The Ontogeny of Human Hepatic Microsomal Glucose-6-Phosphatase Proteins

A. Burchell,1,2 L. Glibb,1 I. D. Waddell,1 M. Gilles,2 and R. Hume2

We have studied 250 human liver biopsy samples to determine the ontogeny of the microsomal glucose-6-phosphatase (EC 3.1.3.9) system. Human hepatic glucose-6-phosphatase enzyme activity develops at 11 weeks' gestation and slowly increases to ~10% of adult activity at term. In the first week after birth, activity rises to adult values. Increases in enzyme activity coincide with increasing concentrations of the glucose-6-phosphatase enzyme protein. The phosphate/pyrophosphate transport protein (T2) of the human hepatic glucose-6-phosphatase complex develops at a different rate from that of the enzyme. Our study shows that the development of rat and human glucose-6-phosphatase activities are completely different. We conclude that deficiencies of the proteins in the microsomal glucose-6-phosphatase complex can be diagnosed with much more certainty perinatally than prenatally.

Additional Keyphrases: liver - heritable disorders - glycogen storage diseases - fetal status - catalytic vs immunoreactivity of enzymes

Hepatic microsomal glucose-6-phosphatase (EC 3.1.3.9; D-glucose-6-phosphate phosphohydrolase) plays an important role in the homeostatic regulation of blood glucose concentrations (1, 2). The complete absence of the hepatic glucose-6-phosphatase enzyme is a severe metabolic disorder (von Gierke's disease, or type 1a glycogen storage disease) that usually manifests early in infancy (3). Recent biochemical and genetic evidence verifies the existence of a multimeric glucose-6-phosphatase complex in the endoplasmic reticulum (see ref. 4 for a review). This complex includes at least five different polypeptides: the glucose-6-phosphatase enzyme, with its active site inside the lumen of the endoplasmic reticulum; a regulatory Ca2+-binding stabilizing protein; and three transport proteins, termed T1, T2, and T3, which allow glucose-6-phosphate, phosphate (and pyrophosphate), and glucose, respectively, to cross the membrane of the endoplasmic reticulum. Three of the proteins—the enzyme, the stabilizing protein, and T2—have been purified and shown to be different polypeptides (5–8). The classical deficiency of the enzyme itself is now termed type 1a glycogen storage disease (3), whereas type 1b is caused by a deficiency of T1 (9–12) and type 1c is deficiency of T2 (13–15). Only one deficiency of the stabilizing protein, type 1aSP, has been reported (16), but as yet none of a deficiency of T3 (presumably type 1d). The importance of early diagnosis of these deficiencies has been highlighted by the discovery of type 1 glycogen storage disease(s) in cases of sudden infant death syndrome (12).

For type 1 glycogen storage diseases to be diagnosed with any certainty in early infancy, the normal developmental profile of the human hepatic proteins required for glucose-6-phosphatase activity must be known. However, the development of human hepatic glucose-6-phosphatase activity has not been determined because, until very recently, very large amounts of liver were needed for the complete kinetic analysis of microsomal glucose-6-phosphatase activity.

We have therefore used a microassay technique (17) and monospecific antibodies to individual proteins of the glucose-6-phosphatase complex to determine the developmental profile of human hepatic glucose-6-phosphatase activity from early in gestation to adulthood. The results demonstrate that the development of glucose-6-phosphatase activity in human liver is very different from the pattern previously found in rat liver (18, 19).

Materials and Methods

4-Chloro-1-naphthol, glucose 6-phosphate (monosodium salt), mannose 6-phosphate (monosodium salt), and pre-stained molecular-mass markers were obtained from the Sigma Chemical Co., Poole, Dorset, U.K. Biotinylated anti-sheep antibody and streptavidin-linked peroxidase complex were purchased from Amersham International PLC, Amersham, U.K. Empigen BB (N-laurel-N, N-di-methylglycine) was the kind gift of Albright and Wilson, Whitehaven, U.K. Nitrocellulose was obtained from Schleicher and Schuell, Dassell, F.R.G.

Liver Samples

Liver tissue was obtained from the following:

- Fetal abortuses (n = 92) from therapeutic termination of pregnancy (11–26 weeks' gestation) by extra-amniotic prostaglandins at the Simpson Memorial Maternity Pavilion, Edinburgh. Fetuses with malformations or chromosomal anomalies were excluded. All abortuses were refrigerated at 4°C until collection, within 3 h, for immediate dissection and subcellular fractionation at this site

- Premature infants (n = 33; 22–37 weeks' gestation) who died after delivery and as a consequence of immature pulmonary function, intraventricular hemorrhage, or necrotizing enterocolitis

- Term infants (n = 7; 37–42 weeks' gestation) who died of perinatal asphyxia, either in the second stage of labor or in the immediate newborn period

- Infants (n = 53) who died suddenly and unexpectedly in the first year of life

- Five infants who in the first year of life had a liver biopsy for investigation of hyperbilirubinemia or hypoglycemia. One infant was diagnosed as having Crigler–Najjar syndrome, one as having phosphofructokinase deficiency; no definite diagnosis was made in the remaining three cases.

- Adult samples (n = 80) were small portions of wedge or needle-biopsy samples obtained for the investigation or

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1 Centre for Perinatal and Paediatric Research, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, U.K.
2 Department of Child Life and Health, University of Edinburgh, Edinburgh EH3 9EF, U.K.
3 Address correspondence to this author at the Department of Obstetrics & Gynaecology, University of Dundee.

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treatment of the original condition for which the patient was referred. We assessed and scored liver histology as normal (grade 1) to severely diseased (grade 5) and then used only liver samples of grade 1.

Preparation and Assay of Human Hepatic Microsomes

Using fresh unfrozen hepatic needle or wedge biopsies, we prepared microsomes as previously described (14). Glucose-6-phosphatase activity was assayed at 30 °C in the human hepatic microsomal fractions with glucose 6-phosphate, mannose 6-phosphate, and pyrophosphate as substrates (17). Microsomes were disrupted with Histone 2A5 (20). Glucose-6-phosphatase activity is expressed as μmol/min per milligram of microsomal protein (U/mg). We assayed nonspecific hydrolysis of glucose 6-phosphate as described previously (21). Microsomes isolated from homogenates (untreated microsomes) are a mixture of intact microsomes, in which the membrane acts as a selective permeability barrier, and disrupted microsomes, in which the enzyme has free access to its substrates (22). We estimated the proportions of the two forms by assaying mannose-6-phosphatase activity (using 1 mmol of mannose 6-phosphate per liter as the substrate), which is expressed only in disrupted microsomes (22). All the intact values we report have been corrected for any microsomal disruption, as described previously (17), which eliminates the large errors in activity measurements that occur if even a small proportion of the vesicles are disrupted. Protein concentrations were measured by the method of Peterson (23).

Ethical approval for this study was given by the Paediatric/Reproductive Medicine Ethics of Medicine Research Sub-Committee of the Lothian Health Board and by the Tayside Health Board Ethical Committee.

Immunoblot Analysis

IgG was partially purified from antisera by (NH₄)₂SO₄ fractionation (24). For sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, we used 7–16% gels (25). Proteins separated on SDS–polyacrylamide gels were electrophoretically transferred to nitrocellulose as described by Towbin et al. (26) [in the presence of Empigen BB detergent, 10 mL/L (27), when we were immunodetecting T₉]. The Western blots were immunostained with sheep IgG previously shown to be monospecific for either the polypeptide containing the active site of the glucose-6-phosphatase enzyme (8, 28, 29) or the phosphate/pyrophosphate transport protein, T₉ (7). Immunoreactive polypeptides were made visible by a biotin–streptavidin peroxidase-linked detection system with 4-chloro-1-naphthol as the substrate (28).

Results

Human hepatic microsomal glucose-6-phosphatase protein and enzyme activity both appear at 11 weeks of gestation (Figure 1) and gradually increase with gestational age until term. Within the first three to four days after birth, in term infants, the glucose-6-phosphatase enzyme activity increases dramatically to adult concentrations (Figure 2), i.e., 0.30 U/mg of microsomal protein. Kₘ for the glucose-6-phosphatase enzyme remains constant throughout development (from 11 weeks' gestation to adult) at a mean value of 0.80 mmol/L (SEM 0.04, n = 222).

The activity of glucose-6-phosphatase in intact microsomes (Figure 3) is similar to that of the glucose-6-phosphatase enzyme (which is measured in disrupted microsomes; Figure 1), except that in each individual liver sample the Vₗₘₐₓ value in intact microsomes is always lower than the value in disrupted microsomes. In contrast, activity of the glucose-6-phosphatase enzyme measured with the alternative substrate pyrophosphate shows a somewhat different pattern of development in intact microsomes (Figure 4, bottom). In disrupted microsomes, the pattern is almost identical to that with glucose 6-phosphate as substrate (Figure 4, top).

Discussion

High concentrations of the glucose-6-phosphatase complex have been found only in liver, kidney, and pancreatic
Fig. 2. The development of the human hepatic microsomal glucose-6-phosphatase enzyme after birth
$V_{max}$ values in post-mortem infant samples (8) and infant liver biopsy samples (9) (see text)

Fig. 3. The development of the human hepatic microsomal glucose-6-phosphatase activity in intact microsomes during gestation

Fig. 4. The development of human fetal hepatic microsomal phosphatase activity measured with pyrophosphate substrate
(Top) Activity in disrupted microsomes and (bottom) in intact microsomes
deficiencies of the transport proteins of the glucose-6-phosphatase complex.

Disruption of hepatic microsomes removes the controlling influence of transport proteins, $T_1$, $T_2$, and $T_3$, and allows direct measurement of the activity of the glucose-6-phosphatase enzyme. In disrupted rat liver microsomes, we previously showed that the glucose-6-phosphatase enzyme can be first demonstrated five days before birth (17 days' gestation) and that concentrations remain low until birth (19). Therefore in the rat, glucose-6-phosphatase develops in the late fetal cluster of enzymes (34, 35). In contrast, we have found (Figure 1) that the glucose-6-phosphatase enzyme in human liver first appears as early as 11 weeks' gestation, when the liver first starts to produce glucose (36). At term, hepatic glucose-6-phosphatase enzyme activity in humans increases rapidly and reaches adult values at about three to four days after birth (Figure 2). In the rat during the first few days after birth, the activity of glucose-6-phosphatase overshoots to concentrations several times higher than adult values (18). In human liver the $K_m$ of glucose-6-phosphatase enzyme stays constant from 11 weeks of gestational age to adulthood. This, together with the fact that the increasing concentrations of enzyme protein match its increasing activity (Figure 1, bottom), suggests that all the activity changes reflect the presence of different amounts of the polypeptide containing the active site of the glucose-6-phosphatase enzyme.

In intact microsomes, assays with glucose 6-phosphate as substrate are a measure of the combined rates of the glucose-6-phosphatase enzyme and the three transport pro-
Transport is rate limiting, and \( T_2 \) has been demonstrated to be the transport protein that is rate limiting (37) in the assay conditions used here. The activity values in Figure 2, therefore, reflect the combined values of \( T_2 \) and the enzyme. The values are similar but somewhat lower than those in Figure 1 (top). This merely reflects that \( T_2 \) is rate limiting and suggests that \( T_2 \) and the enzyme develop at the same time and at the same rate.

In disrupted microsomes the measurement of glucose-6-phosphatase enzyme activity with pyrophosphate as substrate (Figure 4, top) gives, as expected, very similar results to Figure 1 (top). In contrast, in intact microsomes assayed with pyrophosphate substrate, the values obtained are the combined rates of \( T_2 \) and the enzyme. Because \( T_2 \) is rate limiting with this substrate, the values reflect the transport capacity of \( T_2 \) (Figure 4, bottom). Although Figure 4 (bottom) appears to have fewer data points than (e.g.) Figure 1 (top), most of the values early in gestation are zero or extremely close to zero, so that many points coincide near the origin. The data in Figure 4 (bottom) suggest that \( T_2 \) develops later in gestation than the other proteins of the glucose-6-phosphatase system and that, in most cases, appreciable concentrations of \( T_2 \) start to appear only between 20–24 weeks of gestation, which is when the human fetal liver production of glucose reportedly reaches full activity (36). Immunoblot analysis with antibodies monospecific for \( T_2 \) demonstrate that the \( T_2 \) protein is absent from human fetal liver samples where \( T_2 \) capacity is zero (data not shown).

The ontogenies of the rat and human hepatic glucose-6-phosphatase systems are very different. Because of the large changes in glucose-6-phosphatase activity that occur in the first few days after birth, diagnosis of type 1 glycogen storage disease should not be attempted in the first week after birth. However, because glucose-6-phosphatase enzyme activity remains constant after the first week until adulthood, diagnosis of type 1a glycogen storage disease after birth is much easier than previously thought (based on developmental studies in the rat). Recently Golbus et al. (33) have suggested that prenatal diagnosis of type 1a glycogen storage disease is possible with use of fetal liver biopsy samples. Our study shows that the range of glucose-6-phosphatase enzyme activity is quite wide in prenatal liver samples (Figure 1, top). Therefore, we suggest that prenatal diagnosis of type 1a glycogen storage disease should not be attempted unless the laboratory has developed a reference range based on a very large number of age-matched control samples. In addition, care should be taken in diagnosing type 1c glycogen storage disease, because the \( T_2 \) protein develops considerably later than the other glucose-6-phosphatase proteins. We recommend that prenatal diagnosis of this latter disorder not be attempted with fetal liver biopsy samples.

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