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Original article

Intracerebral cell transplantation therapy for murine GM1 gangliosidosis

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Abstract

We performed a cell transplantation study to treat the brain involvement in lysosomal storage diseases. We used acid β-galactosidase knock-out mice (BKO) from C57BL/6 as recipients. To minimize immune responses, we used cells derived from transgenic mice of C57BL/6 overexpressing the normal human β-galactosidase. Fetal brain cells (FBC), bone marrow-derived mesenchymal stem cells (MSC), and mixed FBC and MSC cells were prepared and injected into the ventricle of newborn BKO mouse brain. The mice were examined at 1, 2, 4, and 8 weeks and 6 months after injection. In each experiment, the injected cells migrated into the whole brain effectively and survived for at least 8 weeks. Decrease in ganglioside GM1 level was also observed. FBC could survive for 6 months in recipient brain. However, the number of transplanted FBC decreased. In the brains of MSC- or mixed cell-treated mice, no grafted cells could be found at 6 months. To achieve sufficient long-term effects on the brain, a method of steering the immune response away from cytotoxic responses or of inducing tolerance to the products of therapeutic genes must be developed.

Keywords: GM1-gangliosidosis; Cell transplantation; Fetal brain cell; Mesenchymal stem cell

1. Introduction

Enzyme replacement therapy (ERT), hematopoeitic stem cell transplantation (HSCT), and gene transfer have been studied in animals and in humans with lysosomal storage disease (LSD). ERT is now available clinically for Gaucher disease, Fabry disease, Pompe disease, and MPS I, II, and VI in many countries, and has been successful in visceral organs. HSCT is also effective against the somatic involvements in Gaucher disease and MPS I, II, and VI. However, HSCT exhibits little efficacy in conditions such as Fabry disease and Pompe disease, when enzyme secretion from donor cells is poor or the uptake of enzyme proteins by the affected host cells is inadequate. In addition, efficacy in individual organs differs markedly, in both ERT and HSCT, depending on accessibility of blood flow and the density of mannose-6-phosphate receptors. Neither HSCT nor ERT exhibits efficacy against the brain involvement in Gaucher or MPSs because of the poor access due to the blood–brain barrier.

Many experimental studies have been carried out, involving methods such as gene therapy [1–5], cell

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therapy [6–9], or intrathecal administration of enzymes [10,11], for treatment of the brain in LSDs. Such treatments were able to overcome the blood–brain barrier to access brain tissue and exhibit considerable efficacy in brain. However, it is difficult to maintain such efficacy for long periods of time. Repetition of these treatments is not practical because intracranial administration is required for them. On the other hand, the usefulness of intravenous administration is limited because of the blood–brain barrier, except in newborn mice which have an immature barrier. It has been reported that intravenous administration of extremely high doses of enzymes [12–14] or of enzymes that remain in the circulation for long periods [15,16] yielded slight passage through the blood–brain barrier, though with increase in the risk of immune response.

Oral administration of small molecules would be a good and convenient method of treatment of the brain for prolonged periods, such as substrate reduction therapy with *N*-butyldeoxynojirimycin or *N*-butyldeoxygalactonojirimycin for glycosphingolipidoses [17–19] or genistein for mucopolysaccharidoses [20], and chemical chaperone therapy for Fabry disease [21] or GM1-gangliosidosis [22]. However, the efficacy of substrate reduction therapies has thus far been quite limited, and chemical chaperone therapies are not applicable for every type of gene mutation.

GM1 gangliosidosis is an LSD and a progressive neurological disease in humans caused by a genetic defect of lysosomal acid β-galactosidase, which hydrolyses the terminal β-galactosidic residue of ganglioside GM1 and other glycoconjugates. The defects in β-galactosidase activity result in accumulation of ganglioside GM1 in various organs, especially the brain, causing progressive neurodegeneration. In our previous study [2], we injected recombinant adenovirus encoding mouse β-galactosidase cDNA intravenously in β-galactosidase-deficient newborn mice, and showed that vector-mediated β-galactosidase-producing brain cells could reduce ganglioside GM1 accumulation. We showed that β-galactosidase enzyme protein could be secreted as well as taken up by the brain cells and function effectively. However, the efficacy obtained was transient. If sufficient amounts of the defective enzyme could be permanently secreted by cells in the brain, injury of the brain could be prevented. To examine the possibility of long-term cell treatment of the brain in LSDs, we carried out a transplantation experiment in the brain of a GM1-gangliosidosis mouse model (acid β-galactosidase knock-out mouse) using fetal brain cells (FBC) and mesenchymal stem cells (MSC) from bone marrow. These cells used for transplantation were derived from mice of the same genetic background as recipient mice except for possession of the human β-galactosidase gene.

2. Materials and methods

2.1. Knock-out and transgenic mice

A mouse model of GM1 gangliosidosis (BKO mouse) was generated by targeting of the β-galactosidase gene at exon 15 in ES cells as previously described [23]. Newborn mice were obtained by mating heterozygous female mice with homozygous male mice. Identification of newborn mutants was accomplished by quantitative analysis of β-galactosidase activity in tail tip homogenates on the day of birth. Mice with high β-galactosidase activity (TG mice) [24] were generated by introducing the human β-galactosidase gene as a transgene in ES cells obtained from the BKO mouse, which has several copies of the human β-galactosidase gene without the mouse β-galactosidase background. Age-matched wild-type mice of C57BL/6 strain were used as a control.

2.2. Cell preparations for transplantation

Cultured mesenchymal stem cells (MSC) were obtained from the bone marrow of the tibias and femurs of 5–8 month-old TG mice according to the method of Meirelles et al. [25] with some modifications. Dulbecco's modified Eagle's medium (DMEM: Sigma Chemical Co., St Louis, MO) containing 10% fetal bovine serum (Medical and Biological Laboratories, Nagoya, Japan) was used for culture.

Fetal brain cells (FBC) were obtained from the fetal cerebral cortex of TG mice at 13 days of gestation according to the method of Meberg and Miller [26]. The brain tissue was disrupted in a Pasteur glass pipette by gentle stroking several times (uncultured FBC), and then cultured for 4 h in Neurobasal medium (Invitrogen, Carlsbad, CA, No. 12348-07) containing 2 mM glutamine and 10% FBS, followed by two days in Neurobasal medium containing 2 mM glutamine and B27 supplement (Invitrogen, No. 14175-095) (cultured FBC).

2.3. Transplantation of cells into newborn mouse brain

Each BKO mouse received a single injection of 0.5–1.0×10^5 of the cells prepared as described above in the right cerebral ventricle from 24 to 48 hours after birth. Study groups were as follows: uncultured FBC ($n = 18$), cultured FBC ($n = 10$), MSC ($n = 17$), and mixed MSC and FBC (1:1) ($n = 15$). Mice of each experimental group were divided into three subgroups for X-gal staining, β-galactosidase assay and ganglioside GM1 analysis. Mice were examined at one, two, four, and eight weeks and 6 months after injection as shown in Table 1.

For biochemical analysis, mice were anesthetized with diethylether and the blood was washed out with normal saline by perfusion through the heart, and the

brains were removed and kept at −80 °C until use. For histological studies, the brains were fixed by perfusion through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (PB) for 20 min., after washing out the blood with normal saline. To obtain frozen sections, the brains were placed in 0.1 M phosphate buffer pH 7.4 containing 30% sucrose, and frozen in liquid nitrogen.

All surgical and care procedures were carried out in accordance with the Guidelines for Use and Care of Experimental Animals approved by the Animal Committee of Osaka City University School of Medicine.

2.4. X-Gal staining

Frozen sections (16 μm thick) were reacted with X-gal using the β-gal staining Kit (Invitrogen Corp., Carlsbad, CA) to visualize β-galactosidase activity.

2.5. β-Galactosidase assay

β-Galactosidase activity was analyzed in the tissue homogenate with the artificial substrate 2 mM 4-methylumbelliferyl β-galactoside at pH 4.0 in 0.1 M sodium citrate-phosphate buffer according to the method described by Suzuki [27]. Protein was analyzed using the Bio-Rad protein assay system (Bio-Rad Laboratories, Hercules, CA) with the method of Bradford [28].

2.6. Analysis of ganglioside GM1

Amounts of ganglioside GM1 were measured by immunoblot assay using anti-GM1 ganglioside monoclonal antibody (Code: 370685, Seikagaku Corp., Tokyo, Japan) by the method of Michikawa et al. [29] with some modifications.

Brain tissue cells were disrupted by sonication and solubilized in 20 mM Tris–HCl buffer pH 8.0 containing 137 mM NaCl, 10% glycerol, and a protease inhibitor cocktail (Complete, Mini, Cat No. 11836153001, Roche Diagnostics, Mannheim, Germany). Five micrograms of tissue protein was applied onto Trans-Blot Transfer Medium Pure Nitrocellulose Membrane (0.45 μm pore size, Code: 162-0117, Bio-Rad Laboratories) through the slots of a Bio-Dot SF Microfiltration Apparatus (Bio-Rad Laboratories). The membrane was reacted with anti-GM1 ganglioside monoclonal antibody diluted 1:500, after blocking with 5% skim milk in PBS solution for 1 h at room temperature, and then with horseradish peroxidase-linked anti-mouse IgG sheep antibody (Code: NA931, GE Healthcare UK Ltd., Buckinghamshire, UK) diluted 1:1,000. The washing solution used was 0.1 M Tris buffered saline pH 7.5 containing 0.1% Tween 20 (TTBS). Bound antibody was detected using ECL after reaction with ECL™ Western Blotting Detection Reagents (Code: RPN2209, GE Healthcare UK Ltd.) and visualized on X-ray film. Densitometric quantification of immunoreactive signal was performed using the Kodak Digital Science™ EDAS 120 system with 1D Image Analysis software (Eastman Kodak Company, NY). The values obtained were compared with those of quantification of histological immunoreactivity with Leica Control Software as previously described [30], and the same ratios were obtained among the samples (data not shown). The assay was performed three times and in duplicate for each sample independently, and mean values were calculated.

3. Results

3.1. X-Gal staining

Layered staining of the transplanted cells was observed over the entire ventricular surface on both sides of the cerebral hemispheres in treated mice at one week after injection (data not shown). Positive cells had spread into the brain tissue by two weeks (Fig. 1c and f) in the mice treated with cultured FBC ($n = 1$), uncultured FBC ($n = 1$), and MSC ($n = 2$) in the same amounts. The cells had spread further and had reached every part of the brain by 4 weeks in the mice of all experimental groups (Fig. 1d, g and i). Less positive cells were found in the mice treated with MSC ($n = 3$) or mixed MSC and FBC ($n = 3$) (Fig. 1g and i) than in the mice treated with cultured ($n = 3$) or uncultured FBC ($n = 3$) (Fig. 1d). The number of the X-Gal positive cells increased gradually until 4 weeks after injection in every experimental mouse. At 8 weeks after injection, positive cells still existed in the cultured FBC- ($n = 3$) and uncultured FBC-treated ($n = 3$) mice (Fig. 1e) in the same numbers with a similar distribution as at 4 weeks. However, a significant decrease in number of positive cells was found at 8 weeks in the mice treated with MSC ($n = 3$) or mixed MSC and FBC ($n = 3$) (Fig. 1h and j). In the mice treated with mixed MSC and FBC, positive cells existed in higher numbers in deep areas than in the mice treated with MSC alone. In the mice treated with cultured ($n = 2$) and uncultured FBC ($n = 2$), small numbers of positive cells with strong staining still existed in many parts of the brain, especially around the striatum and lateral globs pallidus (Fig. 1k and l), at 6 months after injection. No grafted cells were found in the mice treated with MSC ($n = 1$) or mixed MSC and FBC ($n = 1$) at 6 months. No significant differences were noted among the mice within each experimental group at each stage.

3.2. β-Galactosidase activity

The β-galactosidase activity in FBC and MSC derived from TG mice were 214.5–227.5 nmol/mg/h ($n = 4$) and 143.0–121.4 nmol/mg/h ($n = 3$), respec-

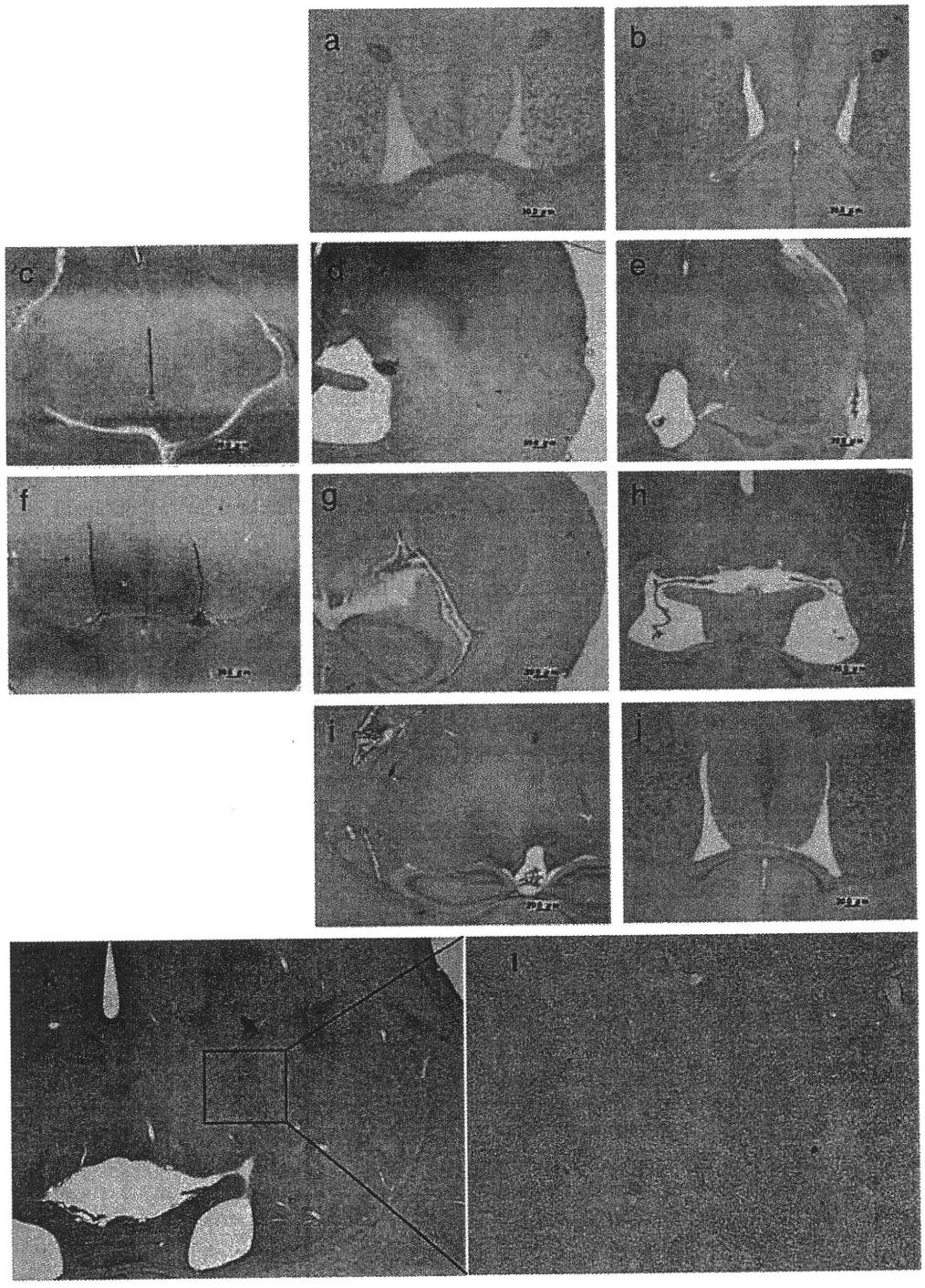

Fig. 1. X-Gal staining of brain coronal sections at +0.8 mm to −2.0 mm of bregma. (a and b) Non-treated BKO mouse at 4 and 8 weeks old, respectively; (c–e) Treated with FBC at 2, 4, and 8 weeks after injection; (f–h) Treated with MSC at 2, 4, and 8 weeks after injection; (i and j) Treated with mixed MSC and FBC at 4 and 8 weeks after injection; (k) FBC-treated brain at 6 months after injection; (l) Magnification of figure k. Positive cells had spread into the brain tissue by two weeks (c and f). The cells had spread further by 4 weeks (d, g and i). Less positive cells were found in the mice treated with MSC or mixed MSC and FBC (g and i) than in the mice treated with FBC (d). At 8 weeks, positive cells still existed in FBC-treated mouse (e) as at 4 weeks (d). A significant decrease in number of positive cells was found at 8 weeks in the mice treated with MSC (h) or mixed MSC and FBC (j). Strong positive staining cells still existed at 6 months in the brain of FBC-treated mouse (k and l).

tively, while the activity in FBC and in MSC derived from wild-type mice were 54.9–69.1 ($n = 2$) and 63.0 ($n = 1$), respectively.

The results of brain β-galactosidase activity in transplantation experiments are shown in Table 2. Increases in β-galactosidase activity were found in the brains of each experimental group at 4 weeks after injection. Activity in the FBC-treated mice was definitely increased at 4 weeks as well as at 8 weeks, while activity at 8 weeks in the MSC-treated mice and mixed MSC and FBC-treated mice was almost the same level as that in the untreated mice. These findings were consistent with those in the X-Gal staining study.

3.3. Immunoassay of ganglioside GM1

Immunoassay of accumulated ganglioside GM1 was performed for each mouse using anti-GM1 ganglioside monoclonal antibody. Values are ratios to the amounts in age-matched normal control mice. The results are shown in Fig. 2 and Tables 3. At 4 weeks after injection, remarkable decrease in ganglioside GM1 accumulation

Table 1
Mouse numbers used for each experiment.

Time after injection	1 week	2 weeks	4 weeks	8 weeks	6 months
	[X-Gal staining]				
Uncultured FBC	1	1	3	3	2
Cultured FBC	1	1	3	3	2
MSC		2	3	3	1
Mixed MSC and FBC			3	3	1
	[β-galactosidase activity]				
Uncultured FBC			2	2	
Cultured FBC					
MSC			2	2	
Mixed MSC and FBC			2	2	
	[Immunoblot assay of ganglioside GM1 amount]				
Uncultured FBC			1	1	1
Cultured FBC					
MSC			2	2	
Mixed MSC and FBC			2	2	

Table 2
β-Galactosidase activity.

	4 weeks	8 weeks
Age-matched normal control (mean ± SD)	197 ± 61 ($n = 7$)	159 ± 56 ($n = 7$)
Non-treated (mean ± SD)	4.38 ± 0.35 ($n = 5$)	4.10 ± 0.47 ($n = 5$)
Treated with uncultured FBC	Mouse 1	Mouse 7
	Rt: 6.65[a]	Rt: 4.94
	Lt: 5.31[a]	Lt: 6.03[a]
	Mouse 2	Mouse 8
	Rt: 7.36[a]	Rt: 5.58[a]
	Lt: 5.33[a]	Lt: 5.05[a]
Treated with MSC	Mouse 3	Mouse 9
	Rt: 6.30[a]	Rt: 4.13
	Lt: 5.95[a]	Lt: 3.67
	Mouse 4	Mouse 10
	Rt: 5.74[a]	Rt: 4.19
	Lt: 5.12[a]	Lt: 5.05[a]
Treated with mixed MSC and FBC	Mouse 5	Mouse 11
	Rt: 5.80[a]	4.13 (mix of both hemispheres)
	Lt: 5.40[a]	
	Mouse 6	Mouse 12
	Rt: 5.06	Rt: 4.85
	Lt: 4.52	Lt: 5.02

Values are in nmol/mg/h. Each sample was tested in duplicate and results are mean values. Rt, right hemisphere; Lt, left hemisphere.
[a] Increase of activity over mean + 2SD of non-treated mice.

Table 3
Immunoblot assay of ganglioside GM1 amount.

	4 weeks	8 weeks	6 months
Age-matched non-treated (range)	2.65–3.55 ($n = 3$)	4.98–5.28 ($n = 3$)	7.58 (n = 1)
Treated with uncultured FBC	Mouse I	Mouse VI	Mouse XI
	Rt: 1.42[a]	Rt: 2.30[a]	Rt: 6.18[b]
	Lt: 1.80[a]	Lt: 2.44[a]	Lt: 6.40[b]
Treated with MSC	Mouse II	Mouse VII	
	Rt: 1.82[a]	Rt: 5.30	
	Lt: 1.31[a]	Lt: 5.23	
	Mouse III	Mouse VIII	
	Rt: 1.40[a]	Rt: 4.40[b]	
	Lt: 1.34[a]	Lt: 4.73[b]	
Treated with mixed MSC and FBC	Mouse IV	Mouse IX	
	Rt: 1.33[a]	Rt: 4.55[b]	
	Lt: 1.34[a]	Lt: 4.78[b]	
	Mouse V	Mouse X	
	Rt: 1.78[a]	Rt: 4.45[b]	
	Lt: 1.62[a]	Lt: 4.58[b]	

Values are ratios to those for age-matched control mice. Each sample was tested in duplicate for three times and results are mean values. Rt, right hemisphere; Lt, left hemisphere.
[a] Remarkable decrease.
[b] Slight decrease of ganglioside GM1 compared with non-treated mice.

was found in the mice of every group. However, at 8 weeks, decrease was detected only in the mouse treated with FBC. Efficacy was still noted at 6 months after injection in FBC-treated mouse. These findings were consistent with those for X-Gal staining (Fig. 1) and β-galactosidase activity (Table 2).

4. Discussion

Two therapeutic methods, HSCT and ERT, are clinically available for LSDs. However, neither is markedly effective in the brain. A number of experiments in animal models have been carried out on the treatment of brain in LSDs. Each revealed some efficacy in the brain, though it was transient and incomplete. Sufficient enzyme expression throughout life is needed in the brain. Thus, permanent engraftment of enzyme-secreting cells in the brain, or permanent expression of an exogenous gene with a vector or as an integrated gene might eliminate the brain involvement in LSDs.

However, the immune responses of host animals are among the most difficult problems to overcome in this respect [31–33]. Although the brain, which is sequestered from systemic immune responses, is thought to exhibit little immune response, elimination of cells expressing a therapeutic transgene occurs in the brain. We speculate that innate inflammatory immune responses are stimulated to kill such cells, not necessarily with the induction of a linked adaptive immune response. When host brain cells express a therapeutic transgene mediated by a viral vector, the host cells themselves will be eliminated, possibly resulting in acceleration of neuronal cell death in neurodegenerative disorders. Transplantation of cells having the same genetic information as the host animals with LSD except for expression of a deficient enzyme protein would thus be a good method of treatment for avoiding the elimination of host neuronal cells and curing diseased host cells.

We performed cell transplantation into the brain of β-galactosidase-deficient mice to study the usefulness of long-term engraftment for supplementation of deficient enzyme protein. To minimize the immune responses in the recipient β-galactosidase knock-out mice, we used cells of mice with the same genetic background as the recipient except for possession of copies of the human β-galactosidase gene.

Initially, in the transplantation experiment, we used FBC from transgenic mice expressing the human β-galactosidase gene. The cells could grow in an environment similar to that of the recipient organ in which they were originally growing. The cells spread into the brains and the cell number increased at least until 4 weeks. They grew very successfully for at least 8 weeks and survived for 6 months or more. However, the number of engrafted cells had decreased significantly at 6 months, while the size of the brain had increased. The decrease in ganglioside GM1 accumulation was also marked until 8 weeks after transplantation. However, at 6 months, this decrease was far less pronounced, with re-accumulation of ganglioside GM1. After the cells were engrafted and the cell number was increased by the cell division in the recipient brain, they were depleted. The mechanism of depletion of transplanted cells involved immunological rejection, although the transplanted cells were very similar genetically and physiologically to the recipient.

Next, we performed a transplantation experiment using MSCs obtained from the bone marrow of the

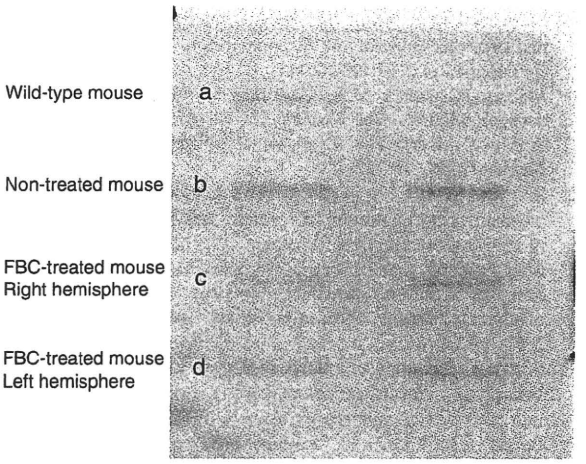

Fig. 2. Immunoblot assay of ganglioside GM1 in brain homogenate at 8 weeks after treatment. Performed in duplicate as shown in two slots for each sample. (a) Wild-type mouse; (b) Non-treated mouse; (c and d) Right and left hemisphere, respectively, of a mouse treated with FBC. The immunoreactivity against ganglioside GM1 antibody in the treated brain (c and d) was less than non-treated brain (d). The accumulated amounts of ganglioside GM1 were calculated in the ratio to the age-matched wild-type mouse (a) from the densitometric quantification signals. These values were shown in Table 3.

same mice expressing the human β-galactosidase gene. MSCs were obtained using the method of plastic adherence. This relatively crude procedure produces a heterogeneous population including multipotential MSCs. These crude cells were used to avoid depletion of potentially important cells and for ease of preparation for clinical application. The cells spread into the brains and the cell number increased similarly to FBC transplantation experiment until 4 weeks. However, decrease in number of engrafted living cells and efficacy in preventing accumulation of ganglioside GM1 were observed in the examination of 8-week-old treated mice.

A number of studies on neural transdifferentiation have been reported [34–37]. Some have reported that neural transdifferentiation of MSCs is induced by cell fusion with host neuronal cells [38–41]. We therefore used mixed FBC and MSC cells to stimulate cell fusion. More engrafted cells were found in the deep areas of the mouse brains treated with mixed cells than in the brains treated with MSC alone. However, no fused cells could be identified. The long-living cells were probably transplanted FBC themselves.

Decrease of ganglioside GM1 was observed even though the increase of the β-galactosidase activity was so small. Similar efficacy was shown previously in our gene therapy experiment [2]. On the other hand, we observed a general depletion of the transplanted cells over time in the BKO mouse brains. The transplanted cells survived in early stage and the number increased by cell division, then, died. This was likely caused by immunological rejection, even though we used fetal brain cells (FBC) from mice with the same genetic background for transplantation. We speculated that immunological reaction occurred because these cells expressed the therapeutic enzyme protein which the host animals did not have. The same has been reported in the transplantation of autogenous cells expressing an exogenous therapeutic gene [33]. The grafted cells were gradually depleted because of immunological rejection by the host animals. To avoid deleterious immune attack and to achieve sufficient long-term efficacy in brain, development of methods to steer the immune response away from cytotoxic responses or to induce tolerance to the products of therapeutic genes is needed [42,43].

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Inhibition of autophagosome formation restores mitochondrial function in mucolipidosis II and III skin fibroblasts

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ABSTRACT

Mucolipidosis II and III are progressive lysosomal storage disorders caused by a deficiency of *N*-acetylglucosamine-1-phosphotransferase, leading to massive accumulation of undigested substrates in lysosomes (inclusion bodies) in skin fibroblast. In this study, we demonstrated accumulation of autolysosomes and increased levels of p62 and ubiquitin proteins in cultured fibroblasts. These autophagic elevations were milder in mucolipidosis III compared with mucolipidosis II. Mitochondrial structure was fragmented and activity was impaired in the affected cells, and 3-methyladenine, an inhibitor of autophagosome formation, restored these. These results show for the first time autophagic and mitochondrial dysfunctions in this disorder.

1. Introduction

Mucolipidosis (ML)[2] II and III are autosomal recessive diseases caused by a deficiency of UDP-*N*-acetylglucosamine:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase) and is characterized clinically by developmental delay and dysostosis multiplex, which overlap partially with mucopolysaccharidoses [1]. GlcNAc-phosphotransferase is composed of six subunits $\alpha_2\beta_2\gamma_2$. The α and β subunits are encoded by a single gene *GNPTAB* and the γ subunit by *GNPTG* [2,3]. Mutations in *GNPTAB* cause both severe type ML (ML II alpha/beta, MIM #252500) and attenuated type ML (ML III alpha/beta, MIM #252600), and mutations in *GNPTG* cause only attenuated type ML (ML III gamma, MIM #252605) [3–6]. GlcNAc-phosphotransferase acts in the first step of synthesizing the mannose 6 phosphate (M6P) recognition marker on lysosomal enzyme proteins, which is recognized by M6P receptor for targeting to the lysosome [7]. In ML patients, lysosomal enzymes lack M6P residues and are hypersecreted into the extracellular space and body fluids instead of being targeted to the lysosome. One of the most characteristic features of these diseases is the presence of numerous phase-dense "inclusion bodies" in patients' skin fibroblasts, which are thought to be lysosomes filled with undigested compounds. However, their contribution to the pathology of ML II and III are still unclear.

Macroautophagy (hereafter referred to as autophagy) is a lysosomal degradation pathway that is essential for cellular survival [8]. Autophagy not only provides nutrients during fasting but also maintains inner cellular routine turnover by degrading misfolded proteins and damaged organelles such as mitochondria, peroxisomes and endoplasmic reticulum. Recent reports show abnormal lysosomal storage blocks the autophagy pathway and the ubiquitin pathway in lysosomal storage diseases [9,10], but the connection between inclusion body formation and the autophagy pathway in ML II and III cells is not known. In this study we found accumulation of autolysosomes followed by mitochondrial dysfunction in ML fibroblasts, and impaired mitochondrial function was restored by 3-MA, an inhibitor of autophagosome formation. Moreover, these autophagic aberrations indicated some correlation with the severity of clinical phenotypes.

2. Materials and methods

2.1. Cell culture

Human skin fibroblasts from a normal control and ML II, ML III patients were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and Antibiotic–Antimycotic (GIBCO, Grand Island, NY, USA) with the informed consent of patients. ML II skin fibroblasts had homozygous mutation of c.3565C>T (p.R1189X) in the *GNPTAB* gene,

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[2] *Abbreviations used:* ML, mucolipidosis; GlcNAc-phosphotransferase, UDP-*N*-acetylglucosamine:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase; M6P, mannose 6 phosphate; LC3, microtubule-associated protein 1 light chain 3; Lamp-2, lysosomal associated membrane protein-2; 3-MA, 3-methyladenine; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Ub, ubiquitin.

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and ML III had compound heterozygote mutations of c.1120T > C (p.F374L) and c.3565C > T (p.R1189X) in the *GNPTAB* gene. These mutations are common in Japanese patients, as reported in the previous paper [11].

2.2. Antibodies and reagents

Polyclonal anti-LC3 (PD014), polyclonal anti-p62/SQSTM1 (PM045) (MBL Co. Ltd., Nagoya, Japan), polyclonal anti-beclin-1 (H-300), monoclonal anti-Ub (P4D1), polyclonal anti-β-tubulin (H-235), monoclonal anti-Lamp-2 (H4B4), polyclonal anti-cathepsin B (S-12), polyclonal anti-cathepsin D (H-75) (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA), monoclonal anti-Tim23 (611223) (BD Biosciences, San Jose, CA, USA), monodansylcadaverine (MDC), and 3-methyladenine (3-MA) (Sigma–Aldrich, St. Louis, MO, USA) were purchased.

2.3. Protein extraction and immunoblotting

Protein extraction from cultured human skin fibroblasts and immunoblotting was performed as described previously [12]. Briefly, equal amounts of proteins (10–20 μg) were electrophoresed in acrylamide gels and subjected to immunoblotting. After incubation with HRP-conjugated secondary antibodies, the membranes were developed using ECL plus reagent (GE Healthcare) and images were captured using X-ray film (RX-U; Fujifilm Co., Tokyo, Japan).

2.4. Fluorescence staining and microscopy

Immunofluorescence staining was performed as described previously [12]. Briefly, cells on coverslips were incubated with primary antibodies against LC3 (1:100), Lamp-2 (1:100), beclin-1 (1:100), ubiquitin (1:100), p62 (1:500), or Tim23 (1:100) for 60 min at room temperature (RT) or 4 °C overnight, and bound antibodies were detected using Alexa Fluor-conjugated secondary antibodies (1:2000 dilution with 0.1% BSA in PBS) for 60 min at RT. For autophagic vacuole labeling, cells were incubated with 50 μM MDC for 10 min at 37 °C. For lysosome staining, cells were incubated with LysoTracker Red DND-99 (100 nM, Molecular Probes Inc., Eugene, OR, USA) for 60 min at 37 °C. For mitochondria staining, cells were incubated with MitoTracker Red CMXRos (100 nM, Molecular Probes Inc.) or JC-1 (3 μM, Molecular Probes Inc.) for 20 min at 37 °C. All fluorescence images were acquired using a fluorescence microscope (Leica DMIRE2; Leica Microsystems, Wetzlar, Germany) or a confocal laser scan microscopy system (Leica TCS SP-2; Leica Microsystems).

2.5. Cathepsin B enzyme activity

The activity of cathepsin B was measured using Magic Red Cathepsin B detection kit (Immunochemistry Tech. LLC, Bloomington, MN, USA). Briefly, cells were prepared on the 96-well cultured plate and incubated with fluorogenic substrate for cathepsin B ($(MR-(RR)_2)$). Fluorescence was measured using a fluorescence mul-

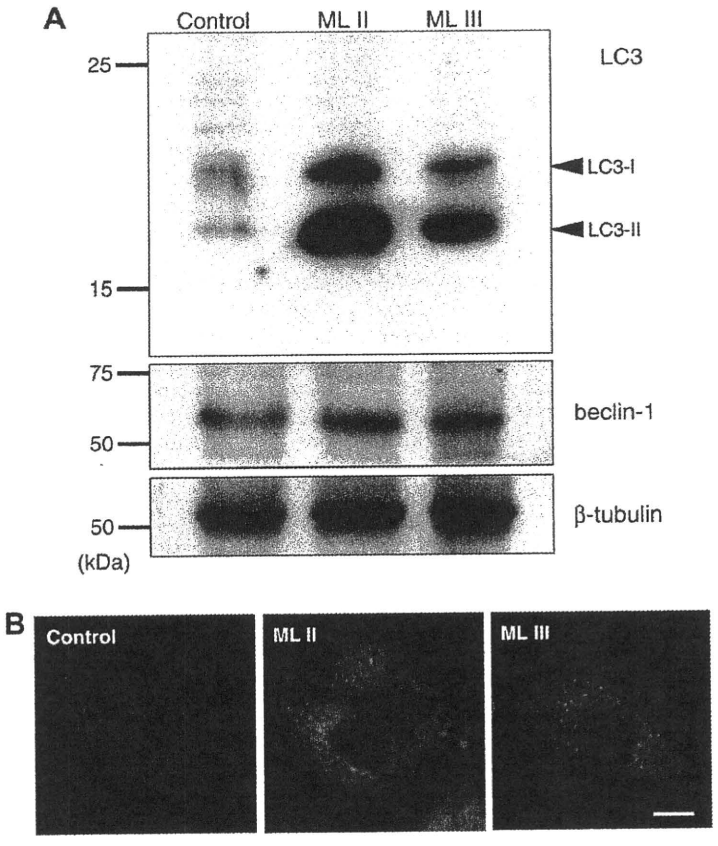

Fig. 1. Elevation of autophagosome formation in ML fibroblasts. (A) Anti-LC3 and beclin-1 immunoblotting of lysates from control, ML II and ML III skin fibroblasts. Densitometric image analysis shows 1.479, 13.862 and 9.672 for LC3-II/LC3-I (average intensities of at least two independent examinations), and 1.028, 0.938 and 0.954 for beclin-1/β-tubulin (average intensities of at least three independent examinations), in control, ML II and ML III, respectively. (B) MDC staining. (C and D) Immunofluorescence of cellular distribution of LC3 with LysoTracker (C) and LC3 with Lamp-2 (D). Bar = 10 μm.

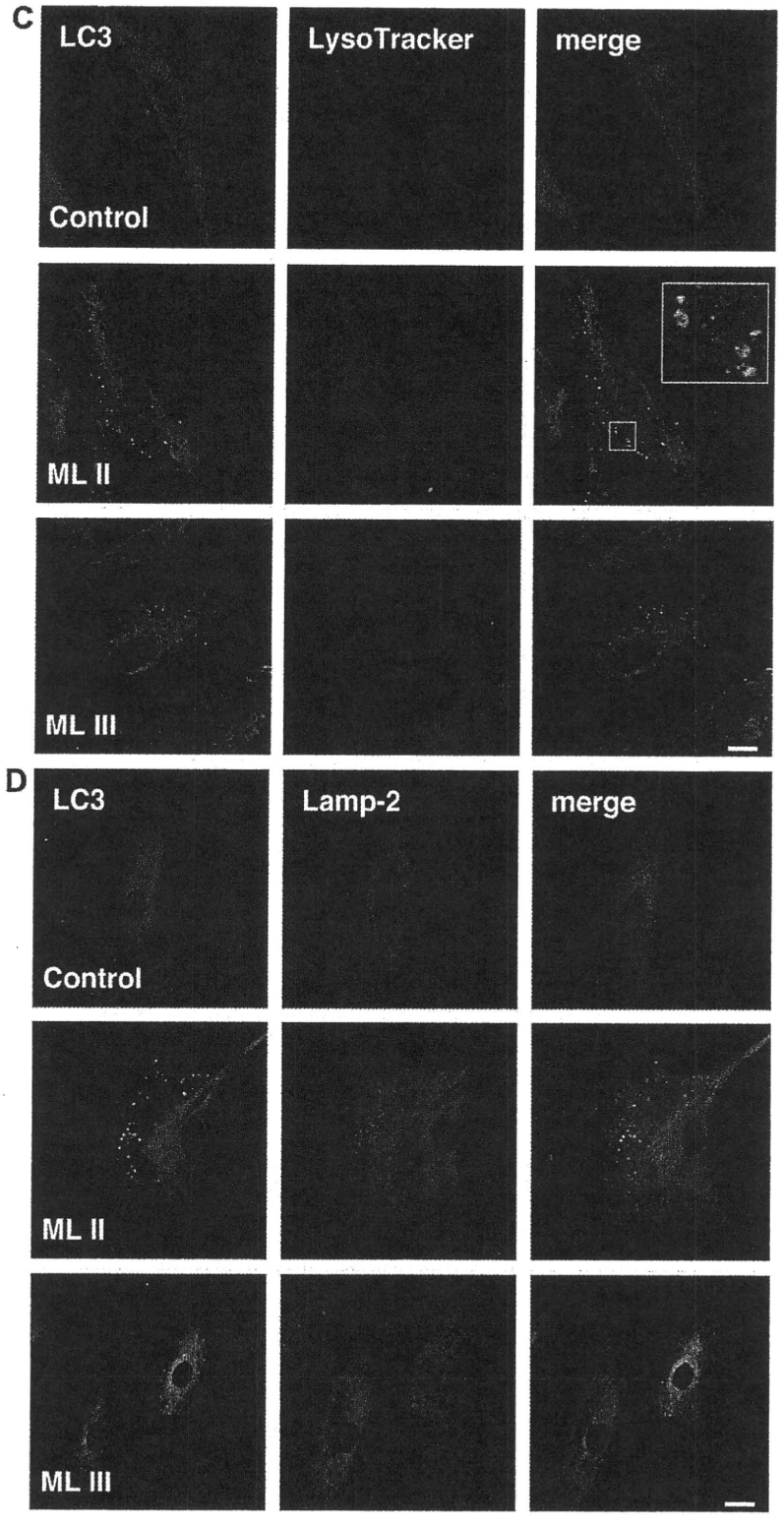

Fig. 1 (*continued*)

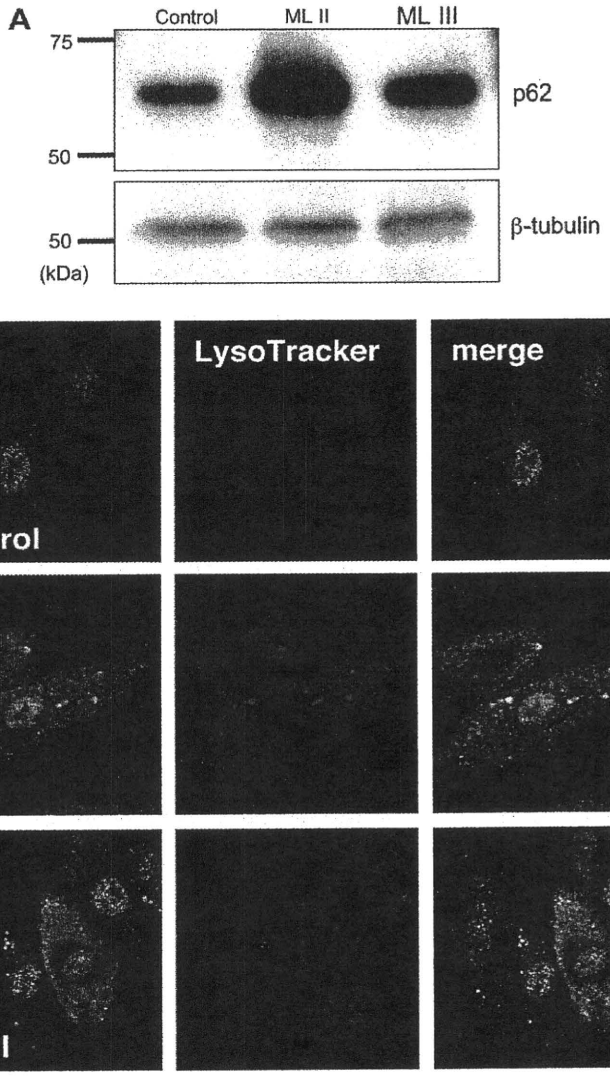

Fig. 2. Accumulation of p62 and ubiquitin-positive inclusions in ML fibroblasts. (A) Immunoblotting analysis with anti-p62. (B) Immunofluorescence showed significant accumulation of ubiquitin-positive inclusions colocalized with LysoTracker in ML cells. Bar = 10 μm.

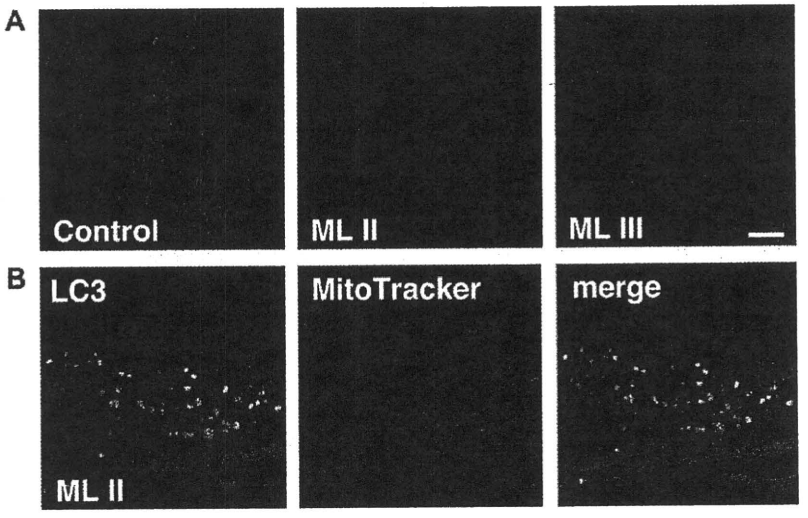

Fig. 3. Mitochondrial impairments in ML fibroblasts. (A) Morphological analysis with MitoTracker-labeled mitochondria. (B) Fluorescence staining with anti-LC3 and MitoTracker revealed that the proportion of tubular-shaped mitochondria was decreased in LC3-positive vesicle rich regions in ML cells. Bar = 10 μm.

ti-well plate reader (excitation 550 nm/emission 640 nm; PerSeptive Biosystems, Framingham, MA, USA).

3. Results

3.1. Increase in autophagosomes and autolysosomes in ML fibroblasts

First, the level of autophagy was assessed in ML fibroblasts. Using Western blotting analysis, the level of LC3-II protein, a membrane bound specific autophagosome marker [8], was found to be increased markedly in the lysate from ML II fibroblasts compared with that in control fibroblasts (Fig. 1A). A modest increase in LC3-II expression was also observed in the lysate from ML III cells. However, the level of beclin-1, a regulator of autophagy, was not elevated in ML II or III cells. The elevation of autophagosome formation was consistent with the observation in ML fibroblasts labeled with MDC, a selective marker for autolysosomes (Fig. 1B). The subcellular localization of autophagosomes was examined using confocal microscopy, and it was found that the number of LC3-positive structures was increased in the cytosol of ML II and III cells, and these LC3-positive structures, especially large vesicles, partly colocalized with LysoTracker and Lamp-2 positive vesicles (Fig. 1C and D). These large circular LC3-positive structures were often seen in ML II cells compared with control and ML III cells.

3.2. Accumulation of ubiquitinated proteins and p62 proteins in ML skin fibroblasts

Recently, p62 protein has been suggested to interact with ubiquitinated proteins and LC3, which may regulate the selective autophagic clearance of protein aggregates [13]. Next, we examined the levels of p62 and ubiquitinated proteins. Immunoblotting analysis showed p62 protein accumulated in the lysates from ML fibroblasts (Fig. 2A). Immunostaining confirmed that ubiquitin (Ub)-positive aggregates were co-labeled with LysoTracker-positive structures in ML cells (Fig. 2B).

3.3. Mitochondrial dysfunction and its restoration by inhibition of autophagy in ML II and III fibroblasts

Autophagic delivery to lysosomes has been shown to be the major pathway in mitochondrial turnover [8]. It was hypothesized that constitutive activation of autophagic formation could affect mitochondrial turnover and impair its function. To address this hypothesis, morphological analysis was performed using MitoTracker Red CMXRos, a membrane potential-dependent fluorescent dye. As shown in Fig. 3A, thick tubular structures of mitochondria were stained by MitoTracker in control cells, whereas thinner tubules and fragmented structures were observed ML II and III fibroblasts (Fig. 3A). These results were the same as in the previous report [14]. When cells were stained using MitoTracker and anti-LC3, MitoTracker-positive tubular structures were decreased near LC3-positive granules in the cytosol of ML fibroblasts, although these did not colocalize (Fig. 3B).

To determine whether the suppression of autophagy recover mitochondrial impairments in ML cells, the effect of 3-MA, an inhibitor of autophagosome formation [15], on ML skin fibroblasts was investigated. When cells were treated with 5 mM 3-MA for 16 h, MitoTracker-labeled mitochondria morphology showed no significant difference from the control cells, whereas thin or fragmented mitochondria seemed to be restored in ML cells (Fig. 4A). Impaired mitochondrial membrane potentials in the affected cells were repeatedly confirmed by staining with JC-1, another mitochondrial membrane sensor, and they recovered significantly after incubation with 3-MA (Fig. 4B).

3.4. Cathepsin B and D in ML II and III fibroblasts

In normal human fibroblasts, cathepsin B and D proteins were transported from the Golgi complex to the lysosome via M6P-dependent pathway [7]. Immunofluorescence analysis showed that cathepsin B and D proteins were colocalized with Lamp-2-positive structures in control fibroblast, whereas these proteins were observed diffuse and partly in the perinuclear, Golgi patterns

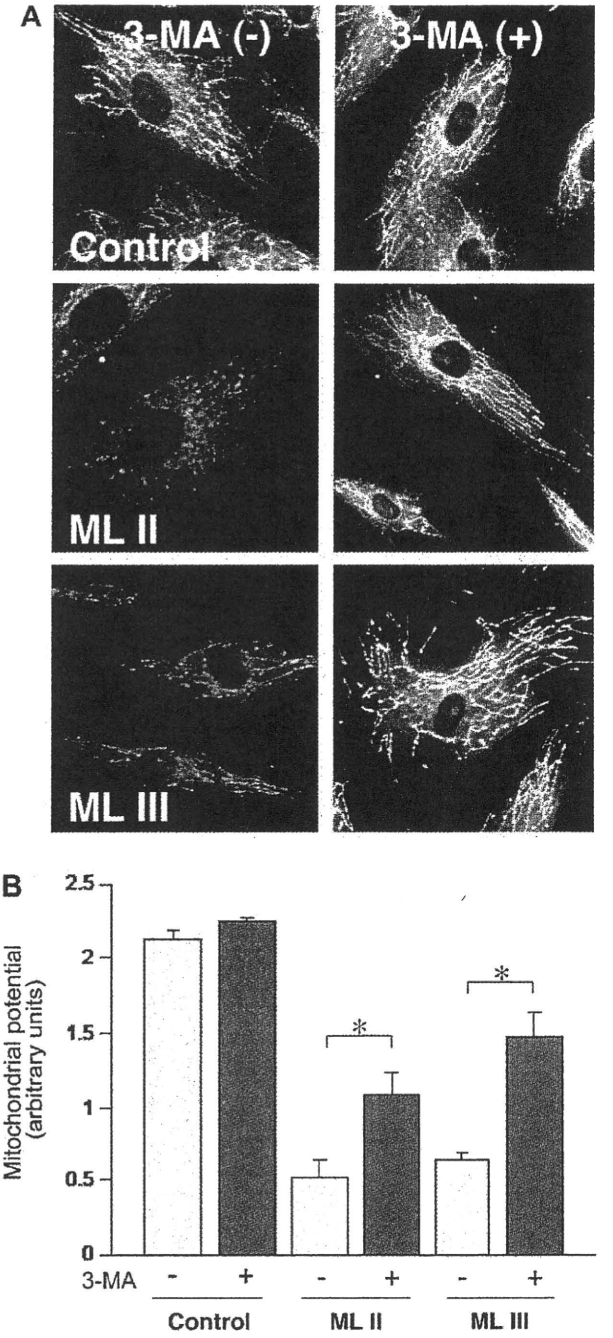

Fig. 4. Restoration of mitochondrial activity by autophagosome inhibitor. Cells were cultured with or without 5 mM 3-MA for 16 h and stained using MitoTracker. DMSO was used as a vehicle (A) MitoTracker staining. (B) The ratio of green and red fluorescence was determined from 10 independent images each of JC-1 labeled-cells. Values are means ± SEM by paired *t*-test. * $p < 0.05$.

(Fig. 5A). The activity of cathepsin B clearly decreased in ML II and III cells, compared to the control (Fig. 5B). Inhibition of autophagy by 3-MA had no effects on subcellular localization of cathepsin B and D proteins (data not shown) and the activity of cathepsin B.

4. Discussion

In this study, accumulation of autolysosomes was observed in ML II and III skin fibroblasts using immunoblotting analysis and immunostaining with anti-LC3. Colocalization studies with LC3, LysoTracker, and Lamp-2 showed that fusion of autophagosomes with late endosomes/lysosomes was not blocked in ML cells. In addition, the accumulation of p62 and ubiquitinated proteins in ML cells suggested a decreased ability to degrade endogenous substrates for autophagy. These findings indicated the impairment of the clearance of autolysosomes in ML II and III skin fibroblasts. Autophagic impairments have been reported in other lysosomal storage disorders [16]. However, the induction of autophagy (marked by beclin-1 activation) differs between these diseases. Elevation of beclin-1 expression has been observed in several cholesterol and sphingolipid storage diseases [17] but not in ML II and III skin fibroblasts. Beclin-1 is thought to be a positive regulator of the autophagic pathway and it has been shown recently to have multiple functions by forming three different complexes with Vps34 [18]. ML II and III skin fibroblasts have many inclusion bodies filled with undegraded substrates, and these contents have been partially characterized [19]. It is possible that these storage materials complicatedly involves in the downstream pathways of autophagy.

Autophagy is a degradative pathway with major roles in the quality control of bulk cytosolic organelles at steady state [8]. In the present study, the numbers of enlarged vesicles, regarded as autolysosomes, increased remarkably in affected cells. Mitochondrial fragmentation and loss of membrane potential was observed in ML cells, and mitochondrial structure seemed to be excluded especially in autolysosome rich regions from the morphological results. There was a possibility that mitochondria are directly impaired by increased autophagosome formation, because inhibiting the formation of autophagosomes by treating with 3-MA for 16 h lead to the recovery of mitochondrial structure and membrane potential. Mitochondrial impairment in lysosomal storage diseases is considered as a secondary accumulation of

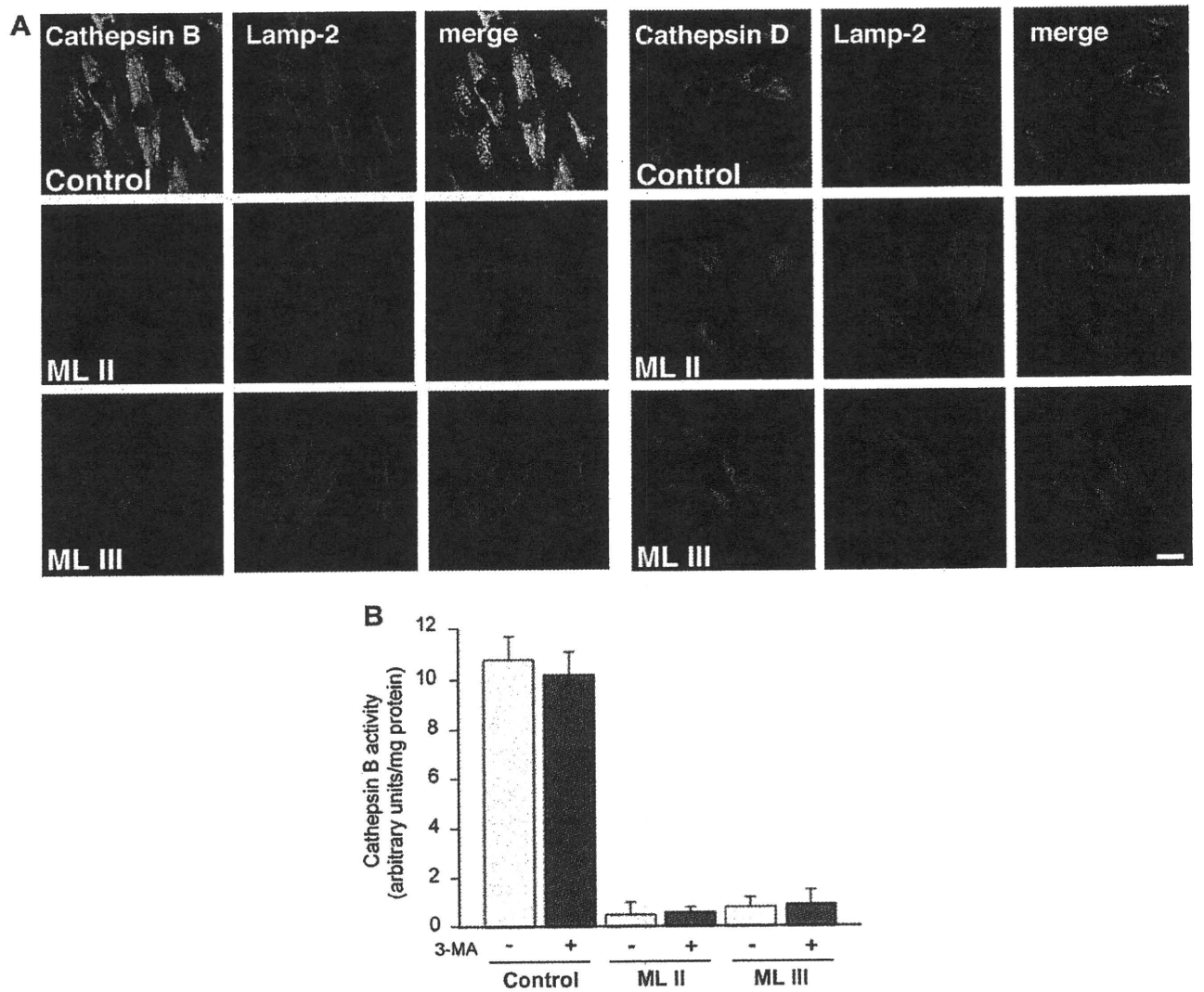

Fig. 5. Subcellar localization and activities of cathepsin B and D. (A) Immunofluorescence of cellular distribution of cathepsin B and D with Lamp-2. Bar = 10 μm. (B) Enzyme activity for cathepsin B. Values are means ± SEM by paired *t*-test.

abnormal mitochondria caused by the defective autophagic degradation pathways [16]. It is suggested that temporary mitochondrial recovery by blocking autophagy finally results in mitochondrial dysfunction over long periods through the secondary accumulation of abnormal mitochondria followed by cell death.

Cathepsin B and D are the main lysosomal aspartic proteases, which are translocated from the Golgi complex to the late endosomes and lysosomes via M6P-dependent manner [20]. In this study we showed defective cathepsin B and D in ML fibroblasts. Previous studies have reported that autophagy was involved in the pathogeneis of the mouse model of neuronal ceroid lipofuscinoses, which is caused by the mutation in cathepsin D or B/L gene [21]. We propose that the mechanism leading to autophagosome accumulation in ML fibroblasts may at least in part share the common pathway in neuronal ceroid lipofuscinoses. It is also indicated that the 3-MA effect to mitochondria is not derived from the restored cathepsins' activities nor normalization of targeting of cathepsins to lysosomes.

Mitochondrial dysfunction is associated with neurodegenerative and neuromuscular diseases [22,23]. According to these pathological conditions, mitochondrial dysfunction ultimately leads to apoptosis. However, cytochrome-c oxidase deficiency, aberrant mTOR signaling, or active cell death were not detected in steady state cultures of ML cells (data not shown), probably because cultured fibroblasts can dilute accumulating cytosolic contents by cell division. On the other hand, ML skin fibroblasts show very low viability against freezing stock. There is a possibility that mitochondrial function is partially compensated for by regular cell proliferation. Further studies are essential to examine the physiological relevance of these results in ML.

Furthermore, it was also found that the elevated levels of LC3, p62, and ubiquitinated proteins correlated with the clinical findings, though lysosomal enzyme activities or phosphotransferase activities did not correlate with the clinical phenotypes [24]. Recently, enzyme replacement therapy and bone marrow transplantation have been developed as possible therapies for lysosomal storage disorders [25,26]. The present findings may provide significant new insights regarding cellular phenotype and clinical phenotype correlations.

In conclusion, the present provides the first characterization of autophagic impairments accompanied by mitochondrial alterations in cultured ML II and III skin fibroblasts and these impairments were temporarily rescued by blocking autophagy. These findings raise the possibility of exploring new therapeutic options by modulating of inclusion body formation and autophagic impairments in ML II and ML III patients.

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The transcriptional repressor RP58 is crucial for cell-division patterning and neuronal survival in the developing cortex

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ABSTRACT

The neocortex and the hippocampus comprise several specific layers containing distinct neurons that originate from progenitors at specific development times, under the control of an adequate cell-division patterning mechanism. Although many molecules are known to regulate this cell-division patterning process, its details are not well understood. Here, we show that, in the developing cerebral cortex, the RP58 transcription repressor protein was expressed both in postmitotic glutamatergic projection neurons and in their progenitor cells, but not in GABAergic interneurons. Targeted deletion of the *RP58* gene led to dysplasia of the neocortex and of the hippocampus, reduction of the number of mature cortical neurons, and defects of laminar organization, which reflect abnormal neuronal migration within the cortical plate. We demonstrate an impairment of the cell-division patterning during the late embryonic stage and an enhancement of apoptosis of the postmitotic neurons in the *RP58*-deficient cortex. These results suggest that RP58 controls cell division of progenitor cells and regulates the survival of postmitotic cortical neurons.

Introduction

Glutamatergic cortical neurons are generated from progenitor cells in the cortical germinal zone and migrate radially in an inside-to-outside gradient. The earliest neurons form the preplate (together with the Cajal-Retzius cells) and the neurons born subsequently migrate past the earliest-born neurons to intercalate within the preplate, divide it into the marginal zone (MZ; layer 1) and the subplate (layer 6b), and form the lower layers of the cortical plate (CP). Late-born neurons then migrate past the early-born neurons to form the upper layers of the CP, beneath the MZ. In contrast, GABAergic neurons and Cajal-Retzius cells are generated from progenitor cells outside the neocortex, in the ganglion eminence and in the cortical hem, respectively, and migrate tangentially into the neocortex (Bayer and Altman, 1991; Allendoerfer and Shatz, 1994; Molyneaux et al., 2007). The radial glial progenitors (RGPs) in the ventricular zone (VZ) give rise to cortical neurons, while the progenitor cells in the subventricular zone (SVZ) produce a substantial number of upper-layer neurons (Smart and McSherry, 1982; Tarabykin et al., 2001; Sugitani et al., 2002). Some of the SVZ progenitor cells are intermediate progenitors (IMPs), which originate from the VZ and produce neurons by dividing limited times (Noctor et al., 2004; Haubensak et al., 2004; Miyata et al., 2004). In the hippocampus, pyramidal neurons of the Cornu Ammonis (CA) are generated from the VZ of the hippocampus, whereas the precursors of the granular neurons of the dentate gyrus (DG) originate in the neuroepithelium near the cortical hem, migrate towards the anlage of

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