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ORIGINAL ARTICLE

Manifestations of Fabry disease in placental tissue

A. C. Vedder · A. Strijland · M. A. vd Bergh Weerman · S. Florquin · J. M. F. G. Aerts · C. E. M. Hollak

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Summary Fabry disease is an X-linked lysosomal storage disorder caused by deficiency of the lysosomal enzyme α-galactosidase A. Manifestations of the disease in placental tissue have been reported only twice. We report for the first time on the biochemical, histological and genetic features of two cases: placenta A derived from a mother heterozygous for Fabry disease who gave birth to a hemizygous son, and placenta B obtained from a healthy mother who carried a heterozygous daughter. Biopsies of placentae A, B and of four healthy controls were taken directly after birth. Assessment of α-galactosidase A (α-Gal) activity was performed both in fetal leukocytes (derived from umbilical cord blood) and in the biopsy specimens. The tissue was further examined by electron microscopy, immunohistochemistry and biochemical analysis for the presence of storage material (ceramide trihexoside (CTH)). In placenta A, characteristic zebra bodies reflecting accumulated storage material were seen in all biopsies evaluated. CTH values were markedly elevated as compared to the controls and α-Gal activity in both fetal leukocytes and placental tissue was very low. Placenta B showed no storage material at all. CTH values were within the control range. α-Gal activity ranged from intermediate to near normal; enzyme activity in fetal leukocytes was significantly decreased. As placental tissue is mainly derived from fetal cells, we may conclude that, in a boy suffering from Fabry disease, extensive storage of CTH is already present at birth. As complications develop only around the age of 10 years, it may be not the CTH itself but secondary processes that cause cellular and organ damage.

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A. C. Vedder (✉)
Department of Internal Medicine/Endocrinology and Metabolism, Academic Medical Center, Amsterdam, The Netherlands;
Department of Biochemistry, Academic Medical Center, Amsterdam, The Netherlands
e-mail: a.c.vedder@amc.uva.nl

C. E. M. Hollak
Department of Internal Medicine/Endocrinology and Metabolism, Academic Medical Center, Amsterdam, The Netherlands

A. Strijland · J. M. F. G. Aerts
Department of Biochemistry, Academic Medical Center, Amsterdam, The Netherlands

M. A. vd Bergh Weerman · S. Florquin
Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands

Fabry disease (McKusick 301500) is an X-linked lysosomal storage disorder caused by deficiency of the lysosomal enzyme α-galactosidase A (α-Gal A) (Brady et al 1967; Kint 1970). Symptoms and complications occur owing to the accumulation of specific glycosphingolipids (mainly globotriaosylceramide (GL-3 or CTH)) in lysosomes of many cells in the body. Classically, males with Fabry disease present with acroparaesthesias, angiokeratoma and reduced sweat function at young age. Later in life they develop complications mainly of vascular origin comprising progressive renal insufficiency, cardiac infarction or hypertrophy, arrhythmias and cerebral infarctions (Desnick et al 2001). Females tend to show a more protracted course, but may suffer from severe complications as well. The disease is rare, with an incidence around 0.2–0.8 per 100 000 newborns (Meikle et al 1999; Poorthuis et al 1999). There is a striking variance in the severity of symptoms and complications between patients, even within monozygotic twins (Redonnet-Vernhet et al 1996). No clear genotype–phenotype correlation has been demonstrated, although it is suggested that patients with no residual

enzyme activity may suffer from renal insufficiency earlier than those with residual α-Gal A activity (Branton et al 2002). Enzyme supplementation therapy has been available for a few years, resulting in a decrease of CTH accumulation in renal, cardiac or skin tissue (Eng et al 2001; Schiffmann et al 2001) and some symptomatic improvement. It is believed that reducing or clearing the amount of storage will halt progression of the disease. However, the relation between the amount and localization of storage and disease complications is unknown. It is possible that there is already extensive storage of CTH early in life or even prenatally, while symptoms develop much later. To the best of our knowledge, manifestations of Fabry disease in placental tissue have been reported only twice (Elleder et al 1998; Popli et al 1990). In the first case report, Popli and colleagues describe a placenta of a heterozygous mother who carried a healthy child. Electron-microscopic examination showed densely packed, concentric lamellar inclusions, characteristic for Fabry disease in the placental decidual cells. The fetal portion of the placenta was reported to be free of storage material. Early storage in the fetal part of the placenta has been described for a male Fabry fetus (Elleder et al 1998), but relationship with genetic abnormalities, residual enzyme activity and biochemical measures of storage has so far not been reported.

In the current study, we report for the first time on the biochemical, histological and genetic features of two cases: placenta A is derived from a mother heterozygous for Fabry disease who gave birth to a hemizygous son, and placenta B is obtained from a healthy mother who carried a heterozygous daughter.

Cases and methods

Cases

Placenta A is derived from a 23-year-old woman diagnosed as heterozygous for Fabry disease in 1994 following a positive family history for this disease. She suffered from acroparaesthesias from her early teens. So far, she developed no further complications, and no enzyme therapy has been initiated. Enzyme analysis in leukocytes showed α-Gal A activity in the normal range (26.2 nmol/h per mg protein; reference 17–55). Mutationanalysis of the α-Gal A gene revealed a T deletion at position 7299 resulting in a frameshift.

After an uneventful pregnancy this woman delivered a son; she gave consent for histological and biochemical studies of the placental tissue. The placenta weighed 575 g, had a centrally inserted umbilical cord and showed no macroscopic abnormalities. Blood of the umbilical cord was collected immediately after birth, revealing a leukocyte α-Gal A activity of 2.4 nmol/h per mg protein (reference 33–77), confirming Fabry disease.

Placenta B is derived from a nonaffected mother who had been carrying the child of a male hemizygous for Fabry disease. The 39-year-old father was diagnosed with Fabry disease at the age of 23 years following renal biopsy for undiagnosed renal failure that showed severe crescentic glomerulonephritis as well as lysosomal deposits, suggestive for α-Gal A deficiency. Leukocyte enzyme activity was 0.8 nmol/h per mg protein (reference 17–55). Mutation analysis showed an A- to-G base substitution at postion 10601, resulting in an (asparagine to serine) amino acid substitution at postion 298. As his renal function deteriorated, enzyme replacement therapy was started in November 2002.

A daughter was born following vacuum extraction because of fetal distress. The pregnancy had been uneventful. Since Fabry disease is inherited in an X-linked pattern, the girl was considered to be a carrier. This was confirmed by enzyme analysis, revealing a decreased α-Gal A activity in leukocytes obtained from umbilical cord blood (24.2 nmol/h per mg protein; reference 33–77). The mother gave consent for histological and biochemical studies of the placental tissue. The placenta weighed 525 g, had a centrally inserted umbilical cord and showed no abnormalities.

Placentae

Directly after birth, blood of the umbilical cord was collected for assessment of α-Gal A activity in fetal leukocytes. The placentae of the two patients and of four healthy controls were examined and weighed. Biopsies of 1 cm^3 were taken from the cord, and the maternal and fetal parts of the placenta and were either stored at −80°C or fixed in Karnovsky's fixative for electron microscopy (EM).

Electron microscopy

Biopsies were analysed electron-microscopically for the presence of storage material. After fixation, the material was postfixed in 1% osmium tetroxide, block-stained with 1% uranyl acetate, one-step dehydrated in dimethoxypropane and embedded in epoxy resin LX-112. Light microscopy sections were stained with toluidine blue. EM sections were stained with tannic acid, uranyl acetate and lead citrate, followed by examination in a Philips CM10 (FEI).

Immunohistochemistry

The possible accumulation of CTH was examined by immunohistochemistry. Cryostat sections (4 μm) were fixed in acetone and pre-incubated with 10% normal goat serum, followed by incubation with rat IgM monoclonal anti-CTH antibody (clone 38-13, Immunotech, Marseille, France) overnight at 4°C. Endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 in methanol for

20 min. Subsequently, antibody binding was detected with PowerVision Poly-HRP-conjugated goat antibodies specific for rat IgM (ImmunoVision Technologies, ImmunoLogic, Duiven, The Netherlands). Peroxidase activity was detected with 3-amino-9-ethylcarbazole (Sigma St. Louis, MO, USA) and 0.03% H_2O_2 . Negative controls were performed by replacing the first-step antibody by isotype-matched and species-matched antibodies that do not bind to human tissue (Dako, Glostrup, Denmark).

Lipid extraction and analysis

Samples were stored at −80°C before use. Glycolipid was extracted from the placental tissue according to Bligh and Dyer (Bligh et al 1959) following homogenization of the tissue by grinding with a mortar and pestle at liquid nitrogen temperature and sonification. The chloroform phase was applied to a 500 mg chloroform-equilibrated LiChrolut RP18 column (Merck). Bound glycolipids were eluated with acetone–methanol (9:1) v/v and dried under nitrogen. CTH was isolated on HPTLC (HP-TLC-silicagel 60) with development solvent of $CHCl_3$–MeOH–H_2O (50:21:3 by vol). Lipids were identified with orcinol staining and quantified using reference standards by density scanning (Scanning Quantity One) and expressed as nmol/mg of protein measured by a bicinchoninic acid (BCA) assay (Smith et al 1985).

Enzyme activity assay

The enzymatic activity of α-galactosidase A was measured as described (Mayes et al 1981). Briefly, 20 µl of sample was incubated at 37°C for 1 h with 100 µl reaction mixture, containing 4-methylumbelliferyl-α-D-galactopyranoside (4-MU-galactopyranoside; Sigma) at a final concentration of 3.5 mmol/L in 100 mmol/L citrate–200 mmol/L phosphate buffer pH 4.6. *N*-Acetylgalactosamine (100 mmol/L) was included in the reaction mixture to inhibit α-*N*-acetylgalactosaminidase activity. Reactions were terminated by the addition of 2.5 ml 300 mmol/L glycine–NaOH buffer, pH 10.6 and fluorescent 4-methylumbelliferone was measured with a fluorimeter (Perkin-Elmer, Norwalk, CT, USA) at 366/445 nm.

Results

Electron microscopy

In placenta A, storage material (zebra bodies) was present in cells of all biopsies evaluated. Lamellar inclusions were seen in endothelial cells (Figure 1A) as well as vascular smooth-muscle cells (Figure 1B) of both the vein and artery of the umbilical cord. In addition, depositions were seen in pericytes of both the maternal (Figure 1C) and the fetal placental tissue. In placenta B, however, no storage material was seen in either the placental tissue or the umbilical cord after extensive analysis (not shown).

Immunohistochemistry

Immunohistochemistry confirmed the electron-microscopical findings, showing positive staining for CTH in placenta A (Figure 1D) and absence of storage material in placenta B (Figure 1E). Figure 1F shows a control placenta.

CTH in placenta and umbilical cord

CTH values in placental and cord tissue of the hemizygous child were significantly higher (11.5 and 16.1 nmol/mg, respectively) than values measured in control tissue (ranging from 4.2 to 5.9 nmol/mg in placental tissue and from 5.9 to 10.2 nmol/mg in cord tissue) (Figure 2). However, CTH in tissue of the heterozygous girl was not elevated (5.4 and 9.0 nmol/mg, respectively).

α-Galactosidase A activity

Enzyme activity was very low in both placental and cord tissue of the hemizygous child (1.2 and 0.1 nmol/h per mg, respectively) compared to tissue from healthy controls (ranging from 10.3 to 13.5 nmol/h per mg for placental tissue and from 3.6 to 9.9 nmol/h per mg for cord tissue) (Figure 3). Analysis of tissue of the heterozygous girl, however, revealed decreased activity in placental tissue (6.5 nmol/h per mg) but near-normal level of activity in cord tissue (3.3 nmol/h per mg).

Discussion

We found that extensive storage and very low α-Gal A activities are present in placental tissue of a mother heterozygous for Fabry disease who gave birth to a hemizygous son. In contrast, no storage material and a slightly decreased enzyme activity was seen in the placenta obtained from a healthy mother who had been carrying a heterozygous girl.

The storage seen in placenta A probably reflects the presence of CTH storage in the organs and tissues of the hemizygous child. The fact that extensive storage is already present at an early age is supported by previous case reports describing storage of CTH in corneal (Tsutsumi et al 1984) and

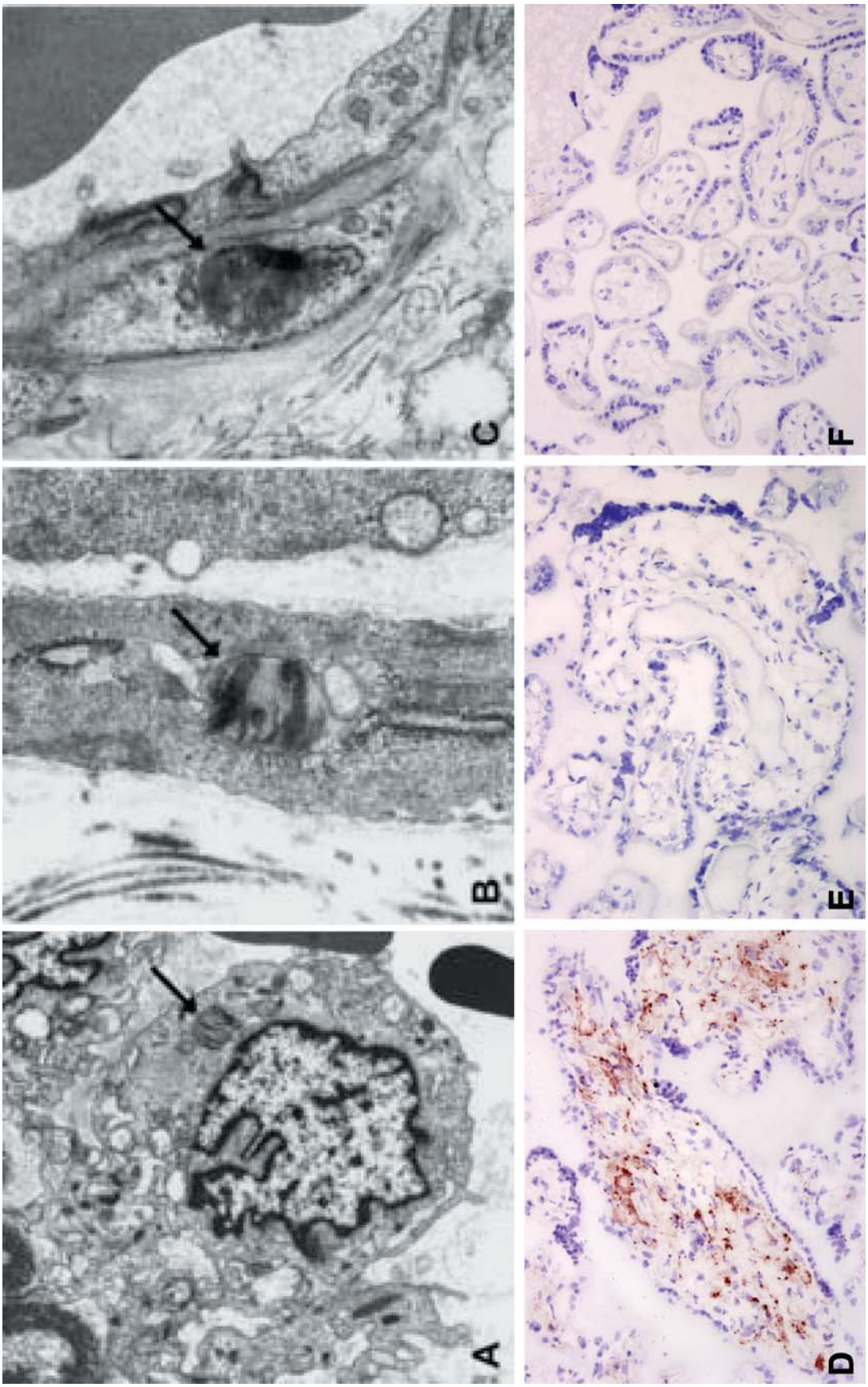

Fig. 1 (A) Endothelial cell with zebra body (arrow), periodicity 5 nm. (Original magnification ×18 000). (B) Vascular smooth-muscle cell with zebra body (arrow) in vein of umbilical cord. (Original magnification ×80 000). (C) Maternal placental tissue; capillary with pericyte showing storage material (arrow). (Original magnification ×32 000). (D–F) Immunohistochemistry showing positive staining for CTH in placenta A (D) and absence of storage material in placenta B (E) and control tissue (F). (Original magnification ×200).

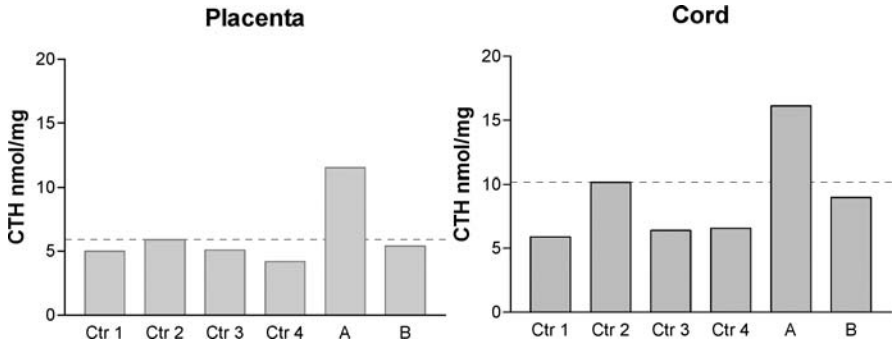

Fig. 2 CTH values in placental and cord tissue

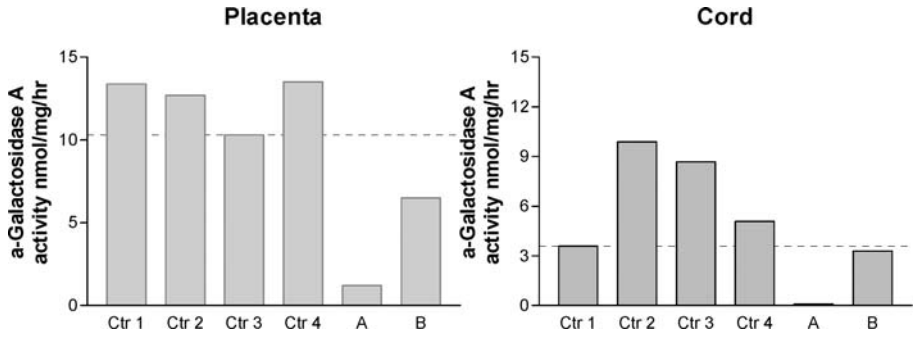

Fig. 3 α-Galactosidase A activity in placental and cord tissue

renal fetal tissue (Elleder et al 1998). If it is hypothesized that the amount of storage is directly related to pathology, one might argue that enzyme therapy should already be initiated prenatally in a mother carrying a hemizygous fetus, resulting in administration of the enzyme product through the mother. However, no information is available on enzyme therapy during pregnancy and it is not clear whether the enzyme (product) passes the placental barrier.

A different, more plausible, explanation could be that CTH itself does not cause major cellular or organ damage. As signs and symptoms in a hemizygous patient start to evolve only around the age of 10 years, it is hypothesized that the presence of CTH results in activation of secondary processes causing the complications seen in Fabry disease.

Since enzyme therapy has shown to effectively reduce storage of CTH, these findings prompt the question of when to start enzyme therapy. We assume that early start of treatment will prevent development of complicatons later in life, although no long-term data are yet available. Ethical aspects need to be weighed carefully. Is it justifiable to start biweekly infusions in a young male who is not expected to suffer from complaints before his early teens? It seems clear that establishing storage of CTH in tissue samples does not in itself justify the initiation of therapy.

Placenta B showed no storage material at all. This may be either the result of the residual α-Gal A activity present in the female fetus or the effect of transport of maternal α-Gal A through the placenta. A definite conclusion that there is no storage of CTH in the tissues of the female fetus cannot be made, however, since in heterozygous females the process of lyonization defines the residual enzyme activity present. This process, in which one of the two X chromosomes in each cell is randomly inactivated early in embryonic development (Lyon 1963), may have influenced the residual α-Gal A activity in the placenta.

Our findings suggest the presence of extensive storage in a hemizygous boy at very early age. As complications only develop around the age of 10 years, it may be not the CTH itself but the secondary processes that cause cellular and organ damage. Future studies may elucidate pathophysiological mechanisms that will ultimately guide in the correct timing of initiation of enzyme therapy.

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