Tamoxifen and vitamin E treatments delay symptoms in the mouse model of Niemann-Pick C

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Abstract. Niemann-Pick C disease (NPC) is an irreversible neurodegenerative disorder without current treatment. It is the result of deficient intracellular cholesterol movement. We investigated the effects of tamoxifen and vitamin E (D-alpha tocopherol) treatment on patterns of weight loss and motor function in the mouse model of Niemann-Pick C disease (Npc1−/− mice). Tamoxifen has multiple metabolic effects, including reducing oxidative damage, while vitamin E primarily has this property. Npc1−/− mice were identified and treatment was initiated at an approximate age of 21 days. Tamoxifen suspended in peanut oil was administered via intraperitoneal injection (weekly, at a dose calculated to deliver 0.023 μg/g/day). Vitamin E (25 IU) was administered orally via gavage once a week. Weight loss and Rota-Rod performance were analyzed by using Kaplan-Meyer survival curves. Tamoxifen treatment by itself significantly delayed weight loss (an endpoint of neurodegeneration) in male and female mice compared to untreated controls. Motor function was evaluated by performance on a Rota-Rod. Tamoxifen maintained Rota-Rod performance for about an extra week. Vitamin E treatment significantly delayed weight loss in females only. Rota-Rod performance was maintained slightly longer in mice treated with vitamin E. Simultaneous use of both treatments did not delay weight loss longer than tamoxifen-only treatment but had a greater effect than either treatment alone on Rota-Rod performance and demonstrated a significant positive effect on the early “learning curve” portion of the Rota-Rod evaluations. We found significant but relatively small improvements in rate of disease progression by treating Npc1−/− mice with tamoxifen and/or vitamin E. Some sex differences in response and an early improvement in Rota-Rod performance suggest areas for further study.

Key words: mice, neurodegeneration, Niemann-Pick C, Rota-Rod, tamoxifen, vitamin E.

Introduction

Niemann-Pick disease type C (NPC) is a panethnic autosomal recessive disorder of unknown pathogenesis (Vincent et al. 2003). A major biochemical finding in this disorder is the intracellular accumulation of unesterified cholesterol within lysosomes and the Golgi apparatus. These findings have prompted the conclusion that NPC is a disorder of intracellular cholesterol trafficking (Patterson et al. 1995). The NPC1 gene has recently been cloned in man (Carstea et al. 1997) and mice (Loftus et al. 1997), and the predicted protein was found to contain a sterol-sensing domain consensus site and other motifs. This suggests a direct causative role for a mutant NPC1 product in the altered cholesterol movement in NPC. In spite of this likely role, the pathophysiological basis for the symptoms present in NPC is unknown and gangliosides accumulate as well (Zervas et al. 2001).

Previous studies with npe1−/− mice revealed a time-dependent accumulation of unesterified cholesterol in every organ except the brain. Subsequently, however, it was found that the brain’s apparent failure to accumulate cholesterol was due to

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a balance of neuronal accumulation and non-neuronal loss due to demyelination (Dietschy and Turley 2001). Treatment of NPC patients with agents that lower somatic cholesterol has not had significant effects on the neurological symptoms (Patterson et al. 1993), although dimethyl sulfoxide showed clinical improvement in one patient (Sakuragawa et al. 1988), and cholestyramine and lovastatin had short-term benefits as assessed by magnetic resonance imaging (Sylvain et al. 1994). Nifedipine and probucol two agents that effectively reduce liver cholesterol did not alter the progression of CNS disease in \( \text{npc1}^{-/-} \) mice (Erickson et al. 2000). It is not certain whether most lipid-lowering drugs successfully permeate the blood-brain barrier, but a recent study found that intra-peritoneal delivery of cholesterol-mobilizing cyclodextrins decreased liver cholesterol storage in \( \text{npc1}^{-/-} \) mice, but this route or intrathecal delivery had only slight effects on onset of neurological symptoms (Camargo et al. 2001).

In this study we analyzed the effects of tamoxifen and vitamin E on the course of the disease in \( \text{Npc1}^{-/-} \) mice. Although tamoxifen is mostly known as an anti-estrogen used in the treatment and prevention of breast cancer, it has multiple other effects relevant to NPC. Tamoxifen retards glycososphingolipid metabolism (Cabot et al. 1996) by inhibiting ceramide glycosylation (Lavie et al. 1997). It has lysosomotropic properties, altering vesicular transport recycling and secretory pathways (Altan et al. 1999), which could accentuate or ameliorate the NPC1 defect in cholesterol transport. Treatment with class 2 amphiphiles mimics the \( \text{NPC1} \) cellular phenotype and endogenous amphiphiles could be dislodged by tamoxifen (Lange and Steck 1998). It inhibits glutamate-induced mitochondrial depolarization (Hoyt et al. 2000) and lowers serum cholesterol (Bilimoria et al. 1996). Orally administered tamoxifen demonstrated a marked decrease in the development of lipid lesions in apolipoprotein E knockout mice (Reckless et al. 1997). Finally, it can reduce oxidative damage (Custodio et al. 1994). Vitamin E was chosen as another anti-oxidant.

Material and methods

Animals

\( \text{Npc1}^{\text{N11H}} \) mutant mice from the BALB/cJ background were maintained by brother-sister mating of heterozygous animals. Animals were kept at the University of Arizona Animal Care Facility (PHS Assurance No. A-3248-01) on mouse chow containing 6% fat (or 10% for breeding mothers) and water \( \text{ad libitum} \). At weaning (at about 21 days of age), tail tips were removed from mice and DNA was prepared. Polymerase chain reactions (PCRs) to identify genotypes at the \( \text{Npc1}^{\text{N11H}} \) locus were performed using the primer pairs described in footnote 28 of Loftus et al. (1997).

For PCRs we used 10 mmol/L Tris, pH 8.3, 50 mmol/L KCl, 2.5 mmol/L Mg\(^2+\); 200 \( \mu \)mol/L dNTPs, 1.25U T\( _{aq} \) polymerase, and 1 \( \mu \)mol of each primer. DNA (20-40 ng) was added at 85°C, and cycles of 30 s at 95°C, 30 s at 61°C, 1 min at 72°C \( \times \) 35, and 10 min at 72°C were used. The products were separated on 1.2% NuSieve agarose gels.

Drugs

Two different drugs were investigated as a treatment in disease progression. Tamoxifen (desicate, 99% concentrate, from Sigma-Aldrich, St. Louis, MO.) was prepared by dissolving 16 \( \mu \)g of the drug per 1 ml of peanut oil at room temperature (23°C). Following dissolution, tamoxifen was filtered with the 0.8/0.2 \( \mu \)m pre-filter, filter combination (Gelman, Suporfilter #4905) and transferred to a sterile container. Peanut oil used as a control was simply filtered with a 0.8/0.2 \( \mu \)m filter and transferred to a sterile container. D-alpha tocopherol (brand “Natural Liquid Vitamin E”), was purchased from Solgar (Leonia, NJ, http://www.solgar.com).

Treatment

The \( \text{Npc1}^{-/-} \) mice were weaned, genotyped and separated according to sex when they were approximately 21 days old. Treatment was initiated at this time. The mice were divided into four groups: control, tamoxifen treated, vitamin E treated, and tamoxifen plus vitamin E treated. Mice receiving tamoxifen were injected intraperitoneally with the drug suspended in peanut oil on a weekly basis with a dosage delivering 0.023 \( \mu \)g/g/day (0.01 c.c./g. and assuming linear release). Mice receiving vitamin E were given 70 \( \mu \)l (25 IU) of D-alpha tocopherol through oral administration via a pipette on a weekly basis. Control mice were either injected with plain peanut oil (with a volume equal to tamoxifen treated mice) or were given no treatment.
**Evaluation criteria**

All mice were evaluated using two criteria: weight loss and Rota-Rod performance. Mouse body weights were recorded on a Monday, Wednesday, Friday schedule. When the weight of any mouse dropped to 80% of its maximum value, rapid deterioration and disease progression were observed. This threshold was used as the weight failure point, when found in 2 consecutive measurements, for Kaplan–Meyer analysis, and mice were euthanized shortly thereafter.

Each mouse was evaluated weekly on a Rota-Rod test instrument (Ugo Basile, NY, USA). The Rota-Rod was driven at a constant rate of 25 rpm. The mouse was given 3 trials on the Rota-Rod, and the maximum trial time was recorded. Mice that could not remain on the Rota-Rod for 10 seconds were considered to have failed.

**Analysis**

Weights and Rota-Rod times for days on which measurements were not taken were calculated via linear interpolation. The data were then analyzed by using Kaplan-Meyer survivability plots, as well as intra-group averaging for the Rota-Rod data. The Kaplan-Meyer plots show the percentage of mice considered viable versus age. In the case of fluctuation around a failure point (80% of maximal weight; < 10 seconds for 3 trials for Rota-Rod), the last time when the mouse crossed the threshold was used as the age of failure, i.e. a mouse might re-gain some weight or improve on the Rota-Rod for a measurement before again dropping below the threshold. In the case of a mouse’s death before reaching the weight failure point, the day of death was considered the failure point. The median age of failure was then used as an estimate of the survivability of the group. The chi-squares compare the Kaplan-Meyer product for each group.

**Results**

Control mice did not show any variation in disease progression (according to the criteria measured), comparing those treated with plain peanut oil versus those with no treatment. Consequently, no distinction was made between the two groups.

**Weight**

A pilot study with tamoxifen injected subcutaneously showed a significant delay in the time of weight loss (data not shown). However, some leakage along the needle track led to questions about precise dosage. Hence the current study with intraperitoneal injections was performed.

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**Table 1. Median weight failure points in days from Kaplan-Meyer survival plots**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>N</th>
<th>Median weight failure</th>
<th>95% CI</th>
<th>$\chi^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td>59</td>
<td>57</td>
<td>61</td>
<td>8.97</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>8</td>
<td>69</td>
<td>56</td>
<td>79</td>
<td>12.07</td>
</tr>
<tr>
<td>Control female</td>
<td>12</td>
<td>59</td>
<td>55</td>
<td>62</td>
<td>2.31</td>
</tr>
<tr>
<td>Vitamin E females</td>
<td>5</td>
<td>70</td>
<td>67</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Control male</td>
<td>12</td>
<td>58</td>
<td>57</td>
<td>63</td>
<td>5.51</td>
</tr>
<tr>
<td>Vitamin E male</td>
<td>5</td>
<td>57</td>
<td>56</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>24</td>
<td>59</td>
<td>57</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Tamoxifen &amp; vitamin E</td>
<td>7</td>
<td>67</td>
<td>60</td>
<td>72</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. Median Rota-Rod failure points in days from Kaplan-Meyer survival plots**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>N</th>
<th>Median Rota-Rod failure</th>
<th>95% CI</th>
<th>$\chi^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>58</td>
<td>56</td>
<td>61</td>
<td>4.03</td>
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<tr>
<td>Tamoxifen</td>
<td>8</td>
<td>64</td>
<td>51</td>
<td>71</td>
<td>5.87</td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>58</td>
<td>56</td>
<td>61</td>
<td>8.13</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>7</td>
<td>65</td>
<td>62</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>58</td>
<td>56</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Tamoxifen &amp; vitamin E</td>
<td>7</td>
<td>69</td>
<td>58</td>
<td>74</td>
<td></td>
</tr>
</tbody>
</table>
The median weight failure points from Kaplan-Meyer plots are presented in Table 1. The experimental and control groups did not show any sex differences (except with vitamin E) in disease progression and are grouped together. The control mice showed a median failure age of 59 days (57–61 days, 95% CI). Mice treated with tamoxifen showed a significant improvement over control mice. There was a 10-day improvement over controls with a median failure age of 69 days (52–79 days, 95% CI; Table 1). Female mice treated with vitamin E showed the most significant sex difference in disease progression. Male mice showed no improvement when treated with vitamin E, having a median failure age of 57 days (56–58 days, 95% CI). By contrast, females showed significant improvement, surpassing the control mice by 11 days with a median failure age of 70 days (67–74 days, 95% CI). In comparison to the tamoxifen group, the males in this group fared worse while the females fared about the same. Mice given both treatments also showed a significant difference in disease progression, beating their control counterparts by 8 days (6072 days, 95% CI, Table 1). However, the combined treatment was perhaps slightly less efficacious than tamoxifen alone.

Rota-Rod

The median Rota-Rod failure results are presented in Table 2. Control mice showed no significant sex differences (58 days, 5661 days 95% CI). Among tamoxifen-treated mice, there was a slight delay before failure on the Rota-Rod (64 days, 5171 days 95% CI). Mice treated with vitamin E showed a 7-day improvement over controls, with a median failure age of 65 days (62–66 days, 95% CI). Mice treated with vitamin E were not significantly different from mice treated with tamoxifen. Mice given both treatments, showed an 11-day increase over controls with a median failure age of 69 days (5874 days, 95% CI; Table 2). In addition, these mice showed a significant increase in absolute Rota-Rod performance (time spent on rotating rod) in the early stages of disease progression (Figure 1).

Discussion

It is now clear that the neurodegeneration seen in NPC is an autonomous process in the central nervous system. The impact of visceral pathology on the neurodegeneration in NPC was studied by Loftus et al. (2002). They reintroduced the wild-type NPC1 gene into npc1/-/- mice by targeting its expression primarily to the CNS through the use of the prion protein promoter. Interestingly, neurodegeneration was prevented, life span was normalized, and the sterility of npc1/-/- mice was corrected. The rescue did not completely rectify the accumulation of GM2 or GM3 gangliosides in some neurons and glia (Loftus et al. 2002). This observation is in agreement with other evidence that these higher order gangliosides are not obligatory players in NPC neurodegeneration (see below). Moreover, the persistence of visceral pathology in the “rescued mice” reinforces the notion of an autonomous pathological process occurring in the NPC brain. The cause of neuronal death is unclear. As a means of identifying the mode of neuronal death in NPC, Erickson and Bernard (2002) overexpressed Bcl2, an anti-apoptotic protein in transgenic mice, using the neuronal specific enolase promoter. Bcl2 prevents developmental programmed cell death, and
neuronal death caused by a variety of stimuli. Cross breeding the neuronally-expressing Bcl2 mice with npe1<sup>−/−</sup> resulted in overexpression of Bcl2 in npe1<sup>−/−</sup> mouse brain neurons, but neuronal death was not spared (Erickson and Bernard 2002). When the mice were treated with minocycline, a tetracycline analog that crosses the blood-brain barrier, and reduces neuronal death in ischemia and Huntington and Parkinson diseases, neurodegeneration proceeded as in untreated mice. This, and normal caspase1 levels in npe1<sup>−/−</sup> mouse brain, have suggested that neuronal death in NPC does not proceed by a Bcl2 and minocycline-inhibitable apoptotic pathway.

Thus, attempts to slow the progression of the disease with therapies targeted at other pathophysiological mechanisms seem warranted. As mentioned in the introduction, tamoxifen has a number of effects that could potentially ameliorate the neurodegeneration of NPC. The multiple properties of tamoxifen may be relevant to its use in the current “cocktail” now in clinical trials for amyotrophic lateral sclerosis (Muscular Dystrophy Association – http://www.azstarnet.com/star/amyotrophic lateral sclerosis (Muscular Dystrophy Association – http://www.azstarnet.com/star/Wed/30806FHmain.html).

The mode of action of tamoxifen in ameliorating the symptoms of Npc1<sup>−/−</sup> is moot. The effects of tamoxifen on ceramide glycosylation (Cabot et al. 1996, Lavie et al. 1997) could still be relevant to NPC. There is substantial accumulation of GM2, GM3, and other glycosphingolipids in the NPC brain, which has prompted studies of the role of these lipids in NPC neuropathogenesis. Taniguchi et al. (2001) found that GM1 accumulates primarily in neurons and astrocytes of npe1<sup>−/−</sup> mouse brain, whereas GM2 accumulates in neurons and macrophages. GM1, which is normally localized to synaptosomal membranes of neurons, accumulates in the cytoplasm and other dendrites, whereas GM2, which is typically absent from the neuronal soma, accumulates in perinuclear vesicles. In view of the postulated role of these sphingolipids in neurite outgrowth and dendritogenesis, these subcellular alterations alone may contribute to neuronal dysfunction.

A genetic approach aimed at reducing the levels of gangliosides by mating npe1<sup>−/−</sup> mice with mice carrying a targeted mutation in the β-1-4GalNAc transferase gene responsible for synthesis of GM2 and higher order gangliosides, successfully reduced CNS accumulation of GM2 and glycolipids GA1 and GA2, but did not improve the clinical phenotype or neuronal pathology of the npe1<sup>−/−</sup> mice (Liu et al. 2000).

A re-investigation of these mice showed that unesterified cholesterol accumulation in the cerebral cortex, the hippocampus, and other subcortical regions was markedly decreased and that a few mice lived longer than controls (Gondre-Lewis et al. 2003). A pharmacological approach was used to target another key synthetic enzyme higher upstream in the glycosphingolipid synthetic pathway: glucosylceramide synthase. Oral administration of the inhibitor of this enzyme, N-butyldeoxynojirimycin, to npe1<sup>−/−</sup> mice and cats resulted in reduced ganglioside accumulation in the brain, accompanied by a modest delay in onset of neurological dysfunction and death of the animals, and reduced Purkinje cell loss (Zervas et al. 2001).

We found that tamoxifen treatment significantly delayed the characteristic loss of weight that occurs in Npc1<sup>−/−</sup> mice (Table 1). This effect was somewhat greater in males than in females (which is not surprising given other sex differences in the symptoms of disease) (Erickson et al. 2002) but not significantly so and our results are from both sexes pooled. Tamoxifen by itself had a smaller effect on Rota-Rod performance, which assesses coordination versus ataxia. These results could suggest that the characteristic loss of weight is not merely due to an inability to feed from the overhead water and pellet containers. In contrast to our finding of a slight benefit of intraperitoneal tamoxifen on neurodegeneration in Npc1<sup>−/−</sup> mice, intracranial administration of tamoxifen to rat pups from 6 to 9 days of age inhibited Purkinje dendritic outgrowth (Sakamoto et al. 2003). Thus, of these 2 drugs, vitamin E might be more appropriate for further study.

Vitamin E has long been known as an antioxidant. Recent research has focused on particular pathways that are affected by it, e.g. signaling pathways (Rimbach et al. 2002) or superoxide production (Ulker et al. 2003). We found no effect of vitamin E in male mice, while female mice showed a significant delay in weight loss. However, sex differences were insignificant for Rota-Rod performance so that data could be pooled and vitamin E treated mice showed a mild improvement in this parameter.

Effects of tamoxifen plus vitamin E were generally similar to those of tamoxifen or vitamin E for both growth and motor performance. The combined treatment was slightly better than either alone for Rota-Rod performance but not better for weight maintenance. Perhaps it is the combined...
anti-oxidant influence of these 2 drugs which is significant.

When analyzing Rota-Rod average performance (Figure 1) instead of Rota-Rod “time of failure” (Table 2), an enhancing effect of the combination of tamoxifen and vitamin E on the early “learning” part of the curve was seen. Using Rota-Rod in the constant speed mode, normal mice improve their performance in the first sessions as they learn to walk on the rotating rod but not on the accelerating Rota-Rod (Jones et al. 1968). Only Npc1-/- treated with tamoxifen and vitamin E show such an early learning component. This effect suggests that this drug combination may be delaying the dementia that is seen in humans but has been little studied in mice (Voikar et al. 2002).

Conclusions

We have found significant but relatively small improvements in rate of disease progression by treating Npc1-/- mice with tamoxifen and/or vitamin E. The mechanism may be due to the combined anti-oxidant effect of these 2 agents. Some sex differences in response and an early improvement in Rota-Rod performance suggest areas for further study.

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REFERENCES


