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Plasma chitotriosidase and CCL18: Early biochemical surrogate markers in type B Niemann–Pick disease

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Summary: Type B Niemann–Pick disease (NPD) is a nonneuronopathic lysosomal storage disorder which is characterized by accumulation of sphingomyelin-laden macrophages. The availability of plasma markers for storage cells may be of great value in facilitating therapeutic decisions. Given the similarity of the storage cells in NPD and Gaucher disease, we studied Gaucher plasma markers (chitotriosidase and CCL18) in two siblings homozygous for the R228C mutation in acid sphingomyelinase (ASM) and a type B course of NPD. The older sibling, first examined at the age of 9 months, showed marked hepatosplenomegaly and pulmonary involvement. The younger sibling has mild asymptomatic hepatosplenomgaly at the age of 5 months. Analysis of plasma specimens revealed markedly increased levels of chitotriosidase and CCL18 in the older sibling. In the younger child also, plasma chitotriosidase and CCL18 were clearly elevated above normal values almost immediately after birth and rapidly increased further. Histochemistry confirmed production of CCL18 by foam cells. In conclusion, plasma chitotriosidase and CCL18 may also serve as markers for the formation of pathological lipid-laden macrophages in type B NPD, in analogy to Gaucher disease. The availability of sensitive plasma surrogate markers may be of great value for monitoring the efficacy of enzyme supplementation therapy that is currently being developed.

A reduced acid sphingomyelinase (ASM; EC3.1.4.12) activity results in lysosomal storage of sphingomyelin, particularly in the monocyte–macrophage system (Schuchman and Desnick, 1995). Two different disease manifestations are caused by inherited ASM deficiencies: type A Niemann–Pick disease (NPD) and type B

NPD (McKusick 607616). In type A NPD the residual ASM activity is generally less than 5% of normal, whereas in type B NPD patients residual enzyme activity may be slightly higher. Whereas type A NPD is invariably a fatal disorder of early infancy characterized by failure to thrive and a rapid progressive neurodegenerative course, type B shows a wide and heterogeneous spectrum of clinical manifestations. Characteristic symptoms are hepatosplenomegaly and progressive pulmonary complications. The histological hallmark of types A and B NPD is the lipid-laden foam cell, often referred to as the Niemann–Pick cell. A similar type of storage cell accumulates in tissues of patients suffering from Gaucher disease (lysosomal glucocerebrosidase deficiency). Gaucher cells are also derived from tissue macrophages and massively accumulate glucosylceramide. In contrast to Gaucher cells, the slightly smaller Niemann–Pick cells stain negative with PAS reagent but do stain positive for lipids with Sudan black and Oil red (Schuchman and Desnick, 1995).

The elucidation of the molecular basis of type B NPD and the cloning of the ASM gene (Quintern et al 1989) have further stimulated the long-standing efforts by Desnick, Schuchman and co-workers to develop a therapeutic intervention for the nonneuronopathic form of NPD. A highly effective treatment has been developed for type I Gaucher disease that is based on chronic intravenous supplementation with recombinant glucocerebrosidase (Barton et al 1991). Targeting of therapeutic enzyme to macrophages in Gaucher patients is promoted by the use of glucocerebrosidase modified in its glycans to expose terminal mannose moieties, favouring uptake by mannose receptor-containing monocytes–macrophages. A similar approach is currently being developed for type B NPD using recombinant human sphingomyelinase (Miranda et al 2000).

The experience with Gaucher disease has demonstrated the importance of surrogate markers of disease that can assist in clinical decision making (Aerts et al 2003; Hollak et al 2001). In Gaucher patients' plasma, markers for the presence of storage cells have been found, such as a 1000-fold increased activity of chitotriosidase (Hollak et al 1994). *In situ* hybridization and histochemical analyses have demonstrated that chitotriosidase is synthesized by activated (lipid-laden) macrophages in Gaucher disease (Boot et al 1995). Besides clinical symptoms, plasma chitotriosidase helps guide the decision making on initiation of therapeutic intervention, monitoring of efficacy of therapy and optimization of individual dosing regimens (Aerts et al 2003; Hollak et al 2001). A serious drawback of chitotriosidase as surrogate disease marker is the relatively common deficiency of the enzyme (Boot et al 1998). Very recently, another Gaucher cell marker, CCL18, has been identified by a proteomics-based survey of serum specimens of symptomatic Gaucher patients (Boot et al 2004). Increased expression of CCL18 mRNA had earlier been observed in an investigation on differentially expressed genes in spleen of Gaucher patients (Moran et al 2000). The chemokine CCL18, which is massively released by Gaucher cells, is about 50-fold increased in plasma and can serve as a useful alternative surrogate marker for Gaucher cells in those patients who lack chitotriosidase for genetic reasons. Elevated levels of chitotriosidase in NPD have been described in two studies (den Tandt and van Hoof 1996; Guo et al 1995), although the relationship with clinical disease parameters has not been investigated.

Given the similarity between the Gaucher cell and the Niemann–Pick storage cell, we have investigated in detail two siblings with biochemically and genetically confirmed type B NPD. It is demonstrated that plasma chitotriosidase and CCL18 can serve as surrogate markers for the presence of pathological cells in type B NPD. The availability of suitable plasma markers for storage cells should help in developing and optimizing enzyme therapy for type B NPD. Moreover, plasma chitotriosidase and CCL18 could be important tools to document the natural course of type B NPD.

PATIENTS AND METHODS

Patients: Two siblings were diagnosed with type B NPD; the older sibling was diagnosed at the age of 9 months and the younger sibling directly after birth, as antenatal screening was refused by the parents. The patients have now been followed for periods of 16 months and 5 months, respectively, by monthly investigations.

The diagnosis of NPD in both children was established by a markedly reduced acid sphingomyelinase activity in leukocytes and fibroblasts, determined with both the ^{14}C-labelled natural substrate sphingomyelin and the substrate 6-hexadecanoylamino-4-methylumbelliferyl-phosphorylcholine (HMU), and was confirmed by ASM mutation analysis. Additional laboratory measurements included liver enzymes (transaminases) and haematological parameters. A chest radiograph was examined in both children. The sizes of liver and spleen were obtained ultrasonographically by measuring the maximal liver height in the midclavicular line and in the plane crossing the vena cava inferior and by determining the longest diameter of the spleen. In the older child, a bone marrow aspiration was performed at the time of diagnosis and at the age of 2 years.

Laboratory assays: Chitotriosidase activity was measured using 4-methylumbelliferyl-β-D-chitotriose as substrate as described (Aguilera et al 2003). Hexosaminidase was measured using 4-methylumbelliferyl-β-D-*N*-acetylglucosaminide as substrate (de Groot et al 1980). Chemicals were obtained from Sigma, St Louis, MO, USA. Plasma CCL18 was determined by ELISA (Biosource, Camarillo, CA, USA) as described earlier (Boot et al 2004).

Normal values of CCL18 and chitotriosidase were obtained for different age groups (0–1 year, 1–3 years, 3–5 years). Blood samples were obtained from children referred to the Academic Medical Centre for diagnostic purposes not related to lysosomal storage diseases and after informed consent.

Samples of aspirated bone marrow were suspended in RPMI medium. After removal of erythrocytes by centrifugation of the suspension over a Ficoll density gradient, cytospins were prepared of the remaining bone marrow cells. Cytospins were dried in air and subsequently fixed in acetone. After preincubation in 10% normal goat serum, the samples were incubated with mouse monoclonal antibodies directed against CCL18. Endogenous peroxidase activity was blocked by incubation with 0.03% H_2O_2 and 0.1% NaN_3. The slides were incubated with rabbit anti-mouse

IgG. Binding of rabbit antibodies was detected by subsequent incubation with goat anti-rabbit IgG-poly-HRP antisera (Immunologic, Duiven, The Netherlands), followed by incubation with 3-amino-9-ethylcarbazole. The nucleated cells were counterstained using haematoxylin. In addition, cytospins were stained according to Giemsa.

RESULTS

Case presentation

Case 1: Patient 1, the first child of second cousins of Turkish ancestry, presented at the age of 9 months with feeding problems. The family history was unremarkable. On physical examination, the girl showed marked hepatosplenomegaly. Psychomotor development was normal and no neurological abnormalities were observed.

Additional laboratory investigation revealed an elevation of AST (1796 U/L) and ALT (1402 U/L). In a bone marrow film, lipid-laden foam cells were observed, typical for NPD. Leukocyte ASM was 0.05 nmol/h per mg using the natural substrate (normal values 1.2–5.2 nmol/h per mg) and 4.6 nmol/17 h per mg using the HMU substrate (normal values 10–54 nmol/17 h per mg). Fibroblast ASM was undetectable and 10 nmol/h per mg for the natural substrate and HMU substrate, respectively (normal values 106–420 and 200–500 nmol/h per mg). ASM deficiency was confirmed by DNA analysis of the ASM gene, revealing a homozygous R228C mutation. This mutation has previously been described in a patient with type B NPD (Simonaro et al 2002).

Table1 Plasma levels of chitotriosidase, CCL18 and hexosaminidase in patients 1 and 2, in comparison to the median level and range in controls

Age[a]	*Chitotriosidase* (nmol/h per ml)	*CCL18* (ng/ml)	*Hexosaminidase* (nmol/h per ml)
Case 1			
1 y 7 mo	2700	1046	3004
1 y 11 mo	2876	1204	2620
2 y 1 mo	3121	1136	2416
2 y 2 mo	2762	1154	2473
Controls 1–3 years ($n = 4$)	49 (41–59)	40 (35–44)	1099 (734–2142)
Case 2			
1 mo	408	–	2122
2 mo	284	99	1596
4 mo	641	227	1561
5 mo	1018	340	1603
Controls 0–12 months ($n = 4$)	52 (21–92)	21 (10–37)	1550 (1034–2278)

[a] y, year(s); mo, month(s)

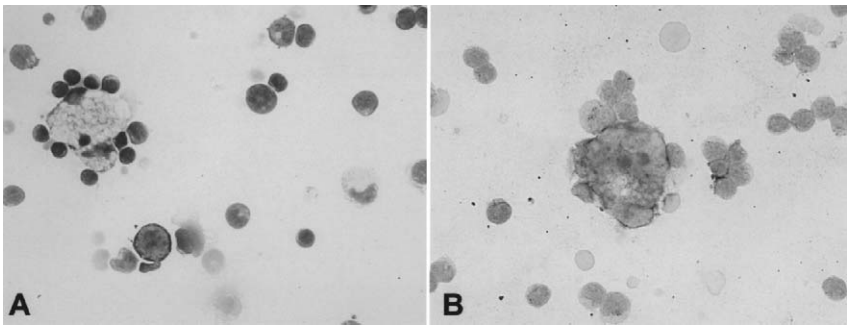

Figure 1 Immunohistochemical staining of foam cells with anti-CCL18 antibodies. Bone marrow aspirate was obtained and histochemistry was performed as described in Patients and Methods. A: Giemsa staining; B: Immunohistochemical detection; original magnification × 40.

At the age of 2 years, psychomotor development is still normal. The size of the spleen has been progressively increasing, whereas the liver size has remained relatively stable. Chest radiography demonstrated diffuse reticulonodular pulmonary infiltration, without clinical signs of dyspnoea. The child suffers from episodic thrombocytopenia, occasionally with a concomitant leukopenia and anaemia. Because of the pancytopenia, a bone marrow aspiration was repeated at the age of 2 years.

Case 2: Patient 2, the second child of the same parents, was born after an uncomplicated pregnancy and delivery. The parents refused antenatal screening. Postnatal leukocyte ASM was 0.1 nmol/h per mg (natural substrate) and 2.3 nmol/17 h per mg (HMU substrate). DNA analysis revealed the same homozygous R228C mutation. Currently, at the age of 5 months, the child is developing well and on physical examination only mild hepatosplenomegaly is observed, which was confirmed by abdominal ultrasound.

Biochemical analysis

Analysis of the plasma specimens revealed elevated levels of chitotriosidase and CCL18 in both children (Table 1). The first sample in the younger child was obtained at the age of 1 month and already showed clearly elevated levels, while the child was still completely asymptomatic. At the age of 4 months, plasma chitotriosidase and CCL18 rapidly increased further in this child (Table 1). However, levels are still below those of chitotriosidase and CCL18 in samples of the older sister. Her plasma chitotriosidase and CCL18 have remained stable over time from the age of 1.7 years (Table 1).

A bone marrow aspirate was obtained from the older patient and analysed by immunohistochemistry with anti-CCL18 antibodies. As shown in Figure 1, foam cells stained strongly positive with the antibody specifically directed towards CCL18.

DISCUSSION

Chitotriosidase and CCL18 are massively produced and secreted by lipid-laden macrophages in Gaucher patients, resulting in markedly increased plasma levels. A more than 100-fold increase in plasma chitotriosidase was also observed in cholesteryl ester storage disease, another condition characterized by accumulation of lipid-laden histiocytes. Previous studies indicated that plasma chitotriosidase is also abnormally high in type B NPD (den Tandt and van Hoof 1996; Guo et al 1995). Our present study confirms this observation. In plasma of two siblings with type B NPD, chitotriosidase was clearly elevated above normal values. The increase was most pronounced in the older child, who had already developed marked hepatosplenomegaly. In the younger child who was still virtually free of any clinical signs, an abnormally high plasma chitotriosidase was already detected at the age of 1 month. During the relatively short period of evaluation of the siblings, an increase in plasma enzyme level was noted in the younger child, while the level appeared stable in the older child.

By analogy with Gaucher disease, plasma CCL18 was increased together with chitotriosidase. This suggests that NP storage cells, like Gaucher cells, also secrete the chemokine CCL18. In view of this finding and the supposed role for CCL18 in promoting interaction of T cells, B lymphocytes and antigen-presenting cells, it will be of interest to investigate more closely the incidence of gammopathies in type B NPD patients. In Gaucher disease, gammopathies occur commonly and may in some cases develop into myeloma or eventually cause lethal amyloidosis. Indeed, one case of combined amyloidosis and type B NPD has been reported in the literature (Zhou et al 1995). Immunohistochemistry on a bone marrow aspirate of the older sibling with type B NPD revealed expression of CCL18 in foam cells, similar to that in Gaucher cells.

Our observations suggest that Niemann–Pick storage cells, like Gaucher cells, secrete chitotriosidase and CCL18 into the circulation. The overproduction of chitotriosidase and CCL18 by storage macrophages is almost a universal phenomenon. For example, in affected siblings from families with cholesteryl ester storage disease (CESD) prominent elevations in plasma CCL18 (A. Groener, unpublished observations) and chitotriosidase (vom Dahl et al 1997) were observed. A full survey of plasma chitotriosidase and CCL18 abnormalities in patients suffering from various storage disorders seems warranted. It is far from clear whether glucosylceramide itself is directly involved in this mechanism. A direct role for glucosylceramide in chitotriosidase expression cannot be excluded on the basis of the present findings since secondary accumulation of glucosylceramide and gangliosides in type B NPD has been described (Vanier 1983). Accumulation of glycosphingolipids has also recently been reported for atherosclerotic foam cells, which are also known to produce chitotriosidase (Boot et al 1999).

Our study suggests that plasma chitotriosidase and CCL18 may be of value in monitoring lipid storage cells in type B NPD. It will be of particular interest to document carefully, in a larger series of type B NPD patients, the changes in plasma chitotriosidase and CCL18. This would provide valuable new information on the

natural course of this disorder. Moreover, plasma chitotriosidase and CCL18 can most likely serve as important surrogate disease markers that will assist the development and optimization of treatment by enzyme supplementation therapy.

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