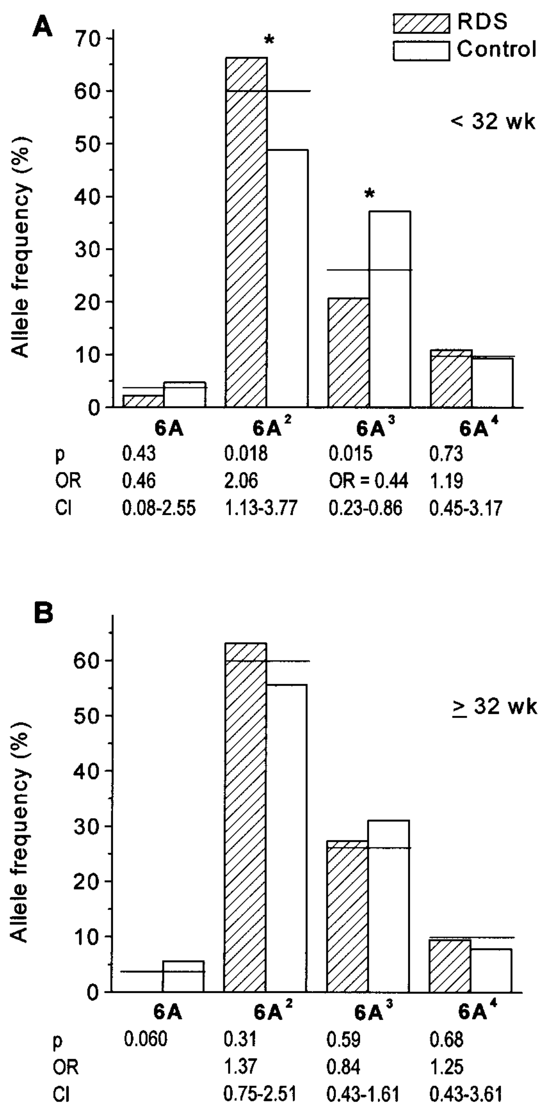

Figure 2 *A*, SP-A1 allele distribution in 46 infants with RDS and 43 controls born at gestation <32 wk. *B*, SP-A1 allele distribution in 42 infants with RDS and 45 controls born at gestation ⩾32 wk. Horizontal lines represent allele frequencies of term infants. The *P* values, ORs, and 95% CIs, which illustrate the association of individual alleles with RDS, are shown below the histograms. An asterisk (*) above the histogram indicates $P < .05$.

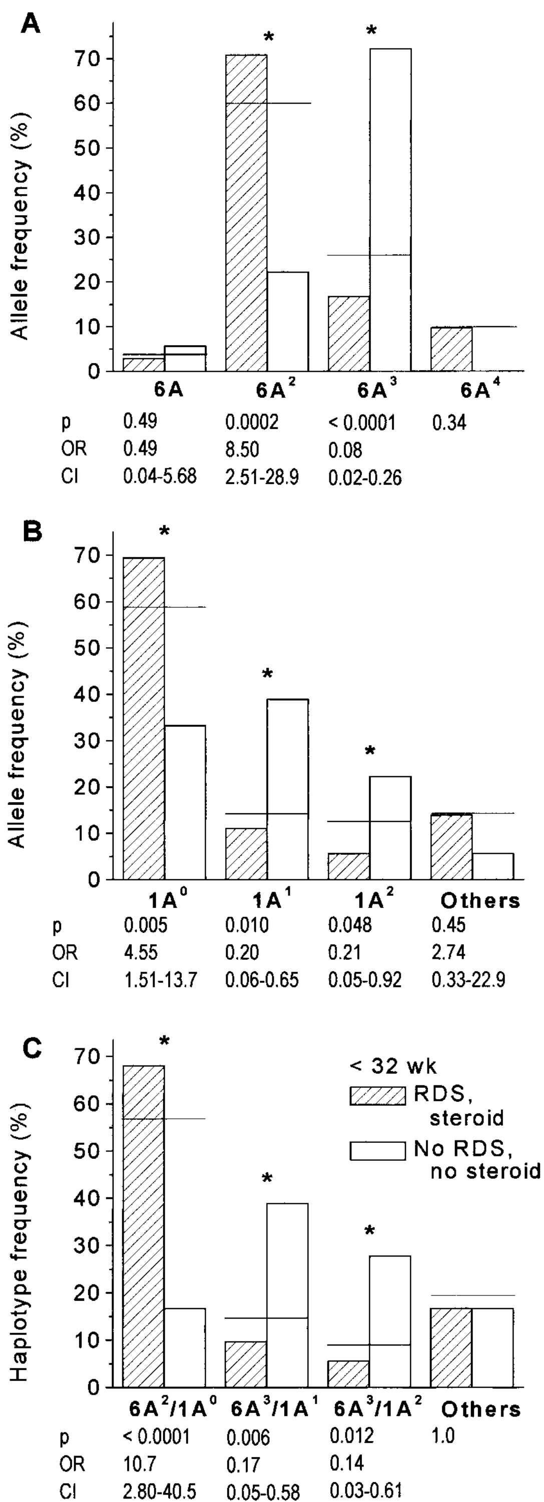

Figure 3 *A,* Distribution of the SP-A1 alleles. *B,* Distribution of SP-A2 alleles. *C,* Distribution of SP-A1/SP-A2 haplotypes in the two groups of premature infants born at gestation <32 wk. RDS, antenatal glucocorticoid ($n = 36$); no RDS, no glucocorticoid ($n = 9$). Horizontal lines represent allele or haplotype frequencies of term infants. The P values, ORs, and 95% CIs, which illustrate the association of individual alleles with RDS, are shown below the histograms. An asterisk (*) above the histogram indicates $P < .05$.

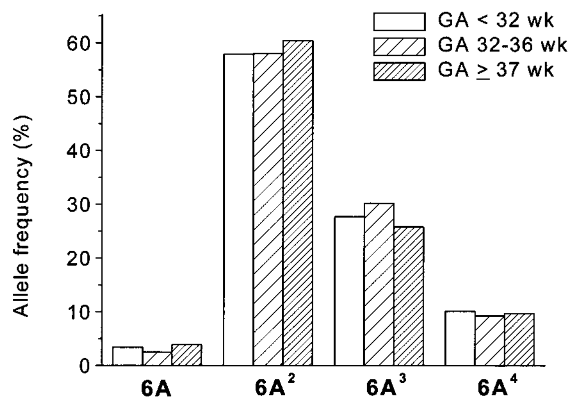

Figure 4 SP-A1 allele distribution in infants divided into the following subgroups defined by the length of gestation at birth: <32 wk ($n = 89$); 32–36 wk ($n = 81$); ⩾37 wk ($n = 231$).

fered remarkably in these two groups (.72 [in those with RDS] vs. .17 [in controls]; $P < .0001$). In the controls (i.e., those that did not receive steroid therapy and did not have RDS develop; gestation <32 wk), the $6A^2$ frequency was lower ($P = .016$) and the $6A^3$ frequency was higher ($P < .0001$) than the corresponding frequencies in infants born at term. Distribution of SP-A2 alleles was different as well (fig. 3*B*). In the controls (i.e., those that did not receive steroid therapy and did not have RDS develop; gestation <32 wk), the $1A^0$ frequency was lower ($P = .005$) and the $1A^1$ ($P = .010$) and $1A^2$ ($P = .048$) frequencies were higher than the corresponding frequencies in the infants with RDS, and the $1A^0$ frequency was lower than that in the infants born at term ($P = .030$).

Figure 3*C* shows the distribution of the haplotypes between those small premature infants that received steroid therapy and had RDS develop and those that did not receive prenatal steroid therapy and did not have RDS develop. Besides $6A^2/1A^0$ ($P < .0001$), both $6A^3/1A^1$ ($P = .006$) and $6A^3/1A^2$ ($P = .012$) had different distributions in infants with RDS and controls.

SP-A1 Genotypes and Causes of Premature Birth as Determinants of RDS

SP-A allele distribution was studied in relation to the length of gestation. None of the SP-A alleles or haplotypes was associated with the degree of prematurity in the population evaluated in the present study ($n = 401$). Figure 4 shows the results for the SP-A1 gene. There were no detectable differences in the distribution of the SP-A1 or SP-A2 alleles, among infants born prematurely as a result of spontaneous vaginal birth ($n = 36$; twin pregnancies excluded), PROM ($n = 38$; twin pregnancies excluded), twin pregnancy ($n = 42$), or preeclampsia ($n = 25$; twin pregnancies excluded) (data not shown).

To ascertain the differences in the distribution of SP-

A alleles in infants with RDS compared with paired controls, we used conditional logistic-regression analysis. We tested the hypothesis of whether an allele of SP-A influences the odds of RDS, regardless of the etiology of prematurity. The following conditions that resulted in premature birth were studied: spontaneous premature vaginal delivery, PROM, twin pregnancy, and preeclampsia. Multiple pregnancy increased the odds of RDS, whereas spontaneous premature birth tended to reduce the odds of RDS (tables 2 and 3). Heterozygous status of $6A^2$ (table 2) or $6A^3$ (table 3) did not influence the odds of RDS. In contrast, the homozygous $6A^3$ genotype reduced the odds of RDS, regardless of the cause of premature birth (table 3). With the exception of prematurity associated with spontaneous vaginal delivery, the homozygous $6A^2$ genotype increased the odds of RDS (table 2).

SP-B Δi4

It has been suggested elsewhere (Floros et al. 1995) that there is an association between SP-B Δi4 and either RDS or prematurity. We studied the frequency of Δi4 variants in the Finnish population. As shown in table 4, the frequencies of the deletion and, especially, the insertion variants were low. No detectable differences in the frequencies of SP-B Δi4 alleles were evident in either a comparison of infants with RDS and controls or a comparison of premature infants and term infants (data not shown).

Discussion

RDS is a multifactorial disease that principally affects infants that are born prematurely. The results of previous studies have pointed to surfactant proteins A and B having a role in the genetic susceptibility to RDS (Floros et al. 1995; Kala et al. 1998). Taking advantage of the homogeneity of the Finnish population, we have studied the associations between SP-A alleles and RDS. In the present study, we genotyped 88 infants with RDS and 88 control infants that were matched pairwise for length of gestation, sex, and prenatal glucocorticoid therapy. We showed that RDS was associated with SP-A1 alleles ($P = .017$), with a homozygous SP-A1 genotype ($P = .009$), and with an SP-A1/SP-A2 haplotype ($P = .018$). Certain SP-A alleles can be viewed as susceptibility ($6A^2$) or protective ($6A^3$) factors for RDS. Heterozygous status of any SP-A allele did not affect the odds of RDS. In contrast, the homozygous $6A^3$ genotype significantly decreased the odds of RDS, whereas the homozygous $6A^2$ genotype increased the odds of RDS, even after adjustment for potential confounding variables. The data from the present study are consistent with previous findings from a study of white American subjects with RDS (Kala et al. 1998), in that they underline the role of the SP-A locus in the susceptibility to RDS in the white population. Finally, the present study demonstrates the influence of steroid treatment and prematurity on the association between SP-A alleles and RDS.

The results of animal studies in which SP-A–deficient mice were evaluated have raised questions about whether SP-A proteins have a role in the maintenance of alveolar stability, which is compromised in RDS. SP-A–deficient mice born at term do not have respiratory failure develop (Korfhagen et al. 1996; Ikegami et al. 1998), unlike SP-B–deficient humans or mice (Nogee et al. 1994; Tokieda et al. 1997; Lin et al. 1998). RDS is mainly associated with prematurity, however, and, although SP-A may not be essential for the respiratory function at term, it may be important for alveolar stability after premature birth. We postulate that SP-A proteins have a role in the pathogenesis of RDS.

The risk of RDS increases as a function of the degree of prematurity, presumably because of insufficient differentiation of the surfactant system (Gluck et al. 1971).

Table 2

Conditional Logistic-Regression Analysis of the Factors Associated with the Odds of RDS among Premature Infants: Causes of Premature Birth and SP-A1 Allele $6A^2$

	Results of Conditional Logistic-Regression Analysis in Premature Infants					
	Any Genotype		With $6A^2$/X vs. X/X Genotype[a]		With $6A^2$/$6A^2$ vs. X/X Genotype[a]	
Cause of Prematurity	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Spontaneous vaginal birth	.46 (.19–1.07)	.070	1.47 (.64–3.41)	.37	2.29 (.93–5.66)	.071
PROM	.53 (.23–1.23)	.14	1.82 (.81–4.13)	.15	3.01 (1.22–7.44)	.017
Twin pregnancy	4.50 (1.17–17.29)	.029	1.53 (.66–3.52)	.32	2.99 (1.19–7.51)	.020
Preeclampsia	1.48 (.61–3.57)	.38	1.72 (.76–3.88)	.19	2.64 (1.09–6.39)	.031
All causes			1.77 (.79–3.97)	.16	2.71 (1.12–6.52)	.026

Note.—In the series of conditional logistic-regression models, individual perinatal disease (yes or no) and/or $6A^2$ genotype (no, heterozygote, or homozygote) were the independent variables. The known confounding variables—length of gestation, fetal sex, and antenatal glucocorticoid treatment—were matched in each stratum.

[a] X = any allele other than $6A^2$.

Table 3

Conditional Logistic-Regression Analysis of the Factors Associated with the Odds of RDS among Premature Infants: Causes of Premature Birth and SP-A1 Allele $6A^3$

	Results of Conditional Logistic-Regression Analysis in Premature Infants					
	Any Genotype		With $6A^3$/X vs. X/X Genotype[a]		With $6A^3/6A^3$ vs. X/X Genotype[a]	
Cause of Prematurity	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Spontaneous vaginal birth	.44 (.19–1.02)	.056	.86 (.42–1.78)	.69	.24 (.06–.89)	.033
PROM	.47 (.19–1.14)	.094	.76 (.36–1.59)	.46	.16 (.04–.64)	.009
Twin pregnancy	4.03 (1.08–15.04)	.038	.79 (.38–1.64)	.52	.19 (.05–.74)	.016
Preeclampsia	1.82 (.73–4.54)	.20	.79 (.38–1.62)	.52	.19 (.05–.72)	.015
All causes			.82 (.40–1.68)	.59	.21 (.06–.77)	.019

Note.—In the series of conditional logistic-regression models, individual perinatal disease (yes or no) and $6A^3$ genotype (no, heterozygote, or homozygote) were the independent variables. The known confounding variables—length of gestation, fetal sex, and antenatal glucocorticoid treatment—were matched in each stratum.

[a] X = any allele other than $6A^3$.

Low SP-A protein level in lung effluent before (Hallman et al. 1988) and shortly after birth (Moya et al. 1994) is closely associated with RDS. Similarly, low levels of SP-A are found in the lungs of infants that have died of RDS (deMello et al. 1989) or that have had chronic lung disease develop (Hallman et al. 1991). It is interesting that the SP-A mRNA levels are shown to differ among adult humans (Floros et al. 1991; Karinch et al. 1997). The $6A^26A^2/1A^01A^0$ genotype, which was associated with RDS in the present study, correlated with low mRNA levels (Karinch et al. 1997). One may propose that an infant with this genotype has an insufficient

Table 4

SP-B Δi4 Polymorphism: Δi4 Allele Distribution in Infants with RDS and Controls

Group and SP-B Δi4 Polymorphism	No. (%) of Δi4 Alleles in Infants with RDS[a]	No. (%) of Δi4 Alleles in Controls[b]	*P*	OR (95% CI)
	n = 88	*n* = 88		
All pairs:				
Invariant	155 (88.1)	157 (89.2)	.74	.89 (.46–1.73)
Deletion	18 (10.2)	15 (8.5)	.58	1.22 (.60–2.51)
Insertion	3 (1.7)	4 (2.3)	1.0	.75 (.16–3.38)
	n = 46	*n* = 43		
Gestation <32 wk:				
Invariant	80 (87.0)	78 (90.7)	.43	.68 (.27–1.76)
Deletion	9 (9.8)	7 (8.1)	.70	1.22 (.44–3.44)
Insertion	3 (3.3)	1 (1.2)	.62	2.87 (.29–28.1)
	n = 42	*n* = 45		
Gestation ⩾32 wk:				
Invariant	75 (89.3)	79 (87.8)	.76	1.16 (.46–2.96)
Deletion	9 (10.7)	8 (8.9)	.69	1.23 (.45–3.35)
Insertion	0	3 (3.3)	1.2	0.15 (.01–2.51)
	n = 36	*n* = 9		
Gestation <32 wk subgroups:				
Invariant	61 (84.7)	15 (83.3)	1.0	1.11 (.27–4.48)
Deletion	8 (11.1)	3 (16.7)	.69	.63 (.15–2.64)
Insertion	3 (8.3)	0	.58	1.86 (.09–37.7)

[a] In the subgroups of infants born at gestation <32-wk, the infants with RDS (*n* = 36) had received steroid therapy.

[b] In the subgroups of infants born at gestation <32-wk, the controls (*n* = 9) had no RDS and had not received steroid therapy.

amount of SP-A at a critical stage of development, compared with those infants with other genotypes. According to the findings of the present study, the critical stage in development occurs considerably before term. The importance of the SP-A genotype, in relation to individual predisposition to RDS, was supported by the results of a comparison of premature infants that, despite having received steroid therapy, had RDS develop and controls that had not received steroid therapy before premature birth. In infants without RDS, the frequencies of alleles $6A^2$ and $6A^3$ were remarkably low (22.2%) and high (72.2%), respectively. The opposite was true in the group of infants that, despite having received glucocorticoid therapy, had RDS develop (frequency 70.8% [for those with $6A^2$] and 16.7% [for those with $6A^3$]). SP-A2 alleles and SP-A haplotypes were also strikingly associated with RDS (fig. 3). Similar to the findings of the present study, the SP-A2 allele $1A^0$ tended to be increased in the white population with RDS, as shown by Kala et al. (1998). The SP-A1 and SP-A2 genetic loci are linked, and $6A^2/1A^0$ is the major haplotype (Floros et al. 1996; the present study).

Infants who did not have RDS develop, despite prematurity and lack of steroid therapy, had a low frequency of the $6A^2$ allele and a high frequency of the $6A^3$ allele. Of relevance is the finding, from in vitro studies, that the responses of the $6A^2$ and $6A^3$ alleles to glucocorticoid treatment were different (Hoover and Floros 1999). Although neither the mechanisms involved nor how these observations reflect the in vivo situation are presently understood, these observations support the notion that the genotype of the individual plays a role in the pathogenesis of RDS and/or in the response to glucocorticoid therapy. Moreover, the observation that the $6A^26A^2/1A^01A^0$ genotype correlates with low levels of SP-A mRNA (Karinch et al. 1997) supports data from the present study. Inactivation of surfactant by plasma proteins is an attractive pathophysiologic mechanism. The high-permeability lung edema caused by increased alveolar and capillary permeability, which is significantly associated with the degree of prematurity, is a characteristic feature in cases of RDS (Jobe 1994). Many plasma components inactivate the surface tension–lowering property of lung surfactant. The surfactant concentration and the quality of surfactant significantly influence the inactivation. SP-A improves the resistance of surfactant against surfactant inhibitors in vitro (Cockshutt et al. 1990; Venkitaraman et al. 1990; Hallman et al. 1991). Furthermore, according to the findings from an experimental study in premature rabbits (Yukitake et al. 1994), the addition of SP-A to the lipid extract of exogenous surfactant protected the surfactant against the inhibitory effects of plasma.

The surface tension–promoting effect of SP-A depends on the presence of SP-B, a hydrophobic surfactant protein that is essential for alveolar stability (Hawgood and Clements 1990; Nogee 1994). SP-B Δi4 has an association with RDS or prematurity in the American population (Floros et al. 1995). In the Finnish population, the frequencies of the deletion and insertion variants were low compared with those in the American population, and no associations with RDS or prematurity were detectable (table 4). This does not rule out the possibility that SP-B gene variability affects the incidence or severity of RDS. Similar to the results of a previous study demonstrating racial differences in the allelic distribution at the SP-B gene locus (Veletza et al. 1996), the results of the present study underline the importance of population homogeneity in studies associating gene variation with a disease.

The allele $6A^2$, for which there was an association with increased odds of RDS, is the most-common SP-A1 allele. Although the $6A^2$ allele may associate with lack of surface activity in premature infants that were previously destined to die, it can be advantageous in some other respects. For example, $6A^2$ may be beneficial in host defense or inflammatory lung disease (reviewed by Eggleton and Reid 1999).

In summary, we have shown here that the SP-A gene locus plays a role in the genetic predisposition to RDS. This is evident among premature infants born at gestation <32 wk. The mechanisms by which either the $6A^2$ allele contributes to or the $6A^3$ allele protects infants from RDS remain to be clarified.

Acknowledgments

We are grateful to Ms. Mirkka Parviainen and Ms. Maarit Hännikäinen, for excellent technical assistance, and to Juha Turtinen, M.Sc., Prof. Charles Berry (University of California, San Diego), and Pekka Uimari, Ph.D., for their skillful advice regarding statistics. We are grateful to Drs. Marja-Leena Pokela and Outi Tammela, for the collection of blood specimens; to Professor Mikael Knip, for the contribution of dried blood samples; and to Ms. Eija Rautio, for collection of clinical data. This study was supported by grants from the Foundation for Pediatric Research (to M.R. and M.H.), the Academy of Finland (to M.R. and M.H.), and Biocenter Oulu (to M.H.) and by grant R37 HL34788 from the National Institutes of Health (to J.F.).

Electronic-Database Information

The accession number and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for RDS [MIM 267450])

References

Avery ME, Mead J (1959) Surface properties in relation to atelectasis and hyaline membrane disease. Am J Dis Child 97:517–523

Bruns G, Stroh H, Veldman GM, Latt SA, Floros J (1987) The 35 kd pulmonary surfactant-associated protein is encoded on chromosome 10. Hum Genet 76:58–62

Cockshutt AM, Weitz J, Possmayer F (1990) Pulmonary surfactant-associated protein A enhances the surface activity of lipid extract surfactant and reverses inhibition by blood proteins in vitro. Biochemistry 29:8424–8429

Crouch EC (1998) Collectins and pulmonary host defense. Am J Respir Cell Mol Biol 19:177–201

Crowley PA (1995) Antenatal corticosteroid therapy: a meta-analysis of the randomized trials, 1972 to 1994. Am J Obstet Gynecol 173:322–335

de la Chapelle A (1993) Disease gene mapping in isolated human populations: the example of Finland. J Med Genet 30:857–865

deMello DE, Hayman S, Phelps D, Floros J (1993) Immunogold localization of SP-A in lungs of infants dying from respiratory distress syndrome. Am J Pathol 142:1631–1640

deMello DE, Phelps DS, Patel G, Floros J, Lagunoff D (1989) Expression of the 35-kDa and low molecular weight surfactant-associated proteins in the lungs of the infants dying with respiratory distress syndrome. Am J Pathol 134: 1285–1293

DiAngelo S, Lin Z, Wang G, Phillips S, Rämet M, Luo J, Floros J (1999) Novel, non-radioactive, simple and multiplex PCR-cRFLP method for genotyping human SP-A and SP-D marker alleles. Dis Markers 15:269–281

Eggleton P, Reid KB (1999) Lung surfactant proteins involved in innate immunity. Curr Opin Immunol 11:28–33

Farrell PM, Wood RE (1976) Epidemiology of hyaline membrane disease in the United States: analysis of national mortality statistics. Pediatrics 58:167–176

Fisher JH, Kao FT, Jones C, White RT, Benson BJ, Mason RJ (1987) The coding sequence for the 32,000-dalton pulmonary surfactant-associated protein A is located on chromosome 10 and identifies two separate restriction-fragment-length polymorphisms. Am J Hum Genet 40:503–511

Floros J, DiAngelo S, Koptides M, Karinch AM, Rogan PK, Nielsen H, Spragg RG, et al (1996) Human SP-A locus: Allele frequencies and linkage disequilibrium between the two surfactant protein A genes. Am J Respir Cell Mol Biol 15:489–498

Floros J, Hoover RR (1998) Genetics of the hydrophilic surfactant proteins A and D. Biochim Biophys Acta 1408: 312–322

Floros J, Kala P (1998) Surfactant proteins: molecular genetics of neonatal respiratory diseases. Annu Rev Physiol 60: 365–384

Floros J, Phelps DS, deMello DE, Longmate J, Harding H, Benson B, White T (1991) The utility of postmortem lung for RNA studies; variability and correlation of the expression of surfactant proteins in human lung. Exp Lung Res 17:91–104

Floros J, Veletza SV, Kotikalapudi P, Krizkova L, Karinch AM, Friedman C, Buchter, et al (1995) Dinucleotide repeats in the human surfactant protein B gene and respiratory distress syndrome. Biochem J 305:583–590

Gluck L, Kulovich MV, Borer RC Jr, Brenner PH, Anderson GG, Spellacy WN (1971) Diagnosis of the respiratory distress syndrome by amniocentesis. Am J Obstet Gynecol 109: 440–445

Graven SN, Mesenheimer HR (1965) Respiratory distress syndrome and the high risk mother. Am J Dis Child 109: 489–494

Hafez M, El-Sallab SH, Khashaba M, Risk MS, el-Morsy Z, Bassiony MR, el-Kenawy F, et al (1989) Evidence of HLA-linked susceptibility gene(s) in respiratory distress syndrome. Dis Markers 7:201–208

Hallman M (1992) Antenatal diagnosis of lung maturity. In: Robertson B, Van Golde LMG, Batenburg JJ (eds) Pulmonary surfactant: from molecular biology to clinical practice. Elsevier, Amsterdam, pp 425–458

Hallman M, Arjomaa P, Mizumoto M, Akino T (1988) Surfactant proteins in the diagnosis of fetal lung maturity. I. Predictive accuracy of the 35 kD protein, the lecithin/sphingomyelin ratio, and phosphatidylglycerol. Am J Obstet Gynecol 158:531–535

Hallman M, Merritt TA, Akino T, Bry K (1991) Surfactant protein A, phosphatidylcholine, and surfactant inhibitors in epithelial lining fluid: correlation with surface activity, severity of RDS, and outcome of small premature infants. Am Rev Respir Dis 144:1376–1384

Hawgood S, Clements JA (1990) Pulmonary surfactant and its apoproteins. J Clin Invest 86:1–6

Hoover RR, Floros J (1999) SP-A 3′-UTR is involved in the glucocorticoid inhibition of human SP-A gene expression. Am J Physiol 276:L917–L924

Ikegami M, Korfhagen TR, Whitsett JA, Bruno MD, Wert SE, Wada K, Jobe AH (1998) Characteristics of surfactant from SP-A–deficient mice. Am J Physiol 275:L247–L254

Jobe AH (1994) Surfactant function and metabolism. In: Boynton BB, Carlo WA, Jobe AH (eds) New therapies for neonatal respiratory disease. Cambridge University Press, New York, pp 16–35

Kala P, Ten Have T, Nielsen H, Dunn M, Floros J (1998) Association of pulmonary surfactant protein A (SP-A) gene and respiratory distress syndrome: interaction with SP-B. Pediatr Res 43:169–177

Karinch AM, deMello DE, Floros J (1997) Effect of genotype on the levels of surfactant protein-A mRNA and on the SP-A2 splice variants in adult humans. Biochem J 321:39–47

Karinch AM, Floros J (1995) 5′ splicing and allelic variants of the human pulmonary surfactant protein A genes. Am J Respir Cell Mol Biol 12:77–88

Korfhagen TR, Bruno MD, Ross GF, Huelsman KM, Ikegami M, Jobe AH, Wert SE, et al (1996) Altered surfactant function and structure in SP-A gene targeted mice. Proc Natl Acad Sci USA 93:9594–9599

Lankenau HM (1976) A genetic and statistical study of the respiratory distress syndrome. Eur J Pediatr 123:167–177

Lin Z, deMello DE, Wallot M, Floros J (1998) An SP-B gene mutation responsible for SP-B deficiency in fatal congenital alveolar proteinosis: evidence for a mutation hotspot in exon 4. Mol Genet Metab 64:25–35

Merritt TA, Soll RF, Hallman M (1993) Overview of exoge-

nous surfactant replacement therapy. J Intensive Care Med 8:205–228

Moya FR, Montes HF, Thomas VL, Mouzinho AM, Smith JF, Rosenfeld CR (1994) Surfactant protein A and saturated phosphatidylcholine in respiratory distress syndrome. Am J Respir Crit Care Med 150:1672–1677

Myrianthopoulos NC, Churchill JA, Baszynski AJ (1971) Respiratory distress syndrome in twins. Acta Genet Med Gemellol (Roma) 20:199–204

Nogee LM, Garnier G, Deitz HC, Singer L, Murphy AM, deMello DE, Colten HR (1994) A mutation in the surfactant protein B gene responsible for fatal neonatal respiratory disease in multiple kindred. J Clin Invest 93:1860–1863

Olowe SA, Akinkugbe A (1978) Amniotic fluid lecithin/sphingomyelin ratio: comparison between an African and North American community. Pediatrics 62:38–41

Peltonen L, Pekkarinen P, Aaltonen J (1995) Messages from an isolate: lessons from the Finnish gene pool. Biol Chem Hoppe Seyler 376:697–704

Richardson DK, Torday JS (1994) Racial differences in predictive value of the lecithin/sphingomyelin ratio. Am J Obstet Gynecol 170:1273–1278

Tokieda K, Whitsett JA, Clark JC, Weaver TE, Ikeda K, McConnell KB, Jobe AH, et al (1997) Pulmonary dysfunction in neonatal SP-B-deficient mice. Am J Physiol 273: L875–L882

Veletza SV, Rogan PK, TenHave T, Olowe SA, Floros J (1996) Racial differences in allelic distribution at the human pulmonary surfactant protein B locus. Exp Lung Res 22:489–494

Venkitaraman AR, Hall SB, Whitsett JA, Notter RH (1990) Enhancement of biophysical activity of lung surfactant extracts and phospholipid-apoprotein mixtures by surfactant protein A. Chem Phys Lipids 56:185–194

Voss T, Melchers K, Scheirle G, Schäfer KP (1991) Structural comparison of recombinant pulmonary surfactant protein SP-A derived from two human coding sequences: implications for the chain composition of natural human SP-A. Am J Respir Cell Mol Biol 4:88–94

Wang G, Phelps DS, Ustead TM, Floros J. Human SP-A protein variants derived from one or both genes stimulate TNF-α production in the THP-1 cell line. Am J Physiol Lung Cell Mol Physiol (in press)

Yukitake K, Brown CL, Schlueter MA, Clements JA, Hawgood S (1994) Surfactant apoprotein A modifies the inhibitory effect of plasma proteins on surfactant activity in vivo. Pediatr Res 37:21–25