LUNG GROWTH FACTORS IN THE AMNIOTIC FLUID OF NORMAL PREGNANCIES AND WITH CONGENITAL DIAPHRAGMATIC HERNIA

Running head: Prenatal lung growth factors in CDH

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ABSTRACT

Aim: Respiratory failure secondary to pulmonary hypoplasia is the main cause of death in congenital diaphragmatic hernia (CDH). Lung growth is regulated by growth factors (GFs), whose imbalances is reported in pathological conditions. We measured amniotic fluid levels of GFs, regulating lung development, in pregnancies with CDH and compared them with normal gestations.

Methods: Amniotic fluid was collected at amniocentesis and delivery from 4 women carrying fetuses with CDH and 12 with normal pregnancy. GFs were isolated and quantified. Same GFs were measured in lung biopsies collected during autopsy of 3 newborns dead of CDH.

Results: Impairment expression of lung GFs in the amniotic fluid of CDH pregnancies in comparison with normal was found. FGF10, FGF7, VEGF and TGFβ were decreased at amniocentesis, while PDGF increased. While FGF10 and PDGF tended to normalize at delivery, EGF increased and TGFβ was still decreased. Same GFs were similarly expressed in both lungs of babies dead of CDH.

Conclusion: Anomalies in lung GFs expression of embryos and fetuses with CDH can be detected by measuring their levels in the amniotic fluid during pregnancy. Further investigation would help to correlate prenatal expression of GFs and clinical outcome of babies with CDH after birth.

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a human malformation that causes high mortality in newborns, because of severe respiratory failure secondary to pulmonary hypoplasia and long-term pulmonary morbidity in survivors. Although widely studied in the experimental model of CDH [1-4], the pathogenesis of lung hypoplasia associated with CDH remains unclear. The studies based on teratogenetic animal models of malformations may be criticized because it is not known whether the anomalies found are to direct effects of the chemical used or to the malformations induced by it. On the other hand, human studies are difficult to realize for ethical and technical reasons. Lung development is regulated by complex tissue interactions mediated by growth factors that promotes cell proliferation and differentiation, such as FGF, EGF, VEGF and PDGF, and others that oppose these effects, such as TGFβ [5]. Their imbalances have been reported in various pathological lung conditions [6]. As growth factors are diffusible proteins, we hypothesized that abnormal expression of growth factors that regulate lung development may be reflected in a different concentration of the same proteins in the amniotic fluid during pregnancy.

The purpose of this study is to gain further insight the role of lung growth factors in the pathogenesis of pulmonary hypoplasia in CDH and to explore the possibility of identifying prenatal predictive values of the clinical outcome of newborns with CDH.

MATERIAL AND METHODS

Study groups: After approval by the Institutional Research Commitee (licence number: 22-11), all women with a spontaneous pregnancy, in which an advanced-level ultrasound (US), performed in our Institution between August 2011 and December 2012, revealed CDH and amniocentesis was indicated, were enrolled in the cases group (A). Twelve women with a spontaneous pregnancy and a routine anatomy US without anomalies who performed amniocentesis, as per advice based on maternal age or as per maternal decision, in our Institution in the same period of time, were randomly enrolled in the controls group (B).

Demographic characteristics, obstetrical variables such as parity, women habits during pregnancy, mode of delivery, obstetrical complications, indication of amniocentesis and gestational age when it was accomplished, fetal karyotype, newborn data such as weight and general conditions at birth were collected.

ELISA analysis in the amniotic fluid: The supernatant of the centrifuged amniotic fluid collected at amniocentesis was snap-frozen and stored at -80°C.

At delivery, 20 ml of amniotic fluid were collected in both groups from the amniotic cavity without contamination with maternal blood. The supernatant was snap-frozen and stored at -80°C. The samples contaminated with blood were discarded.

After assessing the protein content of the amniotic fluid collected during amniocentesis and delivery with the Lowry protein assay (Sigma, Trieste, Italy) and calibrating the samples to enable a balanced comparison between groups, fibroblast growth factor 10 (FGF10), fibroblast growth factor 7 (FGF7), epidermal growth factors (EGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and transforming growth factor β (TGFβ) levels were quantified using ELISA kits (Tema Ricerca ref: CSB-E14965H, RAY-ELH-FGF7-001, RAY-ELH-EGF-001, EH2VEGF, CSB-E04701H, EIA-1864, Bologna, Italy) in both groups of pregnant women. Concentration of amniotic fluid growth factors were expressed in pg/ml.

ELISA analysis in lung biopsies: Biopsies from both lungs were collected during autopsy of newborns who had died of CDH. The lung tissue was homogenized in lysis buffer (1 mmol/l sodium vanate; 1% SDS; 0.01 mol/l Tris-HCl, pH 7.4) with protease inhibitors. The protein content was assessed using the Lowry protein assay (Sigma, Trieste, Italy). FGF10, FGF7, EGF, VEGF, PDGF and TGFβ levels were quantified by ELISA (Tema Ricerca ref: CSB-E14965H, RAY-ELH-FGF7-001, RAY-ELH-EGF-001, EH2VEGF, CSB-E04701H, EIA-1864, Bologna, Italy). All procedures were carried out strictly following the instruction offered with the ELISA kit. Concentration of lung growth factors were expressed in pg/ml.

Statistical methods: The results were expressed as percentages or as means±SD and the groups were compared by Anova tests with a threshold of significance at $p<0.05$. Tukey's range test was used as post-hoc test.

RESULTS

Study groups data: Between August 2011 and December 2012, the advanced-level US confirmed in 4 women the diagnosis of left CDH.

The mean maternal age of the study group (A) was 30.25 years, being two of them 22 and 23 years old, while the others 38 years old. All women were Caucasian. Three of them were nulliparous, two of them had one previous miscarriage. None of them smoked or drank alcohol during pregnancy. None of them had problems of obesity. All of them had unlabored elective cesarean deliveries at term (mean 38 weeks) and the procedure was performed without complications. Gestational age mean at the time of amniocentesis was 18 weeks. The fetal karyotype was 46,XY in all cases without any chromosomal abnormalities. The mean weight of the newborns was 2.8 kg (Table 1). In all newborns the diagnosis of left CDH was confirmed at birth. They were intubated immediately after birth and high-frequency oscillatory ventilation was started.

The mean maternal age of the control group (B) was 37.5 years. All women were Caucasian. Five of them were nulliparous, four of them had previous miscarriages (3 in one woman, 2 in another and 1 in the remaining two). None of them smoked or drank alcohol during pregnancy. None of them had problems of obesity. All of them had unlabored elective cesarean deliveries at term (mean 39 weeks) and the procedure was performed without complications. Advanced maternal age was the most frequent indication for amniocentesis. Gestational age mean at the time of amniocentesis was 17 weeks. The fetal karyotype was 46,XY in 6 cases and 46,XX in the remaining, without chromosomal abnormalities in all of them. The mean weight of the newborns was 3.3 kg (Table 1). All the newborns were healthy at birth and had no respiratory problems.

No significant differences were found among the groups with respect to parity, habits during pregnancy, mode of delivery, obstetrical complications, gestational age at amniocentesis and newborn weight.

ELISA analysis in the amniotic fluid: Growth factors were detectable in most but not all amniotic fluid samples. All samples of amniotic fluid collected at delivery in women with a fetus with CDH had no detectable levels of FGF7 and VEGF. Also the samples of amniotic fluid from delivery of normal pregnancies showed no VEGF (Figure 1 B and E).

When FGF10 was quantified, decreased levels were noted in amniotic fluid of CDH pregnancies at both endpoints, even if the difference was not significant. FGF10 was the most concentrated growth factor both at the time of amniocentesis and delivery in all pregnancies (Figure 1 A).

A similar trend of decrement was seen in FGF7 levels of amniotic fluid from CDH pregnancies at amniocentesis. At the end of the pregnancy, FGF7 was lower in control amniotic fluid and no detectable in CDH pregnancies (Figure 1 B).

The levels of EGF were very low in both groups at the time of second trimester amniocentesis. At delivery, EGF significantly increased in pregnancies with CDH in comparison with controls (Figure 1 C).

PDGF was significantly increased in the second trimester CDH amniotic fluid in comparison with controls, while its levels decreased at the end of the pregnancies and were very similar in CDH and control amniotic fluid (Figure 1 D).

VEGF significantly decreased in the CDH second trimester pregnancies. VEGF was the less concentrated growth factor both at the time of amniocentesis and delivery in all pregnancies (Figure 1 E).

TGFβ levels were significantly decreased in the amniotic fluid of CDH pregnancies both at amniocentesis and at delivery (Figure 1 F).

The levels of the growth factors showed neither any consistency nor any statistically significant association with parity, gestational age at amniocentesis and newborn weight.

ELISA analysis in lung biopsies: Three of the babies born with left CDH died in the first week of life due to the impossibility to achieve the physiological stabilization. Lung biopsies were

collected at autopsy. Growth factors were detectable in all lung samples. Interestingly, no significant differences were found in the expression of lung growth factors in the right and the left lung in all cases. EGF had the lowest concentration in both lungs, while VEGF the highest. FGF10 and FGF7 had concentration lower than 100 pg/ml (Figure 2).

DISCUSSION

Congenital diaphragmatic hernia is a malformation that still causes high mortality in newborns mainly because of severe respiratory failure secondary to pulmonary hypoplasia. The pathogenesis of lung hypoplasia has not been fully understood. It has been suggested that the abnormalities in bronchial innervation described in infants with CDH [1] might contribute to the pulmonary developmental deficiency. The role fulfilled by neural tissue in early gestation seems to be secretion of trophic factors for the smooth muscle that indirectly contributes to lung development. In fact, it has been proposed that the smooth muscle spontaneous contractions produce a rhythmic mechanical stimulus across the airway wall and the adjacent parenchyma that contributes to normal airway differentiation and branching by inducing expression of growth factors [7,8].

Given these observations, we expected that the expression of the growth factors that regulate branching and control airway size and cell fate in the developing lung may be impaired in fetuses and infants with CDH. Lung development is the result of the balanced action of growth factors that promotes cell proliferation and differentiation, such as FGF, EGF, VEGF and PDGF, and others that oppose these effects, such as TGFβ. At the end of development, these signals maintain cellular activities at equilibrium and preserve normal lung structure and function [5,9]. Abnormal expression of these factors and their inappropriate signalling activation cause pathological lung condition. Growth factors are diffusible proteins and we hypothesized that their abnormal expression may be reflected in a different concentration of them in the amniotic fluid during pregnancy. Therefore, we determined the concentration of lung growth factors in the amniotic fluid at amniocentesis and at delivery in pregnancies with a diagnosis of CDH and compared them with normal pregnancies. We considered a panel of growth factors involved in all phases of lung development in an attempt to highlight specific abnormalities in the process of lung formation. We could enroll only four cases in the study group during the study period. Despite this, the two groups were not significantly different with respect to parity, habits during

pregnancy, mode of delivery, obstetrical complications, gestational age at amniocentesis and newborn weight.

Growth factors were not detectable in all amniotic fluid samples. Surprisingly, we could not find VEGF in the amniotic fluid of delivery both in CDH and normal pregnancies. The absence of VEGF in samples of amniotic fluid has been previously reported [10]. VEGF is a strong promoter of angiogenesis and its signalling is responsible for the differentiation of embryonic mesenchymal cells into endothelial cells; the interaction between the epithelium and the mesenchyme contribute to lung neovascularisation that is crucial in normal lung development [9,11,12]. The concentration of VEGF was significantly decreased in the amniotic fluid from amniocentesis performed in women carrying a fetus with CDH. It is well known that the development of vascular bed is defective in CDH [13]. Although VEGF is not only expressed in the lung, its lower levels could account for the decreased number of vessels per unit in CDH lung.

FGF10 is essential for lung branching morphogenesis having a central role in inducing the spatial coordinates for patterning the epithelial tubules [5]. The FGF10 levels were decreased in the amniotic fluid of CDH pregnancies at the time of amniocentesis and tended to normalize at the end of pregnancy. FGF7 promotes epithelial growth and citodifferentiation and it is expressed at highest levels at late stages [5]. FGF7 had decreased levels at amniocentesis and was not detectable at delivery in the amniotic fluid of pregnancies with CDH. Both findings were consistent with the hypothesis that the lung development is delayed in animals and infants with CDH [1]. On the other hand, EGF levels were significantly increased at term in pregnancies with CDH. EGF positively modulates embryonic lung branching morphogenesis and regulates type 2 alveolar cell proliferation [5] and our result could be either the consequence of the delayed in lung development or an attempt to compensate the lower levels of FGF family proteins. A similar intent to counteract the decreased expression of growth factors which promote branching, could be seen in the increased values of EGF and PDGF at the time of delivery. PDGF signalling

212 regulates epithelial DNA synthesis and early branching during embryonic life and it is essential
213 for the ontogeny of pulmonary alveolar myofibroblasts, elastin synthesis and hence
214 alveolarization in postnatal life [5].
215 TGFβ opposes FGF and EGF effects preventing local budding and maintaining proximal airways
216 in an unbranched form by suppressing epithelial cell proliferation and by promoting synthesis of
217 extracellular matrix components around airways. Its levels were significantly decreased in the
218 amniotic fluid of CDH pregnancies at both endpoints. This finding could support the idea that
219 lung development is delayed in CDH and therefore TGFβ concentration is still low when a
220 normal lung development needs it to increase.
221 In conclusion, the expression of lung growth factors is impaired in the amniotic fluid of CDH
222 pregnancies in terms of decreased levels of proteins that regulate the branching or high levels of
223 proteins that modulate early branching when the corresponding control values have already go
224 down. At the same time the levels of growth factors were similar in both lungs of newborns dead
225 of CDH. The fact that the lung ipsilateral and the contralateral to the hernia were equally affected
226 is consistent with the pattern of lung innervation abnormalities found in infants with CDH [1] and
227 further supports the concept of pulmonary hypoplasia as a primary developmental defect rather
228 than the result of lung compression during development.
229 The present study has several weak points that should be acknowledged. In the first place, the
230 reduced number of CDH pregnancies weakens our conclusion and invites further confirmation of
231 our findings in the future. Moreover, the lack of a control group to compare the results of lung
232 biopsies analysis led us to hypothetical speculations about their being different from the control.
233 Third, the management during resuscitation and ventilation of CDH newborns might have
234 affected the expression of lung growth factors. Forth, we were not able to correlate prenatal
235 expression of the growth factors and clinical outcome of the babies after birth because the
236 management was different for each patient. Collection of more specimens is desirable to

investigate if the levels of some of the growth factors could be a predictor of the entity of postnatal lung deficiency in babies with CDH.

Nevertheless, our results demonstrate an impairment in lung growth factors expression of embryos and fetuses with CDH, which can be detected by measuring their levels in the amniotic fluid during pregnancy.

DECLARATION OF INTEREST STATEMENT

The authors report no declarations of interest, nor financial assistance.

LIST OF ABBREVIATIONS

CDH: congenital diaphragmatic hernia

EGF: epidermal growth factors

ELISA: Enzyme-Linked ImmunoSorbent Assay

FGF7: fibroblast growth factor 7

FGF10: fibroblast growth factor 10

GF: growth factors

PDGF: platelet-derived growth factor

VEGF: vascular endothelial growth factor

TGFβ: transforming growth factor β

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Table 1

Control and study groups data.

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Figure 1

Amniotic fluid levels of FGF10 (A), FGF7 (B), EGF (C), PDGF (D), VEGF (E) and TGFb (F) at the time of amniocentesis and delivery in pregnancies with a fetus with CDH and in normal ones (# $p<0.05$ vs control of the same endpoint).

Figure 2

Lung levels of FGF10 (A), FGF7 (B), EGF (C), PDGF (D), VEGF (E) and TGFb (F) in right and left lung of babies dead of CDH (# $p<0.05$ vs control).

Vanessa Candilera performed the experiments and wrote the draft of the manuscript.

Carlo Bouchè collected the amniotic fluid and analyze the data.

Jurgen Schleef analyzed the data.

Federica Pederiva designed the study, analyzed the data and supervised the experiments.