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Definitive Prenatal Diagnosis for Type III Glycogen Storage Disease

Bing-Zhi Yang,* Jia-Huan Ding,* Barbara I. Brown,† and Yuan-Tsong Chen*

*Department of Pediatrics, Duke University Medical Center, Durham, NC; and †Department of Biological Chemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis.

Summary

Prenatal diagnosis for type III glycogen storage disease was performed by using (1) immunoblot analysis with a polyclonal antibody prepared against purified porcine-muscle debranching enzyme and (2) a qualitative assay for debranching-enzyme activity. Cultured amniotic fluid cells from three pregnancies (three families in which the proband had absence of debrancher protein) were subjected to immunoblot analysis. Two unaffected and one affected fetus were predicted. In addition, cultured amniotic fluid cells from nine pregnancies (eight families) were screened with a qualitative assay based on the persistence of a polysaccharide that has a structure approaching that of a phosphorylase limit dextrin when the cells were exposed to a glucose-free medium. This qualitative assay predicted six unaffected and three affected fetuses. All predictions by either method were confirmed postnatally except for one spontaneously aborted fetus. Our data indicate that a definitive diagnosis of type III glycogen storage disease can be made prenatally by these methods.

Introduction

Deficiency of glycogen-debranching-enzyme activity causes type III glycogen storage disease (GSD-III) (Cori disease or limit dextrinosis). Although generally patients with GSD-III are clinically less affected than patients with glucose-6-phosphatase deficiency (type I glycogen storage disease), the GSD-III patients may have severe hypoglycemia and seizures. Overt liver cirrhosis can occur, and gross cardiac involvement may lead to death (Miller et al. 1972; Starzl et al. 1973; Fellows et al. 1983; Olson et al. 1986). In adults, progressive myopathy leading to total muscular paralysis has been reported (Moses et al. 1986). Many families desire prenatal diagnosis but since debranching-enzyme activity is relatively low in cultured amniocytes or villi, a definitive prenatal diagnosis of GSD-III using an enzyme-activity assay is technically difficult (van Diggelen et al. 1985; Maire et al. 1989; Shin et al. 1989).

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Address for correspondence and reprints: Y. T. Chen, M.D., Ph.D., Department of Pediatrics, Duke University Medical Center, P.O. Box 3028, Durham, NC 27710.

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In GSD-III, most patients have a deficiency of debranching-enzyme activity in both liver and muscle (subtype IIIa), while some patients have enzyme deficiency confined to the liver (subtype IIIb). Rarely, patients have only transferase deficiency (subtype IIId). We recently reported immunoblot analyses of debranching enzyme in 41 GSD-III patients using polyclonal antibodies raised against porcine-muscle debranching enzyme (Ding et al. 1990). In both IIIa and IIIb patients, the debranching-enzyme protein is either absent or greatly reduced. Since IIIa and IIIb represent the majority of GSD-III patients (93% in our series) and the debranching enzyme is normally expressed in cultured amniotic fluid cells, we proposed and described a case in which western blot analysis was used for prenatal diagnosis (Ding et al. 1990). We now extend our investigation of the prenatal diagnosis by western blot to three additional families. We also report prenatal testing using a qualitative assay of debranching enzyme described elsewhere for skin fibroblasts and amniocytes (Brown and Brown 1982). This method takes advantage of the fact that, when cells are exposed to a glucose-free medium, glycogen rapidly decreases to a very low level in normal cells but the polysaccharide persists in

the GSD-III cells. By either immunoblot or the qualitative assays, GSD-III can be diagnosed prenatally.

Material and Methods

Patients

Eleven families at risk for GSD-III requested prenatal diagnosis. In each family, the proband had hypoglycemia, hepatomegaly, and failure to thrive in early childhood, and most probands also had a high blood creatine phosphokinase. The diagnosis of GSD-III in the probands had been previously made by demonstrating deficient debranching-enzyme activity in liver and/or muscle biopsy samples.

Immunoblot Analysis

Up to 40 μg of protein was applied to 7% SDS-PAGE. The proteins were electroblotted onto nitrocellulose paper and probed with antiserum prepared against purified porcine-muscle debranching enzyme. The antiserum reacts against debranching enzyme with a single precipitation line and inhibits the enzyme activity (Chen et al. 1987). The detailed procedures of antiserum preparation and western blot analysis have been published previously (Chen et al. 1987; Ding et al. 1990).

Qualitative Assay for Debranching Enzyme in Cells Cultured With and Without Glucose

Early-passage amniotic fluid cell cultures were received from referring medical centers and continued in Eagles minimal essential medium with 10% FCS, 100 units penicillin/ml, and 100 μg streptomycin/ml. For analysis, medium with and without 5 mM glucose and containing 10% dialyzed FCS was added to paired flasks of cells at near confluency and the cells harvested 16–18 h later. The cells were sonicated in saline, aliquots were boiled for 1 min and the glycogen content was measured (Brown and Brown 1982; Brown 1985). In some instances the polysaccharide was degraded by phosphorylase, free of the debranching enzyme, and the yield of glucose-1-phosphate was measured (Brown and Brown 1982; Brown 1985).

Results

Prenatal Diagnosis for Type III Glycogen Storage Disease by Immunoblot Analysis

Immunoblot analyses of glycogen-debranching enzyme were performed in three families at risk for GSD-IIIa. In each family, the absence of debranching-enzyme protein was first documented in the proband. In fami-

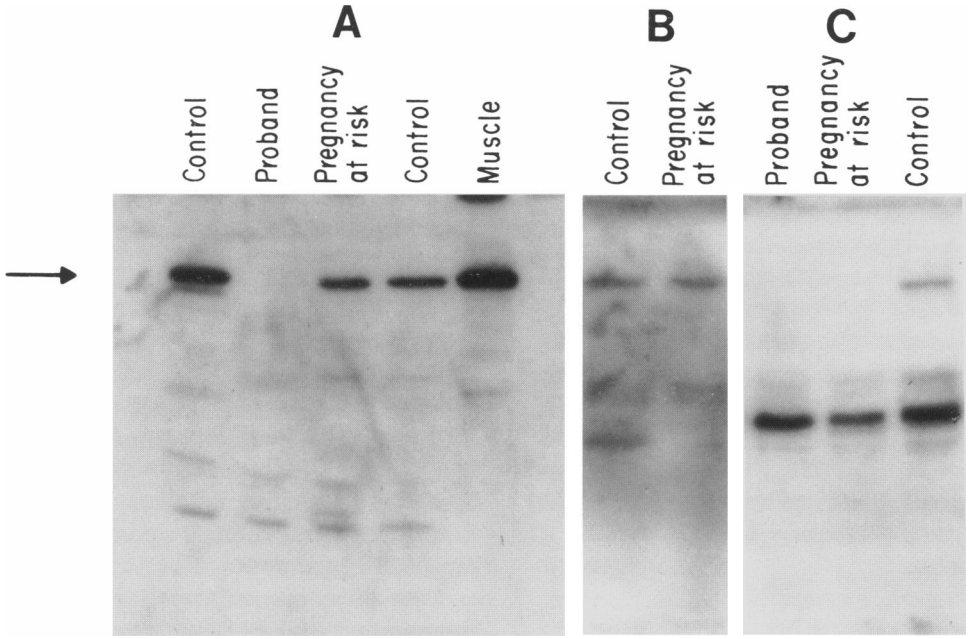

Figure 1 Immunoblot analysis of debrancher protein in pregnancies at risk for GSD-IIIa. The lanes were all loaded with 40 μg protein, except for the muscle lane, which contained 4 μg protein. The control and proband in family A, as well as the proband in family C, were from lymphoblastoid cells. All other controls and pregnancies at risk were from cultured amniotic fluid cells. The arrow indicates the position of the debranching enzyme. The lower molecular weight bands represent nonspecific bands recognized by antiserums.

Table 1

Effect of Overnight Exposure of Cultured Cells to Glucose-free Medium on their Glycogen Content and Structure

Cell Sample	N	Without Glucose		With Glucose	
		Glycogen (μmol polymeric glucose /mg protein)	G-1-P[a] (%)	Glycogen (μmol polymeric glucose /mg protein)	G-1-P[a] (%)
Amniocytes:					
Control	8	.01–.05		.33 ± .12	32.5 ± 1.0
At risk of GSD-III:					
Unaffected	6	.01–.03		.33 ± .10	27.4 ± 6.7
Affected:					
Case 1	1	.17	6.7	.25	32.0
Case 2	1	.35	10.0	.50	29.0
Case 3	1	.41		.51	
Fibroblasts:					
Control	30	.08 ± .07	30.5 ± 2.4[b]	.71 ± .24	30.0 ± 3.5
GSD-III	29	.55 ± .23	7.9 ± 4.6	.85 ± .32	23.4 ± 7.0

[a] Glucose-1-phosphate formed from endogenous polysaccharide by phosphorylase, free of the debranching enzyme, expressed as a percentage of total polysaccharide.

[b] Polysaccharide content in excess of 0.1 μmol/mg protein is required to determine accurately the G-1-P yield, hence not all 30 samples are included.

lies A and B, cultured amniotic fluid cells demonstrated a debranching-enzyme band of the same size and similar intensity as the control cultures, thus predicting an unaffected fetus (fig. 1). In family C, amniotic cells showed an absence of debranching enzyme and an affected fetus was predicted (fig. 1). The family C elected to continued the pregnancy and the baby was born at term. In all three cases, the diagnoses were confirmed postnatally both clinically and by laboratory findings. The affected baby at 1 month of age already had hepatomegaly, fasting hypoglycemia, and elevation of liver transaminases. No symptoms of GSD-III developed in two other cases (ages 3 and 2½ years) predicted to be unaffected. In family A, the positive debranching-enzyme band was also demonstrated postnatally by immunoblot analysis on the lymphoblastoid cells established after birth.

Qualitative Assay for Debranching-Enzyme Activity in Cultured Amniotic Fluid Cells

Eight families at risk for GSD-III (including two pregnancies in one family) were screened by the qualitative assay for debranching enzyme, based on the persistence of a polysaccharide that has a structure approaching that of a phosphorylase limit dextrin in cultures which have been exposed overnight to glucose-free media (Brown and Brown 1982; Brown 1985). Minimal or undetectable levels of glycogen were found in six amniocyte cultures at risk harvested in the absence of media glucose, although cells harvested from the respective paired flasks containing complete media had glycogen contents equal to that of the control cultures (table 1). It was predicted that these six fetuses were not affected by GSD-III since debranching enzyme was present. No symptoms of GSD-III have developed in five of the six individuals predicted to be unaffected. (The sixth fetus spontaneously aborted several weeks after the amniocentesis.) Since all of the cultures had been established elsewhere and then sent to us for analysis, samples of amniocytes not at risk for GSD-III were requested and served as the control amniocytes recorded in table I.

Although chorionic villi cells derived from pregnancies not at risk for GSD-III have also been shown to deplete their glycogen when cultured in the absence of media glucose, no pregnancies at risk have been monitored using cultured chorioni villi.

Amniocytes from the other three pregnancies showed a retention of polysaccharide (table 1), allowing the prediction of GSD-III, which was confirmed after the birth of each infant. Skin fibroblasts derived from two of these infants confirmed the persistence of a phosphorylase limit dextrin on exposure to glucose-free media, and all three manifested the clinical symptoms of GSD-III at an early age. Whenever possible, it is recommended that the glucose-1-phosphate yield from the

phosphorylase degradation of the endogenous polysaccharide be determined as well as the glycogen content. In those instances where there may be little difference between the glycogen contents, the low yield of glucose-1-phosphate from the polysaccharide in the glucose-free sample compared to that of the sonicate derived from the glucose-containing media provides another measure of the enzymatic defect in GSD-III cell lines.

In addition, amniotic fluid cells from one pregnancy at risk, in which immunoblot predicted an unaffected fetus (fig. 1, family B), were also assayed by the qualitative assay. The polysaccharide decreased from 0.28 μmole polymeric glucose/mg protein in complete medium to 0.03 in glucose-free medium. Thus, both methods predicted an unaffected fetus.

Included in table I are data accumulated since 1983 for skin fibroblasts analyzed by this method. These values supplement those previously reported (Brown 1985). The 29 GSD-III fibroblast lines came from patients whose percutaneous liver biopsies may have provided inadequate amounts of tissue for definitive typing of GSD, from siblings at risk, or from adults previously diagnosed at an early age without enzymatic assay as having von Gierke disease.

Discussion

The use of Western blot analysis or a qualitative assay for debranching-enzyme activity of cultured amniotic fluid cells is illustrated for prenatal diagnosis of GSD-III in 12 pregnancies at risk. In all but one case, which was spontaneously aborted, the diagnosis was confirmed postnatally. Both western blot and the qualitative assay are likely to be applicable to pregnancies at risk for types IIIa and IIIb, since absence of debranching enzyme can be demonstrated in skin fibroblasts from both subtypes. The western blot, however, cannot be used for diagnosis of type IIId, as cross-reactive material for debranching enzyme is present in those patients.

The tests are also likely to be applicable to chorionic villi cells since debranching enzyme is expressed in these cells (van Diggelen et al. 1985; Ding et al. 1990). Maire et al. (1989) and Shin et al. (1989) have each reported the diagnosis of one GSD-III fetus in the first trimester of pregnancy using chorionic villi samples. The former authors measured the glucose yield from phosphorylase limit dextrins as the method for assaying the debranching enzyme. The method was based on previous demonstrations that sonicates of GSD-III fibroblasts fail to form more glucose from added phosphorylase limit dextrin than from added glycogen as substrates. A positive net difference (limit dextrin glucose minus glycogen glucose) in glucose yield was obtained with control fibroblasts or cultured amniotic fluid cells (Brown and Brown 1982). Subsequently, it was found that variable results were obtained when different phosphorylase limit dextrin preparations of varying quality were used as substrate. With one sample of limit dextrin, no differences in glucose yield from the two substrates was noted with control fibroblasts. Because of the relatively low content of debranching enzyme in amniocytes (and fibroblasts) and the consequent need for a relatively high protein concentration (3–5 mg/ml reaction mixture) in the assay system, the use of the differential yield method may be technically difficult. To increase sensitivity, a fluorescent assay system, to measure the glucose released, may be helpful (Maire et al. 1989). As for the prenatal diagnosis reported by Shin et al. (1989), the authors appeared to use the incorporation of [^{14}C] glucose into glycogen for assaying the debranching enzyme. The method however, is known to give false negative results in GSD-III fibroblasts and leukocytes (Gutman et al. 1985).

The glycogen content of amniocytes was only about one-half that of fibroblasts harvested from complete media, that is, with glucose present (table 1). Part of this difference may relate to the passage number, or the degree of confluency, of the amniocyte cultures, since, in the interest of providing information promptly, assays were carried out as soon as sufficient material was visible in the flasks. When it was possible to continue the amniocytes in culture for a longer period of time, the glycogen content per milligram of protein often increased, although levels equal to that commonly found in fibroblasts were rarely attained.

The immunoblot analysis described here is a specific, sensitive, and reliable method for prenatal testing and requires only a small amount of cells—one T_{25} flask. The obvious limitations of the method are the general lack of availability of the antiserum and the fact that not all types of GSD-III can be diagnosed. The test cannot be offered to a family in which the proband has cross-reactive material for debranching enzyme.

On the other hand, a qualitative assay of debranching-enzyme activity when cells are cultured with and without glucose is simple and should be able to detect all types of GSD-III. The test, however, requires a greater number of viable cells (minimum of two T_{25} flasks, and preferably more) and requires paired flasks for comparison. In conclusion, our data indicate that by either immunoblot or the qualitative assay, GSD-III can be diagnosed prenatally.

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