3'-phosphodiesterase (CNPase), and proteolipid protein (PLP) that mainly stain the processes of oligodendrocytes and myelin, GST-π clearly reveals the cell soma and some processes and appears suitable for morphological investigation of oligodendrocytes.

Oligodendroglial morphology was examined in the cerebral cortex since unlike those in the white matter oligodendrocytes in the cerebral cortex is less compact distributed which makes the morphological examination much easier to perform. To quantitatively evaluate the aberrations in the morphology of oligodendrocytes, the severity of morphological changes were divided to four grades based on their morphologies in the normal and untreated twitcher mice at P40 after GST-π-immunostaining: Grade 1, spherical, oval or slightly polygonal soma (Fig. 6A). This is typical normal morphology and almost all oligodendrocytes in the normal mouse show this morphology. Grade 2, slightly polygonal soma with one or two swelling-like structures (Fig. 6B, arrowhead) at the soma or the proximal processes. This morphology may be abnormal but could also be seen in oligodendrocytes of the normal mouse at low frequency, so it represents normal or slightly abnormal morphology. Grade 3, markedly altered morphology including enlarged soma, irregularly thick processes and various sizes of swellings at different sites of the processes (Fig. 6C, arrowheads). The morphologies of this grade are never seen in oligodendrocytes of the normal mouse, while most oligodendrocytes in the twitcher at P40 show these morphologies. Grade 4, severe aberrant morphology with shrunken soma and/or fragmented processes (Fig. 6D). Oligodendrocytes displaying this morphology probably correspond to the apoptotic oligodendrocytes described previously [8].

The summary of morphological analysis of oligodendrocytes was shown in Table 1. Around P40, more than 93% of the cerebral cortical oligodendrocytes (GST-π⁺ cells) in the untreated twitcher mice showed severe aberrant morphologies (Grade 3–4) (Figs. 6C and D). As viral infection controls, neonatal twitcher mice received LHCAL injection were analyzed for morphologies of transduced oligodendrocytes (β-gal⁺/GST-π⁺ cells) at P38–39. More than 86% β-gal⁺/GST-π⁺ cells in these mice had severe abnormal morphology (Grade 3–4). This sug-

gests that virus vector injection itself does not alter the morphology of oligodendrocytes in the twitcher mice.

In contrast, dramatic morphological improvement in oligodendrocytes was observed in the twitcher mice that received LHCAGm. About 83% of GALC expressing-oligodendrocytes (myc⁺/GST-π⁺ cells) exhibited completely normal morphology (Grade 1) (arrows in Figs. 6G–J). Only less than 5% of myc⁺/GST-π⁺ cells had typical abnormal morphology such as swellings at the soma or processes (Grade 3). And none was classified to Grade 4. The percentages of cells of each grade within myc⁺/GST-π⁺ cells in these mice were significantly different from that in untreated twitcher mice or twitcher mice received LHCAL injection ($P < 0.0001$, χ^2 test). Despite the significant normalization of myc⁺ oligodendrocytes in LHCAGm transduced twitcher mice, the untransduced (myc-negative) oligodendrocytes located nearing showed aberrations in morphology (arrowheads in Figs. 6H and J). When morphological analysis was performed on GST-π⁺ cells in the cerebral cortex of these mice regardless of myc-immunoreactivity, the percentages of cells of each grade were undistinguishable from those in untreated twitcher controls. This indicates that the persistent expression of GALC in a small number of glial cells did not result in diffuse correction of neighboring oligodendrocytes in the twitcher brain.

Discussion

At present hematopoietic stem cell transplantation is the only available treatment for GLD, especially the late-onset form [4]. At the mean time, gene therapy is another promising strategy to treat inherited disorders like GLD which results from mutations of a single gene. Basic studies on the understanding of the effects of transduced GALC in myelin-forming cells are important in the assessment of the potential of gene therapy in GLD. Previous experiments that introduced GALC gene into cultured oligodendrocytes derived from twitcher showed appropriate localization of the enzyme and normal morphology with highly branched processes in some transduced oligodendrocytes [16,17]. In the present study, an in vivo model for transducing oligodendro-

Table 1
Morphological analysis of oligodendrocytes in the cerebral cortex

Morphological grading	Wild type	Untreated twitcher	Twitcher-LHCAL	Twitcher-LHCAGm	
	GST-π⁺ ($n = 3$)	GST-π⁺ ($n = 4$)	β-gal⁺/GST-π⁺ ($n = 3$)	myc⁺/GST-π⁺ ($n = 4$)	GST-π⁺ ($n = 3$)
1	99.6 ± 0.3% (3281)	1.8 ± 0.3% (28)	5.3 ± 1.9% (10)	83.5 ± 1.3% (101)	1.0 ± 0.3% (19)
2	0.4 ± 0.3% (14)	4.4 ± 1.2% (69)	8.6 ± 1.2% (19)	11.9 ± 1.2% (15)	3.2 ± 0.9% (60)
3	0% (0)	85.8 ± 1.5% (1385)	73.8 ± 0.8% (160)	4.6 ± 1.2% (5)	87.8 ± 1.1% (1708)
4	0% (0)	8.1 ± 1.3% (132)	12.3 ± 1.3% (28)	0% (0)	8.0 ± 1.3% (154)
Total	100% (3295)	100% (1614)	100% (217)	100% (121)	100% (1941)

Data is presented as mean ± SE. In parentheses are the sums of cell numbers counted from 3 or 4 animals.

cytes with GALC was developed and results showed that the morphology was completely corrected in most of the transduced twitcher oligodendrocytes. This suggested that GALC may play an important role in the maintenance of the normal morphology of oligodendrocytes in vivo and provided direct evidence for the usefulness of gene therapy in GLD.

It is known that many lysosomal enzymes can be secreted from normal cells and taken up by enzyme-deficient cells, and this process, called cross-correction, occurs either by direct cell-to-cell transfer or cell surface receptor-mediated endocytosis. Cross-correction is an important concept in gene therapy strategies to treat lysosomal storage diseases since the non-transduced affected cells could also be corrected by this mechanism. And it is also the rationale for the use of hematopoietic stem cell or intracerebral cell transplantation in treating lysosomal diseases that affects the CNS such as Krabbe disease. Previous studies demonstrated that GALC can be secreted into culture medium from normal or over-expressing cells and can be incorporated by several types of enzyme-deficient cells including oligodendrocytes, astrocytes and Schwann cells in vitro [17,36,37]. In the present study, we initially expected that positive signal could be detected in the neural cells neighboring the transduced cells. However, the myc-tag-immunostaining in the brain always showed sharp boundaries between positive and negative cells and we could not observe traces of immunoreactive signals in the brain cells neighbor, even adjacent to positive glial cells above background (Figs. 6G -and I). Moreover, despite of the significant morphological correction of myc⁺ twitcher oligodendrocytes, we could not observe phenotypic changes in oligodendrocytes located nearing positive oligodendrocytes (e.g., the cell on the left upper hand of Fig. 6H pointed by arrowhead). Thus we did not obtain histological evidence for cross-correction of GALC in vivo. However, we cannot rule out the possibility that the level of GALC expression in vivo in our study was relatively low and the amount of enzyme supplied to surrounding cells was insufficient to achieve morphological restoration or to be detected by the immunostaining. Another possible reason could be that the cross-correction process of human GALC is insufficient in the mouse cells. For this, a specifically designed study should be needed to determine the evidence and implications of cross-correction of GALC in vivo.

Stable transduction of oligodendrocytes is essential for successful examination of the in vivo effects of the transgene. To transduce oligodendrocytes, we chose SVZ injection of retrovirus at neonatal period. This is because that in murine, gliogenesis takes place largely in early postnatal life and most oligodendroglial progenitor cells are located in the SVZ at neonatal period. These mitotically active progenitor cells could be infected by retrovirus, migrate away from the SVZ and differentiate into mature oligodendrocytes. Results showed that the neonatal injection of retrovirus into SVZ has significant advantages over direct injection of viral vectors into brain parenchyma to transduce mature oligodendrocytes. The latter usually transduces various types of cells concentrated at the injection sites and leads to obvious trauma and inflammatory reactions making the morphological analysis difficult to perform. Comparing with the infectious titer of LHCAGm the transduction level in this study was low (up to 10 myc⁺ cells were observed in the cerebral cortex per section). The presence of inhibitory factors (non-transducing viral particles and free viral envelope proteins) in the viral stocks may be one of the reasons for the discrepancy between the low in vivo transfectability and the viral titer [38].

Sensitive localization of the protein products of transgene in vivo is an important issue in evaluating the effects of the transgene. Although polyclonal antibodies against GALC have been used to identify GALC-expressing cells in culture systems and tissues previously [17,39], our results showed epitope tagging is also useful. Immunohistochemistry using commercially available antibodies to myc-tag gave to sensitive detection of transduced GALC with satisfactory signal-to-noise ratio in the brain tissues. Epitope-tags at the C-terminus of GALC protein did not interfere with lysosomal targeting and the bioactivity of transduced GALC as demonstrated by transient expression study and [³H]GalCer loading study. Since the antibodies to epitope-tag are specific to the product of the transgene and do not cross-react with endogenous GALC, this method will particularly be useful in gene therapy experiments using normal cells or animals and the patient's cells that produce enzymatically defective but antigenic GALC protein.

Previous studies suggested that GALC protein is synthesized as 80–90 kDa precursor which is then cleaved to yield the 50 and 30 kDa subunits [19,20,31–33]. Consistently, cell extracts of transduced mouse fibroblasts in this study also showed bands at 80–90 and 30 kDa in Western blot analysis. However, the signal of the band near 30 kDa was very weak comparing with bands at 80–90 kDa. Two possible explanations of this finding are: (i) After the precursor being cleaved to 50 and 30 kDa subunits, the myc-epitope was removed from 30 kDa subunit either through proteolytic cleavage at the carboxyl terminus or non-specific degradation. (ii) Precursor form of human GALC protein could not be effectively cleaved to two subunits in the mouse fibroblasts because of species-specific or cell type-specific processing. Further experiments such as Western blot analysis of cell extracts using antibodies recognizing GALC protein or enzyme assay on lysosomal fraction from these cells is needed to clarify this question.

Transfection study in Bosc23 and NIH/3T3 cells showed that "original ATG" gave rise to similar or higher level of expression of GALC than "Kozak ATG"

or "1st ATG." While Chen et al. [19] have reported that modification of the sequences surrounding the initiation codon to more consensus one (A in position +4 to G) resulted in improved expression of GALC in COS-1 cells. This discrepancy suggested that although the sequence around the initiation codon in GALC gene is not optimal for translation according to Kozak rule, when GALC cDNA was driven by a strong heterologous promoter the expression level may be largely influenced by expression vectors and the host cell types rather than ATG surrounding sequence. In our earlier study [37], we reported higher GALC expression in "Kozak ATG" than "original ATG" as unpublished data, however by repeated experiments thereafter we found it should be revised.

Taken together, introduction of retrovirus into the SVZ of neonatal twitcher mouse resulted in stable transduction and expression of GALC in twitcher oligodendrocytes as analyzed by RT-PCR and immunohistochemistry and significant phenotypic improvements were achieved in these transduced oligodendrocytes. Obviously, the morphological aberrations and degeneration of twitcher oligodendrocytes in the CNS [7,8,40] or in culture systems [41,42] are caused by metabolic perturbation most likely the accumulation of psychosine, thus, morphological correction of transduced oligodendrocytes in this study probably reflects normalized biological functions of these cells. Further studies will be conducted to investigate whether and how these morphological corrections are related to functional improvements and myelin sheath preservation. We believe these basic studies will be useful for the development of future gene therapy approaches to treat this severe and fatal leukodystrophy.

Acknowledgments

References

[1] D.A. Wenger, K. Suzuki, Y. Suzuki, K. Suzuki, Galactosylceramide lipidosis: globoid cell leukodystrophy (Krabbe disease), in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill, New York, 2001, pp. 3669–3694.

[2] T. Miyatake, K. Suzuki, Globoid cell leukodystrophy: additional deficiency of psychosine galactosidase, Biochem. Biophys. Res. Commun. 48 (1972) 538–543.

[3] K. Suzuki, Twenty-five years of the psychosine hypothesis: a personal perspective of its history and present status, Neurochem. Res. 23 (1998) 251–259.

[4] W. Krivit, E.G. Shapiro, C. Peters, J.E. Wagner, G. Cornu, J. Kurtzberg, D.A. Wenger, E.H. Kolodny, M.T. Vanier, D.J. Loes, K. Dusenbery, L.A. Lockman, Hematopoietic stem-cell transplantation in globoid-cell leukodystrophy, N. Engl. J. Med. 338 (1998) 1119–1126.

[5] K. Suzuki, K. Suzuki, The twitcher mouse: a model for Krabbe disease and for experimental therapies, Brain Pathol. 5 (1995) 249–258.

[6] N. Sakai, K. Inui, N. Tatsumi, H. Fukushima, T. Nishigaki, M. Taniike, J. Nishimoto, H. Tsukamoto, I. Yanagihara, K. Ozono, S. Okada, Molecular cloning and expression of cDNA for murine galactocerebrosidase and mutation analysis of the twitcher mouse, a model of Krabbe's disease, J. Neurochem. 66 (1996) 1118–1124.

[7] S.M. LeVine, M.V. Torres, Morphological features of degenerating oligodendrocytes in twitcher mice, Brain Res. 587 (1992) 348–352.

[8] M. Taniike, I. Mohri, N. Eguchi, D. Irikura, Y. Urade, S. Okada, K. Suzuki, An apoptotic depletion of oligodendrocytes in the twitcher, a murine model of globoid cell leukodystrophy, J. Neuropathol. Exp. Neurol. 58 (1999) 644–653.

[9] A.M. Yeager, S. Brennan, C. Tiffany, H.W. Moser, G.W. Santos, Prolonged survival and remyelination after hematopoietic cell transplantation in the twitcher mouse, Science 225 (1984) 1052–1054.

[10] P.M. Hoogerbrugge, K. Suzuki, K. Suzuki, B.J. Poorthuis, T. Kobayashi, G. Wagemaker, D.W. van Bekkum, Donor-derived cells in the central nervous system of twitcher mice after bone marrow transplantation, Science 239 (1988) 1035–1038.

[11] K. Suzuki, P.M. Hoogerbrugge, B.J. Poorthuis, D.W. Bekkum, K. Suzuki, The twitcher mouse. Central nervous system pathology after bone marrow transplantation, Lab. Invest. 58 (1988) 302–309.

[12] D.A. Wenger, E.Y. Snyder, R.M. Taylor, M.T. Vanier, M.A. Rafi, P. Luzi, J. Datto, Neural stem cells for the treatment of the twitcher mouse model of Krabbe disease, Am. J. Hum. Genet. 65 (1999) A116.

[13] J.S. Shen, K. Watabe, T. Ohashi, Y. Eto, Intraventricular administration of recombinant adenovirus to neonatal twitcher mouse leads to clinicopathological improvements, Gene Ther. 8 (2001) 1081–1087.

[14] C. Fantz, D.A. Wenger, M. Sands, Neonatal and in utero gene transfer utilizing adeno-associated virus and lentiviral vectors for the treatment of Krabbe's disease, Mol. Ther. 3 (2001) S228.

[15] Y.P. Wu, E.J. McMahon, J. Matsuda, K. Suzuki, G.K. Matsushima, K. Suzuki, Expression of immune-related molecules is down-regulated in twitcher mice following bone marrow transplantation, J. Neuropathol. Exp. Neurol. 60 (2001) 1062–1074.

[16] E. Costantino-Ceccarini, A. Luddi, M. Volterrani, M. Strazza, M.A. Rafi, D.A. Wenger, Transduction of cultured oligodendrocytes from normal and twitcher mice by a retroviral vector containing human galactocerebrosidase (GALC) cDNA, Neurochem. Res. 24 (1999) 287–293.

[17] A. Luddi, M. Volterrani, M. Strazza, A. Smorlesi, M.A. Rafi, J. Datto, D.A. Wenger, E. Costantino-Ceccarini, Retrovirus-mediated gene transfer and galactocerebrosidase uptake into twitcher glial cells results in appropriate localization and phenotype correction, Neurobiol. Dis. 8 (2001) 600–610.

[18] G.K. Matsushima, M. Taniike, L.H. Glimcher, M.J. Grusby, J.A. Frelinger, K. Suzuki, J.P. Ting, Absence of MHC class II molecules reduces CNS demyelination, microglial/macrophage infiltra-

tion, and twitching in murine globoid cell leukodystrophy, Cell 78 (1994) 645–656.

[19] Y.Q. Chen, M.A. Rafi, G. de Gala, D.A. Wenger, Cloning and expression of cDNA encoding human galactocerebrosidase, the enzyme deficient in globoid cell leukodystrophy, Hum. Mol. Genet. 2 (1993) 1841–1845.

[20] N. Sakai, K. Inui, N. Fujii, H. Fukushima, J. Nishimoto, I. Yanagihara, Y. Isegawa, A. Iwamatsu, S. Okada, Krabbe disease: isolation and characterization of a full-length cDNA for human galactocerebrosidase, Biochem. Biophys. Res. Commun. 198 (1994) 485–491.

[21] M. Kozak, The scanning model for translation: an update, J. Cell Biol. 108 (1989) 229–241.

[22] N. Sakai, H. Fukushima, K. Inui, L. Fu, T. Nishigaki, I. Yanagihara, N. Tatsumi, K. Ozono, S. Okada, Human galactocerebrosidase gene: promoter analysis of the 5'-flanking region and structural organization, Biochim. Biophys. Acta 1395 (1998) 62–67.

[23] J.S. Shen, X.L. Meng, T. Yokoo, K. Sakurai, K. Watabe, T. Ohashi, Y. Eto, Widespread and highly persistent gene transfer to the CNS by retrovirus vector in utero: implication for gene therapy to Krabbe disease, J. Gene Med. (in press).

[24] T. Yoshimatsu, M. Tamura, S. Kuriyama, K. Ikenaka, Improvement of retroviral packaging cell lines by introducing the polyomavirus early region, Hum. Gene Ther. 9 (1998) 161–172.

[25] N.E. Bowles, R.C. Eisensmith, R. Mohuiddin, M. Pyron, S.L. Woo, A simple and efficient method for the concentration and purification of recombinant retrovirus for increased hepatocyte transduction in vivo, Hum. Gene Ther. 7 (1996) 1735–1742.

[26] S.W. Levison, J.E. Goldman, Both oligodendrocytes and astrocytes develop from progenitors in the subventricular zone of postnatal rat forebrain, Neuron 10 (1993) 201–212.

[27] S. Raghavan, A. Krusell, Optimal assay conditions for enzymatic characterization of homozygous and heterozygous twitcher mouse, Biochim. Biophys. Acta 877 (1986) 1–8.

[28] T. Kobayashi, N. Shinnoh, Y. Kuroiwa, Metabolism of galactosylceramide in the twitcher mouse, an animal model of human globoid cell leukodystrophy, Biochim. Biophys. Acta 879 (1986) 215–220.

[29] T. Kobayashi, N. Shinnoh, Y. Kuroiwa, Metabolism of ceramide trihexoside in cultured skin fibroblasts from Fabry's patients, carriers and normal controls, J. Neurol. Sci. 65 (1984) 169–177.

[30] L.W. Swanson, Brain Maps: Structure of the Rat Brain, Elsevier Science, Amsterdam, 1998.

[31] Y.Q. Chen, D.A. Wenger, Galactocerebrosidase from human urine: purification and partial characterization, Biochim. Biophys. Acta 1170 (1993) 53–61.

[32] N. Sakai, K. Inui, M. Midorikawa, Y. Okuno, S. Ueda, A. Iwamatsu, S. Okada, Purification and characterization of galactocerebrosidase from human lymphocytes, J. Biochem. (Tokyo) 116 (1994) 615–620.

[33] S. Nagano, T. Yamada, N. Shinnoh, H. Furuya, T. Taniwaki, J. Kira, Expression and processing of recombinant human galactosylceramidase, Clin. Chim. Acta 276 (1998) 53–61.

[34] S. Szuchet, The morphology and ultrastructure of oligodendrocytes and their functional implications, in: H. Kettenmann, B.R. Ransom (Eds.), Neuroglia, Oxford University Press, New York, 1995, pp. 23–43.

[35] F.A. Tansey, W. Cammer, A pi form of glutathione-S-transferase is a myelin- and oligodendrocyte-associated enzyme in mouse brain, J. Neurochem. 57 (1991) 95–102.

[36] M.A. Rafi, J. Fugaro, S. Amini, P. Luzi, G. de Gala, T. Victoria, C. Dubell, M. Shahinfar, D.A. Wenger, Retroviral vector-mediated transfer of the galactocerebrosidase (GALC) cDNA leads to overexpression and transfer of GALC activity to neighboring cells, Biochem. Mol. Med. 58 (1996) 142–150.

[37] J.S. Shen, K. Watabe, X.L. Meng, H. Ida, T. Ohashi, Y. Eto, Establishment and characterization of spontaneously immortalized Schwann cells from murine model of globoid cell leukodystrophy (twitcher), J. Neurosci. Res. 68 (2002) 588–594.

[38] S.P. Forestell, E. Bohnlein, R.J. Rigg, Retroviral end-point titer is not predictive of gene transfer efficiency: implications for vector production, Gene Ther. 2 (1995) 723–730.

[39] R. De Gasperi, V.L. Friedrich, G.M. Perez, E. Senturk, P.H. Wen, K. Kelley, G.A. Elder, M.A. Gama Sosa, Transgenic rescue of Krabbe disease in the twitcher mouse, Gene Ther. 11 (2004) 1188–1194.

[40] H. Takahashi, H. Igisu, K. Suzuki, K. Suzuki, The twitcher mouse: an ultrastructural study on the oligodendroglia, Acta Neuropathol. (Berl) 59 (1983) 159–166.

[41] H. Nagara, H. Ogawa, Y. Sato, T. Kobayashi, K. Suzuki, The twitcher mouse: degeneration of oligodendrocytes in vitro, Brain Res. 391 (1986) 79–84.

[42] H. Ida, F. Kawame, S.U. Kim, Y. Eto, Abnormality in cultured oligodendrocytes and Schwann cells isolated from the twitcher mouse, Mol. Chem. Neuropathol. 13 (1990) 195–204.

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Research Article

Widespread and highly persistent gene transfer to the CNS by retrovirus vector *in utero*: implication for gene therapy to Krabbe disease

Jin-Song Shen [1], Xing-Li Meng [1], Takashi Yokoo [1 2], Ken Sakurai [1 3], Kazuhiko Watabe [1 4], Toya Ohashi [1 3 *], Yoshikatsu Eto [1 3]

[1]Department of Gene Therapy, Institute of DNA Medicine, The Jikei University School of Medicine, Tokyo, Japan
[2]Department of Internal Medicine, The Jikei University School of Medicine, Tokyo, Japan
[3]Department of Pediatrics, The Jikei University School of Medicine, Tokyo, Japan
[4]Department of Neuropathology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan

email: Toya Ohashi (tohashi@jikei.ac.jp)

*Correspondence to Toya Ohashi, Department of Gene Therapy, Institute of DNA Medicine, The Jikei University School of Medicine, 3-25-8 Nishi-Shinbashi, Minato-Ku, Tokyo 105-8461, Japan.

KEYWORDS

in utero • gene therapy • retrovirus • central nervous system • lysosomal storage disease • twitcher

ABSTRACT

Background
Brain-directed prenatal gene therapy may benefit some lysosomal storage diseases that affect the central nervous system (CNS) before birth. Our previous study showed that intrauterine introduction of recombinant adenoviruses into cerebral ventricles results in efficient gene transfer to the CNS in the mouse. However, transgene expression decreased with time due to the non-integrative property of adenoviral vectors. In this study, in order to obtain permanent gene transduction, we investigated the feasibility of retrovirus-mediated *in utero* gene transduction.

Methods
Concentrated retrovirus encoding the LacZ gene was injected into the cerebral ventricles of the embryos of normal and twitcher mice (a murine model of Krabbe disease) at embryonic day 12. The distribution and maintenance of the transgene expression in the recipient brain were analyzed histochemically, biochemically and by the quantitative polymerase chain reaction method pre- and postnatally.

Results
Efficient and highly persistent gene transduction to the brain was achieved both in normal and the twitcher mouse. Transduced neurons, astrocytes and oligodendrocytes were distributed throughout the brain. The transduced LacZ gene, its transcript and protein expression in the brain were maintained for 14 months without decrement. In addition, gene transduction to multiple tissues other than the brain was also detected at low levels.

Conclusions
This study suggests that brain-directed *in utero* gene transfer using retrovirus vector may be beneficial to the treatment of lysosomal storage diseases with severe brain damage early in life, such as Krabbe disease. Copyright ©

ARTICLE TEXT

Introduction

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Brain-directed *in utero* gene therapy may benefit lysosomal storage diseases that affect the central nervous system (CNS) before birth. Early treatment is crucial for the treatment of these diseases since the deterioration of the nervous system progresses rapidly after birth and severe brain damage can be irreversible. Gene transfer to produce the deficient enzyme in the brain before birth may prevent the onset and progression of the neuropathology in these diseases. Furthermore, the developing embryonic brain offers unique advantages for efficient gene delivery to the CNS. It harbors multipotent neural stem cells which are normally inaccessible for gene transduction after birth but can be transduced effectively at early embryonic stage. The subsequent migration of the progeny of the transduced stem cells following normal development would result in wide dispersion of the transgene in the mature CNS.

Recently, we reported brain-targeted *in utero* gene therapy by adenovirus [1]. Recombinant adenoviral vectors administrated into the cerebral ventricles of mouse embryos successfully transduced neural progenitor cells and led to efficient gene transduction to the mature CNS. When a therapeutic gene was introduced into the brain of a mouse model of storage disease by adenovirus, abnormal metabolic storage in the mutant brain was sufficiently prevented. However, the levels of transgene expression and vector genome in the brain decreased with time due to the non-integrative property of the adenovirus vector. Since persistent correction is required for many genetic disorders, it will be more satisfactory to use integrative viral vectors. Retrovirus vector may be the most suitable candidate at present. It has been extensively investigated and widely used in gene therapy studies including human clinical trials. The virus could integrate into host genomes stably, allowing permanent gene transduction to the target cells and their progeny.

Previous studies [2-4] demonstrated that injection of replication-defective retrovirus into sheep and monkey fetus intraperitoneally resulted in the stable transduction of hematopoietic cells and other tissues for several years without detectable pathology. These studies suggested that the direct injection of retroviral vectors *in utero* could be a safe and effective method to achieve permanent gene transduction. Although the technique of gene transfer to embryonic CNS using retrovirus has been widely used in neurobiological studies, such as lineage analysis [5-7] and gene function studies [8][9], the potential of the retrovirus-mediated *in utero* gene transfer to the brain as a therapeutic approach remains unproven. In this study, we injected high-titer retrovirus into mouse embryonic brain intraventricularly and performed detailed analysis of transgene distribution in the mature brain and a long-term study of the presence/expression of transgene by several histochemical and biochemical methods.

The primary motivation of our study on prenatal gene therapy is from an interest in treating globoid cell leukodystrophy (GLD, Krabbe disease), a genetic demyelinating disease caused by genetic defect of β-galactocerebrosidase (GALC) [10]. In this disease, neurological degeneration occurs before birth and progresses rapidly soon after birth causing early death of patients. The cause of demyelination is suggested to be the result of the degeneration or dysfunction of myelin-forming cells (oligodendrocytes and Schwann cells), caused by an accumulation of psychosine, a toxic metabolite of β-galactocerebrosidase [11]. Our study showed that intraventricular injection of retrovirus *in utero* results in sufficient and stable gene transfer into neural cells including oligodendrocytes in normal and twitcher mice (an authentic murine model of Krabbe disease [12]), suggesting the potential usefulness of this approach in treating Krabbe disease and other genetic neurodegenerative disorders.

Materials and methods

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Plasmids and virus preparation

A retrovirus vector pLHCX (Clontech, Palo Alto, CA, USA) was modified by replacing the cytomegalovirus (CMV) immediate early promoter with 1.7 kb of CAG hybrid promoter (generated by connecting the CMV enhancer and the modified chicken β-actin promoter [13]). The LacZ gene (excised from plasmid pcDNA3.1/Myc-His/*lacZ*; Invitrogen Corp., Carlsbad, CA, USA) was placed under the CAG promoter to generate a retrovirus vector named pLHCAL where a hygromycin phosphotransferase gene was driven by a long terminal repeat (LTR) and the LacZ gene was driven by the CAG promoter. To produce recombinant retrovirus, the ecotropic packaging cell line ΨMP34 [14] was transfected using the calcium phosphate method (Cellphect transfection kit; Amersham Pharmacia, Piscataway, NJ, USA) with pLHCAL. After selection with 0.5 mg/ml of hygromycin B (Wako, Osaka, Japan), a drug-resistant subclone (#44.3) producing the highest titer of LHCAL was isolated and used in this study. Retrovirus was concentrated according the protocol reported by Bowles *et al.* [15]. Briefly, virus-producing cells were grown in 15-cm dishes at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/l) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS), and the culture media were changed (18 ml per dish) when cells were confluent. About 10 h after medium change, the virus-containing supernatant (700 ml) was harvested, filtered through 0.22-μm filters (Stericup; Millipore, Bedford, MA, USA) and centrifuged at 6000 *g* at 4 °C overnight. The pellet were resuspended in 1 ml Hanks' balanced salt solution (HBSS) and stored at -85 °C in small aliquots until use.

Viral titer assay and *in vitro* infection

For the determination of viral titers, viral RNA was extracted from viral stocks and their serial dilutions using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) and eluted in 55 µl of RNase-free water. The reverse-transcription was performed using the SuperScript first-strand synthesis system (Invitrogen) with random hexamer primers in a final volume of 20 µl (8 µl of each viral RNA was used as the template). To determine the copy numbers of viral cDNA, a TaqMan polymerase chain reaction (PCR) for the LacZ gene was performed as described previously [1]. Two microliters of each cDNA was used as the template. As standard controls, 10-fold serial dilutions of known concentration of linearized plasmid pLHCAL were used. The titers of unconcentrated and concentrated viral supernatant were 9.8×10^8 and 3.9×10^{11} particles per ml, respectively.

For the assessment of the effects of viral titers on the transduction efficiency *in vitro*, NIH/3T3 target cells were grown in 24-well plates (NunC, Roskilde, Denmark) in DMEM supplemented with 10% (v/v) FBS. When approximately 60% confluent, cells were incubated with 0.3 ml medium containing 0.01-10 µl of concentrated LHCAL ($n = 3$) overnight at 37 °C in the presence of 8 µg/ml Polybrene (Sigma, Saint Louis, MO, USA). Forty-eight hours after infection, cells were fixed with 0.25% glutaraldehyde in phosphate-buffered saline (PBS) for 5 min on ice, washed twice with PBS and stained with X-gal solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM $MgCl_2$ in PBS) overnight at 37 °C, and the number of positive cells per mm^2 was counted. The numbers counted from three randomly selected places in each well were averaged to obtain a value per mm^2 for each well.

Animals and *in utero* injection

Breeding pairs of C57BL/6 strain and twitcher heterozygotes (C57BL/6J, twi/+) were purchased from CLEA Japan (Tokyo, Japan) and The Jackson Laboratory (Bar Harbor, ME, USA), respectively, and maintained in our laboratory under standard housing conditions. Genetic status for the twitcher mutation was determined by a PCR method using genomic DNA extracted from the clipped tail [16]. Mice were mated and the day when a vaginal plug was found was designated as embryonic day 0 (E0). *In utero* injection was performed as previously described [17]. Briefly, approximately 2 µl HBSS containing retrovirus (titer see results), Polybrene (concentration see results) and 0.01% Fast Green (Sigma) were injected into the cerebral lateral ventricles of embryos with a heat-pulled glass micropipette at E12.

Tissue preparation and X-gal histochemistry

The mice were anesthetized and fixed with transcardial perfusion of chilled 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at the time of analysis. Brains were removed and postfixed in the same fixative overnight at 4 °C and cryoprotected in 15% sucrose in 0.1 M PB. Semi-serial coronal sections were made at a thickness of 20-40 µm using a cryostat. Sections were stained overnight at 37 °C with X-gal staining solution as above and counterstained with hematoxylin/eosin (H&E).

To assess the effects of virus and Polybrene on the brain cells of injected mice, brains of wild-type mice with or without viral infection at E12 were harvested at E17. Coronal slices were made and immersion-fixed in 2% paraformaldehyde and 0.25% glutaraldehyde in PBS for 1 h at 4 °C. After washing with PBS, the brain tissues were incubated in X-gal staining solution overnight at 37 °C with 0.02% NP-40 and 0.01% sodium deoxycholate. After a PBS wash, brain slices were dehydrated, cleared and embedded in paraffin. Coronal sections of 3 µm thickness were made, dewaxed and counterstained with H&E (Figures 2d and 2e).

Immunohistochemistry

Tissues were processed as described above. The cryosections (20 µm thick) were incubated with 0.3% Triton X-100 in 0.1 M PBS (PBST) for 1 h at room temperature (RT), then treated with 0.5% H_2O_2 in PBST for 30 min at RT to eliminate endogenous peroxidase activity. Following incubation with 5% normal goat serum (NGS) in PBST for 1 h at RT, the sections were incubated with rabbit polyclonal antibody to β-galactosidase (β-gal; 5 prime ⟶ 3 prime Inc., Boulder, CO, USA) diluted 1 : 500 in 3% NGS/PBST overnight at 4 °C. The sections were then incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1 : 200 in 3% NGS/PBST for 1h at RT, followed by incubation for 30 min with avidin-biotin complex (ABC) reagent (ABC Elite Kit; Vector Laboratories) at RT. The peroxidase labeling was visualized with 3,3′-diaminobenzidine (DAB) substrate (Vector Laboratories) and sections were dehydrated, cleared and mounted.

For the analysis of proportions of transduced neural cells, the number of each cell type was counted on brain sections stained for β-gal immunohistochemistry. The sections from three 1-month-old recipients were analyzed. The coronal sections corresponding to level 30 in the *'Brain Maps: Structure of the Rat Brain'* by Swanson [18] were used to analyze the cerebral cortex, the hippocampal formation and the diencephalon and level 55 for the brain stem and the cerebellar white matter.

Immunofluorescence was performed as previously described [1][19]. The following primary antibodies were used: rabbit polyclonal anti-phosphorylated histone H3 (Upstate Biotechnology, Lake Placid, NY, USA; 1 : 100 dilution), rabbit polyclonal anti-β-gal (5 prime ⟶ 3 prime Inc.; 1 : 100), mouse monoclonal anti-β-gal (Promega, Madison, WI, USA; 1 : 500), mouse monoclonal anti-neuronal nuclei (NeuN; Chemicon, Temecula, CA, USA; 1 : 100), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; Dako, Glostrup, Denmark; 1 : 300), rabbit polyclonal anti-pi-form of glutathione-S-transferase (GST-π; MBL, Nagoya, Japan; 1 : 500) and rabbit polyclonal anti-myelin basic protein (MBP; Dako; 1 : 500). The secondary antibodies used were: FITC-conjugated goat anti-rabbit IgG and rhodamine-

conjugated goat anti-mouse IgG (Cappel, Aurora, OH, USA; 1 : 100). The sections were viewed under a fluorescence microscope and photographed using an AquaCosmos CCD camera (Hamamatsu Photonics, Hamamatsu, Japan).

β-Galactosidase enzyme assay

β-Gal activities of the recipients' whole brain were measured using the β-galactosidase enzyme assay kit (Promega). The brains were homogenized in 10 volumes of distilled water with a Potter-Elvehjem homogenizer and assayed for β-gal activity according to the manufacture's instructions. The activities were calibrated by subtracting the endogenous cellular β-gal activity in age-matched untreated controls at each time point and expressed as activity per total protein (milliunit/mg). Protein concentrations were determined with the BCA protein assay reagent (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as standard.

Quantitative PCR

The mRNA level of the LacZ gene was quantified by quantitative reverse-transcription (RT)-PCR. Total RNA was extracted from the whole brain of recipients using the RNeasy lipid tissue kit (Qiagen) following the manufacturer's instructions including DNase I treatment. The RT reaction was performed using the SuperScript first-strand synthesis system (Invitrogen) with an oligo(dT) primer. The TaqMan probe and primers specific for the LacZ gene were used as previously described [20]. The quantities of LacZ gene were normalized with endogenous control β-actin gene to account for the variability in the initial concentrations and qualities of template. β-Actin cDNA was estimated by quantitative RT-PCR with SYBR Green dye. The primers used to detect mouse β-actin cDNA designed by Primer Express software (ABI, Tokyo, Japan) were 5$'$-tattggcaacgagcggttc-3$'$ (forward primer) and 5$'$-tggatgccacaggattccat-3$'$ (reverse primer, amplicon length of 78 bp). The PCR reaction was carried out in an ABI Prism 7700 sequence detection system under conditions described previously [1]. Each standard and sample was duplicated. The absence of nonspecific amplification including primer dimer formation was confirmed by dissociation curve analysis immediately after the reaction.

To quantify the amount of integrated provirus DNA in the recipients' brains, quantitative PCR for the LacZ gene was performed as previously described [1].

Analysis of tissue distribution of vector

Multiple tissues were harvested from wild-type recipients and their mothers at analysis. Genomic DNA was extracted using the DNeasy tissue kit (Qiagen). The primers used to detect the LacZ gene and the mouse β-actin gene were the same as that for real-time PCR previously described [1] (amplicon lengths of 122 and 77 bp, respectively). Genomic DNA (300 ng) was added to AmpliTaq Gold Master Mix (ABI) containing 0.2 µM of each primer in a final volume of 50 µl. The PCR reactions were carried out as denaturation and activation at 95 °C for 5 min, followed by 40 cycles (for the LacZ gene) or 30 cycles (for the β-actin gene) of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. Ten microliters of each PCR product were electrophoresed on a 3.5% agarose gel and stained with ethidium bromide. To determine the sensitivity of the PCR condition for the LacZ gene, a standard was generated from the genomic DNA of the brain of heterozygous *Bmp4^{lacZneo}*, a LacZ knock-in mouse strain containing a single copy of the LacZ gene [21][22], serially diluted (10^{-1} to 10^{-5}) with normal mouse genomic DNA. The sensitivity of the LacZ PCR is about 1 copy of LacZ gene in 10^4 genomes.

In one animal (recipient at 15.5 months, animal-1 in Figure 6), tissues were harvested and divided into two parts. One part was used for PCR analysis as described above. The other part of the tissues was embedded in OCT compound. Serial cryosections were made at a thickness of 10 µm. Blood was obtained from a peripheral vessel and bone marrow cells were collected from the femur, tibia and pelvis. Cell suspension of the spleen was prepared by manual dissociation. Mononuclear cells were isolated from the blood, bone marrow and the spleen by Ficcoll-Conray (IBL, Fujioka, Japan) density gradient separation. Smears of mononuclear cells were prepared on MAS-coated slide glasses (Matsunami, Osaka, Japan) and allowed to air dry for 30 min at RT. Cryosections and smears were fixed with 0.25% glutaraldehyde in PBS for 10 min, incubated with X-gal staining solution overnight at 37 °C, and counterstained with H&E and nuclear fast red, respectively. The rest of the mononuclear cells from the bone marrow and the spleen were used for PCR analysis.

Results

Efficient gene transfer into embryonic brain under optimized conditions

It is known that the addition of positively charged compounds, such as Polybrene, during infection facilitates the infection of cells by retrovirus *in vitro*. To determine the satisfactory concentration of Polybrene *in utero*, concentrated LHCAL supplemented with different concentrations of Polybrene (at a final concentration of 0, 40, 80 or 160 µg/ml) was injected into the cerebral lateral ventricles of mouse embryos at E12. The gene transduction efficiency was evaluated by the contents of integrated provirus in the recipients' brains at 5 days after injection (E17). Quantitative TaqMan-PCR for the LacZ gene was used to quantify provirus DNA. Results showed that the transduction level increased with increasing Polybrene concentrations (Figure 1a). There was a linear correlation (r = 0.999) between transduction level and Polybrene concentration in the range 0-80 µg/ml. The level of gene transduction reached a peak at a concentration of 80 µg/ml. However, further increment of the concentration to 160 µg/ml did not increase the transduction efficiency.
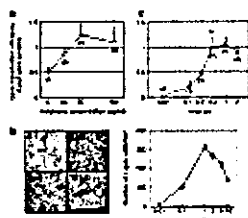
Figure 1. Optimization of transduction conditions. (a) Gene transduction efficiencies when LHCAL supplemented with various concentrations of Polybrene were injected. *P = 0.02 and P = 0.72, when transduction efficiency on Polybrene concentration of 80 µg/ml compared with that on 0 and 160 µg/ml, respectively (Mann-Whitney test). (b) NIH/3T3 cells grown in 24-well plates were infected with various titers of concentrated LHCAL and the transduction efficiencies were evaluated by X-gal staining. Left: Representative photographs of X-gal-stained NIH/3T3 cells. The volumes of virus stock used per well are indicated. Right: Number of β-gal+ cells per mm^2 (n = 3). (c) Gene transduction efficiencies when various amounts (µl) of concentrated virus were injected. **P = 0.77 and P = 0.39, when transduction efficiency on a volume of 0.4 µl/embryo compared with that on 1 or 2 µl/embryo, respectively (Mann-Whitney test). The data are presented as mean ± SE, the numbers in parentheses are numbers of animals studied. Scale bar: 1 mm
[Normal View 33K | Magnified View 52K]

An *in vitro* study using the concentrated LHCAL to infect NIH/3T3 cells showed that excessive titers of viruses do not improve, and even reduce, the infection efficiency (Figure 1b). This result suggests that some factors in virus stock may inhibit infection, especially in high multiplicity of infection (MOI) condition. To determine the satisfactory doses of virus to achieve the highest transduction efficacy *in utero*, serially diluted virus stock (supplemented with 80 µg/ml of Polybrene) was injected into the cerebral ventricles of mouse embryos at E12 and transduction efficiencies were evaluated by the contents of provirus in the recipients' brains as above. Results showed that transduction levels increased with increasing virus doses in the range 0.02-0.4 µl of concentrated LHCAL per embryo, then reached a plateau when 0.4 µl of virus stock was injected. Further increasing of virus to 2 µl did not increase the transduction level (Figure 1c).

The results showed that the satisfactory concentration of Polybrene added to virus is 80 µg/ml and 0.4 µl of concentrated LHCAL (1.6×10^8 particles per embryo) is the minimal dose to achieve the highest infection efficiency *in utero*. This condition was used throughout the following study.

Widespread gene transduction to the CNS by retrovirus *in utero*

To investigate the pattern of gene transduction to the CNS by retrovirus *in utero*, we injected LHCAL to the lateral ventricles of mouse embryos at E12. At E12, many cells lining the ventricular zone are phosphorylated-histone-H3 positive (Figure 2a), indicating they are undergoing active proliferation. This permits the possibility of efficient transduction of these cells, since integration of murine leukemia virus (MLV) DNA into nuclear DNA of cells depends on mitosis and active division of target cells is the prerequisite of highly efficient gene transduction.

Figure 2. Widespread CNS gene transduction after LHCAL injection. (a) Immunofluorescence of brain sections of mouse embryos at E12 stained with anti-phosphorylated histone H3, a mitosis-specific marker. Many ventricular zone cells were phosphorylated histone H3 positive (green) indicating they were in active proliferation (red: propidium iodide nuclear counterstaining). (b-d, f) X-Gal-stained coronal brain sections from the mice that received LHCAL at E12. (b) Cerebral cortex at E17. (c) Cerebellar anlage at E17. Arrowheads, β-gal+ cells inside cerebellum. Arrows, β-gal+ external granular cells. (d, e) Microscopic photographs of paraffin sections of cerebral cortex from an embryo that received LHCAL injection (d) and the untreated litter-mate (e) at E17. (f) Brain sections at P30 listed rostral to caudal. 4V, 4th ventricle; CP, cortical plate; IZ, intermediate zone; LV, lateral ventricle; SVZ, subventricular zone; VZ, ventricular zone. Scale bar: 20 µm in (d, e); 100 µm in (a); 200 µm in (b, c); 1 mm in (f)
[Normal View 52K | Magnified View 92K]

Seventy-one out of 76 embryos (93.4%) who received *in utero* injection of LHCAL were alive when analyzed at E17. For postnatal analysis, the recipients were allowed to be delivered normally and 80% (36/45) of them were alive at postnatal day 3-4 (P3-4). All pups alive at P3-4 (n = 36) survived to weaning and developed normally without noticeable behavioral abnormality. These data indicate that approximately 7% of recipients were dead before E17, and another 13% of recipients died between E17 and P3. The final survival rate of the recipients (80%) was similar to that in the normal development of untreated mice at E19 (67-100%, average 84%) [23], suggesting that the injection did not significantly affect survival rate in these mice. No tumors or other anatomical abnormalities were observed in the recipients' brains.

Efficient gene transfer to the CNS was observed in all recipients examined by X-gal histochemical staining at various time points (n = 8 at E17; n = 26 at 1-14 months). β-Gal+ cells were diffusely distributed throughout the brain of the recipients when analyzed at 5 days after injection (E17) (Figures 2b and 2c). In the cerebellum, in addition to scattered

β-gal+ cells, some external granular cells, the progenitors of the granule cells, were also transduced (Figure 2c, arrows). No obvious difference was observed in the density and the morphology of the cells in the ventricular and subventricular zone of the cerebral cortex between the infected mice and their untreated littermates at E17 (Figures 2d and 2e), suggesting that the injection of virus and Polybrene under the condition used in this study has no significant cytotoxicity on the developing brain cells. At P30, when the migration and differentiation of neural cells are almost completed in the mouse brain, many β-gal+ cells were identified in most regions of the brain although regional differences in the intensities of transduction could be observed (Figure 2f). Most intensive sites of transduction were the cerebral cortex and cerebellar granular layers. A moderate number of transduced cells were distributed in the basal ganglia, hippocampal formation, thalamus and the cerebral and cerebellar white matter. A relatively smaller number of transduced cells were distributed in the brain stem. No positive signal was observed in untreated control brains.

Characterization of transduced neural cells

The cell types of transduced neural cells were identified by their morphology and anatomical locations. Immunohistochemistry against β-gal was used to identify the transduced neural cells. We found that the immunostaining gave more detailed and sensitive detection of positive cells and the distribution pattern of positive cells in the immunostaining was similar to that in X-gal staining.

Many β-gal-immunoreactive neurons with relatively large cell bodies and long processes were distributed in the cerebral cortex, basal ganglia and the hippocampal formation (Figures 3a and 3b). In the cerebellum, numerous immunoreactive granule cells packed in granular layers and some Bergmann glia and Purkinje cells extended processes to the molecular layer (Figure 3c). Transduced oligodendrocytes were distributed both in the gray and white matter throughout the brain and could often be observed as clusters. The myelinating oligodendrocytes had many immunoreactive longitudinal processes arrayed in parallel, connecting to the cell soma by short branches (Figure 3d). The longitudinal processes were presumably the inner and outer cytoplasmic tongues of the myelin sheaths and describe the myelin segments supported by transduced oligodendrocytes. Most transduced astrocytes were distributed in the gray matter having protoplasmic morphologies with highly branched feather-like processes (Figure 3e). Frequently, immunoreactive processes terminated as an end foot on a blood vessel could be seen. The proportion of transduced neural cells in some brain regions is shown in Table 1.

Figure 3. Morphological identification of cell types of transduced cells. (a) β-Gal-immunoreactive cortical neurons. **(b)** β-Gal-immunoreactive granule neurons in the dentate gyrus of the hippocampal formation. **(c)** Numerous cerebellar granule neurons were β-gal-immunoreactive. Note the parallel fibers of granule cells in the molecular layer were also immunoreactive. Arrow, an immunoreactive Bergmann glia. **(d)** A cluster of immunoreactive oligodendrocytes in the white matter of cerebral cortex. Note many immunoreactive longitudinal processes arrayed in parallel describing the myelin segments supported by transduced oligodendrocytes. **(e)** Some immunoreactive protoplasmic astrocytes in the cerebral cortex. Gr, granular layer; Mo, molecular layer. Scale bar: 100 μm in (a-e)
[Normal View 43K | Magnified View 70K]

Table 1. Proportion of transduced neural cells in various brain regions

Cell type	Cerebral cortex[a]	Hippocampal formation	Diencephalon	Brain stem	Cerebellar white matter
Neuron	31.2 ± 1.6% (700)	54.6 ± 2.4% (823)	9.9 ± 1.7% (98)	2.2 ± 1.4% (9)	0% (0)
Astrocyte	53.9 ± 0.7% (1199)	40.2 ± 3.7% (614)	54.3 ± 2.9% (499)	43.3 ± 2.7% (158)	31.0 ± 1.2% (194)
Oligodendrocyte	14.9 ± 1.5% (327)	5.2 ± 1.4% (74)	35.8 ± 3.7% (318)	54.5 ± 2.6% (199)	69.0 ± 1.2% (435)
Total	100% (2226)	100% (1511)	100% (915)	100% (366)	100% (629)

Data is presented as mean ± SE (*n* = 3). In parentheses are the sums of cell numbers counted from three animals.
[a] Including the cerebral white matter.

The morphological identification of cell types was further confirmed by double immunofluorescence for β-gal and cell-type-specific markers (NeuN, a neuronal marker; GFAP, an astroglial marker; GST-π [24] and MBP, oligodendroglial markers) on the recipient brain sections (Figure 4). β-Gal-immunoreactive longitudinal processes of oligodendrocytes were also overlapped with MBP (data not shown).
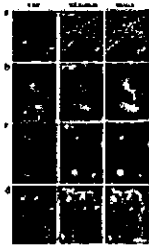

Figure 4. Cell-type identification of transduced cells by double immunofluorescence. (a) β-Gal-immunoreactive cells (green) in the cerebral cortex were NeuN+ (red), indicating they are neurons. (b) A β-gal-immunoreactive cell (red) in the cerebral cortex was GFAP+ (green), indicating it is an astrocyte. It has protoplasmic morphology and has a blood-vessel end foot (arrowheads). (c) β-Gal-immunoreactive cells (red) in the brain stem were immunoreactive for GST-π (green), an oligodendrocyte-associated enzyme, indicating they are oligodendroglia. (d) Some β-gal-immunoreactive cells (red) with processes extended to the molecular layer of the cerebellar cortex were GFAP+ (green), indicating they are Bergmann glia. Scale bar: 40 µm in (a, d); 20 µm in (b, c)
[Normal View 46K | Magnified View 74K]

Long-term gene transfer to the brain

Twenty-six recipients (aged from 1 to 14 months) were examined for long-term expression and the maintenance of transgene by histochemistry, enzymatic assay and PCR-based RNA/DNA analysis.

By X-gal staining, the general distribution patterns of β-gal+ cells in the transduced brains at 1, 3, 6, 9 and 14 months after birth were similar. Although there were some variations in the number of positive cells among animals, the overall transduction level was similar and did not exhibit the trend of decrement with time (Figure 5a). Quantitative assessment of the β-gal activities in brain homogenates revealed that β-gal expression was constant throughout 14 months (Figure 5b, top). The LacZ transcript was also monitored by quantitative RT-PCR using LacZ-gene-specific probe and primers. Similar to protein expression, the β-gal mRNA expression also persisted to at least 14 months after birth (Figure 5b, middle). Brain tissues were also examined for the presence of transduced LacZ gene by quantitative PCR and results showed that the contents of LacZ DNA (i.e. provirus DNA) in the brains were stably maintained for 14 months without decrement (Figure 5b, bottom).

Figure 5. Long-term presence/expression of transgene in recipients' brains. (a) X-gal-stained coronal sections of the brain which received LHCAL at E12. (b) Quantitative analysis of the level of transgene and its expression in the brain at various time points. Top: β-gal enzyme activities in the brain. Middle: LacZ mRNA expression in the brain assessed by quantitative RT-PCR. Bottom: Amount of provirus DNA in the brain assessed by quantitative PCR for the LacZ gene. The numbers in parentheses are numbers of animals studied. Scale bar: 1 mm
[Normal View 61K | Magnified View 91K]

Tissue distribution of viral vectors

To determine whether gene transduction by intraventricular administration of retrovirus was limited to the CNS, PCR analysis was performed on the genomic DNA extracted from different tissues of six recipients (n = 3 at E17; n = 3 at 15.5 months, females) to detect integrated provirus DNA (the LacZ gene). Results showed that, in addition to the brain, multiple tissues including the skin, heart, lung, liver, ovary and bone marrow mononuclear cells from the recipients contained the LacZ sequence, although the positive tissues were not consistent among animals (Figure 6). Kidneys were negative for the LacZ gene in all six recipients examined. To confirm the presence/expression of LacZ gene in these tissues, X-gal staining was performed on the sections of the heart, lung and liver and mononuclear cells of the bone marrow, spleen and the peripheral blood from one recipient (15.5 months, animal-1 in Figure 6). β-Gal+ cells were detected from the heart, liver and bone marrow cells although the transduction level was much lower than that in the brain (e.g., 0–4β-gal+ cells per longitudinal section of the heart) (data not shown). These observations indicate that vectors injected into the cerebral ventricles spread, probably through fetal circulation, to multiple organs of recipients. To further determine whether transplacental transfer of vector from the fetus to the mother occurs, tissues from two mothers whose embryos received intraventricular injection of LHCAL were also tested by LacZ-PCR at 17 days gestation. Several organs including the heart, lung, liver and the kidney from the mothers were positive for the LacZ sequence (Figure 6).

Figure 6. PCR analysis of tissue distribution of viral vector. LacZ-specific PCR (amplicon length of 122 bp) was used to detect provirus DNA in tissues from LHCAL recipients (n = 3 at E17; n = 3 at 15.5 months of age) and mothers (n = 2 at 17 days gestation). Many tissues were positive for the LacZ sequence in addition to the brain
[Normal View 34K | Magnified View 54K]

Observations in twitcher mouse brain that received LHCAL *in utero*

The goal of our study is the application of intrauterine gene therapy to genetic neurodegenerative disorders such as Krabbe disease. The efficient gene transfer into neural cells including oligodendrocytes suggested the possible utility of prenatal gene transfer by retrovirus in the treatment of this kind of disease. As a preliminary study of the next challenge of therapeutic study using this approach in animal models of Krabbe disease, LHCAL was injected into the mouse embryos of pregnant twitcher heterozygotes (twi/+) at gestational day 12. The survival rate of the recipients to weaning was 63.3% (19/30), which is similar to that in the normal C57BL/6 strain (19/30 vs. 36/45, $P = 0.12$, Fisher's exact test). Four homozygous twitcher mice were obtained and gene transduction to the brain was analyzed by β-gal immunohistochemistry ($n = 2$ at P20; $n = 1$ at P30; and $n = 1$ at P41).

Efficient introduction of the LacZ gene into the brain was achieved in all recipient twitcher mice and their unaffected littermates examined (Figures 7a and 7b). The distribution pattern and the composition of transgene positive cells in the twitcher mice were similar to that in the normal mouse strain described above. Consistent with earlier studies [25] [26], morphological alterations including enlarged somas and swellings in processes were recognized in some of transduced oligodendrocytes in the twitcher mice even at P20 (Figure 7d). At P30, many transduced oligodendrocytes in twitchers showed these morphological alterations (88.4% (191/216 cells) in the cerebrum; 82.6% (138/167) in the brain stem; and 79.7% (106/133) in the cerebellar white matter). At P41, abnormal swellings in processes further progressed in number and size generally and unstained vacuoles could be frequently seen in the swellings (Figures 7e and 7f). The changes were shown in most, but not all, transduced oligodendrocytes. Immunoreactive Myelin segments*, however, were still relatively preserved in many oligodendrocytes, despite severe abnormalities of the soma and processes (Figure 7f).
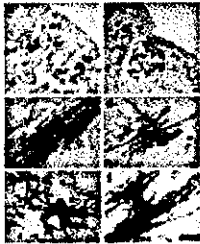

Figure 7. Efficient transduction to oligodendrocytes in the twitcher brain after LHCAL injection. Anti-β-gal immunohistochemistry of the brain sections from the twitcher mice which received LHCAL at E12. (a, b) Overview of widespread gene transduction to the twitcher brain at P30. (a) Cerebral cortex. (b) Cerebellar cortex. (c-f) High-magnitude view of immunoreactive oligodendrocytes from control and the twitcher mice. (c) Wild-type littermate at P41. (d) Twitcher at P20. (e, f) Twitcher at P41. Arrows, unstained vacuoles in the swellings. Scale bar: 200 μm in (a, b); 20 μm in (c-f)
[Normal View 42K | Magnified View 68K]

Discussion

This study demonstrated that direct intraventricular injection of retrovirus *in utero* is a useful strategy to achieve efficient and persistent gene transfer to the brain before birth.

In order to obtain high infection efficiency *in vivo*, retrovirus was concentrated and the transduction conditions were optimized. Although Polybrene has often been used in retrovirus-mediated *in utero* gene transfer to facilitate infection, the concentrations used vary significantly among laboratories [5-9]. Our results showed that the optimal concentration of Polybrene is 80 μg/ml (160 ng per embryo) to achieve the highest transduction efficiency without noticeable toxicity in the embryonic mouse brain. We also showed that excessive titer of retrovirus could not increase or even inhibit the transduction both *in vitro* and *in vivo*. A similar phenomenon was also seen in another *in utero* gene therapy study [3], in that 1000-fold increased titers of retrovirus led to only 2-8-fold increment of transduction efficiency. The discrepancy between the end-point titer and transduction efficiency has been documented by Forestell *et al.* previously [27], and the authors suggested that, in high MOI condition, inhibitors (non-transducing viral particles and free viral envelope proteins) present in the virus stock would compete with transducing virions for receptor-binding sites on the target cells and reduce transduction efficiency. Our results suggest that the satisfactory titer should be determined when retrovirus is used *in utero*.

After *in utero* gene transfer, transgene-expressing neural cells were observed throughout the brain by X-gal histochemistry and immunohistochemistry. Efficient gene transfer was achieved in major types of neural cells (neurons, astrocytes and oligodendrocytes). This suggests a possibility of treating a number of neurological disorders with the involvement of various cell types. Another important finding is the permanent expression of vector-encoded gene within the brain suggesting that retrovirus-mediated *in utero* gene transfer might be a useful approach toward achieving long-term correction of genetic disorders.

There were some differences in the transduction pattern in the brain between the present study and our previous study using adenovirus [1]: (i) In this study, transduced neural cells were widely dispersed spanning the entire depth of the cerebral cortex, while in the previous study using adenovirus the transduced cells were distributed predominantly in certain layers of the cortex (e.g., distributed in layers VI-VI when adenovirus was injected at E12). (ii) Both neurons and glia were transduced in similar proportions in this study, while the majority of the transduced cells were neurons when adenovirus was used. (iii) The levels of transgene and its expression in the brain persisted up to 14 months of age without decrement in this study, while these significantly decreased at about 10 months of age when adenovirus was used. All these differences are most likely attributed to the different properties of the two types of viruses regarding the capacity of integration. In contrast to retrovirus that could permanently transmit the virus genome to all descent cells, episomally presenting adenovirus genome was rapidly diluted following sequential divisions of infected

progenitor cells, thus only the cells undergoing few divisions after infection could contain enough adenovirus genomes and produce detectable level of β-gal. Although the powerful infection capability of adenovirus makes it a useful tool to study *in utero* gene delivery routes and the role of a specific gene, from the therapeutic point of view for most genetic diseases that require lifetime correction, retrovirus may be more desirable.

The embryonic cerebral ventricular system is an ideal route for retrovirus-mediated *in utero* gene transfer to the brain. Firstly, multipotent neural stem cells resident in the ventricular zone at early stages of development could be infected by virions presenting in the ventricular fluid. As most of them are undergoing active proliferation (see Figure 2a), efficient gene transfer with MLV-based retrovirus which requires mitosis for integration would occur. Secondly, most neural cells in the mature brain arise from small numbers of neural stem cells by sequential divisions. Thus, even if small numbers of primitive stem cells were transduced *in utero*, expansion of their progeny could lead to significant amplification of the transgene (e.g., see significant proliferation of β-gal+ cerebellar granule neurons from small number of transduced external granular cells; Figures 2c, 2f and 3c). Thirdly, as the ventricular system is a relatively closed narrow cavity, the ventricular zone could be infected effectively by a relatively small dose of retrovirus. Finally, the cerebral ventricle of a human fetus is easily identified and targeted under ultrasound guidance at an early stage of gestation providing the technical feasibility of clinical application of this approach in the future.

The use of retrovirus (including lentivirus) for *in utero* gene delivery has been investigated in a number of studies previously. Viral supernatant or retroviral producing cells were introduced into rats [28], mice [29-31], dogs [32], sheep [2][3] and monkeys [4][33][34] through various routes, such as intraperitoneal, intrahepatic, intrapulmonary, intramuscular, intra-amniotic and intraplacental routes, prenatally. Transduced tissues varied among these studies. This probably depended on the differences in the species of animal models used, target sites and the time of injection. The injection into certain tissues resulted in relatively site-specific transduction [31][33]. Intraperitoneal injection, in general, resulted in the transduction of nearly all organs and the hematopoietic cells [2-4][32]. Porada *et al.* [2] reported that the expression of transgene in hematopoietic cells persisted more than 5 years in sheep that received a single *in utero* intraperitoneal injection of retrovirus. No adverse effects to the fetuses or the mothers were reported in these previous studies. These investigations indicated that *in utero* introduction of retrovirus vector could be a relatively safe and effective method to achieve persistent transduction of exogenous genes. In addition, Tarantal *et al.* [4] demonstrated that intraperitoneal injection of HIV-1-derived lentivirus resulted in a significantly greater transduction frequency in hematopoietic progenitor cells when compared with a similar titer of MLV-based vectors. In the present study, although MLV-based retrovirus was used, we expect that lentivirus may provide a greater level of gene delivery since it could integrate into both dividing and non-dividing cells.

Although the primary target of this study is the transduction of the CNS, multiple organs were also transduced in the recipients. The wide distribution of the retroviral vector throughout the body suggests that some virions accessed the fetal circulation. We speculate that the blood-cerebrospinal fluid (CSF) barrier (mainly constituted with tight junctions connecting the apical regions of the epithelium of the choroid plexus) may not be fully formed or matured at the injection time and this may allow the transport of virions from the CSF into the circulation. Gene transduction to multiple systems such as the heart, lung, liver and the bone marrow other than the brain may be helpful to the treatment of storage diseases since almost all storage diseases with CNS involvement also affect the peripheral nervous system and/or other non-nervous systems. However, the levels of transduction in these organs were very low in this study and whether it could provide therapeutic benefit is unclear at this time.

The presence of vector sequence in the ovaries of the recipients in this study is of concern regarding the safety of this approach, although germ-line transmission in these animals was not determined. Many previous studies also reported that vector DNA was detected within the gonads of the mouse, dog, sheep and monkey after *in utero* administration of retroviral vectors [2-4][31-33]. Breeding studies and PCR analysis of purified sperm cells, however, showed no evidence of the transduction of germ line in the sheep [2][3]. In our study, vector DNA was also detected in the maternal tissues, suggesting the possibility of transplacental transport of vectors into the maternal circulation. Previous studies of *in utero* gene delivery using retrovirus reported that provirus DNA was detected in maternal tissues of the sheep [2], but not the monkey [4][33], and the reason for these different findings was still not known. One possible explanation may be the structural differences of the placenta among species (mouse, sheep and monkey) which may determine whether or not virions could cross the placenta.

Although we did not find evidence of tumor formation on semi-serial sections from the recipients' brains in this study, it is difficult to exclude the possibility of the presence of tumors of small sizes. Since retroviruses including lentivirus integrate into the host genome in a random manner, there are potential risks of influence on the activity of adjacent genes that could lead to tumor formation or developmental aberrations. This concern will be resolved eventually with the development of a viral vector that enables site-specific integration. Although our study suggests the potential utility of retrovirus-mediated *in utero* gene transfer via the intraventricular route for the treatment of genetic neurological disorders, further extensive and rigorous studies, especially using non-human primate models, will be required to explore the safety and feasibility of this approach in human fetuses.

At present, treatment for Krabbe disease is limited to hematopoietic stem cell transplantation. Neurological manifestations of the patients could be significantly improved following transplantation [35][36]. On the other hand, animal experiments that introduced the GALC gene into twitcher mice and cultured cells by virus vectors or transgenic technology showed various degrees of correction of biochemical, pathological and clinical phenotypes suggesting the potential usefulness of gene therapy strategy in GLD [19][37-39]. The previous virus-mediated gene therapy studies in twitcher mice [19][40] have provided important points: (i) Gene transfer must be initiated from an early stage of disease course. (ii) Enzyme supplied to the brain should be in sufficient levels. (iii) Compared to other models of

storage disease, the twitcher mice may be more difficult to treat because of the rapidly progressed deterioration of the nervous system. *In utero* gene therapy has been considered as an alternative approach to this disease [12][40][41] and viral vectors encoding reporter genes were introduced into the fetus of animal models through an intrahepatic route. Although multiple tissues were transduced in these studies, gene transfer to the brain cells was relatively limited [40][41]. Development of a gene delivery system that could achieve more global, sustained and regulated expression of GALC in brain cells may be needed. Our study demonstrates that intrauterine injection of retrovirus via cerebral ventricles could achieve efficient transfer and persistent expression of exogenous gene throughout the brain. Sufficient gene delivery to oligodendrocytes and the other cell types achieved in the twitcher mice is encouraging and suggests the potential utility of this method in treating GLD. Future investigations will be conducted to explore whether introduction of the GALC gene into the twitcher brain *in utero* could effectively correct the metabolic defect and prevent the morphological abnormality of the oligodendrocytes and the neurological symptoms.

Acknowledgements

REFERENCES

1 Shen J-S, Meng X-L, Maeda H, Ohashi T, Eto Y. Widespread gene transduction to the central nervous system by adenovirus *in utero*: implication for prenatal gene therapy to brain involvement of lysosomal storage disease. *J Gene Med* 2004; **6**: 1206-1215. Links

2 Porada CD, Tran N, Eglitis M, *et al.* In utero gene therapy: transfer and long-term expression of the bacterial neo (r) gene in sheep after direct injection of retroviral vectors into preimmune fetuses. *Hum Gene Ther* 1998; **9**: 1571-1585. Links

3 Tran ND, Porada CD, Zhao Y, Almeida-Porada G, Anderson WF, Zanjani ED. In utero transfer and expression of exogenous genes in sheep. *Exp Hematol* 2000; **28**: 17-30. Links

4 Tarantal AF, O'Rourke JP, Case SS, *et al.* Rhesus monkey model for fetal gene transfer: studies with retroviral-based vector systems. *Mol Ther* 2001; **3**: 128-138. Links

5 Walsh C, Cepko CL. Clonally related cortical cells show several migration patterns. *Science* 1988; **241**: 1342-1345. Links

6 Luskin MB, Pearlman AL, Sanes Jr. Cell lineage in the cerebral cortex of the mouse studied in vivo and in vitro with a recombinant retrovirus. *Neuron* 1988; **1**: 635-647. Links

7 Price J, Thurlow L. Cell lineage in the rat cerebral cortex: a study using retroviral-mediated gene transfer. *Development* 1988; **104**: 473-482. Links

8 Ishibashi M, Moriyoshi K, Sasai Y, Shiota K, Nakanishi S, Kageyama R. Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. *EMBO J* 1994; **13**: 1799-1805. Links

9 Gaiano N, Kohtz JD, Turnbull DH, Fishell G. A method for rapid gain-of-function studies in the mouse embryonic nervous system. *Nat Neurosci* 1999; **2**: 812-819. Links

10 Wenger DA, Suzuki K, Suzuki Y, Suzuki K. Galactosylceramide lipidosis: globoid cell leukodystrophy (Krabbe disease). In *The Metabolic and Molecular Bases of Inherited Disease*, Scriver CR , Beaudet AL , Sly WS , Valle D (eds). McGraw-Hill: New York, 2001; 3669-3694.

11 Suzuki K. Twenty five years of the "psychosine hypothesis": a personal perspective of its history and present status. *Neurochem Res* 1998; **23**: 251-259. Links

12 Suzuki K, Suzuki K. The twitcher mouse: a model for Krabbe disease and for experimental therapies. *Brain Pathol* 1995; **5**: 249-258. Links

13 Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 1991; **108**: 193-199. Links

14 Yoshimatsu T, Tamura M, Kuriyama S, Ikenaka K. Improvement of retroviral packaging cell lines by introducing the polyomavirus early region. *Hum Gene Ther* 1998; **9**: 161-172. Links

15 Bowles NE, Eisensmith RC, Mohuiddin R, Pyron M, Woo SL. A simple and efficient method for the concentration and purification of recombinant retrovirus for increased hepatocyte transduction in vivo. *Hum Gene Ther* 1996; **7**: 1735-1742. Links

16 Sakai N, Inui K, Tatsumi N, *et al.* Molecular cloning and expression of cDNA for murine galactocerebrosidase and mutation analysis of the twitcher mouse, a model of Krabbe's disease. *J Neurochem* 1996; **66**: 1118-1124. Links

17 Tabata H, Nakajima K. Efficient in utero gene transfer system to the developing mouse brain using electroporation: visualization of neuronal migration in the developing cortex. *Neuroscience* 2001; **103**: 865-872.

Links

18 Swanson LW (ed). *Brain Maps: Structure of the Rat Brain*. Elsevier Science B.V.: Amsterdam, 1998.

19 Shen JS, Watabe K, Ohashi T, Eto Y. Intraventricular administration of recombinant adenovirus to neonatal twitcher mouse leads to clinicopathological improvements. *Gene Ther* 2001; **8**: 1081-1087. Links

20 Senoo M, Matsubara Y, Fujii K, *et al*. Adenovirus-mediated in utero gene transfer in mice and guinea pigs: tissue distribution of recombinant adenovirus determined by quantitative TaqMan-polymerase chain reaction assay. *Mol Genet Metab* 2000; **69**: 269-276. Links

21 Lawson KA, Dunn NR, Roelen BA, *et al*. Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev* 1999; **13**: 424-436. Links

22 Miyazaki Y, Oshima K, Fogo A, Ichikawa I. Evidence that bone morphogenetic protein 4 has multiple biological functions during kidney and urinary tract development. *Kidney Int* 2003; **63**: 835-844. Links

23 Muneoka K, Wanek N, Bryant SV. Mouse embryos develop normally exo utero. *J Exp Zool* 1986; **239**: 289-293. Links

24 Tansey FA, Cammer W. A pi form of glutathione-S-transferase is a myelin- and oligodendrocyte-associated enzyme in mouse brain. *J Neurochem* 1991; **57**: 95-102. Links

25 LeVine SM, Torres MV. Morphological features of degenerating oligodendrocytes in twitcher mice. *Brain Res* 1992; **587**: 348-352. Links

26 Taniike M, Mohri I, Eguchi N, *et al*. An apoptotic depletion of oligodendrocytes in the twitcher, a murine model of globoid cell leukodystrophy. *J Neuropathol Exp Neurol* 1999; **58**: 644-653. Links

27 Forestell SP, Bohnlein E, Rigg RJ. Retroviral end-point titer is not predictive of gene transfer efficiency: implications for vector production. *Gene Ther* 1995; **2**: 723-730. Links

28 Clapp DW, Dumenco LL, Hatzoglou M, Gerson SL. Fetal liver hematopoietic stem cells as a target for in utero retroviral gene transfer. *Blood* 1991; **78**: 1132-1139. Links

29 Douar AM, Adebakin S, Themis M, Pavirani A, Cook T, Coutelle C. Foetal gene delivery in mice by intra-amniotic administration of retroviral producer cells and adenovirus. *Gene Ther* 1997; **4**: 883-890. Links

30 Turkay A, Saunders T, Kurachi K. Intrauterine gene transfer: gestational stage-specific gene delivery in mice. *Gene Ther* 1999; **6**: 1685-1694. Links

31 MacKenzie TC, Kobinger GP, Kootstra NA, *et al*. Efficient transduction of liver and muscle after in utero injection of lentiviral vectors with different pseudotypes. *Mol Ther* 2002; **6**: 349-358. Links

32 Meertens L, Zhao Y, Rosic-Kablar S, *et al*. In utero injection of alpha-L-iduronidase-carrying retrovirus in canine mucopolysaccharidosis type I: infection of multiple tissues and neonatal gene expression. *Hum Gene Ther* 2002; **13**: 1809-1820. Links

33 Tarantal AF, Lee CI, Ekert JE, *et al*. Lentiviral vector gene transfer into fetal rhesus monkeys (Macaca mulatta): lung-targeting approaches. *Mol Ther* 2001; **4**: 614-621. Links

34 Bennett M, Galan H, Owens G, *et al*. In utero gene delivery by intraamniotic injection of a retroviral vector producer cell line in a nonhuman primate model. *Hum Gene Ther* 2001; **12**: 1857-1865. Links

35 Krivit W, Shapiro EG, Peters C, *et al*. Hematopoietic stem-cell transplantation in globoid-cell leukodystrophy. *N Engl J Med* 1998; **338**: 1119-1126. Links

36 Kurtzberg J, Richards K, Wenger D, *et al*. Correction of Krabbe disease with neonatal hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2002; **8**: 97-98. Links

37 Matsumoto A, Vanier MT, Oya Y, *et al*. Transgenic introduction of human galactosylceramidase into twitcher mouse: significant phenotype improvement with a minimal expression. *Dev Brain Dysfunct* 1997; **10**: 142-154. Links

38 Luddi A, Volterrani M, Strazza M, *et al*. Retrovirus-mediated gene transfer and galactocerebrosidase uptake into twitcher glial cells results in appropriate localization and phenotype correction. *Neurobiol Dis* 2001; **8**: 600-610. Links

39 De Gasperi R, Friedrich VL, Perez GM, *et al*. Transgenic rescue of Krabbe disease in the twitcher mouse. *Gene Ther* 2004; **11**: 1188-1194. Links

40 Fantz C, Wenger D, Sands M. Neonatal and in utero gene transfer utilizing adeno-associated virus and lentiviral vectors for the treatment of Krabbe's disease. *Mol Ther* 2001; **3**: S228. Links

41 Lai L, Davison BB, Veazey RS, Fisher KJ, Baskin GB. A preliminary evaluation of recombinant adeno-associated virus biodistribution in rhesus monkeys after intrahepatic inoculation in utero. *Hum Gene Ther* 2002; **13**: 2027-2039. Links

Peripheral nerve avulsion injuries as experimental models for adult motoneuron degeneration

Kazuhiko Watabe, Yuichi Hayashi and Yoko Kawazoe
Department of Molecular Neuropathology, Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo, Japan.

Short running title: Peripheral nerve avulsion

Address correspondence to: Kazuhiko Watabe, MD, PhD,
Department of Molecular Neuropathology, Tokyo Metropolitan Institute for Neuroscience,
2-6, Musashidai, Fuchu, Tokyo 183-8526, Japan.
Phone: (81)42-325-3881, ext.4717, FAX: (81)42-321-8678,
E-mail: kazwtb@tmin.ac.jp

ABSTRACT

We have utilized adult rat peripheral nerve avulsion models to evaluate the effects of neuroprotective molecules on motoneuron degeneration. The right facial nerves of adult Fischer 344 male rats were avulsed and adenoviral vectors encoding glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), transforming growth factor-b2 (TGFb2), and growth inhibitory factor (GIF) were injected into the facial canal. The treatment with the vectors significantly prevented the loss of lesioned facial motoneurons, improved choline acetyltransferase (ChAT) immunoreactivity and suppressed the induction of nitric oxide synthase activity in these neurons. In separate experiments, animals were orally administered solution of a neuroprotective compound T-588 after avulsion. Both free oral administration and oral tube administration of T-588 improved the survival of injured motoneurons and ameliorated their ChAT immunoreactivity. These results indicate that the gene transfer of GDNF, BDNF, TGFβ2, and GIF and oral administration of T-588 may prevent the degeneration of motoneurons in adult humans with motoneuron injury and motor neuron diseases.

Key words: avulsion, adenovirus, facial nerve, motoneuron, neurotrophic factor

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder characterized by selective loss of motoneurons leading to progressive paralysis and death by respiratory failure. Approximately 10% of ALS cases are familial that include mutations in the Cu/Zn superoxide dismutase (SOD1) gene and in the ALS2/alsin gene putatively encoding a ras GTPase. Since the development of transgenic (tg) mice expressing human mutant SOD1 that show clinicopathological characteristics comparable to human familial ALS, the mutant SOD1-tg animals have been the most widely used experimental models to elucidate the pathomechanism of and the therapeutic approach against familial ALS as well as sporadic ALS.[1] Although the precise mechanism of motoneuron degeneration in mutant SOD1-tg animals is largely unknown, the mutant SOD1 is thought to have a gain of toxic function. Most cases of ALS, however, are considered to be sporadic and of unknown etiology. With regard to another animal model of adult motoneuron degeneration, avulsion of peripheral nerves exhibits extensive loss of motoneurons in adult rats.[2-12] The mechanism of motoneuron degeneration after avulsion also remains unclear, but peroxinitrite-mediated oxidative damage and perikaryal accumulation of phosphorylated neurofilaments have been demonstrated in injured motoneurons after avulsion.[13] Both of these pathological features have also been shown in spinal motoneurons in mutant SOD1-tg animals as well as in patients with familial and sporadic ALS.[1,14,15] To explore therapeutic strategies against motoneuron injury and motoneuron degeneration such as ALS, we have utilized adult rat peripheral nerve avulsion models and examined the effects of neuroprotective molecules on injured motoneurons.[5-12]

ADULT RAT PERIPHERAL NERVE AVULSION

In neonatal rats, peripheral nerve axotomy (transection) causes extensive motoneuron death through apoptotic process associated with Bax and caspase-3 pathways.[16-19] In adult rats, however, axotomy does not induce significant motoneuron death.[20,21] Adult motoneuron degeneration and death can clearly be demonstrated after avulsion of peripheral (facial, hypoglossal, vagal and spinal) nerves.[2-12] In contrast to axotomy, avulsion causes complete disappearance of peripheral nerve components including Schwann cells that produce several molecules neuroprotective for motoneurons. The precise mechanism of adult motoneuron death after peripheral nerve avulsion remains unclear. Some investigators reported that the degeneration of motoneurons following avulsion is due to apoptosis,[13,22] whereas others described that it more closely resembles necrosis.[23] In our facial nerve and spinal root avulsion models as described below, no typical morphological features of apoptosis were identified in injured motoneurons, as described by other investigators.[21,24] Herpes simplex virus vector-mediated expression of Bcl-2 prevented degeneration of spinal motoneurons after root avulsion,[25] whereas caspase inhibitors failed to rescue avulsed spinal

19

motoneurons in adult rats,[26] suggesting that adult motoneuron death after avulsion is not simply due to an apoptotic mechanism.

We have performed avulsion of facial nerve and cervical spinal root in adult rats. Adult Fischer 344 male rats (12-14 weeks old, 200-250 g) were anesthetized with intraperitoneal injection of pentobarbital sodium (40 mg/kg). As for facial nerve avulsion, the right facial nerve was exposed at its exit from the stylomastoid foramen under a dissecting microscope.[6-11] Using microhemostat forceps, the proximal facial nerve was avulsed by gentle traction and removed from the distal facial nerve (Fig. 1). As for cervical root avulsion, the right seventh cervical segment (C7) nerve was exposed by separating the surrounding cervical muscles and connective tissue until the point where the vertebral foramen was identified.[5] Using microhemostat forceps, the C7 ventral and dorsal roots and dorsal root ganglia (DRG) were avulsed and removed from the peripheral nerve (Fig. 2). After 2-8 weeks, rats were anesthetized with a lethal dose of pentobarbital sodium and transcardially perfused with 0.1 M phosphate buffer, pH 7.4 (PB) followed by 4% paraformaldehyde in 0.1 M PB. The brain stem tissue containing facial nuclei and their intramedullary nerve tracts or the cervical spinal cord tissue was dissected and immersion fixed in the same fixative for 2 hours. The tissues were either cryoprotected, or dehydrated and embedded in paraffin, and serial transverse sections were made. For motoneuron cell counting, every fifth section was picked up and stained with cresyl violet (Nissl staining). In 25 sections, motoneurons having nuclei containing distinct nucleoli on both sides of the facial nuclei or the Rexed's lamina IX of C7 spinal cord were counted. The data were expressed as the mean ± SD and statistical significance was assessed by Mann-Whitney U-test. Two to eight weeks after facial nerve and C7 root avulsion, there was marked atrophy of facial nucleus and C7 spinal ventral horn, respectively, and the loss of motoneurons with prominent gliosis (Fig. 1,2,3). The number of motoneurons gradually decreased and reached 20-30% of contralateral side by 4-8 weeks after avulsion (Fig.4).

ADENOVIRAL GENE TRANSFER OF NEUROPROTECTIVE FACTORS

Several neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), NT-4/5, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), cardiotrophin-1 (CT1), insulin-like growth factor-1 (IGF1), and glial cell line-derived neurotrophic factor (GDNF), have been shown to prevent the death of facial and spinal motoneurons, suggesting them as potential therapeutic agents for motoneuron injury and motor neuron diseases such as ALS.[27,28] Recombinant adenoviral vectors encoding BDNF, NT3, CNTF, CT1, and GDNF cDNAs have been used to protect neonatal rat facial and spinal motoneurons from axotomy-induced death[29-32] as well as spinal motoneurons in the mouse mutant *pmn* (progressive motor neuronopathy).[33,34] In adult rats, as described above, axotomy does not generally induce significant motoneuron death.[20,21]

20

Injured motoneuron death in adult rats can be demonstrated 2-4 weeks after the avulsion of facial and spinal nerves.[2-12] Previously, local administration of BDNF, GDNF, or transforming growth factor-β2 (TGFβ2) protein has been reported to prevent the death of adult rat motoneurons after avulsion of spinal root and hypoglossal nerve.[35-40] We investigated whether adenoviral gene transfer of these neurotrophic factors can prevent the death of facial and spinal motoneurons after avulsion in adult rats.[5-10]

Adenovirus preparation

Replication-defective recombinant adenoviral vectors encoding human GDNF (AxCAhGDNF), mouse BDNF fused with Myc epitope-His/IRES-EGFP at the 3' end (AxCAmBDNFME), rat CNTF and CT1 fused with the mouse nerve growth factor (NGF) signal sequence at the 5' ends (AxCANrCNTF and AxCANrCT1, respectively), human IGF1 (AxCAhIGF1), mouse TGFβ2 (AxCAmTGFβ2), and rat growth inhibitory factor (GIF), also called metallothionein-III (MT-III), fused with Myc epitope at the 3' end (AxCArGIFM) were generated using a cassette cosmid pAxCAwt (TaKaRa, Osaka, Japan) carrying an adenovirus type-5 genome lacking the E3, E1A, and E1B regions to prevent the virus replication (Fig. 5).[5,9,10] The cosmid pAxCAwt contains the CAG (cytomegalovirus-enhancer-chicken b-actin hybrid) promoter on the 5' end and a rabbit globin poly (A) sequence on the 3' end. The cosmids were cotransfected to 293 cells with the adenovirus genome lacking the E3 region.[41] Recombinant adenoviral vectors were propagated and isolated from 293 cells, and purified by two rounds of CsCl centrifugation. A recombinant adenoviral vector encoding bacterial b-galactosidase gene (AxCALacZ) were used as a reporter adenovirus.[42] The ability of recombinant adenoviral vectors to induce expression of neurotrophic factors in vitro was confirmed by Western blot analysis of conditioned media or total cell lysates derived from COS1 cells infected with the vectors.[5,9,10] In vitro neurotrophic activity of conditioned media from COS1 cells infected with the vectors were checked by the survival assays using E14 rat mesencephalic and E15 rat spinal motoneuron cultures and the proliferation assay using an immortalized Schwann cell line IMS32.[5,9,43-45]

Transduction of injured adult motoneurons by adenoviral vectors

Immediately following the avulsion of facial nerve, microsyringe was inserted into the stylomastoid foramen and 20 μl solution of either adenoviral vectors (1 x 10⁸ pfu each for single and combined injection) or phosphate buffered saline (PBS) was injected through the facial canal. The wounds were covered with a small piece of gelatin sponge (Gelfoam; Pharmacia Upjohn, Bridgewater, NJ) and suture closed.[6] As for spinal root avulsion, a small piece of Gelfoam presoaked with 10 μl solution of either adenoviral vectors (1 x 10⁸ pfu) or phosphate buffered saline (PBS) was placed in contact with the lesioned C7 intervertebral foramen.[5]

One week after the avulsion and the treatment of AxCALacZ, injured facial and C7 spinal ventral motoneurons and their axons were stained with X-gal histochemistry.[5,6] This indicates the diffusion of the virus through the facial canal or intervertebral foramen, its adsorption to injured axons, retrograde transport of the virus to soma of the motoneurons, and successful induction of the virus-induced foreign gene in these neurons. Retrograde transport of adenovirus encoding β-galactosidase gene from the proximal stump of axotomized peripheral nerves or from the innervated skeletal muscles to soma of motoneurons and successful b-galactosidase expression in these neurons has been demonstrated in neonatal and adult rodents.[29-32,46,47] Because the avulsion of peripheral nerves can cause significant injured motoneuron death in adult rodents, we consider that facial nerve and spinal root avulsions are excellent model systems to examine the effects of adenoviral vectors expressing various neurotrophic factors and neuroprotective molecules on the survival of adult motoneurons.

In a similar manner, one week after avulsion and the treatment with AxCAhGDNF, AxCAmBDNFME, AxCANrCNTF, AxCANrCT1, AxCAhIGF1, AxCAmTGFβ2, and AxCArGIFM, intense cytoplasmic immunolabeling for GDNF, BDNF/Myc, CNTF, CT1, IGF1, TGFb2, and GIF/Myc, respectively, was demonstrated in 10-20 % of facial motoneurons exclusively on the ipsilateral side of the facial nucleus.[6-10] There was no definite immunostaining for these factors in facial motoneurons on the side contralateral to the adenovirus treatment as well as those in unoperated control animals or operated animals without adenovirus treatment. These results led us to expect the autocrine and paracrine neurotrophic effects of these vectors on injured motoneurons after avulsion.[6-10]

Neuroprotective effects of adenoviral vectors

Four weeks after facial nerve avulsion, the treatment with AxCAhGDNF, AxCAmBDNFME, AxCAmTGFβ2, and AxCArGIFM significantly prevented the loss of facial motoneurons after avulsion as compared to the treatment with PBS or AxCALacZ (Fig. 6). In addition, it has been known that peripheral nerve avulsion as well as axotomy induces rapid decrease of ChAT immunoreactivity in injured adult motoneurons.[38,48] We demonstrated that the treatment with AxCAhGDNF, AxCAmBDNFME, AxCAmTGFβ2, and AxCArGIFM after avulsion attenuated the decrease of ChAT immunoreactivity in lesioned facial motoneurons.[5-10] Similar immunohistochemical results have been demonstrated by local or subcutaneous administration of BDNF and GDNF protein after facial nerve axotomy[48,49] or by continuous intrathecal infusion of BDNF protein after sciatic nerve avulsion[38] in adult rats, indicating neuroprotective effects of these factors on adult motoneurons. Furthermore, AxCAhGDNF, AxCAmBDNFME, AxCAmTGFβ2, and AxCArGIFM suppressed NOS activity in lesioned facial motoneurons after avulsion.[5-10] It has been reported that NOS activity is induced in lesioned adult facial and spinal motoneurons after avulsion, suggesting that the induction of NOS activity plays a significant role in the initiation of adult motoneuron death.[3,50]