

Review

The pathophysiology and mechanisms of NP-C disease

Stephen L. Sturley^{a,b,*}, Marc C. Patterson^{b,c}, William Balch^d, Laura Liscum^e

^a*Institute of Human Nutrition, Columbia University Medical Center, New York, NY 10032, United States*

^b*Department of Pediatrics, Columbia University Medical Center, New York, NY 10032, United States*

^c*Department of Neurology, Columbia University Medical Center, New York, NY 10032, United States*

^d*Department of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037, United States*

^e*Department of Physiology, Tufts University School of Medicine, Boston, MA 02111, United States*

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Abstract

The molecular isolation of NPC1 and NPC2, the genes defective in patients with Niemann–Pick disease type C (NP-C), has heralded in an exponential increase in our understanding of this syndrome and thus of human intracellular sterol transport. Despite this, neither the mechanisms of action nor the substrates for these putative transporters have been defined. In this overview, we describe our perspectives on the current awareness of the genetic determination and cellular biology of this syndrome, with emphasis on the underlying events that lead to neurodegeneration and the manner in which they might eventually be treated.

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1. Introduction

Unesterified sterols modulate the function of all eukaryotic cellular membranes by lending rigidity to the phospholipid bilayer. Optimal membrane function is regulated by maintaining an equilibrium between sterol biosynthesis, uptake, and metabolism. Superimposed upon this is the need to adequately balance sterols with phospholipids, specifically those of the sphingolipid group particularly in plasma membrane sub-domains (“rafts”). In addition, sterols are stored as steryl esters and act as building blocks for bile acids, steroid hormones and, notably, neurosteroids. Niemann–Pick diseases (A, B, and C) are all characterized by defects in the activity of sphingolipid degrading enzymes. The NP-A and NP-B subtypes arise due to mutations in the acid sphingomyelinase (SMase) structural gene [1]. Niemann–Pick disease type C (NP-C) is commonly, and perhaps too simplistically, considered to be a syndrome of defective cholesterol transport from the lysosome with a secondary

defect in SMase activity (reviewed in Refs. [2,3]). This is particularly pronounced in liver and spleen cells, but more deleteriously in brain, where the lipid accumulation correlates with severe neuronal dysfunction that is ultimately fatal.

Our understanding of NP-C has progressed dramatically in the past ~8 years [4] with the strong encouragement and support of family-centered foundations such as the Ara Parseghian Medical Research Foundation. Following isolation of the NPC1 and NPC2 genes [5,6] responsible for this disease and the resulting burgeoning of cell biological data, we now have a reasonable appreciation of its molecular nature. Although we know the players in this game, it is clear that we do not yet know either the rules by which the game is played or even the shape of the ball! In this brief overview, we describe our perspectives on the current state of this field and speculate on the future directions that this research may take.

2. Genetic determination

The isolation of the NPC1 gene, which is defective in the majority of individuals afflicted with this disease, provided

* Corresponding author. Institute of Human Nutrition and Department of Pediatrics, Columbia University, 630 West 168th Street, New York, NY 10032, United States. Tel.: +1 212 305 6304; fax: +1 212 305 3079.

E-mail address: sls37@columbia.edu (S.L. Sturley).

an important molecular key to understanding the disease and the process of subcellular cholesterol transport [7,8]. Some 4 years later, label and colleagues identified NPC2 as the determinant of the rarer form of NP-C and subsequently created a murine model of the disorder (see review Vanier and Millat [9]). The NPC1 and NPC2 proteins share no sequence identity, but both have hallmarks of genes involved in cholesterol homeostasis and likely act in the same pathway. Moreover, both genes have counterparts in virtually all eukaryotes [10], suggesting a primordial function. Thus, the tools are in place to examine how these proteins work, yet many questions remain unanswered.

For example, do genes other than NPC1 or NPC2 cause this syndrome? Probably not; genetic linkage, cell fusion studies [11] and nucleotide sequence analyses [12–16] indicate that the vast majority of causes of NP-C are associated with mutations at the chromosome 18 and 14 loci. However, there are isolated examples of “mystery” forms of the disease that at the nucleotide level do not appear to be NPC1 or NPC2 alleles, at least within coding sequences [13,17]. Furthermore, there is evidence for the presence of modifiers or suppressors of these pathways [18] and rare instances of “mistaken identity” suggest the existence of phenocopies of the NP-C syndrome. Conversely at the cell biology level it is possible to suppress the NPC1 syndrome by mis-expression of mediators of endosomal/lysosomal trafficking ([8,19–21]).

Another major question is what accounts for the surprising soft link between disease alleles and phenotype in NP-C disease. Although some correlation of alleles with either the severe or “variant” disease phenotypes has been suggested, there is no founder effect for most kindreds with this panethnic disorder and most mutations are private. Even within a family, where the NPC1 mutant alleles are identical, there may be striking variation in age of onset, severity of symptoms and progression of the disease. The molecular isolation of the genetic and environmental factors that cause this variation would undoubtedly enlighten our understanding of the mechanism of lipid movement and provide currently untapped targets for therapeutic intervention. Approaches in model systems (yeast, fruit flies, nematodes), which have maintained remarkable conservation of this gene in terms of structure and function, may represent an appropriate and fruitful strategy in this regard [10].

3. Cellular biology

Although the predominant phenotype in NP-C mutant liver cells and fibroblasts is an accumulation of free cholesterol, several classes of sphingolipids also amass [22,23]. In addition, it is clear that gangliosides such as GM1, GM2 and GD3 are transported by the NPC1 defined pathway in cell culture models [24,25]. A striking aspect of NP-C disease is the panoply of biochemical and physiological disturbances that have been reported in the

literature. These include changes in cholesterol and sphingolipid/ganglioside accumulation, membrane microdomains (“rafts”), sphingomyelinase activity, caveolin and annexin II expression, peroxisomal function, copper metabolism, apoptosis, and neurotrophin response. What is/are the primary defect(s) that arise from loss of this gene as opposed to secondary consequences? NPC pathways transport other molecules in addition to sterols and sphingolipids. General markers of endocytosis such as sucrose and DiIC₆ are mislocalized by NPC mutant fibroblasts [26]. Thus, is there one primordial metabolite that *must* be transported by the NP-C pathways? Is there a single toxic metabolite that accumulates in this disorder, the removal of which might represent a therapeutic approach?

With respect to the basic function of this protein in the cell, we know a considerable amount about the subcellular itinerary of the culprit proteins [9,21]. Evolution provides a clue to understanding the mechanism of NPC1 function and pathophysiology. Unlike NPC2, the NPC1 molecule shows remarkable conservation, with bona-fide representatives (sometimes more than one per genome) in yeasts, worms, insects, plants and mammals [10]. The genetic conservation may be even more ancient; Ioannou and colleagues (Ref. [27] and this volume [8]) made the breakthrough observation that NPC1 is a eukaryotic member of the resistance-nodulation-division (RND) family of prokaryotic permeases. These proteins are well studied in numerous bacteria and use proton-motive force to remove hydrophobic molecules from the cell. Expression of NPC1 in *Escherichia coli* facilitated the transport of metabolites such as acriflavine and oleic acid, but not cholesterol or cholesteryl oleate, across the bacterial membrane. In yeast, despite structural and functional conservation with the mammalian protein, the primary role of the NPC1 ortholog is not to transport sterols [10,28]. These studies point to a very basic function of the protein that predates sterol transport and remains elusive. That is not to say accumulation of sterols is irrelevant to the pathophysiology of this disease or that they are not the offending metabolites (as will be discussed at a later point in this overview). However, this protein did not evolve as a low density lipoprotein derived cholesterol transporter as it significantly predates many components of mammalian sterol homeostasis. Admittedly, there are multiple camps that champion different molecules as being the most significant substrate for NPC1. To add to the complexity, the recent identification [29] of the mammalian NPC1 relative, NPC1-L1, as an intestinal transporter of cholesterol suggests that the substrate for this protein could even be tissue-specific. Clearly, any satisfying explanation of NPC1 function must account for all of the observed phenomena, but identification of the primordial cargo molecule for NPC1 could be of practical value, as this so far elusive molecule could represent a prime target for intervention. At present there is no biochemical basis (e.g.,

protein–ligand interaction) for distinguishing fatty acids from sterols or from sphingolipids as substrates. Indeed, other cargos may be relevant; it is possible that defective transmembrane movement of substrate X (where $X \neq$ cholesterol, fatty acids or sphingolipids) could be the underlying trigger to the consequences of loss of the NPC1 proteins. It would be particularly exciting if this substrate were amenable to simple treatment, but the challenge is to identify it.

A unifying hypothesis to the apparent pleiotropy of this syndrome is that NPC1 is a component of a signal transduction cascade. This is suggested by the striking sequence conservation between NPC1 and the proteins that function in the hedgehog morphogen cascade of signal generation for development. Indeed, the strongest full-length homology of NPC1 is with *Patched* (*Ptc*), a protein involved in morphogenesis in *Drosophila* and humans. *Ptc* achieves its biological function, i.e., the induction of genes involved in morphogenesis, via the Sonic Hedgehog signal transduction pathway and a direct interaction with *Smoothened* [30,31]. Despite an immense accumulation of data on this process, no direct link has yet been made between NPC1 and the manner in which *Ptc* works.

Alternatively, the pleiotropy of NPC1 mutant phenotypes may arise from the aberrant initiation of signaling cascades that derive from or respond to sterol (e.g., the sterol regulatory element binding protein SREBP and/or oxysterol LXR/RXR pathways), ceramide- or sphingosine-1-phosphate (e.g., which induces inflammatory responses and proapoptotic events); all of which significantly accumulate in NP-C disease. Indeed, it is clear that these responses are aberrant compared to normal cells [32–34], although it remains to be seen whether intervention at these pathways represents treatments for the disease (see below).

The steps in lipid transport through the lysosomal–endosomal system may be conferred by the interactions of NPC1 with NPC2 and other currently obscure “partners”. However, it is interesting that the NPC2 protein, a member of a large family of molecules that bind many divergent lipids in higher eukaryotes [35], is a demonstrably avid binder of cholesterol, and yet is poorly conserved in simple organisms. It may be that convergent evolution has produced multiple methods to maintain these hydrophobic molecules in a soluble and innocuous form, once they have permeated a membrane in an NPC1-dependent manner. Interestingly, the sequences of mammalian NPC1, NPC1-L1 [36], *C. elegans* NPC1 (a and b) and yeast Ncr1 retain the characteristic NH₂-terminal “NPC1-domain” that distinguishes this gene family from the Patched and SCAP-proteins. The sequence may mediate an association with other components of lipid transport, particularly as in some instances it encompasses a leucine zipper interaction motif.

An exciting new insight regarding the link between NPC1 dysfunction and cholesterol trafficking came from manipulations of small GTPases belonging to the Rab gene

family. This large family of proteins (63 members) control vesicle targeting and fusion between all endomembrane compartments and hence control flow of protein and lipid movement in the cell. Strikingly, overexpression of a subset of Rab proteins has dramatic effects on the NPC1 phenotype. Overexpression of Rab 7 and 9, GTPases, which in cell culture systems regulate trafficking between the cell surface and endosomal compartments, resulted in complete reversal of the cholesterol/sphingolipid accumulation (see [13,14] and [8]). Thus it is apparent that the NPC1 defect leading to accumulation of cholesterol/sphingolipid in unusual late endosomal compartments, can be normalized by altering the dynamics of these Rab regulated pathways. These results suggest that in NPC1 disease, lipid accumulation occurs in response to blocking a function of these critical catalysts of membrane transfer. Alternatively, the steady-state accumulation of cholesterol/sphingolipid is dependent on Rab-independent mechanisms that can be modulated by altering the dynamics of endosomal membrane trafficking pathways. Knowledge of the importance of these Rab-dependent pathways may represent a significant step towards understanding and treating this complex disease.

4. Neurodegeneration

The absence of the NPC1 protein has striking effects on sterol homeostasis in all cells. LDL-derived, and perhaps in some cells endogenously synthesized [37], sterols are mislocalized. As described above, and at multiple points throughout this volume, NP-C disease is not only a cholesterol lipidosis; multiple lipids accumulate in multiple tissues [38]. In the context of the syndrome, this initially presents as hepatosplenomegally in early onset cases, which typically resolves as neurodegeneration becomes symptomatic and ultimately lethal. Thus, to adequately deal with this syndrome, we must understand the processes impacted by NPC1 and NPC2 in the brain. Moreover, any models of the neurodegeneration must explain the marked and selective sensitivity of brainstem and cerebellar neurons to loss of these proteins. Several reviews in this volume [38,39] elegantly define the events that happen in the brains of affected individuals. Although it is clear that Purkinje cells are most compromised in this cerebellar disorder, it remains to be defined how they die [40], although markers of both apoptosis and necrosis have been identified [41]. The events leading to apoptosis and also an ER-stress salvage pathway, known as the unfolded protein response, have all been related to sterol and sphingolipid metabolism. Indeed, cells ablated for NPC1 activity (i.e., heterozygotes or hydrophobic amine drug treated) are protected from the toxic effects of sterols [42,43]. It remains to be determined whether these pathways are directly relevant to the neuropathogenesis associated with homozygous forms of this disorder, although it seems plausible.

5. Therapeutic intervention

As described elsewhere in this volume [44], the NP-C syndrome is a challenge to the physician. Current treatments are largely symptomatic and preventive at best. The unraveling of the genetic basis of this disease created high but unrealistic expectations that effective interventions based on an understanding of fundamental mechanisms would soon follow. It was immediately apparent that protein transduction approaches would not be applicable to NPC1, a multipass-transmembrane protein. Indeed, the results of hepatic and bone marrow transplantation in humans and mice had already suggested as much. In contrast, the soluble, cholesterol binding NPC2 protein appears as an excellent candidate for replacement therapy, either as a recombinant protein or as a protein expressed by donor cells such as mobile monocytes, which could then be secreted and resorbed by neurons. Such a therapeutic trial for NPC-2 disease would be highly desirable but is unlikely given the low frequency of occurrence of this specific disorder. For the more common NPC-1 phenotype, gene therapy or stem cell replacement approaches are clearly the ideal and curative strategy. Gene therapy for this and similar neurodegenerative diseases cannot be pursued until formidable technical barriers are overcome, including bypassing the blood barrier, attaining transduction efficiency, establishing and maintaining appropriate, regulated gene expression and preventing neoplastic transformation.

The limited therapeutic trials that have been performed have been based on efforts to eliminate or limit the putative offending metabolite(s). A clinical trial of cholesterol-lowering therapy did not show evidence of neurologic benefit despite substantial reductions in hepatic unesterified cholesterol in humans. Animal studies have been similarly disappointing [45]. The failure of this approach might reflect the inability of such approaches to change brain cholesterol metabolism or simply that cholesterol is not the offending metabolite.

More recently, the significance of glycosphingolipids in the brain in NP-C disease [46–48] has been reemphasized [38]. The role of specific sphingolipids such as GM2 in promoting morbid anatomic changes has been described in NP-C and related diseases, and the inhibition of glycosphingolipid synthesis by agents such as N-butyldeoxynojirimycin (NB-DNJ), an inhibitor of glucosylceramide synthase, has been tested as treatment [49]. NPC1-mutant mice and cats showed delayed onset of neurological symptoms, increased life span, and reduced ganglioside accumulation. NB-DNJ (miglustat) has been approved in Europe and the United States for treatment of type 1 Gaucher's disease, and is currently in phase I/II trials for patients with NP-C at medical centers in New York and Manchester.

The defect in NP-C results in deficiency states as well as toxic accumulations of metabolites. Thus, the sequestration of sterols at the lysosome has the consequence of

limiting the substrates for pathways such as bile acid metabolism and steroid hormone production. In particular, the synthesis of neurosteroid hormones, dihydroprogesterone and allopregnanolone is markedly compromised in cells and animals lacking NPC1 [50]. Treatment of NPC1 mutant mice with parenteral allopregnanolone resulted in delayed onset of symptoms, prolonged life span and improved neurological function, comparable to that seen with NB-DNJ [50].

In contrast to the above approaches focused on understanding and correcting directly the manifestations of deficient NPC1/2 function, the observation that modulation of Rab-dependent dynamics of the endosomal trafficking pathways can reverse cholesterol/sphingolipid intracellular accumulation, points toward a completely different corrective strategy. This therapeutic strategy would be aimed at modulating Rab-dependent steps, thereby potentially markedly reducing the severity of onset of disease by lowering lipid accumulation in endosomal compartments.

Finally, for the person (scientist or parent) not absorbed by this specific syndrome, it is worth recalling that understanding NP-C disease goes beyond a small community. Late stages in the neuropathy of NP-C and Alzheimer's disease have compelling similarities, particularly with regard to the formation of neurofibrillary tangles consisting of paired helical filaments and hyperphosphorylated tau protein [51,52]. Moreover, there is a marked appreciation of the role of cholesterol homeostasis in AD progression [53]. Similarly, concepts of sterol toxicity and its sequelae with respect to atherosclerosis will likely be impacted by understanding this complex syndrome. Thus NP-C research will impact many more of the population than first thought likely for an orphan disorder.

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