Prenatal diagnosis of cystinosis

Cystinosis was diagnosed in a small quantity of cultured amniotic cells from a 22-week-old fetus by a modified pulse-labeling technique in which intracellular ^{35}S L-cystine retention was measured. As a result of the above finding, the pregnancy was terminated by administration of prostaglandin. The diagnosis was confirmed when the nonprotein-free cystine content of the kidney, liver, placenta, spleen, thymus, and gut, as well as that of a large amount of cultured amniotic cells, was found to be 100-fold higher than normal levels.

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CYSTINOSIS is an enigmatic disorder characterized clinically by the onset of the renal Fanconi syndrome during the first year of life in otherwise normally appearing infants. Despite the control of electrolyte abnormalities, the children grow poorly, develop chronic anemia, and demonstrate progressive renal glomerular insufficiency and end-stage kidney disease by the end of the first decade of life. In tissues of these children a high intracellular content of nonprotein (free) cystine has been demonstrated, which may be manifested on light microscopy as cystine crystals or on electron microscopy as amorphous masses located in subcellular structures resembling lysosomes.

This disease has an autosomal recessive inheritance. It has been observed that peripheral leukocytes or cultured skin fibroblasts of homozygotes contain about 100 times the normal amount of free cystine and that such cells from obligate heterozygotes contain four to five times the normal amount. In the absence of a known enzymatic defect in cystine metabolism, the genotype of cultured cells has been determined by such measurement of cystine content when sufficiently large quantities of cells are available. With small amounts of fibroblasts, pulse-labeling techniques with ^{35}S L-cystine may be used to differentiate between normal and homozygous cells.[1] The latter, when incubated with radioactive cystine, will accumulate much more of the label as cystine, and after the radioactivity is removed from the media will retain the ^{35}S L-cystine within the cystine pool long after it has been metabolically removed by normal cells.

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Abbreviations used
NEM: *N*-ethylmaleimide
MEM: minimum Eagle's medium
PBS: phosphate-buffered saline

Employing the measurement of ^{35}S L-cystine uptake after 48 hours incubation of a minute amount of cultured amniotic fluid cells, Schneider and associates[2] were able to determine prenatally for the first time the existence in utero of a fetus homozygous for cystinosis. We report here the successful diagnosis of cystinosis in a fetus in utero by measurement of the retention of ^{35}S L-cystine within cultured amniotic cells after a short-term 2-hour pulse-labeling incubation followed by a 20-minute period of incubation without the labeled substrate, a technique which can be carried out with greater ease than methods reported previously.[2, 3]

CASE REPORT

This was the seventh pregnancy for a 37-year-old Caucasian woman. She and her husband were unrelated and there was no history in their families of cystinosis, Fanconi syndrome, or other renal disease. Four children are living, three normal girls ages 19, 18, and 10, and a 12-year-old boy with proved nephropathic

cystinosis who had undergone renal transplantation at age 10. Fifteen years ago, a 2-year-old girl died of "pneumonia," and 11 years ago a 4-year-old boy died with a diagnosis of "fever and salicylate poisoning" despite the fact that the mother only administered small amounts of aspirin. Each of the children was said by the mother to have been anemic and tiny, with poor growth and very light hair. In retrospect, these two siblings appear to fit the syndrome of cystinosis and both may have died because of acidosis and dehydration.

The mother presented herself to the genetics clinic at St. Christopher's Hospital for Children, Philadelphia, in the twenty-first postmenstrual week of pregnancy. When told of the possibility of prenatal detection of cystinosis, she elected to undergo amniocentesis which was performed in the twenty-second week of pregnancy. On the basis of the biochemical findings on amniotic cells cultured for 3 weeks (described below), the parents requested termination of pregnancy. This was performed in the twenty-fifth postmenstrual week. After prostaglandin-induced labor, a stillborn male fetus was delivered which was of appropriate size for gestational age and without obvious external abnormalities. Autopsy permission was granted.

MATERIALS

L-Cystine, cysteine •HCl, *N*-ethylmaleimide, and reduced glutathione were purchased from Calbiochem, San Diego, California. ^{35}S L-cystine (specific activity 313 mCi/mM) was obtained from The Radiochemical Centre, Amersham, Bucks, England. Minimum Eagle's medium with Earle salts was obtained from Microbiological Associates, Inc., Bethesda, Md., and fetal bovine serum and glutamine were purchased from Flow Laboratories, Inc., Rockville, Md. Falcon tissue culture glassware, coverslips, and all A.R. grade reagents were purchased through Fisher Scientific Co., Pittsburgh, Pa.

METHODS

The amniotic fluid was processed within 4 hours of amniocentesis according to the method of Greene and associates.[4] Cells were cultured in MEM with Earle salts supplemented with 2mM glutamine and 20% fetal bovine serum and grown for 2 to 3 weeks in 25 cm^2 Falcon flasks under 95:5 air:CO_2 at 37° C. The cells in one Falcon Flask were passaged onto five 22 × 22 mm^2 No. 2 sterile coverslips contained in individual 35 × 10mm Petri dishes by washing the cells with Versene in Puck saline A[5] followed by a 6-minute treatment with a Versene-trypsin solution in Puck saline.[6] Two milliliters of MEM with 20% fetal bovine serum and 2mM glutamine were added to each Petri dish. Incubation was at 37° C under air:CO_2 (95:5) in a humidified chamber. The cells were confluent on the coverslip after 4 days and were refed 24 hours prior to determination of the capacity of the amniotic cells to accumulate cystine. Both normal and cystinotic fibroblast lines were passaged onto coverslips and treated in the same manner as that described above for amniotic cells.

Experimental procedure. Five coverslips confluent with cells were washed three times with 1 ml of 37° C Dulbecco phosphate-buffered saline, pH 7.45, containing 0.1% glucose and immediately transferred to individual Petri dishes containing 1 ml of Dulbecco PBS + 0.1% glucose to which 0.1mM ^{35}S L-cystine (approximately 30 μCi/ml medium) had been added. Incubation was at 37° C for 2 hours with periodic manual swirling. At the end of this pulse-labeling period, each coverslip was removed from its Petri dish, washed carefully three times, and transferred immediately to a new Petri dish containing 2 ml of Dulbecco PBS + 0.1% glucose for an additional 20-minute incubation at 37° C in the absence of labeled substrate. At the end of the second incubation, the washing procedure was the same as previously described by us.[1] Five coverslips were then pooled onto filter paper, carefully broken into small pieces, and transferred immediately with the aid of a glass rod to 5 ml of cold NEM (5 mg/ml) prepared in 0.01M phosphate buffer pH 7.45. These coverslips were homogenized in the NEM solution for 30 seconds (at least ten strokes) and 0.5 ml cold 50% trichloroacetic acid was added. The mixture was thoroughly mixed, transferred to a 40 ml centrifuge tube, and centrifuged for 10 minutes at 1,200 × g. The supernatant was transferred carefully to a 12 × 150 glass tube. Each supernatant was extracted two times with 5 ml of ether to remove unreacted NEM and trichloroacetic acid. After removal of traces of ether under nitrogen, from 10 to 50 μl of the sample were spotted onto Whatman 3MM paper for a 2-hour electrophoretic run at 35° C in 6.8% formic acid in a Gilson high-voltage Electrophorator (4,000 v, 250 ma). Standards of glutathione-NEM, cysteine-NEM, and cystine were spotted between samples on each paper electrophoretic run to make sure cystine separated adequately from the two NEM adducts. Ninhydrin (0.2%) in acetone supplemented with cadmium acetate acid[7] was used as a dip for staining the 2-cm wide strips containing the standards. The radioactive paper strips, each 4 cm wide, were cut into fifty 1 cm sections starting 7 cm from the origin in the direction of the anode and extending 43 cm from the origin toward the cathode. Each 1 × 4 cm strip was placed in a counting vial with 2 ml of phosphor and counted in a liquid scintillation spectrometer as described previously.[1]

Determination of tissue cystine. Sections of organ tissues from the fetus were prepared for amino acid analysis, cell culture, and histologic examination. Tissues for amino acid analyses of approximately 150 to 400 mg wet weight were placed on ice immediately after removal. The precise wet weights were determined and recorded; the tissues were homogenized in either 2 or 3 ml 3%

Table I. Incorporation of label in intracellular components after pulse-labeling with ^{35}S L-cystine

Cells	% of total ^{35}S* in GSH-NEM + CSH-NEM†	% of total ^{35}S* in Cystine	Cystine content (μmoles 1/2 cystine/gm protein)
Fibroblasts			
Normal (4)	89.6 ± 1.5 ¶	2.3 ± 1.5	< 0.07
Cystinotic			
Heterozygous‡	83.9	1.0	
Homozygous	61.9	27.7	19.0
Amniotic cells			
Normal			
I	90.0	1.1	
II	88.6	1.0	< 0.07
III	87.8	1.6	
At risk ‖	56.8	20.1	7.4

*Based on the percentage of the total label recovered in the designated compound after high voltage electrophoresis, cutting the section into strips, and counting as described in text. The total of the percentage of ^{35}S in glutathione, cysteine, and cystine in the cells at risk is less than in normal cells due to significant ^{35}S appearing in other metabolites than those indicated.

†GSH-NEM refers to *N*-ethylmaleimide adduct of reduced glutathione CSH-NEM refers to *N*-ethylmaleimide adduct of cysteine.

‡These cells originated from a skin biopsy taken from the parent of the aborted fetus under study.

¶ Cultured amniotic cells isolated from amniotic fluid after amniocentesis which was homozygous for normal male by conventional karyotyping as determined by P.S. Moorhead, Children's Hospital of Philadelphia.

‖ Mean ± SD.

Table II. Free (nonprotein) cystine in fetal tissue

Tissue	Cystinotic fetus* (μmoles 1/2 cystine /gm protein)	Control fetus† (μmoles 1/2 cystine /gm protein)
Adrenal	None detected‡	0.48
Amnion	1.87	Not reported
Brain		
Cerebrum cortex	None detected‡	Not reported
Medulla	None detected	Not reported
Cerebellum	None detected	Not reported
Thalmus	None detected	Not reported
Gut		
Jejunum	3.50	Not reported
Ileum	5.20	Not reported
Kidney	62.71	0.32 - 0.50
Liver	11.73	0.42 - 0.48
Placenta	9.19	0.13
Spleen	5.88	0.07
Thymus	5.71	0.01-0.14
Thyroid	1.47	0.33

*Tissues from aborted fetus on which prenatal diagnosis of cystinosis was made.

†Normal control values based on tissues from fetuses of approximate postmenstrual age of 18 and 20 weeks as reported by Schneider and associates.[2]

‡Less than 0.07 μmoles 1/2 cystine/gm protein.

sulfosalicylic acid and centrifuged at 2,000 × g for 10 minutes. The extracts were stored at −40° C until analyzed for cystine contents, i.e., for a period not exceeding 2 weeks.

Cultured amniotic cells and fibroblasts were washed and trypsinized as described above. The cell pellet was washed three times with 5 ml of PBS with centrifugation of 6 minutes at 800 × g between each wash. Then the cells were suspended in 0.75 ml of 3% sulfosalicylic acid, frozen, and thawed three times, centrifuged for 10 minutes at 2,000 × g, and the cystine content of the cellular extract determined.

Nonprotein (free) cystine contents of the extracts were based on ion exchange chromatography on a 56 cm acidic-neutral column attached to a standard Beckman Model 120C or 119 Amino Acid Analyzer according to the method for physiologic fluid analyses described in the *Beckman Amino Acid Analyzer Instruction Manual.*

Precipitable cellular protein was determined by the method of Oyama and Eagle[8] and cystine was calculated and recorded as μmoles ½ cystine/gram tissue protein.

RESULTS

A significant difference in retention of ^{35}S in cystine between normal and homozygous cystinotic cells is evident with about 1.5% of the label in cystine in normal fibroblasts as compared with 28% in cystinotic fibroblasts. A direct reflection of ^{35}S accumulation in cystine is the incorporation of label in other intracellular components as evidenced by the appearance of 90% of the label in the *N*-ethylmaleimide adducts of glutathione and cysteine in normal cells and 62% in cystinotic fibroblasts. No significant difference in retention of ^{35}S in cystine by normal cells and those heterozygous for cystinosis exists; however, the percentage appearing in glutathione-NEM + cysteine-NEM is 84% in heterozygotes, a value slightly lower than the 90% observed in normal cells. The genotype of the normal and cystinotic cells was confirmed by assay of intracellular cystine (Table I).

Since the method had shown unequivocally that cells homozygous for cystinosis could be detected, three normal amniotic cell lines and the amniotic cells at risk were assayed for their accumulation of ^{35}S L-cystine in the manner described above. Table I shows that normal amniotic cells retained only a trace amount of the label in cystine (approximately 1%) with approximately 88% appearing in the two intracellular components related to cystine. The amniotic fetal cells at risk showed characteristics typical of cells homozygous for cystinosis with accumulation of 20% of the label in cystine and only 57% in the combined NEM adducts of glutathione and cysteine.

Quantitative cystine analysis of bulk amounts of amniotic cells substantiated the ^{35}S cystine retention data indicating the fetus was homozygous for cystinosis; the elevated cystine content resembling skin fibroblasts homozygous for the disease (Table I). Further confirmation of the diagnosis was obtained when the various fetal tissues were assayed for their cystine content. The analyses are recorded in Table II and show that all of the tissues examined, except the adrenals and the brain, contained elevated levels of cystine. Tissue samples examined by light and electron microscopy have revealed crystals and intracellular inclusions; a report of the histologic findings is in preparation.

DISCUSSION

The method described takes advantage of our previously published observation that cystinotic cells differ from normal cells in that they accumulate labeled cystine after a short period of pulse-labeling with ^{35}S L-cystine.[1] Other workers in their identification of mutant genotypes for cystinosis in utero utilized exposure of cultured amniotic cells to ^{35}S cystine for 2 to 4 days followed by analysis of cellular extracts to determine the ratios of radioactive cystine to glutathione-NEM and cysteine-NEM. To do this, they passed the effluent from an ion exchange column of an amino acid analyzer through an anthracene-packed flow cell placed in the well of a scintillation counter, a method which required special equipment and considerable time. The simplicity of our technique is evidenced by its use of only two short incubations: 2 hours with exogenous ^{35}S L-cystine followed by 20 minutes in the absence of labeled substrate. No specially prepared media are required, a minimum of labeled substrate is utilized, and the complicated separation by ion exchange chromatography is replaced by electrophoresis.

With our procedure, we identified unequivocally the homozygous genotype for cystinosis in utero. We do not believe, however, that our technique would identify heterozygotes. This impression supports the similar conclusion of Schneider and associates[2] and is at variance with that of Schulman and associates[3] who reported the ability to distinguish between normal and heterozygotes for cystinosis with certainty.

The presence of cystine crystals has not been diagnosed clinically until at least 10 weeks after birth.[9-11] Our data show that the free cystine content of most of the organs of the cystinotic fetus is greatly increased before birth. Similarities and differences are evident in comparison of the data on the free cystine in fetal tissues of the aborted 23-week fetus examined by Schneider and his colleagues[2] and the free cystine content of the tissues of the 25-week fetus studied by us. The cystine content of the thymus, the low level of cystine in the thyroid, and the absence of elevated cystine in the adrenals are comparable. Differences are found, however, in the cystine content of kidney and liver. The fetal liver studied by Schneider and associates[2] contained more than three times as much free cystine as the fetal kidney, whereas the fetal kidney studied by us contained more than five times as much free cystine as the liver. A high cystine level in the kidney is not surprising since cystine storage disease is characterized by impairment of both tubular and glomerular kidney functions and lead to the early diagnosis of nephropathic cystinosis. Also, the cystine content of the amniotic cells from the previously studied fetus was approximately one-half of that found in the amniotic cells at risk in this report (i.e., compared with 7.4 μmoles ½ cystine/gm protein).

The biochemical examination of the cystine content of the diseased fetus may indicate a clue of therapeutic importance. Absence of cystine from the adrenal glands despite its presence in other tissues and the known high content of ascorbic acid in the adrenal prompted Kroll and Schneider[12] to test and report the remarkable ability of ascorbic acid to deplete cystinotic fibroblasts of cystine. Although there is no direct evidence that ascorbic acid is causally related to the low cystine content of the adrenal, Kroll and Schneider[12] indicate a therapeutic trial of ascorbic acid in patients with cystinosis may be warranted.

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REFERENCES

1. States B, Harris D, and Segal S: Uptake and utilization of exogenous cystine by cystinotic and normal fibroblasts, J Clin Invest **53**:1003, 1974.
2. Schneider JA, Verroust FM, Kroll WA, Garvin AJ, Horger EO III, Wong VG, Spear GS, Jacobson C, Pellett OL, and Becker FLA: Prenatal diagnosis of cystinosis, N Engl J Med **290**:878, 1974.
3. Schulman JD, Fujimoto WY, Bradley KH, and Seegmiller JE: Identification of heterozygous genotype for cystinosis in utero by a new pulse-labeling technique: preliminary report, J PEDIATR **76**:289, 1970.
4. Greene AE, Toji L, Nichols WW, and Coriell LL: Amniocentesis cells: Culture, cryogenic storage, isozymes and finite life in vitro, In Vitro **9**:156, 1973.

5. Coriell LL: Amniocentesis, *in* Kruse P Jr, and Patterson M Jr, editor: Tissue culture: Methods and application New York, 1973, Academic Press, Inc., Chap 7, p 62.
6. Cox R, Krauss M, Bolis M, and Dancis J: Evidence for transfer of enzyme product as the basis of metabolic cooperation between tissue culture fibroblasts of Lesch-Nyhan disease and normal cells, Proc Natl Acad Sci USA **67:**1573, 1970.
7. Rosenberg LE, Crawhall JC, and Segal S: Intestinal transport of cystine and cysteine in man: Evidence for separate mechanisms, J Clin Invest **46:**30, 1967.
8. Oyama VI, and Eagle H: Measurement of cell growth in tissue culture with a phenol reagent, Proc Soc Exp Biol Med **91:**305, 1956.
9. Schneider JA, Rosenbloom FM, Bradley KH, and Seegmiller JE: Increased free-cystine content of fibroblasts cultured from patients with cystinosis, Biochem Biophys Res Comm **29:**527, 1967.
10. Holtzapple PG, Genel M, Yakovac WC, Hummeler K, and Segal S: Diagnosis of cystinosis by rectal biopsy, N Engl J Med **281:**143, 1969.
11. Hummeler K, Zajac BA, Genel M, Holtzapple PG, and Segal S: Human cystinosis: Intracellular deposition of cystine, Science **168:**859, 1970.
12. Kroll WA, and Schneider JA: Decrease in free cystine content of cultured cystinotic fibroblasts by ascorbic acid, Science **186:**1040, 1974