SHORT COMMUNICATION

Hepatic Primary and Secondary Cholesterol Deposition and Damage in Niemann-Pick Disease

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Niemann-Pick C disease is a neurovisceral disorder caused by mutations in the NPC gene that result in systemic accumulation of intracellular cholesterol. Although neurodegeneration defines the disease’s severity, in most patients it is preceded by hepatic complications such as cholestatic jaundice or hepatomegaly. To analyze the contribution of the hepatic disease in Niemann-Pick C disease progression and to evaluate the degree of primary and secondary hepatic damage, we generated a transgenic mouse with liver-selective expression of NPC1 from embryonic stages. Hepatic NPC1 re-expression did not ameliorate the onset and progression of neurodegeneration of the NPC1-null animal. However, the mice showed reduced hepatomegaly and dramatic, although not complete, reduction of hepatic cholesterol and serum bile salts, bilirubin, and transaminase levels. Therefore, hepatic primary and secondary cholesterol deposition and damage occur simultaneously during Niemann-Pick C disease progression.


Niemann-Pick C disease (NP-C) is a neurovisceral lipid storage disorder affecting 1 in 120,000 live births.1 NP-C is transmitted in an autosomal-recessive manner by mutations in NPC1 (95% of cases) or NPC2 genes. The NPC proteins mediate cholesterol efflux from endosomes and NP-C is characterized by systemic accumulation of unesterified cholesterol and other lipids in lysosomes.

The clinical spectrum of NP-C is highly variable, from a neonatal fatal disorder to adult-onset neurodegeneration. Although neurodegeneration commonly defines NP-C severity, it often is preceded by visceral complications such as cholestatic jaundice and hepatosplenomegaly.1–3 The hepatic disease is especially severe in perinatal and infantile periods; patients with fetal hydrops survive for only a few days and 10% of the neonates with cholestatic icterus die after 3 to 6 months.

The current view is that hepatic and neuronal diseases progress independently in NP-C.4 A mouse with specific loss of neuronal NPC1 recapitulates the neurodegeneration of the NPC1-null animal.5,6 However, although specific neuronal NPC1 re-expression in NPC1-null mice increases survival, neurologic defects still are observed.7,8 Thus, it is likely that neuronal NPC1 delays pathogenesis, however, glial and visceral NPC1 is needed for complete rescue.4

Two transgenic mice were used to argue about the involvement of the hepatic disease in NP-C.4 An Npc1 rescue using a prion-promoter, expressing NPC1 primarily but not exclusively in neurons,9 generated a mouse with

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high levels of neuronal NPC1 and low levels of hepatic NPC1. The animals presented with liver histocytosis but not detectable neurodegeneration, suggesting that neuronal NPC1 ameliorates neurodegeneration even in the presence of hepatic damage. The second strategy used a Tet-inducible Npc1 activated in several visceral tissues but not in neurons. Visceral NPC1 did not ameliorate neurodegeneration.\textsuperscript{10} However, in this study NPC1 re-expression was not liver-specific, hepatic NPC1 levels were not controlled, and NPC1 was not expressed during embryogenesis. Because the most aggressive visceral disease occurs in neonates and Npc1 is required during embryogenesis,\textsuperscript{11} embryonic NPC1 re-expression is necessary to unequivocally define causality of hepatic NPC1 in the complex clinical spectrum of NP-C. Likewise, a single case of hepatic transplantation in an NP-C patient was reported.\textsuperscript{12} After an initial period of stabilization, progression of neurologic symptoms and recurrence of storage material in the transplanted liver were observed, suggesting primary and secondary hepatic damage. To analyze the contribution of hepatic disease in NP-C progression and the sources of primary and secondary hepatic damage, we generated a transgenic mouse with liver-selective expression of NPC1 from embryonic stages.

**Materials and Methods**

**Construction of the Targeting Vectors**

The conditional allele is based on the knockout-first strategy.\textsuperscript{13} The final targeting vector was assembled from two component plasmids using a Gateway-based switching technology\textsuperscript{14,15}; the intermediate targeting vector, which is gene specific and contains the homology arms and the attachment site right (attR)1 and attR2 Gateway sites, and gene targeting element plasmid or the L1L2 plasmid, including a promoterless selection cassette flanked by attachment site left (attL)1 and attL2 Gateway sites (Figure 1A). For the intermediate targeting vector two consecutive recombining steps are required: insertion of an attR1/attR2 zeo-pheS Gateway element into the B6 mouse bacterial artificial chromosome clone containing the Npc1 gene (between exons 1 and 2), and subcloning the modified region of genomic DNA into a Gateway-adapted plasmid backbone. The final targeting vector was assembled in vitro in a two-part Gateway reaction. As a result, the Npc1 gene at intron 1 contains a removable floxed cassette with an acceptor splicing site and a polyadenylation signal that interrupts transcription rendering a NPC1-null animal (referred to hereafter as Npc1\textsuperscript{fl/fl}).

**Embryonic Stem Cell Targeting**

To obtain heterozygous G4 (F1 129S/B6) mouse embryonic stem cells, final targeting constructs were transfected by electroporation using standard procedures. After electroporation, cells were plated in the absence of G418 for 24 hours, and then grown in the presence of 20 µg/mL G418 during 1 week. On day 7, cells were trypsinized and seeded, and colonies were picked in duplicate and placed onto 96-well culture plates. For large-scale screening, one plate was subjected to long-range PCR using primers GF1 (forward, 5’-GGTTGGGTGCTCCGCCGTTG-3’) and LAR3 (reverse, 5’-CACACCGGTTCTTTCTGATCC-3’), whereas the other plate was used to expand the positive clones. Southern blot analysis was performed as a secondary screen. Genomic DNA from embryonic stem cells was digested with EcoRI and probed with a 0.57-kb neo probe, expecting a 7.2-kb fragment for the knock-out allele.

**Generation of NPC1-Null Mice and Genotyping Analysis**

Heterozygous Npc1\textsuperscript{+/-} embryonic stem cell clones were injected into the C57BL/6J blastocysts, selecting a chimeric mouse that transmitted the floxed allele through the germ line. Background purification was obtained by a speed congenics system\textsuperscript{16} through successive interbreeding with C57BL/6J mice. Genomic DNA extraction and PCR genotyping was performed using a commercially available Accustart II Mouse Genotyping Kit (Quanta Bioscience, Gaithersburg, MD). The knock-out alleles were detected using primers up forward, 5’-TCTTGGGGCGTGGTCTCC-CCAAGGAGGAGCCGCCTTTA-3’; up reverse, 5’-CCTCGTCTAGAGCCACCGT-3’; and neomycin resistance, 5’-CGGAATCTGTTTCCGGG-3’; resulting in a 477-bp product from the wild-type allele and a 735-bp product from the targeted allele.
CRE3 primers in two PCR reactions (Figure 1C). All animals received humane care in compliance with institutional guidelines regulated by the European Community. The experimental protocols used were approved by the Animal Care Committee of the Hospital Clinic—Universidad de Barcelona. All mice were housed in groups in cages with a 12-hour light/dark cycle. Food and water were available ad libitum and the weight of the animals (Figure 2) was determined weekly after genotyping.

**Tissue Homogenization**

Liver and brain tissue were homogenized using the Bullet Blender (Next Advance, Cambridge, MA) at 6000 × g for 5 minutes at 4°C in homogenization buffer (10 mMol/L Tris, pH 7.5, 150 mMol/L NaCl, 5 mMol/L EDTA) and a mixture of protease inhibitors. Protein concentration was quantified using the BCA Protein Reagent Assay kit (Pierce Chemical Co., Rockford, IL).

**Western Blot Analysis**

Protein lysates were subjected to SDS—polyacrylamide gel electrophoresis performed in 7.5% polyacrylamide. Proteins were transferred electrophoretically to Immobilon-P Transfer Membranes (Merck Millipore Chemicals and Life Science GesmbH, Madrid, Spain). Membranes were blocked and incubated with a 1:1000 dilution of anti-NPC1 (Abcam, Cambridge, UK) or 1:1000 dilution of anti—glyceraldehyde-3-phosphate dehydrogenase (GenScript USA, Inc., Piscataway, NJ). Membranes were washed and incubated with peroxidase-conjugated secondary antibodies (1:3000; Amersham-Pharmacia, GE Healthcare Europe GmbH, Barcelona, Spain) and developed with ECL reagent (Biological Industries Israel Beit-Haemek Ltd., Kibbutz Beit Haemek, Israel) on Kodak X-OMAT film (Kodak, Rochester, NY). Densitometric analysis was performed using ImageJ version 1.45 (NIH, Bethesda, MD; http://imagej.nih.gov/ij).
Serum Biochemical Analysis

Serum was collected after centrifugation of blood samples at 6000 × g for 1.5 minutes at 4°C in Serum Heparin Separator Tubes (Becton Dickinson, Franklin Lakes, NJ). Serum parameters were determined by the Laboratorio Core facility of Hospital Clínic i Provincial de Barcelona. Briefly, aspartate aminotransferase and alanine aminotransferase were quantified by enzymatic reactions according to the International Federation of Clinical Chemistry method without pyridoxal phosphate. Lactate dehydrogenase was determined by using the pyruvate-to-lactate conversion method kit (Germany Roche Diagnostics GmbH, Nonnenwald, Germany), and high-density lipoprotein cholesterol was measured using a commercial immunoturbidimetric method (Wako Chemicals, Neuss, Germany). Total bilirubin and direct bilirubin levels were quantified by the vanadate oxidation method (total bilirubin and direct bilirubin; Advia Chemistry Systems; Siemens AG, Munich, Germany). Biliary acids were determined by enzymatic reaction and Thio-NADH production (Spinreact,
Girona, Spain). All of the assays were measured on the Siemens Advia 1800 analyzer. The results are expressed as the ratio between the values obtained in Npc1<sup>fl/fl</sup> or Cre-Npc1<sup>fl/fl</sup> mice with respect to the corresponding WT animal (WT and Cre-WT, respectively). At least five mice were analyzed for each parameter and the statistical significance was calculated with the t-test comparing Npc1<sup>fl/fl</sup> versus Cre-Npc1<sup>fl/fl</sup>.

**Liver Cholesterol and Triacylglycerol Determination**

Triacylglycerol content in liver homogenates was determined by an enzymatic method using the Triglyceride Detection Kit (BioSystems, Barcelona, Spain). Heparin cholesterol was quantified by a fluorometric method using the Amplex Red Cholesterol Assay Kit (Life Technologies, Carlsbad, CA). Both methods were used according to the manufacturer’s instructions. The results are expressed as the ratio between the value obtained in Npc1<sup>fl/fl</sup> or Cre-Npc1<sup>fl/fl</sup> mice with respect to the corresponding WT animal (WT and Cre-WT, respectively). At least five mice were analyzed for each parameter and the statistical significance was calculated with the t-test comparing Npc1<sup>fl/fl</sup> versus Cre-Npc1<sup>fl/fl</sup>.

Figure 3: Hepatic study of 9-week-old Cre-Npc1<sup>fl/fl</sup> mice. The results are expressed as the ratio between the values of Npc1<sup>fl/fl</sup> versus WT (black bars) or Cre-Npc1<sup>fl/fl</sup> versus Cre-WT (white bars). The dashed line indicates the value at which there were no differences with the WT. Absolute values corresponding to WT are included in parentheses (see Materials and Methods). A: Percentage of body weight that corresponds to the weight of the liver (liver weight/body weight × 100). B and C: Hepatic levels of cholesterol (B) and triacylglycerol (C). D–H: Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and high-density lipoprotein (HDL). n = 5 (A–H). *P < 0.05, **P < 0.01 by t-test of matched WT.
Results

We generated a transgenic mouse with liver-selective NPC1 expression from embryonic stages to precisely define the extent in which hepatic disease participates in NP-C and evaluate the degree of primary and secondary hepatic damage in NP-C. The strategy (Figure 1) followed three steps: i) generations of mice with the Npc1 gene containing a removable floxed cassette arresting Npc1 transcription (Npc1fl/fl, see Materials and Methods), ii) crossing Npc1fl/fl and Afp-Alb-Cre+−/− mice and selecting for Afp-Alb-Cre+−/− Npc1fl/fl progeny, and iii) back-crossing Afp-Alb-Cre+−/− Npc1fl/fl to generate Afp-Alb-Cre+−/− Npc1fl/fl or Afp-Alb-Cre+−/− Npc1fl/fl progeny (Cre-Npc1fl/fl; see Materials and Methods).20 The Cre-Npc1fl/fl mouse expresses Cre-recombinase under the albumin promoter and the α-fetoprotein enhancer (hepatocyte-specific), removing the floxed cassette to allow Npc1 transcription. In the Cre-Npc1fl/fl mice after 9 weeks the protein levels in the liver were similar to wild-type animals (WT and Cre-WT); however, NPC1 was not expressed in brain (Figure 1D) and other tissues (data not shown). The WT and the Cre-WT mice did not show differences in weight (Figure 2) or in the rest of parameters analyzed in this study. Because differences between the sexes in hepatic cholesterol homeostasis have been proposed, we analyzed females and males separately. The Cre-expression pattern during embryogenesis (embryonic day 15.5) has been shown previously,18,19 and NPC1 already was detected in the liver of newborn Cre-Npc1fl/fl mice (2 days) (Figure 1E). Densitometric quantitation of the Western blot analysis suggested that hepatic NPC1 levels in the Cre-Npc1fl/fl were approximately 60% of the levels in WT mice (WT, 1.99 ± 0.89 arbitrary units; Npc1fl/fl, 1.22 ± 0.64 arbitrary units; n = 3).

When compared with WT, Npc1fl/fl and Cre-Npc1fl/fl mice showed delayed development, weight loss, and signs of ataxia after 8 weeks and died after 10 weeks (Figure 2, A–C). In both strains, the anatomic analysis showed the characteristic loss of Purkinje cells in the cerebellum,21 especially in lobules II to IV (Figure 2, D and E). Thus, re-expression of hepatic NPC1 does not ameliorate progression of the fatal neurodegeneration occurring in NPC1-null mice.

In contrast, the histology of the liver showed an almost normal morphology and, in contrast to Npc1fl/fl, few Cre-Npc1fl/fl hepatocytes accumulated intracellular lipids (Figure 2F). Furthermore, the Cre-Npc1fl/fl mice showed a normal hepatic index, highly reduced—although not completely—hepatic cholesterol levels, and hepatic triglyceride levels similar to WT (Figure 3, A–C). In contrast, the characteristic splenomegaly associated with NP-C was not corrected after hepatic re-expression of NPC1 (×1.65 in Npc1fl/fl and ×1.76 in Cre-Npc1fl/fl; n = 5). The hepatic damage—estimated by serum transaminase, bilirubin, and bile acid levels—was highly but not entirely reduced, suggesting secondary causes of liver disease (Figure 3, D–F). Similarly, cytotoxicity (serum lactate dehydrogenase) was drastically, although not completely, recovered in Cre-Npc1fl/fl mice (Figure 3G), suggesting that although most of the tissue damage recovers after re-expression of hepatic NPC1, cytotoxicity persists in Cre-Npc1fl/fl mice. Finally, we took advantage of Cre-Npc1fl/fl to further analyze the intriguing observation that NP-C patients show low levels of circulating high-density lipoprotein cholesterol, which correlates inversely with NP-C severity and the age of death.22 Indeed, the Npc1fl/fl mice showed a significant reduction of high-density lipoprotein cholesterol, although it was not corrected in Cre-Npc1fl/fl animals (Figure 3H). Thus, we speculate that low high-density lipoprotein cholesterol in NP-C patients is not caused by hepatic malfunctioning but through impaired cholesterol uptake by the lipoprotein in peripheral tissues.

Discussion

We have generated the first NPC1-null mice with liver-selective NPC1 re-expression from embryonic stages. Now, the Cre-Npc1fl/fl mice can be used in neurologic studies to eliminate those symptoms arising from hepatic disease and, as shown here, secondary to the severity of the neurodegeneration in NPC1-null animals. We show that most of the hepatic damage in NP-C is primary and largely is rescued by hepatic-NPC1. However, hepatic cholesterol, serum transaminase, and serum bilirubin levels remain slightly increased in Cre-Npc1fl/fl mice, indicating the existence of extrahepatic factors that could explain the recurrence of liver damage after transplantation.12

Neuronal re-expression of NPC1 does not completely recover neurodegeneration in NPC1-null animals,7,8 suggesting that glial and visceral NPC1 are needed for rescue. The mouse generated here now can be crossed with strains expressing Cre-recombinase under tissue-specific promoters to generate a mouse with NPC1 expression in one or various cell types. These studies undoubtedly will define causes of primary and secondary neuronal toxicity, a central question for understanding the complex clinical spectrum of NP-C and essential for designing more efficient therapeutic approaches.

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