0031-3998/94/3606-0771$03.00/0
PEDIATRIC RESEARCH

Vol. 36, No. 6, 1994
Printed in U.S.A.

Clara Cell Protein in Human Amniotic Fluid: A Potential Marker of Fetal Lung Growth

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ABSTRACT

Clara cell protein (CC16) is a 16-kD protein secreted at the surface of respiratory airways by nonciliated bronchial and bronchiolar cells, including Clara cells. Using the same immunoassay as that recently developed for CC16 in lung lavage, we have measured CC16 in amniotic fluid samples from 100 normal fetuses and 51 fetuses with various pathologies. Ouchterlony immunodiffusion analysis showed a complete identity between CC16 in amniotic fluid and the protein in lung lavages of adults. CC16 was detectable in amniotic fluid from about the 15th wk of pregnancy, then progressively increased until delivery, with a tendency to reach a plateau after the 30th wk. Between the 15th and the 39th wk of pregnancy, the concentration of CC16 in amniotic fluid increased on average 25 times. The sex of the fetus did not influence the concentration of CC16 in amniotic fluid. Compared with expected values, levels of CC16 in amniotic fluid were on average not significantly altered in cases of spina bifida (n = 9), anencephaly (n = 7), and trisomy 21 (n = 6). In contrast, CC16 was on average significantly decreased in cases of diaphragmatic hernia (n = 6), trisomy 18 (n = 14), Turner syndrome (n = 4), and diabetic pregnancy (n = 5). In cases of diaphragmatic hernia, a relation emerged between the concentration of CC16 in amniotic fluid and both the weight of the lungs and the survivorship of the fetuses. The time course of CC16 in amniotic fluid during normal pregnancy and its reduction in pathologies associated with lung hypoplasia suggest that CC16 in amniotic fluid might serve as a marker of bronchial epithelium growth. (***Pediatr Res* 36: 771–775, 1994**)

Abbreviations

CC16, Clara cell protein
RDS, respiratory distress syndrome

CC16 is a lung secretory protein that has been described in rodents and humans (1–5). Initially, CC16 was described as a specific product of the Clara cells, which are nonciliated cells predominantly localized in respiratory and terminal bronchioles (hence the name Clara cell protein). Recent evidence, however, indicates that the protein has a much wider distribution, being synthesized by nonciliated cells in large and small bronchi as well as in bronchioles (6). In addition, the protein is also secreted by the male urogenital tract from puberty on (7). It is identical to protein 1, an α-microprotein isolated from the urine of patients with tubular proteinuria (7), and presents a 61% sequence homology with rabbit uteroglobulin (4, 7, 8). The M_r of human CC16, determined by electrospray ionization/mass spectrometry, is 15 840 (9). The protein is composed of two identical subunits linked together by two disulfide bonds. Although the molecular size of CC16 may vary between animal species—it is 16 908 D in the rat (10)—the abbreviation CC16 will be used hereafter regardless of the species[1].

The exact function of CC16 is yet to be determined. Evidence available to date suggests that it might be an immunosuppressive or antiinflammatory protein protecting the respiratory and urogenital tracts from exaggerated inflammatory reactions (3, 4, 7, 8). This evidence is based on the finding that CC16 inhibits phospholipase A_2 and on the marked sequence similarity with rabbit uteroglobulin, which reportedly has immunosuppressive, antiinflammatory, antiproteinase, and antiphospholipase A_2 activity (4, 8).

Received April 22, 1993; accepted June 23, 1994.
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Supported by the Fonds de la Recherche Scientifique Médicale (Belgium) and the Commission of the European Communities. A.M.B. is Research Director of the National Fund for Scientific Research, Belgium.

[1]Because of anomalous migration properties, CC16 shows by SDS-PAGE an M_r of 10 000. For this reason, CC16 has been misleadingly referred to in the literature as the 10-kD Clara cell protein (CC10).

Using the same immunoassay as that recently applied to adult lung lavage (11), we have studied the time course of CC16 in human amniotic fluid during normal pregnancies and the influence of various fetal pathologies on the secretion of this lung protein in amniotic fluid.

METHODS

Amniotic fluids. The study was carried out on a total of 151 samples of amniotic fluid. With the exception of seven samples obtained at the time of delivery, all samples were collected by amniocentesis for prenatal diagnosis and were obtained from the same medical genetic center. One hundred samples were from pregnancies with a normal karyotype that resulted in the birth of normal healthy babies. They were collected for fetal karyotyping because of advanced maternal age or because of suspected pathology. Fifty-one samples were from abnormal pregnancies including nine cases of spina bifida (mean gestational age, range: 24 wk, 16–37 wk), six cases of diaphragmatic hernia (33 wk, 16–38 wk), 14 cases of trisomy 18 (21.6 wk, 15–36 wk), six cases of trisomy 21 (21.8 wk, 16–32 wk), four cases of Turner syndrome (45,X0) (20 wk, 16–24 wk), seven cases of anencephaly (19.6 wk, 14–22 wk), and five cases from insulin-dependent diabetic women (18.4 wk, 16–25 wk). Gestational age was determined from the first day of the last menstrual cycle and confirmed by ultrasonography. The samples were stored at −20°C for variable periods of time. As shown previously (12), CC16 is stable under these conditions.

Assay of CC16. The concentration of CC16 in amniotic fluid was determined by the same immunoassay as that recently used for the analysis of the protein in bronchoalveolar lavages (11). A detailed description of this immunoassay, which relies on the agglutination of latex particles, in regard to its application to the determination of CC16 (or protein 1) in urine has been published (12). The assay uses the anti–protein 1 antibody from Dakopatts (Glostrup, Denmark) and, as standard, the protein purified in our laboratory (7). CC16 was determined on the supernatant obtained by centrifuging the amniotic fluids at 85–90 × *g* for 10 min. The limit of detection of the assay, defined as the protein concentration agglutinating 10% of the particles, lies around 0.3–0.4 μg/L. However, because amniotic fluids must be diluted at least 40 times to get a linear response, the lowest concentration measurable with precision was 15 μg/L. (This value was adopted for the graphic representation of samples with undetectable CC16.)

Statistical analysis. All statistical tests were done with Statview SE software (13). The effects of gestational age and sex on the concentrations of CC16 (log transformed) were examined by stepwise regression analysis. Expected values were calculated by a polynomial regression. Differences between values during normal pregnancy and those in pathologic pregnancies (expressed as a percentage of expected values and then log transformed) were assessed by an analysis of variance followed by Dunett's multiple comparison test. Results are reported as statistically significant at $p < 0.05$.

RESULTS

Accuracy and specificity of the assay. The accuracy of the assay was tested by adding purified CC16 (7) to six amniotic fluid samples to increase their concentration by 200 μg/L. The analytical recovery (measured within 48 h) averaged 104% (SD, 8%). In Ouchterlony immunodiffusion analysis, CC16 from amniotic fluid showed a complete identity with the protein present in bronchoalveolar lavage of adults or that purified from tubular proteinuria (Fig. 1). Pooled amniotic fluids from five normal fetuses were fractionated by fast protein liquid chromatography on Sephacryl S-200 (Pharmacia-LKB Biotechnology, Uppsala, Sweden), and CC16 was assayed in the eluted fractions by latex immunoassay (12). CC16 eluted as a single component with an apparent size of approximately 16 kD, which was indistinguishable from that of the native protein or the protein present in lung lavage of adults (results not shown).

CC16 in amniotic fluid from normal pregnancies. In stepwise regression analysis, CC16 levels in normal amniotic

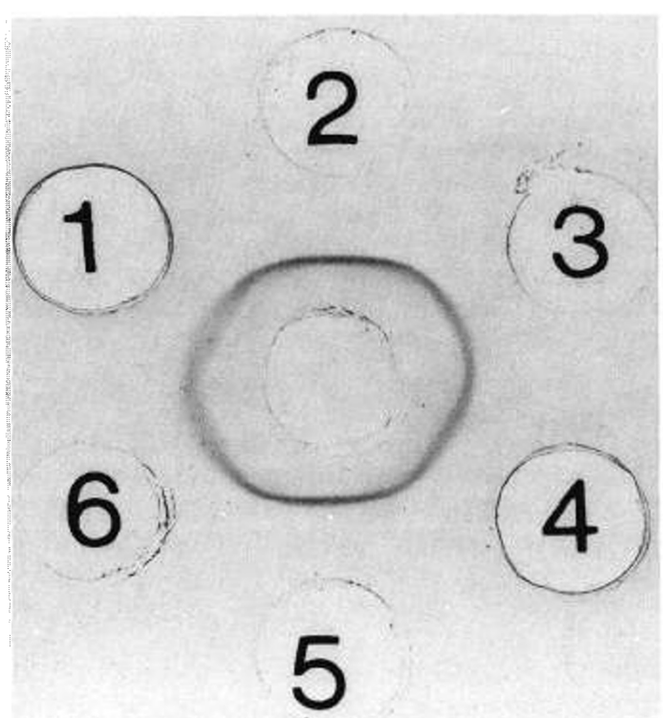

Figure 1. Ouchterlony immunodiffusion analysis of amniotic fluid from two pregnant women (*wells 1* and *4*), purified CC16 (*wells 2* and *5*), and bronchoalveolar lavage fluid from two healthy nonsmokers (*wells 3* and *6*). The central well contained 20 μL of anti-CC16 antibody (anti–protein 1 antibody from Dakopatts), and other wells contained 20 (wells 2 and 5) or 40 μL (wells 3, 4, and 6) of solutions with CC16 concentrations between 10 and 50 mg/L. After 24 h of diffusion, the plate was washed, dried, and stained with Coomassie brilliant blue.

fluid were independent of the sex of the fetus (partial r^2 = 0.015, p = 0.08) but highly correlated with the gestational age (partial r^2 = 0.67, p = 0.0001). The time course of CC16 in amniotic fluid during normal pregnancy is depicted in Figure 2. The protein was detectable on average from the 15th wk of pregnancy, then progressively increased until the delivery, with a tendency to reach a plateau after wk 30. Between the 15th wk and the end of pregnancy, the concentration of CC16 in amniotic fluid was multiplied on average by a factor of 25.

CC16 in amniotic fluid from pathologic pregnancies. Values of CC16 in amniotic fluid from various abnormal pregancies are shown in Figure 3. For the statistical evaluation of these data, we expressed all the results as percentages of the expected values, the latter being derived from the second-degree polynomial of Figure 2. The levels of CC16 in amniotic fluid in cases of spina bifida (geometric mean, 141%), anencephaly (72.4%), and trisomy 21 (111%) were not significantly different from that calculated in controls (108%). In contrast, mean values observed in fetuses with diaphragmatic hernia (26.9%), Turner syndrome (19.7%), or trisomy 18 (50%) or from diabetic mothers (52.4%) were significantly lower than expected whether the comparison was made with normal fetuses or with other abnormal fetuses (101%, n = 22). In view of the variable onset of CC16 in normal amniotic fluid during pregnancy (Fig. 2), CC16 is likely to be a reliable index of fetal lung growth only in samples collected after the 20th wk of pregnancy. To further assess the potential of CC16 to detect abnormal growth, we calculated the lowest 95% probability limit of the expected CC16 values in amniotic fluid of normal fetuses with a gestational age greater than 20 wk (n = 30). The CC16 value was below this threshold (40%) in the three fetuses with Turner syndrome and in four of the five fetuses with diaphragmatic hernia aged greater than 20 wk. CC16 in amniotic fluid was also below this threshold in two of the eight fetuses with trisomy 18. In contrast, no value of CC16 laid below the 95% limit in the 12 other

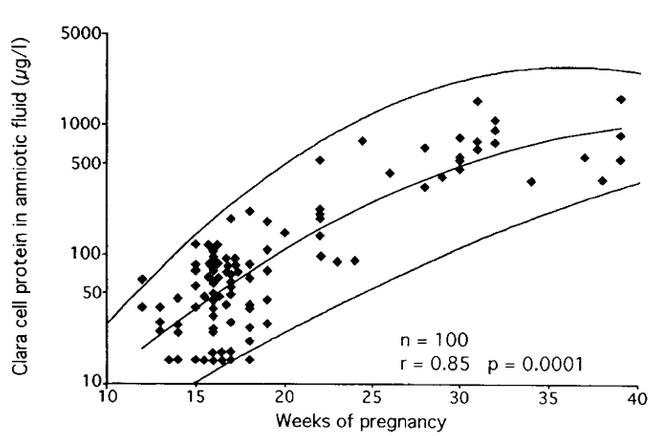

Figure 2. Time course of CC16 in amniotic fluid during normal pregnancy. The equation of the regression line is: log y = −0.31 + 0.15x − 1.7 10^{-3} x^2. The area between the upper and lower lines represents the 90% confidence interval.

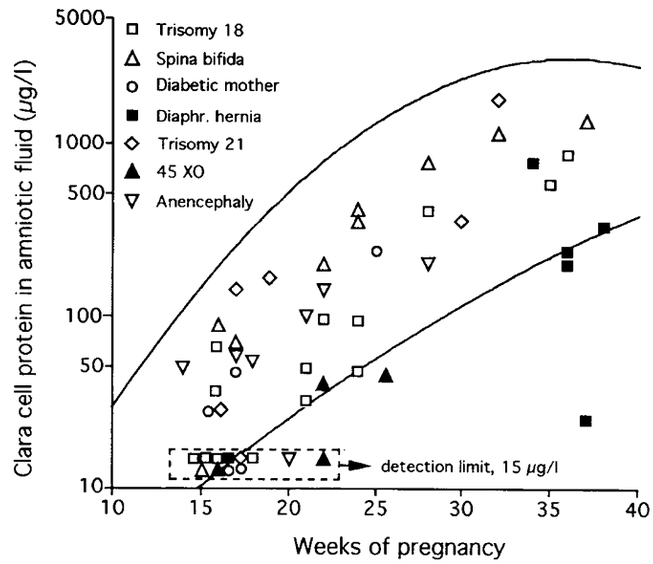

Figure 3. CC16 in amniotic fluid in different types of pathologic pregnancies. The two lines represent the 90% confidence interval of the regression line of Figure 2. *Diaphr.*, diaphragmatic.

fetuses of abnormal pregnancies with a gestational age greater than 20 wk (*i.e.* six with spina bifida, two with trisomy 21, three with anencephaly, and one fetus from a diabetic mother).

Table 1 compares the values of CC16 in amniotic fluid with the total lung weight and the survivorship of fetuses with diaphragmatic hernia and of three other abnormal fetuses for which information on lung weight and survivorship was available. Of the six fetuses with diaphragmatic hernia, five died between 1 and 34 h after delivery despite appropriate medical assistance and one survived. It is interesting to note that the concentration of CC16 in amniotic fluid was normal (109% of the expected value) in the latter case and much lower in other cases, the lowest value (2.5% of the expected value) being observed with the baby who survived only 1 h. All fetuses who died and were autopsied had hypoplastic lungs (total lung weight, 4.1 to 17.2 g; 8.4 to 35% of the expected value). Table 1 also reports values from three other abnormal fetuses, two anencephalic and one with Turner syndrome. The latter subject was of interest because he had a pronounced lung hypoplasia that was mirrored in the level of CC16 in amniotic fluid (16 and 15% of the expected values, respectively). When all cases are combined, a highly significant correlation emerges between CC16 in amniotic fluid and the total lung weight (both expressed as a percentage of the expected value, r = 0.94, p = 0.002, n = 7).

DISCUSSION

CC16 was measured in amniotic fluid with an immunoassay initially developed for a urinary microprotein called protein 1 identical with CC16 (12) and recently applied to the analysis of CC16 in bronchoalveolar lavage (11). A reaction of complete identity was demonstrated

Table 1. *Outcome, lung weight, and CC16 in amniotic fluid in several cases of abnormal pregnancy**

Type of pathology	Birth (wk)	Survival time (h)	Lung weight (g)				CC16 in amniotic fluid		
			Left	Right	Total	% expected	Time (wk)	μg/L	% expected
Diaphr	37	1	2.1	2	4.1	8.4	36	25	2.5
Diaphr	41	2	1.5	6.8	8.3	17	36	235	29
Diaphr	36	24	3	11	14	31.8	16	15.5	32
Diaphr	38	24					36	214	26
Diaphr	39	34	4.7	12.5	17.2	35	38	327	36
Diaphr	38	Survived					34	767	109
Anenceph	29	Abortion	2.5	2.5	5	28	28	198	53
Anenceph	23	Abortion	4.5	5	9.5	86	22	141	93
45,XO	26	Abortion	2.1	2.3	4.4	16	24	45	15

* Diaphr, diaphragmatic hernia; anencephal, anencephaly; 45,XO, Turner syndrome.

by Ouchterlony immunodiffusion analysis between CC16 in amniotic fluid and the protein in lung lavages of adults or that purified from pathologic urine. The protein occurring in these three fluids also showed by gel filtration an identical M_r around 16 000.

At the present time, two sources of CC16 have been identified: nonciliated columnar cells of the bronchial and bronchiolar epithelium, in particular Clara cells (6), and, from puberty on, the male urogenital tract (7). The absence of a sex effect on CC16 in amniotic fluid allows us to rule out the latter source and to conclude that most CC16 in amniotic fluid probably originates from the fetal lung. This interpretation is supported by the fact that the time course of CC16 in normal amniotic fluid is consistent with what is known about the development of Clara cells (14). These cells are thought to develop during the second half of gestation from primitive nonciliated cells of the terminal airways. By 18–19 wk of gestation, the dome-like apical protrusion characteristic of mature cells has formed. Maturation involves a gradual loss of cytoplasmic glycogen and the appearance of electron-dense granules that probably contain CC16. On the other hand, it is well established that by 15–16 wk, the fetus has sufficient breathing activity to move small amounts of amniotic fluid in and out of the respiratory tract, which can explain the release of CC16 into amniotic fluid. The timing of CC16 in human amniotic fluid resembles also that of CC16 in the amniotic fluid and the developing lung of rat. Singh *et al.* (15) have noted in rat a progressive increase of CC16 in both lung and amniotic fluid between d 18 and 21 of gestation. The average increase was only 3.8-fold in amniotic fluid but reached 30-fold in the lung, which is comparable to the factor of 25 observed in the present study for amniotic fluid. In hamsters, too, CC16 was immunolocalized by electron and light microscopy in the fetal lung from d 15 of gestation, and an increasing amount of labeling was noted until birth and during the first few weeks after birth (16).

Results obtained in pathologic pregnancies are also consistent with an exclusively pulmonary origin of CC16 in amniotic fluid. The concentrations of CC16 in amniotic fluid were markedly decreased in fetuses with diaphragmatic hernia, which is a well-known cause of lung hypoplasia (14). The concentration of CC16 in amniotic fluid correlated with both the weight of the lungs and the survivorship of the fetuses with diaphragmatic hernia (Table 1). A pronounced decrease of CC16 was also found in fetuses with Turner syndrome, resulting most likely from lung hypoplasia, inasmuch as the three cases we have examined showed by echography signs of hygroma and lymphedema hindering the development of lungs. In one case, this was confirmed by the anatomopathologic examination (Table 1). It is interesting to note that when one considers only fetuses with a gestational age of more than 20 wk (*i.e.* for whom CC16 can be detected in amniotic fluid) all fetuses with Turner syndrome or a diaphragmatic hernia (with the exception of the subject who survived) had values of CC16 in amniotic fluid below the lowest 95% probability limit, whereas the values for all other abnormal fetuses except two with trisomy 18 fell inside these limits. The mean concentrations of CC16 in amniotic fluid were also slightly lower in the case of trisomy 18 and in diabetic pregnancy, two situations that may be associated with lung hypoplasia or immaturity (14, 17).

Tests of fetal lung maturity presently available measure phospholipids of the pulmonary surfactant in amniotic fluid and aim to assess the risk of the neonatal RDS. The most commonly used test involves the lecithin/sphingomyelin ratio, but other markers are also available such as the concentration of lecithin or phosphatidylglycerol, the foam stability index, or the surfactant apoproteins, which appear to have a better predictability in diabetic pregnancy, a situation associated with a particularly elevated risk of neonatal RDS (18–21). All these tests are based on surfactant constituents—which are deficient in neonatal RDS—and thus reflect the maturation of lung alveoli. Little attention has been paid so far to potential markers of the development of airway cells. The low-M_r antileukoprotease (a protein secreted by both Clara cells and goblet cells) has been shown to be present in bronchiolar epithelium by the 38th wk of gestation, but to our knowledge this protein has not yet been investigated in amniotic fluid (22). CC16 thus seems to be the first potential marker of bronchial epithelium development to have been monitored in human amniotic fluid. The time course of CC16 during normal pregnancy and its reduction in abnormal pregnancies associated with lung

hypoplasia suggest that CC16 in amniotic fluid might be a useful gestational age–dependent marker of lung growth. A practical advantage of CC16 over other lung development markers is its insensitivity to unintentional contamination of the amniotic sample by blood, because CC16 occurs in serum only in trace amounts (9).

Acknowledgments. The authors thank X. Dumont for his expert technical assistance. Dr. J. C. Daniel from Rhône-Poulenc Industrie, France, is gratefully acknowledged for providing us with batches of polystyrene latex particles (ESTAPOR K109).

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