Published in final edited form as:

Neurosci Lett. 2009 March 27; 453(1): 27–30. doi:10.1016/j.neulet.2009.01.072.

Genetic modulation of apoptotic pathways fails to alter disease course in tripeptidyl-peptidase 1 deficient mice

Kwi-Hye Kim^{1,2}, David E. Sleat^{1,3}, Ora Bernard⁴, and Peter Lobel^{1,3}

¹ Center for Advanced Biotechnology and Medicine, University of Medicine and Dentistry of New Jersey - Robert Wood Johnson Medical School, Piscataway, NJ 08854

² Graduate Program in Molecular Biosciences, University of Medicine and Dentistry of New Jersey - Robert Wood Johnson Medical School, Piscataway, NJ 08854

³ Department of Pharmacology, University of Medicine and Dentistry of New Jersey - Robert Wood Johnson Medical School, Piscataway, NJ 08854

⁴ Cytoskeleton and Cancer Unit, St Vincent's Institute, Fitzroy, Victoria 3065, Australia

Abstract

Late-infantile neuronal ceroid lipofuscinosis (LINCL) is a fatal, incurable neurodegenerative disease of children caused by the loss of the lysosomal protein tripeptidyl peptidase 1 (TPP1). Previous studies have suggested that Bcl-2 dependent apoptotic pathways are involved in neuronal cell death in LINCL patients and, as a result, anti-apoptotic treatments that increase Bcl-2 activity have been proposed as a potential therapeutic approach. In this study, we have directly investigated whether targeting anti-apoptotic pathways may be of value in LINCL in a mouse model of this disease that lacks TPP1 and which recapitulates many aspect of the human disease, including a greatly shortened life-span. Our approach was to genetically modify apoptotic pathways and determine the effects of these changes on the severe neurodegenerative phenotype of the LINCL mouse. LINCL mice were generated that either lacked the pro-apoptotic p53 or had increased levels of anti-apoptotic Bcl-2, changes that would exacerbate or ameliorate neuronal death, respectively, should pathways involving these proteins be important. Neither modification affected the shortened life-span of the LINCL mouse. These results suggest that either neuronal death in LINCL does not occur via apoptosis or that it occurs via apoptotic pathways not involving p53 or Bcl-2. Alternatively, pathways involving p53 and/or Bcl-2 may be involved in neuronal death under normal circumstances but may not be the only routes to this end. Importantly, our findings suggest that targeting pathways of cell death involving p53 or Bcl-2 do not represent useful directions for developing effective treatment.

Keywords

neuronal ceroid lipofuscinosis; apoptosis; tripeptidyl peptidase 1; lysosome; Bcl-2; p53

The neuronal ceroid lipofuscinoses (NCLs) are a group of autosomal recessive lysosomal storage disorders (LSDs) that primarily affect children and adolescents. NCLs are characterized

Corresponding address: Dr. Peter Lobel, CABM, 679 Hoes Lane, Piscataway, NJ 08854. Tel: 732-235-5032, FAX: 732-235-4466, Email: E-mail: lobel@cabm.rutgers.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

by profound and progressive neurodegeneration which results in clinical symptoms that include seizures, blindness, mental retardation, ataxia and premature death. At the cellular level, these diseases are characterized by the accumulation of autofluorescent storage material within the cells of affected individuals [8]. One of the most prevalent forms of NCL, the classical late infantile form (LINCL) results from mutations in the gene *TPP1* that encodes the lysosomal protease, tripeptidyl-peptidase 1 (TPP1) [22]. Typically, disease in LINCL patients is marked with the onset of seizures at 2–4 years and overall life expectancy is ~12 years of age. LINCL is always fatal and there is currently no effective treatment for affected individuals.

While neurodegeneration is central to the pathology of the NCLs, the precise cellular mechanisms underlying neuronal death are not known. Apoptosis, necrosis and autophagy have been described as pathways for the death of neurons [17,28] but other mechanisms or combinations of mechanisms may also be involved. Apoptotic cascades have been widely implicated in neuronal death in neurodegenerative disorders such as Parkinson's and Alzheimer's diseases [26] and there are some indications that they may be involved in NCLs. Apoptotic cells identified by TUNEL staining have been reported in the brain of LINCL and juvenile NCL (JNCL) patients [14] and in a mouse model of infantile NCL [9], but were not observed in a mouse model of JNCL and a variant LINCL (CLN6-deficient) mouse [1,19]. In addition, some reports have suggested that alterations in levels of the anti-apoptotic protein Bcl-2 may be associated with NCL diseases [20] but others conclude that this does not appear to be the case [13]. Thus, the role played by apoptotic pathways in neuronal death in the NCLs remains uncertain. However, this remains a question of fundamental importance as pharmacological intervention in apoptotic pathways has been proposed as a therapeutic strategy for these diseases [3,14].

In an initial study to investigate whether apoptotic cell death could be detected in a mouse model of LINCL, we performed immunostaining for a specific marker for this process, activated caspase-3 A. We failed to identify any populations or isolated neurons that specifically expressed this marker to indicate that the neurons are undergoing apoptosis (data not shown). However, a failure to directly observe apoptotic cells cannot rule out the possibility that neurons die by pathways of programmed cell death. A significant problem with this and other approaches using morphological methods on human autopsy or animal samples is that individual neurons appear to be lost over a broad time period (months to years) yet detectable apoptotic events tend to be transient and occur within a relatively narrow time span (hours). When considered with the fact that neuronal death may be spatially restricted, this means that the frequency of cells in the brain undergoing detectable apoptosis at any given time is probably very low and this may account for conflicting conclusions. It is also worth noting that at the time of death or late-stage neurodegeneration, death of all susceptible neurons may be completed, providing another possible reason as to why cells undergoing apoptosis might not be found.

In this study, we therefore directly investigated if intervening in the apoptotic pathways of neurodegenerative LINCL, using available genetically manipulated mouse models, could alleviate the symptoms or prolong the life-span of the LINCL mice. We focused on two molecules that are central to apoptotic pathways, p53 and Bcl-2 which promote or inhibit apoptosis, respectively. Our approach was to generate mice that lacked TPPI in either the absence of p53 or in the presence of increased levels of Bcl-2 in neurons with the rationale that if neurons were undergoing cell death via pathways involving these proteins, then disease in the LINCL mouse should be attenuated in either of these modified genetic backgrounds.

Mice were cared for in accordance with the guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of

New Jersey. Mice that are deficient in p53 [4] were obtained from the Mouse Models of Human Cancers Consortium (Frederick, MD). Transgenic mice overexpressing human Bcl-2 under the regulation of the neuron specific enolase promoter have been characterized, with the transgene being expressed in neurons throughout the brain as determined by immunohistochemistry and western blotting [7]. The protein is functional, with transgene heterozygosity providing protection against a number of neurological insults including ethanol-induced neurotoxicity in the neonatal cerebellum [10] stress-induced exacerbation of infarction size in an ischemic stroke model [2], and retinal ganglion cell death following intraorbital optic nerve transection [15]. *Tpp1* targeted mice (*Tpp1*^{-/-}) have been described previously [24]

Genotypes were determined by PCR analysis of genomic DNA prepared from the tail biopsies (Fig. 1) using primers listed in Table 1. TPPI deficient mice recapitulate many features of human LINCL including tremors, ataxia and seizures that reflect the rapid and severe neurodegeneration [24]. Lifespan of these mice is greatly shortened with a median survival of 138 days in a mixed C57BL6/129Sv genetic background [24]. Survival of TPPI-deficient mice is somewhat strain-dependent with a median survival of 155 days in an isogenic 129Sv background compared to 132 days in an isogenic C57BL/6J background [23]. Thus, phenotype analyses were conducted by comparing littermates with similar genetic backgrounds.

p53 knockout mice, in a mixed 129S7/SvEvBrd × C57BL/6 genetic background, were crossed with *Tpp1*^{-/-} mice in a C57BL/6J × 129Sv mixed background to generate double heterozygotes which were subsequently intercrossed. Progeny that were p53 heterozygotes in *Tpp1*^{-/-} background were identified and used to generate a cohort of p53^{+/+}, p53^{+/-}, and p53^{-/-} littermates on a *Tpp1*^{-/-} background. Transgenic mice expressing Bcl-2 under the regulation of the neuronal enolase promoter (*NSE-bcl-2* mice) [7] in an isogenic C57BL/6 background were crossed with *Tpp1*^{-/-} mice on a C57BL/6J × 129Sv mixed background to generate *Tpp1* heterozygotes, with or without the transgene. These were intercrossed to generate *Tpp1*^{-/-} mice that express normal or elevated levels of Bcl-2. Mice were handled gently to minimize the occurrence of fatal seizures in the *Tpp1*^{-/-} animals. Moribund animals having severe ataxia and tremors restricting food uptake were euthanized under IACUC policy and were scored as dying from disease on the day of euthanasia for the survival analysis.

As reported previously [24], TPPI-deficient animals present initial tremors around 7 weeks of age and develop severe ataxia throughout the course of the disease. There was no obvious deviation in trend of the disease course with the modification of p53 or Bcl-2 expression in *Tpp1*^{-/-} animals as judged from observations made in the routine handling of the animals. In addition, histological examination did not reveal any obvious differences in neurodegeneration (data not shown). Most importantly, Kaplan-Meier survival analysis of *Tpp1*^{-/-} mice in the presence or absence of p53, or with wild-type or elevated Bcl-2 was conducted using Prism v 4.03 (GraphPad Software, San Diego) and is shown in Figure 2. Neither the loss of p53 (Fig. 2A) nor increased levels of Bcl-2 (Fig. 2B) resulted in any significant increase in the lifespan of the TPPI deficient mice, as determined by log-rank test or the Gehan-Breslow-Wilcoxon Test (data not shown). In addition, we generated a small number of *Tpp1*^{-/-} mice that both expressed the Bcl-2 transgene and lacked p53, but there was no obvious effect from manipulating the two apoptotic pathways (137 day median survival, n=6, data not shown).

Apoptosis has been implicated in many neurodegenerative diseases, such as Parkinson disease, Alzheimer disease, and amyotrophic lateral sclerosis [26]. While initial claims of the involvement of apoptosis in these neurodegenerative diseases came from descriptive analyses of human post-mortem brain samples, the advent of mouse models of these diseases and mice with genetically modified apoptotic pathways have facilitated functional investigations. In some cases, altering the expression of pro-survival or pro-apoptotic molecules rescued neurons to some degree. For example, ablation of the proapoptotic Bax or over-expression of Bcl-2

attenuated neurodegeneration in a chemically-induced model of Parkinson disease [25,27]. In a mouse model of amyotrophic lateral sclerosis, transgenic expression of Bcl-2 delayed the onset of the disease and increased the lifespan the mutant mice [12]. In other cases, manipulation of apoptotic pathways has not proved beneficial. For instance, overexpression of Bcl-2 did not alter disease course in a mouse model of Niemann-Pick type C1 disease [6], and in a mouse model of congenital NCL due to cathepsin D deficiency, the loss of Bax was found to have no significant effect on survival despite attenuating some of the neuropathological manifestations of the disease [21]. Our results indicate that neurodegeneration in LINCL can occur by pathways that are independent of p53 or Bcl-2. It is quite possible that neuronal death in LINCL occurs via non-apoptotic pathways (e.g., autophagy or necrosis). However, we cannot formally exclude a contribution of apoptosis towards cell death, but if it does occur, other pathways may be involved. For example, elevated levels of the pro-apoptotic BH3 family members such as Bim, Noxa and Puma can also contribute to neuronal death [5,11,16,18]. Regardless, the findings described here have significant implications for the treatment of LINCL patients. Flupirtine, a non-opioid analgesic that is reported to have anti-apoptotic effects including upregulation of Bcl-2, has been proposed as a potential therapy for NCLs as a pharmacological agent that may delay neuronal death [3]. Our results provide no indication that this therapeutic approach is likely to be successful.

Acknowledgements

This work was supported by National Institutes of Health Grant NS37918 (P.L.) and a research fellowship (K.-H.K.) from the Batten Disease Support and Research Association. We would like to thank Dr. David Sleat for valuable advice and Drs. Eileen White and Richard Nowakowski for the discussions on apoptosis and neuronal death.

References

- Cooper JD, Russell C, Mitchison HM. Progress towards understanding disease mechanisms in small vertebrate models of neuronal ceroid lipofuscinosis. *Biochim Biophys Acta* 2006;1762:873–889. [PubMed: 17023146]
- DeVries AC, Joh HD, Bernard O, Hattori K, Hurn PD, Traystman RJ, Alkayed NJ. Social stress exacerbates stroke outcome by suppressing Bcl-2 expression. *Proc Natl Acad Sci U S A* 2001;98:11824–11828. [PubMed: 11553785]
- Dhar S, Bitting RL, Rylova SN, Jansen PJ, Lockhart E, Koeberl DD, Amalfitano A, Boustany RM. Flupirtine blocks apoptosis in batten patient lymphoblasts and in human postmitotic CLN3- and CLN2-deficient neurons. *Ann Neurol* 2002;51:448–466. [PubMed: 11921051]
- Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS, Bradley A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 1992;356:215–221. [PubMed: 1552940]
- Engidawork E, Gulesserian T, Seidl R, Cairns N, Lubec G. Expression of apoptosis related proteins in brains of patients with Alzheimer's disease. *Neurosci Lett* 2001;303:79–82. [PubMed: 11311497]
- Erickson RP, Bernard O. Studies on neuronal death in the mouse model of Niemann-Pick C disease. *J Neurosci Res* 2002;68:738–744. [PubMed: 12111834]
- Farlie PG, Dringen R, Rees SM, Kannourakis G, Bernard O. bcl-2 transgene expression can protect neurons against developmental and induced cell death. *Proc Natl Acad Sci U S A* 1995;92:4397–4401. [PubMed: 7753817]
- Goebel HH, Wisniewski KE. Current state of clinical and morphological features in human NCL. *Brain Pathol* 2004;14:61–69. [PubMed: 14997938]
- Gupta P, Soyombo AA, Atashband A, Wisniewski KE, Shelton JM, Richardson JA, Hammer RE, Hofmann SL. Disruption of PPT1 or PPT2 causes neuronal ceroid lipofuscinosis in knockout mice. *Proc Natl Acad Sci U S A* 2001;98:13566–13571. [PubMed: 11717424]
- Heaton MB, Moore DB, Paiva M, Gibbs T, Bernard O. Bcl-2 overexpression protects the neonatal cerebellum from ethanol neurotoxicity. *Brain Res* 1999;817:13–18. [PubMed: 9889302]

11. Hetz C, Thielen P, Fisher J, Pasinelli P, Brown RH, Korsmeyer S, Glimcher L. The proapoptotic BCL-2 family member BIM mediates motoneuron loss in a model of amyotrophic lateral sclerosis. *Cell Death Differ* 2007;14:1386–1389. [PubMed: 17510659]
12. Kostic V, Jackson-Lewis V, de Bilbao F, Dubois-Dauphin M, Przedborski S. Bcl-2: prolonging life in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Science* 1997;277:559–562. [PubMed: 9228005]
13. Kurata K, Hayashi M, Satoh J, Kojima H, Nagata J, Tamagawa K, Shinohara T, Morimatsu Y, Kominami E. Pathological study on sibling autopsy cases of the late infantile form of neuronal ceroid lipofuscinosis. *Brain Dev* 1999;21:63–67. [PubMed: 10082255]
14. Lane SC, Jolly RD, Schmechel DE, Alroy J, Boustany RM. Apoptosis as the mechanism of neurodegeneration in Batten's disease. *J Neurochem* 1996;67:677–683. [PubMed: 8764595]
15. Leaver SG, Cui Q, Bernard O, Harvey AR. Cooperative effects of bcl-2 and AAV-mediated expression of CNTF on retinal ganglion cell survival and axonal regeneration in adult transgenic mice. *Eur J Neurosci* 2006;24:3323–3332. [PubMed: 17229081]
16. McNeill-Blue C, Wetmore BA, Sanchez JF, Freed WJ, Merrick BA. Apoptosis mediated by p53 in rat neural AF5 cells following treatment with hydrogen peroxide and staurosporine. *Brain Res* 2006;1112:1–15. [PubMed: 16901471]
17. Mitchison HM, Lim MJ, Cooper JD. Selectivity and types of cell death in the neuronal ceroid lipofuscinoses. *Brain Pathol* 2004;14:86–96. [PubMed: 14997941]
18. Perier C, Bove J, Wu DC, Dehay B, Choi DK, Jackson-Lewis V, Rathke-Hartlieb S, Bouillet P, Strasser A, Schulz JB, Przedborski S, Vila M. Two molecular pathways initiate mitochondria-dependent dopaminergic neurodegeneration in experimental Parkinson's disease. *Proc Natl Acad Sci U S A* 2007;104:8161–8166. [PubMed: 17483459]
19. Pontikis CC, Cella CV, Parihar N, Lim MJ, Chakrabarti S, Mitchison HM, Mobley WC, Rezaie P, Pearce DA, Cooper JD. Late onset neurodegeneration in the *Cln3*^{-/-} mouse model of juvenile neuronal ceroid lipofuscinosis is preceded by low level glial activation. *Brain Res* 2004;1023:231–242. [PubMed: 15374749]
20. Puranam K, Qian WH, Nikbakht K, Venable M, Obeid L, Hannun Y, Boustany RM. Upregulation of Bcl-2 and elevation of ceramide in Batten disease. *Neuropediatrics* 1997;28:37–41. [PubMed: 9151319]
21. Shacka JJ, Klocke BJ, Young C, Shibata M, Olney JW, Uchiyama Y, Saftig P, Roth KA. Cathepsin D deficiency induces persistent neurodegeneration in the absence of Bax-dependent apoptosis. *J Neurosci* 2007;27:2081–2090. [PubMed: 17314303]
22. Sleat DE, Donnelly RJ, Lackland H, Liu CG, Sohar I, Pullarkat RK, Lobel P. Association of mutations in a lysosomal protein with classical late-infantile neuronal ceroid lipofuscinosis. *Science* 1997;277:1802–1805. [PubMed: 9295267]
23. Sleat DE, El-Banna M, Sohar I, Kim KH, Dobrenis K, Walkley SU, Lobel P. Residual levels of tripeptidyl-peptidase I activity dramatically ameliorate disease in late-infantile neuronal ceroid lipofuscinosis. *Mol Genet Metab* 2008;94:222–233. [PubMed: 18343701]
24. Sleat DE, Wiseman JA, El-Banna M, Kim KH, Mao Q, Price S, Macauley SL, Sidman RL, Shen MM, Zhao Q, Passini MA, Davidson BL, Stewart GR, Lobel P. A mouse model of classical late-infantile neuronal ceroid lipofuscinosis based on targeted disruption of the *CLN2* gene results in a loss of tripeptidyl-peptidase I activity and progressive neurodegeneration. *J Neurosci* 2004;24:9117–9126. [PubMed: 15483130]
25. Vila M, Jackson-Lewis V, Vukosavic S, Djaldetti R, Liberatore G, Offen D, Korsmeyer SJ, Przedborski S. Bax ablation prevents dopaminergic neurodegeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *Proc Natl Acad Sci U S A* 2001;98:2837–2842. [PubMed: 11226327]
26. Vila M, Przedborski S. Targeting programmed cell death in neurodegenerative diseases. *Nat Rev Neurosci* 2003;4:365–375. [PubMed: 12728264]
27. Yang L, Matthews RT, Schulz JB, Klockgether T, Liao AW, Martinou JC, Penney JB Jr, Hyman BT, Beal MF. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity is attenuated in mice overexpressing Bcl-2. *J Neurosci* 1998;18:8145–8152. [PubMed: 9763461]

28. Yuan J, Lipinski M, Degterev A. Diversity in the mechanisms of neuronal cell death. *Neuron* 2003;40:401–413. [PubMed: 14556717]

Figure 1.
Genotyping of mouse models used in this study.

Figure 2.

Survival of *Tpp1*^{-/-} mice with modified expression of p53 (Panel A) or Bcl-2 (Panel B). Log-rank test of the survival of *Tpp1*^{-/-} mice that are *p53*^{-/-} and *Tg(NSE-bcl-2)*⁺ were not significantly different from their respective *Tpp1*^{-/-} controls (P values are 0.9280 and 0.6335, respectively). The median survival and number of animals in each group were as follows: Panel A: *p53*^{+/+}, 132 days, n=22; *p53*^{+/-}, 132 days, n=56; *p53*^{-/-}, 131 days, n=19. Panel B: *Tg(NSE-bcl-2)*⁻, 141 days, n=70; *Tg(NSE-bcl-2)*⁺, 141 days, n=62.

Table 1
Primers and PCR product sizes used to determine the genotype of mutant mice.

Primer ID	Primer sequences	PCR product sizes
<i>p53</i>		
p53-501F	AAGGGAGGAAGAAGGAAAGG	570 bp wild type 480 bp mutant
p53-504R	GTCTCTAAGACGCACAAACC	
p53-503neo	AGGACATAGCGTTGGCTAC	
<i>Tg(NSE-bcl-2)</i>		
TgBcl2F	GGGTTTTTCACAGACCGCTT	400 bp transgene
TgBcl2R	TGCAGGGTGTGTTAGCAAAC	
<i>Tpp1</i>		
wtF	ATCTGATGGCTACTGGGTGG	402 bp wild type 328 bp mutant
wtR	CCCCCAAACACTGGAGTAGA	
neo	CCCGGTAGAATTCCGATCAT	