Clara Cell Protein Expression in Human Neonates During Respiratory Distress Syndrome

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Key Words

Neonatology • Respiratory Distress Syndrome • Bronchoalveolar aspirate • Clara Cell • Clara cell protein • Human • Two-dimensional gel electrophoresis

Abstract

Background/Aims: Clara cell protein (cc-10) has been shown to negatively regulate inflammation, protect pulmonary surfactant from degradation in the lung, and administration of this recombinant protein improves the condition of infant respiratory distress syndrome (iRDS), a disease that occurred mainly in preterm infants. In view of the possibility that altered expression of cc-10 might regulate its protective function, we attempted to characterize this protein in infants with iRDS. **Methods:** Using bronchotracheal aspirates from human infants, we analyzed cc-10 in two-dimensional gel electrophoresis (2-DE) by combining immunoprecipitation, carbonyl groups and total protein immunoblotting. **Results:** Seven forms of cc-10 were detected with western immunoblots in infants with iRDS while only four forms were present in neonates who needed mechanical ventilation for other reasons without any lung disease (control group). The overall levels of cc-10 in iRDS were lower

and differences were seen in isoform pattern and distribution. **Conclusion:** Our demonstration that cc-10 is differentially expressed in infants with iRDS may point the way towards one possible mechanism that potentially involves modifications of the protein structure with its anti-inflammatory and surfactant protective function and could be detrimental for this airway disorder.

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Introduction

The search for biomarkers of lung diseases in pulmonary surfactant is a current area of investigation that will contribute to the understanding of pathogenesis and molecular mechanisms. In particular, deficiency of pulmonary surfactant is involved in the pathology of infant respiratory distress syndrome (iRDS), a multifactorial disease occurring mainly in infants born before term presenting microatelectasia, alveolar damage, and is the main cause of respiratory failure and death in these infants [1-4]. Alveolar liquid is composed by phospholipids (90%) and by many proteins, one of them is clara cell-specific

10-kDa protein, also termed uteroglobin, secretoglobin or cc-10, secreted by Clara cells from the respiratory tract [5, 6]. Cc-10 consists of two identical subunits covalently linked by two disulfure bridges, giving rise to a 91 amino acid protein with a molecular weight of ~ 10-12 kDa under non-reducing conditions [5, 7, 8]. The biological function of cc-10 remains incompletely understood, although it has been shown that cc-10 binds to lipid components of the pulmonary surfactant, such as phosphatidilcoline and phosphatidilinositol, suggesting that it may transport or protect these phospholipids from degradation [7]. Furthermore, cc-10 negatively regulates the inflammatory response in the airway by inhibiting phospholipase A₂ activity, enzyme responsible for the release of the precursor of prostaglandin and leukotriene biosynthesis [9], and is also in charge for surfactant phospholipids catabolism. Indeed, it has been demonstrated that cc-10 reduces lung inflammation in premature infants with respiratory distress syndrome [10] and has been found oxidized in premature infants developing Bronchopulmonary Dysplasia, suggesting that cc-10 structure and function are critical for normal bronchoalveolar fluid homeostasis [11]. Recently, cc-10 has been proposed as a lung epithelial marker for acute lung injury [12], and alteration of cc-10 isoform expression was demonstrated in human bronchoalveolar lavage fluid of smoking and non-smoking adults and children with asthma and rhinitis [13]. Taking into account these evidences, the aim of this work was then to identify the cc-10 forms present in bronchotraqueal aspirate samples from human iRDS; the description of cc-10 from these infants may be of great importance because changes in its structure could be involved in the mechanism of action of this protein.

Materials and Methods

Study subjects

The institutional review board at the Instituto Nacional de Perinatología IER approved this study. Ten premature infants (28-35 wk gestation) diagnosed with iRDS according to the classical criteria (prematurity, respiratory distress, requirement of oxygen more than 21% and ventilator support) and without having congenital anomalies or congenital sepsis, were enrolled in this study. Control subjects who needed ventilation for other reasons than any lung disease were five infants (36-39 wk gestation) diagnosed at birth with gastroschisis (a congenital anomaly characterized by a defect in the anterior abdominal wall through which the intestinal contents freely protrude). All these infants were routinely intubated, ventilated and only infants with RDS were treated with exogenous surfactant after

iRDS diagnosis. Neonatal and maternal birth histories were obtained at enrollment and assessed for presence or absence of preeclampsia, chorioamnionitis or gestational diabetes, multiple gestation, preterm labor, exposure to prenatal steroids, gestational age at delivery, birth weight and gender.

Study design

The main study objective was to characterize cc-10 from iRDS in comparison with samples from infants without RDS and having a healthy lung (infants diagnosed with gastroschisis). For this purpose, proteins from bronchotraqueal aspirate samples (collected before exogenous surfactant treatment of iRDS) of eligible infants were analyzed for cc-10 expression by combining immunoprecipitation, two-dimensional gel electrophoresis, carbonyl groups and total protein immunoblotting.

Collection of Bronchotraqueal Aspirate samples

Bronchotraqueal aspirate samples were collected only when traqueal suction was clinically indicated, before exogenous surfactant administration in the case of iRDS and in the first 30 min of intubation. Collection of bronchotraqueal aspirates were done according to previous recommendations [14, 15]. Briefly, after corroborating, by radiographic and clinical method, that the orotraqueal canula was adequately ubicated and under cardiorespiratory monitorization, bronchotraqueal aspirates were obtained under a closed circuit of *ad hoc* aspiration and with a tramp for sterile secretions, using 0.8 ml of normal sterile saline previously warmed to body temperature (37°C). Aliquots were kept at 4°C. Blood tinged aspirates were not used. Samples were immediately centrifuged at 500 x g for 10 min, and aliquots of the cell-free supernatant were stored at -70°C until analysis. Total protein concentration in bronchotraqueal aspirate samples was determined with Sigma Protein Assay (St Louis, MO, USA) according to Bradford [16] and ranged between 0.3–2.5 mg/ml.

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

30-µg total protein from bronchotraqueal aspirate sample was mixed with an equal volume of 2X reducing Laemmli buffer, boiled for 5 min at 100°C and analyzed in 15 % SDS-PAGE at 25 mV in the Mini-Protean III Biorad unit. Proteins were detected by silver staining according to manufacturer's instructions (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The protein patterns in the gels were analyzed as digitalized images and the amount of protein in a band was assessed as background-corrected optical density (OD) expressed as percentage of the cc-10 band per total OD of the sample (% OD).

Immunoprecipitation

50-µg total protein from bronchotraqueal aspirate sample was subjected to immunoprecipitation with rabbit anti-cc-10 antibody H-75 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) coupled to protein A-Sepharose beads (GE Healthcare) overnight at 4°C. After several washings, either reducing 2X Laemmli buffer or 2-D rehydration buffer (8 M urea, 2% CHAPS, 2% Pharmalyte (v/v) 3-10, 0.002% bromophenol blue, DTT is

	Infant Respiratory Distress Syndrome (n=10)	Control group (Gastroschisis) (n=5)
Gestational Age ¹	29.6 ± 2.6	38.2 ± 1.7
Birth Weight, g ¹	1418 ± 616	2760 ± 796
Apgar score at 5 minutes	>7	>7
Hours of life at which the BAL had been collected	First 2 hours	First 8 hours
Number of babies who developed broncho-pulmonary dysplasia	4	0

Table 1. Study Population. ¹Values are means ± SD

added just prior to use: 7 mg DTT per 2.5-ml aliquot of rehydration stock solution, giving a nonlinear pH gradient from 3-10, GE Healthcare) was applied to beads.

Two-dimensional gel electrophoresis (2-DE)

50-µg total protein from bronchotraqueal aspirate sample was first treated with the 2-D Clean-Up Kit (GE Healthcare) to remove contaminants and salts and precipitated proteins were resuspended in 2-D rehydration buffer sample solution. Rehydrated sample was applied to 13 cm- Immobiline nonlinear pH 3-10 Isoelectric Focusing (IEF) strips (GE Healthcare) overnight at room temperature. First-dimension gels were focused to 70,000-V hours on Ettan IPGphor 3 IEF Unit (GE Healthcare) for about 6 h. After IEF, the strips were kept at –80°C or equilibrated twice for 15 min, under gentle shaking first in equilibration buffer (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue from GE Healthcare) containing 20 mM DTT added prior to use and next, in equilibration buffer containing 244 mM iodoacetamide added prior to use (GE Healthcare). Second-dimension 15 % reducing SDS-PAGE gels were run at 14 mA in the Ruby 600 Unit (GE Healthcare) for 4 h. Separated proteins were either detected by deep purple staining according to manufacturer's instructions (GE Healthcare), acquired directly in Typhoon 9410 equipment (GE Healthcare) and visualized with Image Master Platinum 2D software (GE Healthcare) or by Western Blot with anti-cc10 antibody. Molecular mass was determined by using M_r standards in each run (Mark12; 200-2.5, GE Healthcare) and Isoelectric points of the separated proteins were estimated by running pI standards (2D standards; GE Healthcare).

Western Blot

After equilibration of SDS-PAGE gels in transfer buffer, proteins were transferred to nitrocellulose membranes (GE Healthcare) overnight at 4°C. The membrane was blocked with 5 % (w/v) skim milk - 0.05 % tween in Tris Buffer (TBST) for 30 min at room temperature and then incubated for 1 h at room temperature with rabbit anti-cc10 antibody H-75 (Santa Cruz) or rabbit anti-human cc-10 antibody (Biovendor, Brno, Czech Republic). After several washes with TBST, spots or bands were detected by horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA, USA) and visualized by enhanced chemiluminescence (ECL-kit from Pierce, Rockford, IL, USA) on ECL Hyperfilm (GE Healthcare) or acquired directly in Typhoon 9410 equipment.

Derivatization and detection of carbonyl groups by immunoblotting

The method used was a modification of the method described by Conrad et al. [17]. Following the western blot procedure, the nitrocellulose membrane was dried at room temperature and then equilibrated in 20% (v/v) ethanol-80% (v/v) TBS for 5 minutes. The membrane was incubated in 2N HCl for 5 minutes and incubated in a solution of 2,4-dinitrophenylhydrazine (0.5mM) in 2N HCl for exactly 5 minutes each. The membrane was washed in 2N HCl and then in 50% ethanol. The manufacturer's protocol used for protein oxidation identification was the Oxiblot protein oxidation detection kit (Millipore, Billerica, MA, USA). Briefly, the membrane was blocked with 5 % (w/v) bovine serum albumin (Sigma, St. Louis, MO, USA) - TBST for 1 hour at room temperature then incubated with rabbit anti-2-4-dinitrophenyl antibody, washed three times with TBST and incubated with a goat horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG antibody for 1 hour at room temperature. After several washes with TBST, spots were detected by ECL acquired directly in Typhoon 9410 equipment.

Analysis

The pI and Mr of cc-10 spots from bronchotraqueal aspirate samples were compared to the pI predicted by amino acid sequence, published literature and control group samples. The amount of protein in a band/spot was obtained as background-corrected optical density, integrated over all pixels in the band/spot and expressed as arbitrary units (AU) from Image Master Platinum 2D software, by directly acquiring the ECL signal in Typhoon 9410 equipment. The distribution of different forms of cc-10 was calculated by dividing the AU of one form with the Σ AU of all the forms of the protein (% AU).

Results

Identification of cc-10 protein in human iRDS and in individuals with healthy lung

In order to explore if the observed cc-10 alterations were specific to iRDS, and given that it is not ethically possible to obtain bronchotraqueal aspirates from healthy neonates as controls, we decided to use as a comparison for a healthy lung sample, bronchotraqueal aspirates from neonates who needed ventilation for other reasons than

any lung disease (control group). Ten infants were diagnosed with iRDS and five infants with gastroschisis at birth, a congenital anomaly characterized by a defect in the anterior abdominal wall but with a mature and healthy lung (control group). The groups were assessed for differences in gestational age, birth weight, receipt of prenatal steroids, Apgar score at 5 minutes, the hours of life at which the BAL had been collected and the number of babies who eventually developed broncho-pulmonary dysplasia (Table 1). These data showed that control samples came from term infants.

The protein pattern of bronchotraqueal aspirate samples from three different infants diagnosed with iRDS (Fig. 1A, lanes *iDR1*, *iDR2* and *iDR3*) and one gastroschisis patient (Fig. 1A, lane *G*), was analyzed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). On silver-stained gels, a prominent band of an electrophoretic mobility under reducing conditions consistent with a molecular mass of ~ 5 kDa was present in all samples. Although the level of expression varied between subjects, the percentage of this band per total optical density of silver-stained proteins ranged between 5 - 10 % (± 0.5 %). The molecular weight of this band is similar to what is commonly observed for the homodimer of natural and recombinant cc-10 protein, which migrates as a diffuse ~ 5-6 kDa band [7, 18, 19]. This band was also present in silver-stained gels of rabbit, pig and bovine bronchotraqueal aspirates (*data not shown*), which present a high sequence homology and similar biochemical properties with human cc-10 [20, 21]. Moreover, this band disappeared in the SDS-PAGE when the bronchotraqueal aspirate sample from iRDS was previously incubated with a saturating concentration of the antibody against cc-10, further supporting this band to be cc-10 (Fig. 1A, lane *iSDR3* + *Ab CC10*).

Next, bronchotraqueal aspirate proteins separated by SDS-PAGE were transferred to nitrocellulose membranes, analyzed by western blot using specific antibodies against cc-10 and revealed by Enhanced Chemiluminescence (ECL) (Fig. 1B). Only the ~ 5 kDa band was immunoreactive with the antibody to cc-10 from both the gastroschisis (Fig. 1B, lane *G*) and iRDS samples (Fig. 1B, lane *iSDR*), confirming the identity of this protein band to be clara cell protein. Furthermore, the antibody to cc-10 did not recognize any band from a sample of mononuclear cells from peripheral human blood that do not express cc-10 (Fig. 1B, lane *MC*) and thus confirmed the specificity of the antibody. To further validate the identification of cc-10 from iRDS samples, a cc-10 immunoprecipitation was effectuated from

Fig. 1. Expression of cc-10 in human Infant Respiratory Distress Syndrome (iRDS) and gastroschisis. (A) Silver stained – SDS-PAGE or (B) western blot analysis of anti-cc-10 antibody (IB: cc-10) from total proteins in human from iRDS and gastroschisis (G). Total proteins from three distinct patients were analyzed in lanes 1, 2 and 3. Lane 4 depicts total protein from bronchotraqueal aspirate sample from patient 3 previously incubated with a saturating concentration of cc-10 antibody (*iSDR3* + *Ab cc10*). Molecular weight standards are indicated to the left in each figure. In B, the immunoreactive bands from iRDS, G and mononuclear cells from peripheral human blood (MC) were detected with a goat anti-rabbit IgG antibody coupled to horseradish peroxidase and ECL was directly visualized in Typhoon 9410 image station, which gives true linear signals throughout the exposure range. IP: cc-10 depicts cc-10 immunoprecipitation from iRDS sample.

bronchotraqueal aspirates and analyzed by SDS-PAGE and western blot with an anti-cc10 antibody, depicting the band of ~ 5 kDa (Fig. 1B, lane *IP: cc-10*). These results demonstrate the presence of cc-10 in bronchotraqueal aspirate samples from infants with iRDS and gastroschisis (control group), with a molecular weight similar to the predicted by aminoacid sequence and published in the literature. Moreover, cc-10 expression

was found diminished in bronchotraqueal aspirate samples from iRDS in comparison with gastroschisis patients however; the level of expression of cc-10 from gastroschisis patients varied between subjects (10 - 30 % ($\pm$ 13 %, n = 5).

Isoform expression of cc-10 in human iRDS

To further investigate possible modifications in the expression of cc-10 in iRDS, bronchotraqueal aspirate samples were analyzed by two dimensional gel electrophoresis (2-DE), a widely used methodology to analyze protein patterns in various biological materials [22]. Isoelectric focusing from iRDS samples was performed on a wide non-linear pH gradient to separate proteins with isoelectric points (pI) from 3 to 10, and a wide acrylamide concentration (15 %) was utilized to separate proteins with molecular masses (Mr) from 2 to 250 kDa. Oxidation of the sample was avoided by adding a reducing agent (*e. g* dithiothreitol) to the rehydration solution [23]. On these gels, an average of $\sim$ 130 deep-purple stained protein spots were detected in 50 μ g of total protein (Fig. 2). Several spots of the same molecular weight of cc-10 (5.5 kDa) focused at different pI from 3.9 to 6.6 and only one protein spot exhibited an electrophoretic mobility in the second dimension of 5.1 kDa (Fig. 2, see *ellipse with spots I-VII*). The percentage of all cc-10 isoforms per total OD of deep purple-stained proteins was 6.8 % ($\pm$ 0.5 %, n = 10), in accordance with the results from Fig. 1.

Oxidation of cc-10 isoform in human iRDS

There are several modifications that may induce changes in the charge and mass shift of a protein and could explain distinct cc-10 isoforms. Protein oxidation may result in the appearance of more acidic satellite spots towards pH 3 observed after separation by 2-DE. Furthermore, several studies have shown oxidation of proteins in traqueal aspirates from premature infants [24, 25] and in particular, cc-10 was found oxidized in infants who develop Bronchopulmonary Dysplasia [11]. Therefore, we analyzed the possibility that the acidic forms of cc-10 could be oxidized. Protein carbonyl (CO) content is actually the most general indicator and by far the most commonly used marker of protein oxidation [26, 27] because of the early formation and the relative stability of carbonylated proteins. CO groups were detected by derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which leads to formation of a stable dinitrophenyl (DNP) hydrazone product [25], detected in western blot after 2-DE of cc-10

Fig. 2. Localization of the seven forms of cc-10 (I-VII) in human protein patterns from Infant Respiratory Distress Syndrome. Deep purple stained – non-linear two dimensional gel electrophoresis (pH 3-10) of proteins in human bronchotraqueal aspirates from iRDS. The figure is a gel acquired directly in Typhoon 9410 image station, presented as an unmanipulated image in Image Master Platinum 2D software, except for the overall contrast and light, using programs from graphic processing (Adobe Systems, San Jose, CA, USA, Photoshop CS4). The localization of the seven forms of cc-10 (I-VII) is indicated by the elliptic figure. Molecular weight standards are indicated to the right and pH values in the upper part of the figure.

Fig. 3. Post-Electrophoretic identification of oxidized cc-10 forms from Infant Respiratory Distress Syndrome. Western blot analysis of anti-dinitrophenil (DNP) antibody (IB: DNP) on a cc-10 immunoprecipitation (IP: cc-10) priorly derivitized with DNPH (which reacts with aldehydes, ketones, and certain other products of protein oxidation) from human bronchotraqueal aspirates of iRDS. pH values from 3 to 7 are indicated in the upper part of the figure. The cc-10 oxidized form II is indicated by the arrow.

Fig. 4. Expression of cc-10 forms from human Infant Respiratory Distress Syndrome and Gastroschisis. Western blot analysis of anti-cc-10 protein antibody (IB: cc-10) tested against human bronchotracheal aspirates of iRDS (left panel) or gastroschisis (right panel). Cc-10 isoforms with molecular masses from 5.1 to 5.5 kDa and with pI ranging from 4.0 to 6.6 are depicted as I to VII. The molecular weight corresponding to 6 kDa is indicated in the left and the pH values from 3 to 8 are indicated in the upper part of the figure.

Spot No.	pI , M_r (kDa)	iRDS ²	Control group (Gastroschisis) ²
I	4.0, 5.5	+	+
II	4.2, 5.1	+	+
III	4.4, 5.5	+	+
IV	4.5, 5.1	+	-
V	5.0, 5.5	+	-
VI	6.4, 5.1	+	-
VII	6.6, 5.5	+	-

Table 2. Distribution of cc-10 isoforms in bronchotracheal aspirate samples from iRDS and control group

immunoprecipitates. A single spot, corresponding to cc-10 form II in 2-DE (pI 4.2, M_r 5.1), contained DNPH-reactive products of oxidation from iRDS (Fig. 3).

Cc-10 patterns in human iRDS and in individuals with healthy lung

Bronchotracheal aspirates from iRDS and control (Gastroschisis) samples were resolved by two-dimensional gel electrophoresis (2-DE), immunoblotted with cc-10 antibody and revealed by ECL (Fig. 4). Significant differences were found in the number and distribution of cc-10 forms showing seven cc-10 isoforms from iRDS samples and only three forms in the control group (Fig. 4 and Table 2). Three isoforms of M_r 5.1 kDa (II, IV and VI) and four isoforms of 5.5 kDa (I, III, V and VII)

were observed in iRDS, while one isoform of 5.1 kDa (II) and two isoforms of 5.5 kDa (I and III) were present in the control group. These results were confirmed with another anti-cc-10 antibody against recombinant human cc-16 protein (data not shown).

Discussion

Clara cell protein has not been analyzed by 2-DE in bronchotracheal aspirates from human newborn infants before and in this study, distinct forms were found in 2-DE patterns from iRDS and control subjects. The pI and M_r of the different forms of cc-10 matches in general with the predicted by amino acid sequence (UniProtKB/Swiss-Prot P11684 UTER_HUMAN) and reported in literature [5, 13, 19]. However, cc-10 in human adult or children bronchoalveolar lavage fluid was previously identified as two protein spots of pI from 4.8-5.2 and M_r from ~ 7.9 [28] or four protein spots of pI from 4.6-5.2 and M_r of 6 kDa [13]. The divergent expression of cc-10 from our study may be characteristic to the fact that bronchoalveolar aspirates come from human newborns and not children or adults. However, there are other possibilities that may explain these differences including co- and post-translational modifications such as glycosylation, phosphorylation, acetylation, and proteolytic cleavage. Further experiments should be conducted to address this question.

Nevertheless, oxidation of the sample could explain the acidic forms of cc-10 and interestingly, our study showed that the only form found oxidized in infants with iRDS is isoform II. Furthermore, cc-10 protein was found oxidized in tracheal samples from human neonates with Bronchopulmonary dysplasia [11], and our result further characterized that from cc-10 protein it is only one isoform that is found oxidized. Cc-10 has been shown to bind to hydrophobic ligands such as progesterone and prostaglandins [29, 30] and even if its ability to bind to its ligands is not abolished in rabbit oxidized cc-10, it seems to be more kinetically hindered [31]. In particular, these findings and our results suggest that oxidation of cc-10 from human iRDS may affect the binding of cc-10 to its ligands, weakening its suppressive production as well as its scavenging function of inflammatory mediators. Indeed, oxidations of proteins have been observed in tracheal aspirate fluids of prematurely delivered infants and have been associated with adverse pulmonary outcomes [24, 25]. Oxidation of cc-10 may be a common fact in lungs from premature infants and could indicate an oxidant–

antioxidant imbalance occurring in iRDS. This description of cc-10 isoform oxidation in iRDS may point the way to new therapeutic treatments and to development surfactant products that could be administrated in the lungs of those infants to protect them from this oxidant–antioxidant imbalance.

Cc-10 expression was found diminished in iRDS samples in comparison with control subjects, and even though these findings are based on only few individuals and do not allow definite conclusions, the results are of substantial interest in sight of the proposed function of cc-10 as an anti-inflammatory protein in the lung [9, 10]. Clara cell protein has been shown to produce its anti-inflammatory action through the inhibition of phospholipase A₂ enzyme activity, by sparing surfactant phospholipids catabolism and preventing chronic lung disease, such as broncho-pulmonary dysplasia [11]. Interestingly, phospholipase A₂ seems to have the same effect both in the neonatal period and beyond this period as suggested by correlating levels of this enzyme with clinical severity, lung stiffness and need for respiratory support in neonates with iRDS [32] as well as with clinical severity, higher oxygen requirement and more aggressive ventilation in pediatric post-neonatal respiratory distress [33]. The fact that lower levels of cc-10 and a distinct pattern are present in this study may be of significance to the inflammatory process in iRDS since a decreased capacity to reduce lung inflammation will probably predispose these infants to further lung injury. In agreement with our results, other reports have shown lower levels of cc-10 in umbilical cord blood and tracheal aspirate fluid samples from infants developing Bronchopulmonary dysplasia [11, 34, 35]. In addition to the possible augmented inflammation in iRDS samples from a decreased level of cc-10 expression and altered expression, it is also possible that diminished cc-10 levels may also indicate changes in surfactant catabolism. Furthermore, cc-10 production is influenced by gestational age, the available surfactant pool, steroids and especially the degree of prenatal lung inflammation. Cc-10 structure and function are critical for normal bronchoalveolar fluid homeostasis [11] and the divergent cc-10 forms found in infants diagnosed with RDS from this work are probably detrimental to the lung if these modifications alter its function. Indeed, cc-10 role in reducing inflammation through inhibiting phospholipase A₂ and phospholipid catabolism may probably be severely impaired in iRDS, thus contributing to the development of a future chronic lung disease.

However, this study had some limitations. First, the overall number of subjects was modest. Second, it is not possible to determine if altered expression of cc-10 is the cause or consequence of NRDS. Nonetheless, our demonstration that cc-10 protein is diminished and differentially expressed in neonates with RDS may point the way towards one possible mechanism that potentially involves modifications of the protein structure with its anti-inflammatory and surfactant protective functions; and could be disadvantageous to develop other lung diseases.

Conclusion

The identification of distinct cc-10 forms should be the basis for further research aimed at identifying the molecular differences between these isoforms and their potential physiological implications in the process of human iRDS. Altogether, these results should guide further investigations on neonatal respiratory distress pathophysiology by unraveling the composition and function of surfactant components.

Abbreviations

iRDS (Infant Respiratory Distress Syndrome); Clara cell protein (cc-10); SDS-PAGE (Dodecyl-polyacrylamide gel electrophoresis); 2-DE (Two-dimensional electrophoresis); pI (isoelectric point); M_r (molecular weight); OD (optical density); AU (arbitrary units); CO (Protein carbonyl).

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References

- Avery M-E, Mead J: Surface properties in relation to atelectasis and hyaline membrane disease: *AMA J Dis Child* 1959;97:517-523.
- Clements J-A, Avery M-E: Lung surfactant and neonatal respiratory distress syndrome. *Am J Respir Crit Care Med* 1998;157:S59-66.
- Phua J, Badia J-R, Ferguson N-D: Acute respiratory distress syndrome and the Art of War. *Crit Care Med* 2009;37:1798-1799.
- Gunther A, Ruppert C, Schmidt R, Markart P, Grimminger F, Walrmath D, Seeger W: Surfactant alteration and replacement in acute respiratory distress syndrome. *Respir Res* 2001;2:353-364.
- Singh G, Singh J, Katyal S-L, Brown W-L, Kramps J-A, Paradis I-L, Dauber J-H, Macpherson T-A, Squeglia N: Identification, cellular localization, isolation, and characterization of human Clara cell-specific 10 KD protein. *J Histochem Cytochem* 1988;36:73-80.
- Mukherjee A-N, Zhang Z, Chilton B-S: Uteroglobin: a steroid-inducible immunomodulatory protein that founded the Secretoglobulin superfamily. *Endocr Rev* 2007;28:707-725.
- Singh G, Katyal S-L: Clara cells and Clara cell 10 kD protein (CC10): *Am J Respir Cell Mol Biol* 1997;17:141-143.
- Umland T-C, Swaminathan S, Singh G, Warty V, Furey W, Pletcher J, Sax M: Structure of a human Clara cell phospholipid-binding protein-ligand complex at 1.9 Å resolution. *Nat Struct Biol* 1994;1:538-545.
- Levin S-W, Butler J-D, Schumacher U-K, Wightman P-D, Mukherjee A-B: Uteroglobin inhibits phospholipase A₂ activity. *Life Sci* 1986;38:1813-1819.
- Levine C-R, Gewolb I-H, Allen K, Welch R-W, Melby J-M, Pollack S, Shaffer T, Pilon A-L, Jonathan Davis M: The safety, pharmacokinetics, and anti-inflammatory effects of intratracheal recombinant human Clara cell protein in premature infants with respiratory distress syndrome. *Pediatr Res* 2005;58:15-21.
- Ramsay P-L, DeMayo F-J, Hegemier S-E, Wearden M-E, Smith C-V, Welty S-E: Clara cell secretory protein oxidation and expression in premature infants who develop bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 2001;164:155-161.
- Kropfski J-A, Fremont R-D, Calfee C-S, Ware L-B: Clara cell protein (CC16), a marker of lung epithelial injury, is decreased in plasma and pulmonary edema fluid from patients with acute lung injury. *Chest* 2009;135:1440-1447.
- Lindahl M, Svartz J, Tagesson C: Demonstration of different forms of the anti-inflammatory proteins lipocortin-1 and Clara cell protein-16 in human nasal and bronchoalveolar lavage fluids. *Electrophoresis* 1999;20:881-890.
- de Blic J, Midulla F, Barbato A, Clement A, Dab I, Eber E, Green C, Grigg J, Kotecha S, Kurland G, Pohunek P, Ratjen F, Rossi G: Bronchoalveolar lavage in children. *Eur Respir J* 2000;15:217-231.
- Midulla F, de Blic F, Barbato A, Bushz A, Eber E, Kotecha S, Haxby E, Moretti C, Pohunek P, Ratjen F: Flexible endoscopy of paediatric airways. *Eur Respir J* 2003;22:698-708.
- Bradford M-M: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-254.
- Conrad C-C, Talent J-M, Malakowsky C-A, Gracy R-W: Post-Electrophoretic Identification of Oxidized Proteins. *Biol Proced Online* 2000;2:39-45.
- Umland T-C, Swaminathan S, Furey W, Singh G, Pletcher J, Sax M: Refined structure of rat Clara cell 17 kDa protein at 3.0 Å resolution. *J Mol Biol* 1992;224:441-448.
- Bernard A, Dumont X, Roels H, Lauwerys R, Dierynck I, De Ley M, Stroobant V, de Hoffmann E: The molecular mass and concentrations of protein 1 or Clara cell protein in biological fluids: a reappraisal. *Clin Chim Acta* 1993;223:189-191.
- Mantile G, Miele L, Cordella-Miele E, Singh G, Katyal SL, Mukherjee A-B: Human Clara cell 10-kDa protein is the counterpart of rabbit uteroglobin. *J Biol Chem* 1993;268:20343-20351.
- Singh G, Katyal S-L, Brown WE, Kennedy A-M, Singh U, Wong-Chong M-L: Clara cell 10 kDa protein (CC10): comparison of structure and function to uteroglobin. *Biochim Biophys Acta* 1990;1039:348-355.
- Pierce J-D, Fakhari M, Works K-V, Pierce J-T, Clancy R-L: Understanding proteomics. *Nurs Health Sci* 2007;9:54-60.
- Altland K, Becher P, Rossmann U, Bjellqvist B: Isoelectric focusing of basic proteins: the problem of oxidation of cysteines. *Electrophoresis* 1988;9:474-485.
- Gladstone Jr I-M, Levine R-L: Oxidation of proteins in neonatal lungs. *Pediatric* 1994;93:764-8.
- Varsila E, Pesonen E, Andersson S: Early protein oxidation in the neonatal lung is related to development of chronic lung disease. *Acta Paediatr* 1995;84:1296-1299.
- Dalle-Donne I, Rossi R, Giustarini D, Milzani Roberto A, Colombo: Protein carbonyl groups as biomarkers of oxidative stress. *Clin Chim Acta* 2003;329:23-38.
- Levine R-L: Carbonyl modified proteins in cellular regulation, aging, and disease. *Free Radic Biol Med* 2002;32:790-796.
- Wattiez R, Hermans C, Cruyt C, Bernard A, Falmagne P: Human bronchoalveolar lavage fluid protein two-dimensional database: study of interstitial lung diseases. *Electrophoresis* 2000;21:2703-2712.
- Mandal A-K, Ray R, Zhang Z, Chowdhury B, Pattabiraman N, Mukherjee A-B: Uteroglobin inhibits prostaglandin F₂alpha receptor-mediated expression of genes critical for the production of pro-inflammatory lipid mediators. *J Biol Chem* 2005;280:32897-32904.
- Mandal A-K, Zhang Z, Ray R, Choi MS, Chowdhury B, Pattabiraman P, Anil B, Mukherjee A-B: Uteroglobin represses allergen-induced inflammatory response by blocking PGD₂ receptor-mediated functions. *J Exp Med* 2004;199:1317-1330.
- Winkelmann R, Geschwindner S, Haun M, Ruterjans H: Solution structure of the recombinant oxidized rabbit uteroglobin using homonuclear and heteronuclear multidimensional NMR. *Eur J Biochem* 1998;258:521-532.
- De Luca D, Minucci A, Cogo P, Capoluongo E, Conti G, Pietrini D, Carnielli Vand Piastra M: Secretory phospholipase A₂ pathway during pediatric acute respiratory distress syndrome: a preliminary study. *Pediatr Crit Care Med* 2011;12:e20-e24.
- De Luca D, Baroni S, Vento G, Piastra M, Pietrini D, Romitelli F, Capoluongo E, Romagnoli C, Conti G, Zecca E: Secretory phospholipase A₂ and neonatal respiratory distress: Pilot study on broncho-alveolar lavage. *Intensive Care Med* 2008;34:1858-1864.
- Sarafidis K, Stathopoulou T, Diamanti E, Soubasi V, Agakidis C, Balaska A, Drossou V: Clara cell secretory protein (CC16) as a peripheral blood biomarker of lung injury in ventilated neonates. *Eur J Pediatr* 2008;167:1297-1303.
- Schrama A-J, Bernard A, Poorthuis B-J, Zwinderman A-H, Berger H-M, Walther F-J: Cord blood Clara cell protein CC16 predicts the development of bronchopulmonary dysplasia. *Eur J Pediatr* 2008;167:1305-1312.