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Mini Review

Niemann–Pick disease type C

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Niemann-Pick disease type C (NPC) is an autosomal recessive neurovisceral lipid storage with a wide spectrum of clinical phenotypes. At the cellular level, the disorder is characterized by accumulation of unesterified cholesterol and glycolipids in the lysosomal/late endosomal system. Approximatively 95% of patients have mutations in the *NPC1* gene (mapped at 18q11) which encodes a large membrane glycoprotein primarily located to late endosomes. The remainder have mutations in the *NPC2* gene (mapped at 14q24.3) which encodes a small soluble lysosomal protein with cholesterol-binding properties. The identical biochemical patterns observed in NPC1 and NPC2 mutants suggest that the two proteins function in a coordinate fashion. Identification of mutations revealed a complex picture of molecular heterogeneity, allowing genotype - phenotype correlations for both genes and providing insights into structure - function relationships for the NPC1 protein. Although a whole body of evidence suggests that the NPC1 and NPC2 proteins are involved in the cellular postlysosomal/late endosomal transport of cholesterol, glycolipids and other cargo, their precise functions and relationship remain unclear and are currently the subject of intense investigation. These studies, conducted in various models, should ultimately lead to a better understanding of the pathophysiology of NPC and new therapeutic approaches.

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The eponym 'Niemann–Pick disease' encompasses a heterogeneous group of lysosomal lipid storage diseases with autosomal recessive inheritance. Based on the clinical and biochemical study of 18 cases, Crocker proposed in 1961 a classification into four subgroups, types A–D (1, 2). Patients with type A showed a severe neurovisceral disease, while patients with type B had a chronic course with visceral involvement only. The common massive accumulation of sphingomyelin found in extraneural organs of these patients (1) and the later demonstration of a generalized deficiency of acidic (lysosomal) sphingomyelinase (3, 4) established types A and B as primary sphingomyelin storage disorders. Patients with types C and D had a subacute neurological involvement, less-pronounced organomegaly than type A, and, above all, a milder level of lipid storage and no generalized sphingomyelinase deficiency (1, 2, 4). Type D was delineated from the common Acadian ancestry of patients otherwise undistinguishable from type C (and later shown to be allelic to type C) (1, 2).

The metabolic basis of types C and D Niemann–Pick disease remained for a long time an enigma and is not yet fully elucidated, in spite of a reclassification of the disease as a cellular lipid trafficking disorder, following seminal studies by the group of Peter Pentchev in the early 1980s (5, 6). Today, the denomination 'Niemann–Pick disease type C' designates disorders characterized by unique abnormalities of intracellular transport of endocytosed cholesterol with sequestration of unesterified cholesterol in lysosomes and/or late endosomes (5–10). Major advances in the elucidation of the disease have been the description of two genetic complementation groups (11, 12) and the subsequent isolation of the two underlying genes. *NPC1*, located to 18q11, is involved in most families, including those with type D (13, 14). *HE1*/*NPC2*, located to 14q24.3, is involved in very rare families (15–17). Although the precise functions of the NPC1 and NPC2 proteins are still elusive, current knowledge supports the idea that these proteins function at two close steps of the same pathway and that they are involved in the

cellular postlysosomal/late endosomal transport of cholesterol and other cargo (6, 18, 19).

From a practical standpoint, it is essential to remember that a diagnosis of Niemann–Pick disease without specification of a subgroup may lead to erroneous genetic counseling. The current classification opposes two metabolically different groups: Niemann–Pick types A/B, true sphingomyelinase deficiencies due to mutations of the acid sphingomyelinase gene, and Niemann–Pick type C, with alterations of trafficking of endocytosed cholesterol due to either *NPC1* or *NPC2/HE1* mutations. Type D as a distinct entity is no longer justified.

Epidemiology

Niemann–Pick type C disease (NPC) is panethnic. The prevalence in countries from Western Europe (France, UK, and Germany) has been estimated to be approximately 1/120,000 to 1/150,000 living births, based on diagnostic data from our laboratory. This figure is probably an underestimate, due to the fact that atypical phenotypes may not be recognized. Most patients (95% or more) have mutations on the *NPC1* gene. Among them, two genetic isolates have been reported. The first one, in French Acadians originating from Normandie and originally established in Nova Scotia, was initially described as Niemann–Pick type D and is characterized by the G992W mutation (1, 14). Another isolate sharing the I1061T mutation has been reported in Hispanics living in New Mexico and Colorado, with their roots in the Upper Rio Grande valley of the US (20, 21).

Clinical phenotypes and natural history

The wide clinical spectrum of Niemann–Pick C disease, already obvious in Crocker and Farber's series (1), is now well recognized (22–35). Age of presentation may vary from the perinatal period to adult age and results in a highly variable age at diagnosis (Fig. 1). Initial manifestations can be hepatic, neurologic, or psychiatric. The systemic (liver, spleen, and lung) involvement and neurologic disease follow an independent course. Liver involvement of varying severity is often present in the first months of life, when it constitutes the main feature of the disease. A moderate, sometimes transient hepatosplenomegaly is a common finding, but may be absent in 10 to 15% of patients. In typical patients, the neurologic disorder consists mainly of cerebellar ataxia, dysarthria, dysphagia, and progressive dementia. Cataplexy and seizures occur frequently, dystonia and psychiatric disturbances occasionally. The majority of cases show characteristic vertical supranuclear gaze palsy. Delayed motor development and hypotonia followed by pyramidal signs are the main features of a rarer, early onset, and rapidly progressive variant (32). From the current standpoint, no specific feature differentiates NPC1 from NPC2 patients, even though most NPC2 patients described to date suffered an acute, severe form of the disease, with often pronounced pulmonary involvement (16).

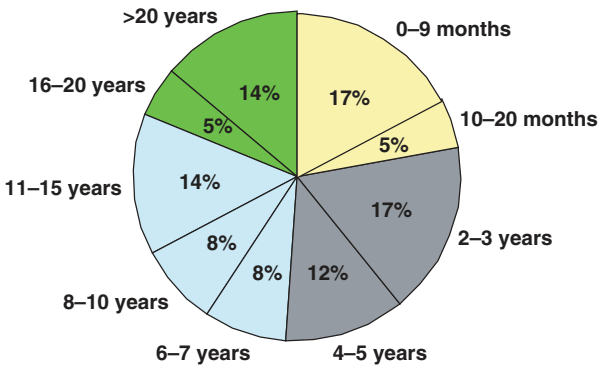

Fig. 1. Age distribution of Niemann–Pick type C patients at the time of diagnosis. The data were compiled from a cohort of 200 patients diagnosed in our laboratory from late 1993–2002.

Except for the rapidly fatal neonatal cholestatic form and the very rare non-neuronopathic adult form, classification of the clinical phenotypes can be made from the age of onset and the presenting neurological symptom (6, 22, 23, 29).

Perinatal presentation

The perinatal period is asymptomatic in about half of the patients. In the other half, liver disease is the major sign. Fetal ascites or fetal hydrops can be observed. Prolonged neonatal cholestatic icterus associated with progressive hepatosplenomegaly is the most common sign (22, 26). Spontaneously resolving by 2–4 months of age in most patients, it may lead to a rapidly fatal liver failure in about 10% of cases with liver disease. Children with this dramatic 'rapidly fatal neonatal cholestatic form' die before the age of 6 months, without neurologic symptoms. Rare cases with a severe neonatal respiratory failure have also been described. The severe fetal or neonatal forms may be associated with neurological forms of varying severity in the same sibship.

Infantile presentations

In infants and young children, isolated hepatosplenomegaly may be the only sign of the disease

for a number of years. NPC should be considered when Niemann–Pick B and Gaucher diseases have been excluded. A less-frequent 'severe infantile neurological onset form' has been identified (about 20% of the cases in a European survey) (22, 25). In those infants, hepatosplenomegaly is almost invariably present. Hypotonia and delay of developmental motor milestones become evident by the age of 12–18 months. Subsequent clinical course includes a loss of acquired motor skills, proportionally less-marked mental regression, followed by pronounced spasticity with pyramidal tract involvement. Intention tremor is frequent, supranuclear gaze palsy usually absent, and seizures uncommon. Many of these children never learn to walk. Imaging shows signs of leukodystrophy. Most patients with this form die before the age of 5.

Classical disease with neurological onset in childhood and adolescence

The most common presentations (about 60–70% of the cases) are the late-infantile and the juvenile neurologic onset forms. Transient neonatal icterus may have occurred. Hepatosplenomegaly could have been noticed since a number of years. In children with a neurologic onset by the age of 3–5 years, the first manifestation is often an ataxic gait, usually associated with spleno- or hepatosplenomegaly. When neurologic onset occurs between 6 and 12 years, poor school performance due to intellectual impairment and impaired fine movements is often the first symptom. Seizures or cataplexy may occasionally be the presenting symptom. Absence of a clinically detectable organomegaly has been reported in at least 10% of the cases. Accompanying or later symptoms include ataxia and dysarthria. Gelastic cataplexy with or without narcolepsy is observed in about 20% of the cases. Resulting falls may lead to injury, and treatment with clomipramine and/or modafinil is indicated. Epileptic manifestations (absences, partial and/or generalized seizures) develop in about half of the 'classic' patients and can become a major problem. Supranuclear vertical gaze palsy (downward, upward, or both) (36, 37) is almost invariably present and often obvious, but may require an examination of voluntary vertical eye saccade movements. Choreoathetoid movements and dystonic postures may occur. As the disease progresses, pyramidal signs and spasticity usually develop. Motor impairment is major, while intellectual decline is more variable. Psychotic episodes may occur. Dysphagia is prominent and gastrostomy often necessary. Many of these patients die in their teenage years, with a trend for patients with a late-infantile onset or intractable epilepsy to die earliest. A number of them, however, survive into their twenties or thirties.

Adult presentations

The usual symptomatology of the neurologic adult onset form (30) is that of an attenuated juvenile form with an insidious onset and progressive dementia. Psychosis is not uncommon and may even be the initial manifestation. Movement disorders and extrapyramidal signs are more frequent than in the juvenile form. Visceromegaly was not clinically detectable in nearly half of the cases, and paresis of the vertical gaze was reported as absent in a number of patients.

In addition, two patients aged 53 and 63 years, with isolated splenomegaly and an unequivocal biochemical and molecular diagnosis of Niemann–Pick C disease, suggest the existence of a non-neuronopathic form of the disease (38–40).

Animal models

Two murine NPC1 models (BALB/c-*npc1*^{nih} and C57BL/KsJ-*npc1*^{spm}), as well as feline and canine NPC1 models, all spontaneous, are known. The seminal role of cholesterol trafficking in the cellular pathology of NPC was first discovered through the investigation of the BALB/c mutant (5, 6). This mouse, which has since been widely used, develops symptoms by 4–5 weeks of age. Initial tremor is followed by hind limb paralysis, poor feeding, and death between 70 and 80 days of age. The *npc1*^{nih} mutation results in protein truncation before the sterol-sensing domain (SSD) and 11 of the 13 transmembrane domains (41). The brain of both mutant mice is smaller than controls, with atrophy of the cerebellum and midbrain region including colliculi (42, 43). Progressive neuronal loss, especially of cerebellar Purkinje cells, is a prominent feature. Demyelination is also present. An NPC2 knock-out mouse is now available and has a phenotype globally similar to that of the NPC1 model (P. Lobel, S. Walkley, M.T. Vanier, unpublished data). The murine models mimic the severe infantile neurological form of human NPC, whereas the feline model, which has a missense (C955S), less-severe mutation, is closer to the juvenile neurological form (44).

Biochemistry

Lipid storage profiles

Although few studies have been conducted on NPC2 patients, the storage profiles appear

similar in NPC1 and NPC2. The pattern of the stored lipids varies according to the tissue of origin (5, 6, 29, 45). Spleen and liver show a moderate accumulation of unesterified cholesterol and sphingomyelin (twofold to fivefold increase), bis(monoacylglycero) phosphate (LBPA), and glycolipids (essentially glucosylceramide, lactosylceramide, and ganglioside GM3), with no compound predominating. These changes are already present in fetal life. In brain tissue, neither sphingomyelin nor cholesterol overtly accumulate, but significant alterations of glycosphingolipids (glucosylceramide, lactosylceramide, and gangliosides GM3 and GM2) have been reported. These alterations are not present in fetal tissues, but appear and gradually increase during the first two postnatal years. Excess GM2 ganglioside has been postulated as a causative of ectopic dendritogenesis and meganeurite formation in several lysosomal storage diseases, including NPC (46, 47). Myelin lipids are markedly affected in the severe infantile form only. It has been recently claimed that, at least in the mouse, a neuronal cholesterol increase could be overlooked by the concomitant decrease in white matter due to extensive demyelination and that there is an increase of cholesterol in the brain of newborn pups (48). No significant cholesterol increase was found in dissected human gray matter from NPC patients (45), in good accordance with the report of a globally normal content of unesterified cholesterol in cultured neurons from *npc*^{nih} mice (49, 50). Nevertheless, a mildly abnormal filipin staining of NPC neurons has been reported (47, 49, 51). Recent data showed that cholesterol accumulated in neuronal cell bodies of NPC mice, but was decreased in distal axons and suggested that this imbalance was due to an impaired trafficking of endogenously synthesized cholesterol (49, 50).

Among other features (6), neuropathology of NPC shows similarities to Alzheimer disease, with the presence of neurofibrillary tangles with paired helical filaments of tau protein (52) and the accumulation of amyloid-β-protein (53). A relationship with abnormalities of cholesterol metabolism is under discussion, as the latter finding may be related to elevated cholesterol levels in late endosomes. A role of the apo E ε4 genotype in accelerating such changes has also been suggested (54).

Cellular cholesterol homeostasis and transport

Initial studies by Pentchev and associates (7–9) and further work from several laboratories (10) demonstrated a disruption in intracellular transport of endocytosed cholesterol in NPC fibroblasts. Internalization, transport to endocytic vesicles, and hydrolysis of low-density lipoprotein (LDL) appear to be normal. However, further transport of unesterified cholesterol to other sites is impaired, in a fashion which is not totally elucidated. As a consequence, free cholesterol accumulates in lysosomes and/or late endosomes (Fig. 2). This anomaly is the most conspicuous specific feature of Niemann–Pick C cells, either NPC1 or NPC2.

The subsequent induction of all LDL-mediated homeostatic responses (more specially, cholesteryl ester formation) is retarded in NPC cells. However, normal homeostatic responses are induced by membrane-permeable oxysterol and by mevalonate, showing that the ability of the cell to respond is maintained (10). Interestingly, a very recent report postulates a role for NPC1 and NPC2 in the regulation of sterol homeostasis through the generation of LDL cholesterol-derived oxysterols (55). NPC is the main metabolic disorder affecting the transport of cholesterol through the late endosomal/lysosomal system and intracellular cholesterol homeostasis

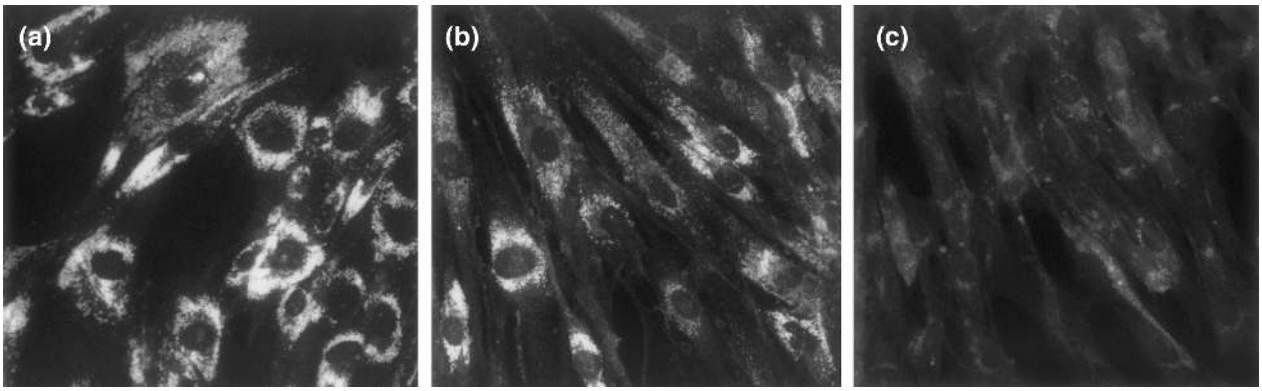

Fig. 2. Accumulation of unesterified cholesterol visualized by filipin staining and fluorescence microscopy in skin fibroblasts cultured in presence of low-density lipoprotein. (a) Classic Niemann–Pick type C (NPC) cell line; (b) variant NPC cell line; (c) normal cell line.

and has been widely used to dissect this pathway. There is, however, still a dispute whether NPC1 facilitates the initial transport of LDL cholesterol to the plasma membrane, or whether LDL cholesterol is first transported to the plasma membrane and then internalized to a NPC1-containing compartment for further disposition. The reader is referred to several recent reviews on the subject (19, 56–59). While the general understanding has been that mutations in NPC1 disrupt trafficking of lysosomal cholesterol to the plasma membrane and endoplasmic reticulum, other studies (60, 61) suggest that NPC1 might in fact intervene in a postplasma membrane-recycling pathway, which, to a certain extent, also involves transport of endogenous cholesterol. The most recent reports have not solved the controversy. Wojtanik and Liscum (62) concluded that in NPC1 cells, LDL cholesterol traffics directly through endosomes to lysosomes, bypassing the plasma membrane and becomes trapped there due to dysfunctional NPC1. Wiegand et al. (63), using a new fluorescent cholesterol analog to study the transport from the plasma membrane to the endoplasmic reticulum and the Golgi complex, obtained data indicating that NPC1 plays an essential role in the distribution of plasma membrane-derived cholesterol. Reid et al. (64) also concluded that endogenously synthesized cholesterol may contribute significantly to the overall cholesterol accumulation observed in NPC in various cell types, including glial cells. As brain cholesterol is essentially acquired from the endogenous pathway (65), elucidation of the precise steps in which the NPC1 compartment intervenes is particularly crucial to better understand the pathophysiology of the neurodegenerative disease.

Lysosomal storage of unesterified cholesterol combined with delayed LDL-mediated regulatory responses appears specific for Niemann–Pick C disease, but may show variable severity (Fig. 2). A variant biochemical phenotype with mild abnormalities has been described (66) and was later ascribed to specific NPC1 mutations (see below). Several abnormalities, such as the sphingomyelin or the free cystine storage, appear to be under the dependence of lysosomal cholesterol storage (6).

Fibroblasts from I-cell disease were shown to behave very similarly to NPC cells (66). A likely explanation is that NPC2, which requires the mannose-6-phosphate marker, cannot be processed normally in such cells. Several pharmacological agents, either hydrophobic amines (67, 68) or progesterone (69), were also shown to induce an NPC phenotype when added to normal fibroblasts, and a number of studies have used this experimental model of NPC.

Molecular genetics

Cell hybridization studies and linkage analysis have established the existence of two genetic complementation groups, NPC1 and NPC2 (11, 12). More than 95% of patients with NPC are linked to NPC1.

NPC1 gene and protein

The *NPC1* gene, mapped to chromosome 18q11-q12, spans 56 kb and contains 25 exons (13, 70, 71). It encodes a 1278 amino acid, integral membrane protein with 13 transmembrane domains, three large luminal hydrophilic loops, one cytoplasmic loop, a luminal amino-terminus, and a cytoplasmic tail with a dileucine motif (72). The region between amino acid residues 615 and 797 shows strong homology to the SSD identified in several other integral membrane proteins that respond to endoplasmic reticulum cholesterol (SCAP and HMG-CoA reductase). The N-terminal luminal loop contains a highly conserved region (amino acids 55–165) with a leucine zipper motif (amino acids 73–94), called NPC1 domain. Finally, structural and mutational studies have emphasized the functional significance of a third domain, a large cysteine-rich luminal loop encompassing amino acids 855–1098 which contains a ring-finger motif and is a likely site for protein–protein interaction (40, 73–75). The native NPC1 is a large transmembrane glycoprotein (170–190 kDa) that resides primarily in late endosomes and interacts transiently with lysosomes and the trans-Golgi network (76, 77).

Spectrum of NPC1 mutations

To date, 133 disease-causing NPC1 mutations have been reported, with a large majority (71%) of missense mutations (13, 14, 20, 40, 70, 71, 73, 74, 78–83) (Fig. 3). These mutations are scattered through the NPC1 gene and affect all functional domains except the leucine zipper motif. More than one-third of the mutations, however, are concentrated within the cysteine-rich luminal loop, with a hot spot within the region between amino acid residues 927 and 958, where 14 different mutations are located. Only three frequent mutations have been described. I1061T, found in patients of Western European descent, accounts for approximately 20% of the alleles in the UK and France and 15% in the USA. It is highly prevalent in patients from the Spanish-American isolate in southern Colorado and New Mexico (20). The two other most recurrent mutations are associated with a mild impairment of cholesterol trafficking (variant biochemical form):

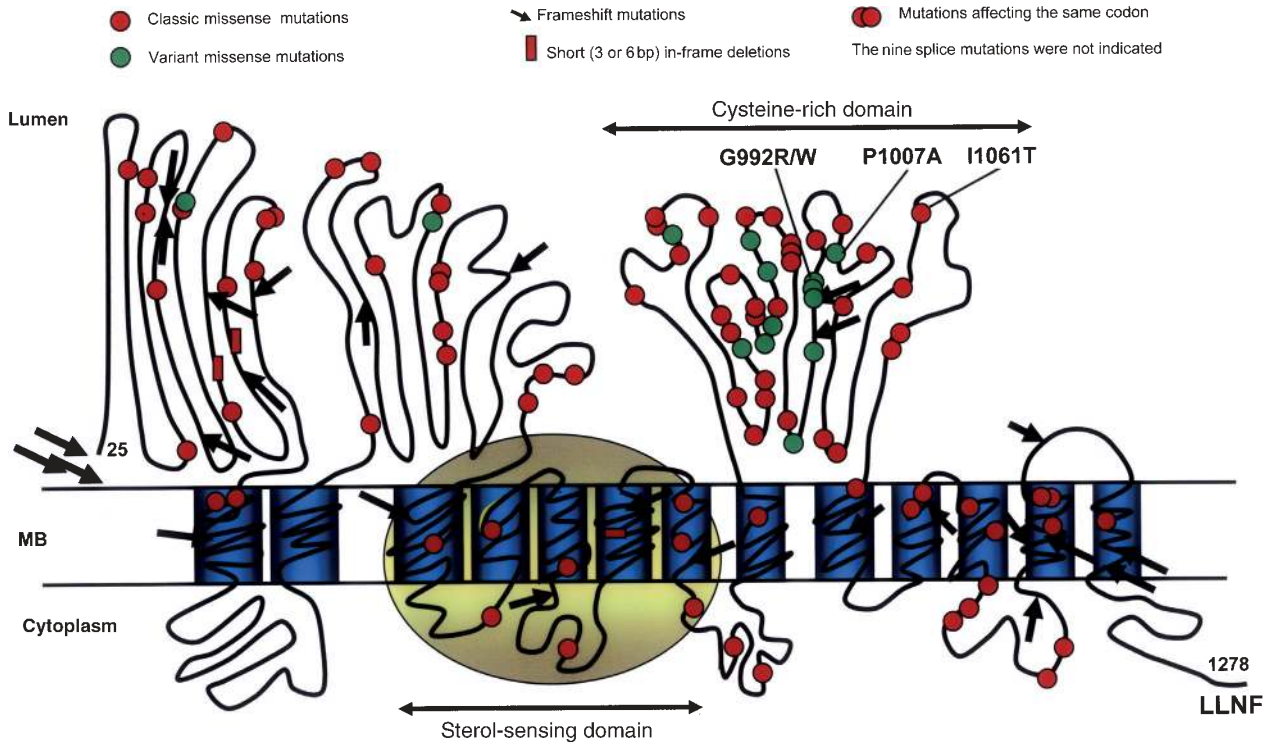

Fig. 3. Topology of *NPC1* mutations, compiled from published data (see text for references). The schematic NPC1 protein model is drawn as proposed by Davies and Ioannou (72).

P1007A ranks as the second most frequent mutation in Europe. G992W is typical of Nova Scotian patients, but rare in other populations, and unexpectedly, was never found so far in a French patient (40, 70, 73, 74, 79–81, 83). Among additional recurrent mutations, some were reported in several studies (R404Q, Y825C, D874V, R934Q, S940L, D948N, S954L, R978C, M1142T, N1156S, and R1186H), while others seem to be prevalent only in Japanese (R518Q) (83) or in Italian patients (P474L) (81). Molecular analysis of NPC1 patients is challenging owing to the size of the gene, the large number of private mutations, and the occurrence of numerous polymorphisms (see below). Further, by primary cDNA sequencing, mutations leading to very unstable transcripts may be overlooked (81).

NPC1 genotype–phenotype correlations

NPC1 mutations identified in patients and expression studies of mutant cDNAs have provided some insight into structure–function relationships for this protein. The correlations found by several groups suggest that three NPC1 domains are functionally critical: the SSD, the large luminal cysteine-rich loop, and the luminal NPC1 domain. All mutations located in the SSD, including missense mutations, appear very deleterious, corresponding in the homozygous state to a lack of mature NPC1 protein and to a very severe biochemical and clinical phenotype (40, 83). Expression studies of SSD mutants confirmed the crucial role of this domain (84). The cysteine-rich luminal loop contains approximately 50% of all described missense mutations (including the three more common mutations), associated with variable clinical and biochemical phenotypes. In the homoallelic state, I1061T correlates with the juvenile neurological onset form, but fatal perinatal liver disease has also occurred. Patients heteroallelic for I1061T have presented with all clinical phenotypes except the severe infantile neurologic onset form (20, 40). Mutations, associated with the biochemical variant biochemical phenotype (mild alterations of cellular cholesterol trafficking), were located in the cysteine-rich loop, with two exceptions (40, 73, 74, 80, 81) (Fig. 3). Compilation of published data shows a clustering within a small region of this loop (Fig. 3). Homozygous P1007A and V950M corresponded to adult neurological onset forms. Homozygous G992R was found in a 63-year-old woman with a non-neuronopathic form. The presence of a single variant allele seems sufficient to confer the variant biochemical phenotype, which is often associated with slowly progressing juvenile or adult onset forms, although this is not invariably the case (40, 85). On the other hand,

the classic biochemical phenotype is consistently observed in the most severe neurological forms, but can also be associated with any clinical form of the disease, including one adult without neurological manifestations. From the scanty clinical data available, NPC1 domain mutations (C63R, Q92R, C113R, and T137M) seem correlated with the severe infantile neurological form of the disease (74, 79, 80, 86). Overexpressed C113R protein was not localized to late endosomes, but rather to endoplasmic reticulum, Rab7-negative endosomes and cell surface (86). Expression studies of mutants affecting either conserved cysteine residues or the leucine zipper motif of this domain resulted in inactive proteins targeted to lysosomal membranes encircling cholesterol-laden cores (84, 87). In summary, while the NPC1 genotype is reasonably predictive of the severity of the neurological course, perinatal liver involvement is independent of the NPC1 mutation, and the factors responsible for its expression remain unknown.

NPC1 polymorphisms

More than 50 exonic and intronic SNPs have been described (70, 71, 80–83). The most prevalent polymorphisms are Y129Y, H215R, P237S, I642M, I858V, N931N, and R1266Q. Surprisingly, I642M was described as a disease-causing mutation in one patient (78). The P237S SNP was first reported to be a deleterious mutation, as it was never identified in 200 normal samples (40, 83), then reclassified as a polymorphism (68). Recent studies showed that in the Finnish and Swedish population, 5% of the alleles carried the P237S substitution, and several NPC1 patients have been reported with two mutations in addition to the P237S substitution (74, 86). Overexpression of P237S substitution definitely revealed that P237S is a benign polymorphism and not a deleterious mutation (86).

NPC2 gene and protein

Using a proteomic approach, *HE1*, a gene mapped to chromosome 14q24.3, which encodes a protein initially described as a major secretory protein present in the human epididymis, was found mutated in NPC2 patients (15) and thus renamed *NPC2*. It is 13.5 kb long and comprises five exons. The mature 132 amino acid *NPC2* gene product is a soluble glycoprotein ubiquitously expressed in all tissues studied (15) (Millat and Vanier, unpublished data). NPC2 binds the mannose-6-phosphate receptor and comigrates with lysosomal markers on sucrose gradients (15). In normal fibroblasts, NPC2 immunoreactivity has been reported to be distributed to the trans-Golgi network, Lamp-1-positive endosomes, and Lamp-1-negative peripheral organelles (86). A potential role of NPC1 as a regulator of NPC2 transport has been suggested (86). The porcine HE1 homolog was reported to bind cholesterol with macromolar affinity (88), a property that was seminal in identifying HE1 as a candidate *NPC2* gene product. A recent, more refined work (89) has provided evidence for a much higher affinity binding and identified a hydrophobic cholesterol-binding pocket around amino acid K97 (corresponds to K116 when counted from the initiation codon) by a site-directed mutagenesis approach. An independent study on the high-resolution crystal structure of bovine NPC2 confirmed these data and revealed a loosely packed region in the protein interior proposed to represent the incipient cholesterol-binding site (90). NPC2 has a 63% homology with the Der p 2 dust-mite allergen. Although crystal structures of NPC2 and Der p 2 show important differences, these proteins could define a family with a particular strategy for hydrophobic ligand binding. It has been postulated that NPC2 would require a conformational change to bind cholesterol (90). On the basis of evolutionary analysis and mutagenesis, three other regions of the NPC2 protein emerged as putatively important for function, including one for efficient secretion (89).

Only a few NPC2 cases (11 families) are known to date, all showing marked abnormalities of cellular cholesterol processing. None of the currently known *NPC2* mutations involves any of the four conserved domains discussed above, but molecular studies have shown very good genotype/phenotype correlations (15–17). Most described NPC2 patients had a neonatal onset with severe (often lethal) respiratory or hepatic manifestations and/or severe neurological disease with death before 4 years of age. Nearly all were found to have nonsense or frameshift mutations, and E20X accounts for nearly half of the mutant alleles so far published. Two sisters with juvenile neurological onset and a slow course had a splice mutation, leading to multiple transcripts and a less-severe protein dysfunction (16). Two other siblings with a slowly progressive, adult onset neurological disease had a missense mutation (17). With increasing number of NPC2 families, it becomes obvious that the clinical heterogeneity observed in NPC1 also applies to the NPC2 group.

Putative function(s) of the NPC1 and NPC2 proteins

The precise functions of the NPC1 and NPC2/HE1 proteins have not yet been fully elucidated

(6, 18, 19, 91). The NPC1 compartment appears to be a dynamic, sterol-modulated sorting organelle. Studies using a green fluorescent protein (GFP)-fusion NPC1 protein in living fibroblast cultures have revealed that this compartment undergoes rapid movements that are markedly impaired in *NPC1*-mutant cells. The process involves the production of tubulovesicular structures, which show loss of flexibility and slower rate of movement in the mutant cells (92, 93). Although the exact scheme remains unclear and some results are contradictory, a whole body of experimental data provide evidence for a role of NPC1 in regulating or mediating retrograde transport of multiple lysosomal cargo in the late endosomal/lysosomal pathway (6, 77). Apart from cholesterol (see above), glycolipids are also candidates for transport under the dependency of NPC1 (94, 95), especially considering that they are, together with cholesterol and caveolin, raft components. The NPC1 compartment is enriched in glycolipids, and internalization of GM2 ganglioside into endocytic vesicles was found to require functional NPC1 protein (95). Independently, it has been shown that the endosome-to-Golgi transport of sphingolipids can be blocked by high levels of intracellular cholesterol (96). Regarding the function of NPC1, challenging data have been published based on homologies of NPC1 with prokaryotic permeases of the resistance-nodulation-division (RND) family, indicating that NPC1 protein is a transmembrane molecular pump which, from transport studies in *Escherichia coli*, could act as a permease-transporting fatty acids across cellular membranes (91). How this reconciles with a role of NPC1 in cellular redistribution of endocytosed cholesterol and of glycolipids is not yet understood (19). The regulation of subcellular lipid transport may in fact be more complex. There is strong evidence that the NPC1 and NPC2 proteins must function in a closely related fashion, because comparative studies between *NPC1* and *NPC2* mutants found no qualitative difference in their ability to respond to exogenous LDL cholesterol loading and in their tissue lipid storage (12). A role for both NPC1 and NPC2 in the regulation of sterol homeostasis through the generation of LDL cholesterol-derived oxysterols has also been proposed (55). Recent data (89) have confirmed that NPC2 specifically binds cholesterol with high affinity. Several proteins residing in the endosomal–lysosomal system, NPC1, NPC2, but also MLN 64 and MENTHO (97, 98), may be sequentially or alternatively involved in the facilitation of cellular cholesterol movements (19). It has been postulated (19) that NPC1 activity may depend on prior action of NPC2 to insert sterol into the endosomal/lysosomal membrane. Recent progress on structure and localization of the NPC2 protein should facilitate further understanding of this process.

While the precise cause of the neurodegenerative syndrome in NPC remains unknown, it is well established that defects in either NPC1 or NPC2 protein cause a 'traffic jam' of lipids in the late endosome (58). Getting the lipids moving is thus a challenge in NPC research. Exciting data along this line have recently been published by two independent groups. Pagano and associates (99) have shown that the intracellular trafficking of cholesterol and glycolipids in NPC fibroblasts can be dramatically improved by the overexpression of Rab7 or Rab9, and Ioannou and associates (100) have obtained quite similar results with Rab9. Although these data raise a number of yet unresolved questions (101), they may suggest new avenues for therapy.

Diagnosis

Suspecting a diagnosis of NPC is easy in patients with the most typical symptoms, such as combined splenomegaly, ataxia, and vertical gaze palsy. But strikingly different clinical presentations exist, especially in infants and neonates, and neurologic onset may be delayed until adolescence or adulthood. NPC is often not considered in cases without organomegaly, a not infrequent situation in older children and adults. No screening test in urine and blood is reliable for the diagnosis of NPC, but chitotriosidase activity in serum is usually moderately (10- to 20-fold) elevated. Acid sphingomyelinase activity is always normal in leukocytes (which allows the exclusion of Niemann–Pick B or A) and inconstantly partially deficient in cultured fibroblasts. Foam cells and sea-blue histiocytes are usually present in bone marrow but may be lacking. When present, foam cells stain positive with filipin. Ultrastructural studies on conjunctival, skin, or liver biopsies can provide strong support to the diagnosis, but false-negative results may occur on liver biopsy (26). Analysis of the lipids in a liver biopsy may also be inconclusive. Imaging and neurophysiologic studies are non-specific (102). Magnetic resonance imaging and computerized tomography scans may be normal or show cerebellar or cortical atrophy, or, in the severe neurological infantile form, white matter changes.

The discovery of abnormal cellular cholesterol processing established the rationale for specific tests to diagnose either NPC1 or NPC2 patients. The procedure requires living cultured cells,

generally skin fibroblasts. The definitive diagnosis is best achieved by the demonstration of a lysosomal accumulation of unesterified cholesterol, as shown by an intense perinuclear fluorescence after staining with filipin (a polyene antibiotic that binds to the hydroxyl group of unesterified cholesterol to form a fluorescent complex) (Fig. 2), coupled to the study of intracellular cholesterol homeostasis as defined by the early rate (4–6 h) of LDL-induced cholesteryl ester formation (66). In both tests, cells are first cultured in a lipoprotein-deficient medium, then challenged with an LDL-enriched medium. The filipin test appears more specific (impaired esterification may occur in other disorders) and is more sensitive. Most patients (80 to 85%) show pronounced abnormalities for both the filipin test and the esterification test (classical phenotype). The remaining 15–20% (variant phenotype) show a milder accumulation with the filipin test, combined with esterification rates 30 to 80% of normal. The diagnosis of variant cases is difficult and will be missed by simple filipin staining of cells cultured in regular medium, often sufficient to pick up classic cases. Note that, as discussed earlier, fibroblasts from I-cell disease will behave very similarly to classic Niemann–Pick C cells (66).

Similar tests can be used for prenatal detection of affected fetuses (either NPC1 or NPC2), using preferably cultured chorionic villus cells and eventually amniotic fluid cells, provided clear-cut abnormalities have been demonstrated in the index case, thus excluding families in which the proband has a variant biochemical phenotype (103). If mutations have been identified in the proband, prenatal diagnosis is preferably achieved using molecular genetic testing. Uncultured chorionic villi is the material of choice. This approach provides a faster answer, and is applicable to variant families (104). Over 200 pregnancies at risk for NPC have been monitored worldwide, most by the conventional biochemical method. Detection of heterozygotes is not fully reliable using cellular biology methods, but identification of mutations in probands allows precise genotyping in their relatives.

Treatment

There is as yet no specific treatment for NPC. Hepatic transplantation corrected liver dysfunction but did not influence the progression of neurologic disease. Suggested benefit of a single study of fetal liver cell transplantation in the *npc*^{nih} mouse model (105) is inconclusive, because genotyping of the treated mice was not feasible when the study was done. From the follow up of patients by one of us, no long-term benefit of this approach could be achieved when applied to human patients. Similarly, bone marrow transplantation in humans (106) and animal models has not significantly influenced neurologic progression.

Pharmacological trials have so far had two metabolic targets: reduce the cellular influx of cholesterol or reduce the influx of less-complex glycolipids. A study of a low-cholesterol diet and varying combinations of cholesterol-lowering agents (lovastatin, niacin, and cholestyramine) produced marked diminution in hepatic storage of unesterified cholesterol (107), but there was no clear evidence of benefit from this therapy in the long run (6, 29). Subsequent studies in the murine and feline models of NPC using a variety of cholesterol-lowering strategies have shown no evidence of amelioration of the neurologic disease (108, 109). The potential implication of glycolipids in the pathophysiology of NPC, especially brain dysfunction, provided the rationale for trials of substrate balance therapy using inhibitors of glucosylceramide synthase. Crossbreeding of *npc*^{nih} mice with essentially asymptomatic GM2 synthase-deficient mice did not lead to clinical improvement of the npc mice (51). No comment was made regarding the status of dendritic/meganeurite formation in the double-mutant mice. On the other hand, oral administration of *N*-butyldeoxynojirimycin in *npc*^{nih} mice led to a 20% delay in the onset of symptoms and increased survival by a similar factor (110). Trials with *N*-butyldeoxynojirimycin therapy (OGT 918) have been conducted for treatment of patients with type I Gaucher disease (111). This drug (Miglustat, Zavesca®) has recently been approved for this indication in the European Community, Israel and the USA. A clinical trial with this agent is currently underway in NPC. In the recent 2nd International Conference on Niemann–Pick type C disease, another therapeutic trial in the mouse using the neurosteroid ALLO, 3α 5α tetrahydroprogesterone was reported to delay the onset and progression of the neurological symptoms and to increase the number of Purkinje cells (112).

No attempt to gene therapy applicable to humans has been reported so far. The NPC2 protein is soluble, binds the mannose-6-phosphate receptor, and its addition to the culture medium of cells was shown to correct the cellular cholesterol accumulation (15). Strategies under development for lysosomal enzyme deficiencies should therefore be applicable to this disorder. An additional problem arises with the NPC1

protein (involved in the vast majority of patients), as it does not appear to be secreted and recaptured. Recently, selective overexpression of an *NPC1* transgene in the brain of npc^{nih} mice using a prion-driven promoter was shown to rescue both the neurodegeneration and the neurological symptoms in the transgenic animals (113). The study confirmed the independence of neural and visceral pathology and showed that gene overexpression was well tolerated.

To develop new therapeutic strategies, it will be crucial to better understand the NPC pathophysiology. Ongoing studies from many groups are aiming to more precisely define the function(s) of the NPC1 and NPC2 proteins as well as their impact on other proteins involved in the regulation of cellular cholesterol trafficking. Work is also actively pursued to unravel the mechanisms of glycolipid accumulation. More investigations in neurons and glial cells are needed, as progressive neurological degeneration is the main feature of the disease. Additional mice models carrying specific NPC1 mutations leading in humans to the most common juvenile phenotype or to a variant biochemical phenotype could prove useful to better approach the molecular mechanisms of neurodegeneration and define the nature of the primary offending metabolite.

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