



DETECTION OF SPECIFIC SURFACTANT PROTEINS IN GASTRIC ASPIRATES FROM PREMATURELY BORN CHILDREN AFTER CORTICOSTEROID THERAPY

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ABSTRACT

Purpose: Alveolar surfactant (AS) components, including the specific surfactant proteins (SPs) SP-A, SP-B, SP-C, and SP-D, provides stability during the dynamic process of inhalation/exhalation. In prematurely born children different respiratory pathologies due to surfactant components deficiency, like Neonatal Respiratory Distress Syndrome, can be observed. Administration of corticosteroids to pregnant women at risk of preterm birth is an established intervention in clinical practice. In this study, we analyzed gastric aspirates (GAs), as alternative samples of tracheal aspirates and amniotic fluids for AS maturity determination. Samples were taken from prematurely born babies after antenatal corticosteroid therapy (CST) of pregnant women and were analyzed for the presence of specific surfactant proteins.

Materials and Methods: Clinical samples of gastric aspirates were collected in the first minutes after delivery by using of a nasogastric tube and were analyzed by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot assays for detection of SP-A, SP-B, and SP-C.

Results: Our results showed the expression of different isoforms of each specific surfactant protein (SP) in all GA samples, depending on the stage of maturation.

Conclusions: Our results showed that CST plays a role in AS components production and maturation. Moreover, GA can be considered as an adequate sample for assessment of surfactant maturity at birth.

Keywords: surfactant proteins, prematurely born children, corticosteroid therapy, SDS-PAGE, Western blot,

INTRODUCTION

Worldwide reproductive problems are increasing and the risk of preterm delivery is high. Each year, 15 million babies are born preterm [1]. According to the World Health Organization, preterm is defined as babies born alive before 37 weeks of gestation [2]. During pregnancy, the structural and metabolic development of the lungs is not synchronized. The initial stage of lung development in human starts between 16 to 26 weeks of gestation. Alveolarization proceeds from 32-34 weeks of gestation to childhood [3].

Neonatal Respiratory Distress Syndrome (NRDS) is one of the most common lung disorders in prematurely born infants and causes increasing difficulty in breathing. NRDS develops due to primarily or secondary deficiency, or inactivity of alveolar surfactant (AS) components [4].

Alveolar surfactant provides lung stability during the dynamic process of breathing by maintaining low surface tension to prevent the collapse at the end of exhalation. Surfactant is synthesized and secreted by type II alveolar epithelial cells, also called pneumocytes, which differentiate between 24 and 34 weeks of gestation [4, 5]. AS consists of lipids, mainly phosphatidylcholine, and surfactant proteins. Four of the surfactant proteins, SP-A, SP-B, SP-C and SP-D, named according to the order of detection, are specific for AS [3]. SPs are classified into two major structural and functional groups. In the first group are involved SP-A and SP-D and in the second group – SP-B and SP-C. SP-A is the most abundant of the specific surfactant proteins in human lung and along with SP-D are hydrophilic, collagenous C-type lectins. They are involved in the regulation of surfactant structure, host defense against different pathogens, and recognition and clearance of apoptotic cells [6]. Many lung diseases are associated

with abnormal SP-A and SP-D expression in both bronchoalveolar lavage fluid and serum, genetic polymorphisms, and gene mutations of SP-A and SP-D. It is found that infants with NRDS have relatively reduced levels of surfactant proteins including SP-A [7]. SP-B and SP-C are small hydrophobic proteins that play an important role in surface film formation and stability, surfactant homeostasis and function after birth. They contribute to the reduction of alveolar surface tension and facilitate the adsorption of surfactant phospholipids to the air-water interface and stabilize the interfacial lipid layer during film compression [8, 9]. Complete deficiency of SP-B is characterized by the absence of lung compliance and results in lethal NRDS [10]. SP-C deficiency can provoke chronic pneumonitis in infancy and adults as histologically heterogeneous interstitial lung diseases [11].

One of the most common treatments for prevention of NRDS is the antenatal corticosteroid therapy (CST) with betamethasone or dexamethasone to accelerate fetal organ maturation. Both drugs in their active form cross the placenta and have nearly identical biologic activity [12]. Corticosteroids appear to increase surfactant production by both transcriptional and post-transcriptional mechanisms, enhancing the rate of phosphatidylcholine and fatty acid biosynthesis in the fetal lung, and stimulating SPs expression and mRNA transcripts stabilization [13,14].

This study aimed to analyze for the first time the presence of specific surfactant proteins in gastric aspirates (GA) from premature newborns after antenatal corticosteroid therapy of pregnant women. Thus, GAs could be used as alternative samples of tracheal aspirates and amniotic fluids for diagnostic of NRDS in prematurely born infants as non-invasive and less traumatic probing.

MATERIALS AND METHODS

Clinical samples

The patient population included prematurely born infants of 30–34 weeks of gestation without congenital anomalies, born after *in vitro* fertilization and with realized antenatal corticosteroid therapy of the pregnant women. Gastric aspirates were collected in the first minutes after delivery by a routine rapid, easy and non-invasive procedure by using of a nasogastric tube. GAs were stored in sterile test-tubes at -20°C. The samples were collected after informed parental consent.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (12%) [15] under reducing (in the presence of β -mercaptoethanol) and non-reducing conditions was carried out for the separation of surfactant proteins in 7 representative GA samples. The code numbers of the samples are: 4108, 4101, 4899, 2180, 5241, 5243, and 4099. The gels were stained by Coomassie Brilliant Blue G-250.

Western blot assay

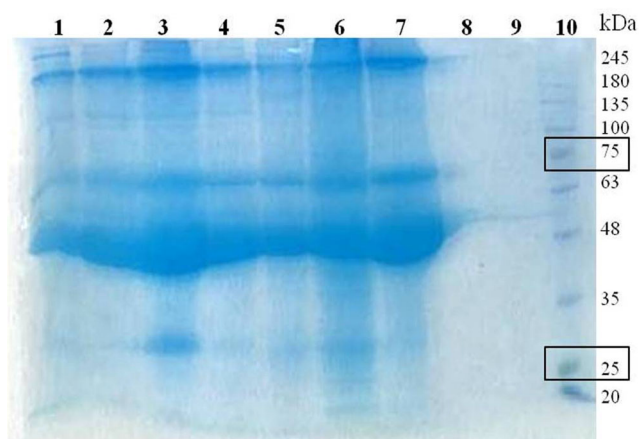
Western blot assay was performed by transfer of separated proteins (under reducing conditions) on the nitrocellulose membrane (0.45 μ m, AmershamTMProtran[®]Western blotting membranes) by wet electroblotting for 3h/200 mA. As for a blocking agent, 5% non-fat dry milk in TTBS solution (Tris Buffered saline, pH 7.6, containing 0.05% Tween 20) was used. Specific primary rabbit anti-human IgG antibodies (Abcam plc, UK) were used for recognition of SP-A, pro-surfactant B protein, and pro-surfactant protein C. Secondary antibody was goat anti-rabbit IgG-HRP (Abcam plc, UK). For detection of surfactant proteins, diaminobenzidine tablets with metal enhancer were used.

RESULTS AND DISCUSSION

In the past, most common tests for lung maturity assessment involved lecithin: sphingomyelin ratio, the concentration of phosphatidylglycerol, and lamellar body count, mostly in amniotic fluid and tracheal aspirates [16]. The collection of these samples is invasive and harmful for pregnant women and the fetus. The analysis of gastric aspirates from prematurely born infants is a new, adequate and non-invasive approach. The fetus swallows amniotic liquid and pulmonary fluids, secreted by lung during embryonic development. Thus, the GA is rich in different components of amniotic, pulmonary and gastric fluids [17]. For this reason, we analyzed GAs for detection of specific surfactant proteins SP-A, SP-B, and SP-C from prematurely born infants after antenatal betamethasone therapy. The separation of the proteins in GA was carried out by 12% SDS-PAGE under non-reducing and reducing conditions followed by wet Western blotting. The blots were probed with specific polyclonal antibodies against human SP-A, pro-SP-B, and pro-SP-C. It is known that the specific surfactant proteins are expressed as larger precursor peptides that undergo several proteolytic processing and post-translational steps to produce mature forms. Maturation of the surfactant is a dynamic process, regulated by many hormones, characterized by changes in molecular composition [3].

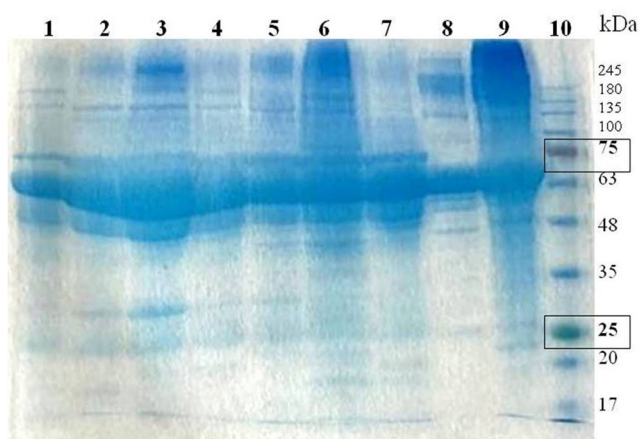
Our results showed different electrophoretic profiles of the analyzed proteins in GA samples from premature newborns depending on the conditions, reducing and non-reducing (Fig. 1 and Fig. 2). As expected, the electrophoretic profile under non-reducing conditions (Fig. 1) revealed the presence of albumin (67 kDa), which is the main nonspecific protein in gastric aspirates. Of the specific AS proteins, the highest concentration was detected for the most abundant multimeric SP-A with molecular weight about 48 kDa in all GA samples, as compared with the standard SP-A in lanes 8 and 9. In most of GA studied (lanes 3, 5, 6, 7) protein bands of about 28-30 kDa were visualized, probably corresponding to pro-SP-C. The electrophoretic profile reflected the complicated multimeric structure of the specific surfactant proteins, their different expression rate and post-translational modifications [3].

Fig. 1. SDS-PAGE (12%) of GAs under non-reducing conditions: 1 - 4108; 2 - 4101; 3 - 4899; 4 - 2180; 5 - 5241; 6 - 5243; 7 - 4099; 8, 9 - standard SP-A (oligomer and monomer forms); 10 - protein standards.



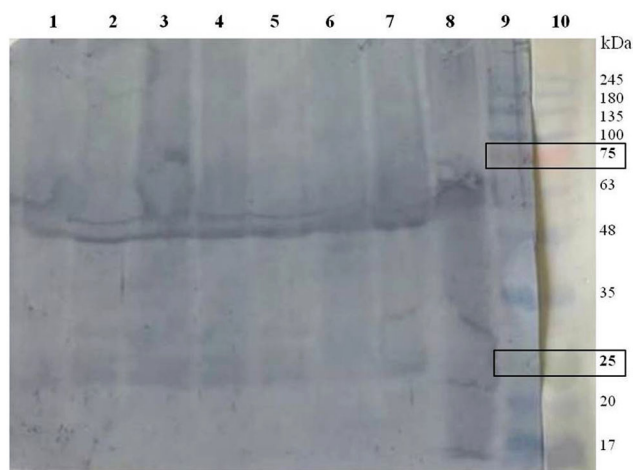
Due to the differences in the structure of the specific surfactant proteins, we conducted a SDS-PAGE analysis of the same GA samples under reducing conditions. The results are presented in Fig. 2. Under reducing conditions, we detected a lot of different mature and immature forms of surfactant proteins depending on the stage of maturation and glycosylation of the molecules, with the most abundant of them was SP-A. It is known that SP-A is the largest surfactant protein. Its mature form (octadecamer with a molecular weight of 630 kDa) consists of 18 polypeptide chains bound by many disulfide bridges [8, 18]. In our samples, a monomer (about 25 kDa) and partially reduced dimer forms of SP-A (48, 55, 66 kDa) in all GA samples were registered (Fig. 2). As with the healthy full-term children (data not shown), the effect of CST led to normal expression of SP-A in prematurely born children.

Fig. 2. SDS-PAGE (12%) of GAs under reducing conditions: 1 - 4108; 2 - 4101; 3 - 4899; 4 - 2180; 5 - 5241; 6 - 5243; 7 - 4099; 8 - bovine serum albumin; 9 - human serum albumin, 10 - protein standards.



To confirm our suggestions about the presence of the specific surfactant proteins in GA samples studied we conducted Western blot assays for detection of SP-A, pro-surfactant SP-B and pro-surfactant SP-C. Our results showed the presence of SP-A dimer forms of 55 kDa, SP-A monomer form at 35 kDa in 4108 sample, and monomer form in the other GA with a molecular weight between 27-35 kDa (Fig. 3). Our findings were in agreement with those by Floros et al. [19], who reported that the simplest polypeptide chain if reduced to its most native form, would yield a band of 35 kDa monomeric SP-A. In addition, native SP-A depending on the degree of post-translational modification migrates as a triplet at 28-36 kDa under reducing conditions and as disulfide-bonded oligomers at a higher molecular weight under non-reducing conditions [20].

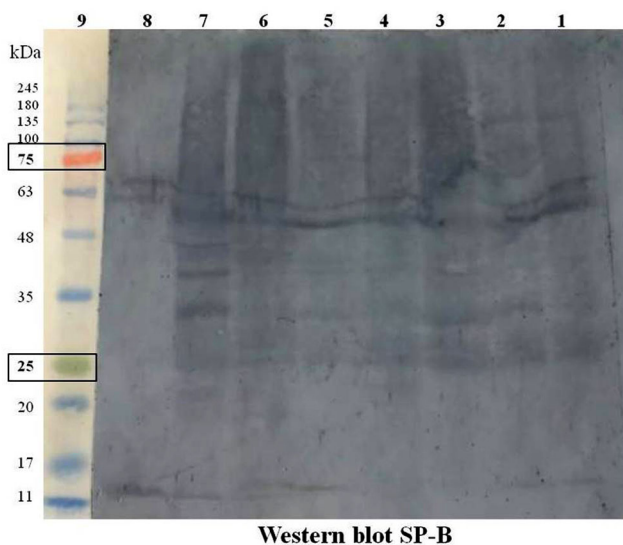
Fig. 3. Western blot assay of SP-A: 1 - 4108; 2 - 4101; 3 - 4899; 4 - 2180; 5 - 5241; 6 - 5243; 7 - 4099; 8 - standard SP-A; 9, 10 - protein standards.



Western blot SP-A

SP-B is synthesized as a 40-kDa precursor molecule and undergoes maturation to 8 kDa peptide [21, 22]. The Western blot analysis of all GAs studied for the presence of SP-B revealed mainly the precursor form of the protein with size between 25-45 kDa, showing different stages of maturation (Fig. 4). The most full range of SP-B in different maturation isoforms was observed in lane 7. In addition to GA samples taken after antenatal CST, in this immunoblot, we included a sample of baby with clinically diagnosed NRDS (Fig. 4, lane 8), where the above mentioned forms of SP-B were not detected.

Fig. 4. Western blot assay of pro-SP-B: 1 - 4108; 2 - 4101; 3 - 4899; 4 - 2180; 5 - 5241; 6 - 5243; 7 - 4099; 8 - NRDS; 9 - protein standards.



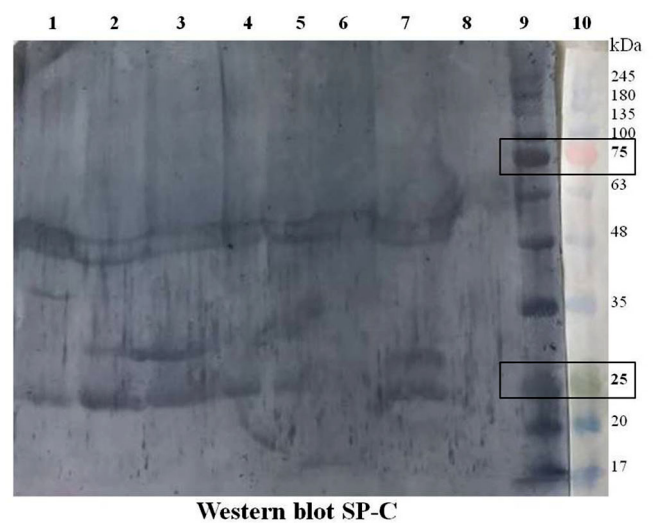
SP-C is a small, uniquely hydrophobic peptide. It is expressed as a 197 amino acid preproprotein of approximately 21 kDa that consists of three distinct domains: 1) a short 23 amino acid N-terminal peptide; 2) a hydrophobic helical transmembrane domain which corresponds to the mature, secreted form of SP-C, and 3) a 14 kDa C-terminal peptide. Pro-surfactant protein C is processed to a 3.7-5 kDa mature protein [23, 24].

The profile of pro-SP-C in the analyzed clinical samples showed again different immature protein forms with a molecular weight in the range of 25 kDa in most of the lanes (Fig.5). Only in sample 5243, pro-SP-C was not detected, probably as a result of the weaker effect of CST. In addition, in lanes 2, 3, and 7 higher molecular weight isoform of pro-SP-C, most probably due to the post-translational modifications, was observed. Our findings are in agreement of those reported by other authors for the presence of different forms of pro-SP-C [25].

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Fig. 5. Western blot assay of pro-SP-C: 1 - 4108; 2 - 4101; 3 - 4899; 4 - 2180; 5 - 5241; 6 - 5243; 7 - 4099; 8 - standard SP-A; 9, 10 - protein standards.



In summary, our results showed the expression of many different immature forms of three of the specific surfactant proteins in all gastric aspirates from prematurely born children after corticosteroid therapy. The presence of SPs is a marker for lung maturity in infants involved in this research. Therefore, this study confirmed the positive effects of corticosteroids administration in pregnant women of a high risk of preterm delivery and NRDS triggering to save the children and to support their normal development after birth. Moreover, we proved that gastric aspirates are a reliable clinical sample for assessment of lung maturity by detection of the main specific surfactant proteins.

CONCLUSIONS

Early diagnostics of lung maturity in risk newborns is crucial for the prompt postnatal therapy and for a chance to improve the quality of their life. Until now lung maturity is determined by invasive and traumatic sample collection of amniotic fluid from mothers and tracheal aspirates from the newborns. Gastric aspirates collection is fast, simple, non-invasive procedure, realized in the first minutes after the delivery. Our results proved that GAs can be used as an adequate and reliable approach for assessment of surfactant maturity at preterm birth. In prematurely born children the antenatal corticosteroid therapy benefits the surfactant production and normal lung development. The electrophoretic and immunoblot analyses from premature babies showed the presence of the specific surfactant proteins tested in all gastric aspirates confirming the positive effects of CST.

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