Lab. Anim. Res. 2010: 26(3), 301-305

Laboratory Animal Research

http://submission.kalas.or.kr

Laser Capture Microdissection Reveals Specific Genes Related to Purkinje Cell Death in the Leaner Mice

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The leaner mouse carries a mutation in the gene encoding the α1A subunit of P/Q-type calcium channels. Leaner mice exhibit extensive cerebellar granule and Purkinje cell loss that results in cerebellar dysfunction. A previous study suggested that a small population of leaner Purkinje cells undergo apoptosis, however the cell death mode of the rest of degenerating Purkinje cells has not been identified. In order to investigate the mechanisms underlying leaner Purkinje cell death, gene arrays that contain 243 cell death related genes were carried out. To increase the chance of detecting Purkinje cell specific genes, laser capture microdissection was employed to obtain Purkinje cell enriched samples. The gene array analysis revealed several potential genes that are involved in autophagic cell death pathway including cathepsin D, a key lysosomal protease that triggers autophagic degradation. Further analysis on LC3, which is a hallmark for autophagic cell death showed that leaner Purkinje cells are degenerating via autophagic process. The present study provides evidence that calcium channel defects trigger different modes of neurodegeneration in the cerebellum.

Key words: Cerebellum, autophagy, calcium channel mutation, cathepsin D, LC3

Received 14 August 2010; Revised version received 15 September 2010; Accepted 18 September 2010

Mutations in P/Q-type calcium channels in humans cause channelopathies such as episodic ataxia type-2, familial hemiplegic migraine and spinocerebellar ataxia type-6 (Ophoff *et al*., 1998). Moreover, P/Q-type calcium channel mutations have been suggested to be a new etiology for inherited human epilepsy (Burgess and Noebels, 1999).

The leaner mouse (tg^{la}/tg^{la}) carries an autosomal recessive mutation in the gene encoding the α1A subunit of P/Q-type calcium channels (Fletcher *et al*., 1996). The α1A subunit is the pore-forming subunit of P- and Q-type calcium channels and it is highly expressed in Purkinje and granule cells in the cerebellum.

The leaner mouse exhibits severe ataxia, absence seizures and paroxysmal dyskinesia (an intermittent movement disorder). Electrophysiological studies on the leaner cerebellum revealed that the amplitude of calcium current is significantly decreased in Purkinje cells (Dove *et al*., 1998). Leaner Purkinje cells display reduced calcium buffering capacity, which may cause failure of calcium homeostasis and neurodegeneration (Dove *et al*., 2000).

Previous histopathological study showed that up to 50% of Purkinje cells are lost from the leaner cerebellum (Herrup and Wilczynski, 1982). A more recent study using Fluoro-Jade staining, which specifically detects degenerating neurons regardless of their cell death modes, demonstrated that the leaner Purkinje cell death peaks at around postnatal day 40-50 (Frank-Cannon, 2007). It is interesting to note that a small number of dying Purkinje cells are found to be dependent on caspase-3 activation (Frank-Cannon, 2007), which suggests that at least some of the leaner Purkinje cells degenerate via apoptosis. However, the types of cell death mode that are involved in the rest of Purkinje cells that are lost from leaner cerebellum are still unclear.

To investigate gene expression related to cerebellar Purkinje cell neuro-degeneration in the leaner mouse, cDNA gene array analyses were carried out. We employed laser capture microdissection (LCM) to procure cerebellar Purkinje cell enriched samples to increase the chance of detecting Purkinje

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cell specific genes differently regulated in the leaner mouse cerebellum (Nahm *et al*., 2002).

Materials and Methods

Animals and RNA extraction

C57BL/6J wild type (+/+) and leaner (tg^{la}/tg^{la}) mice at postnatal day 40 or 50 were used for all experiments. All procedures for animal use were approved by Institutional Animal Care and Use Committee at Konkuk University. Cerebella were removed from anesthetized mice and frozen immediately using powdered dry ice, then stored at −70°C until used.

Three wild type and three leaner cerebella were used for LCM. Frozen coronal cerebellar sections (12 μm) were prepared using a cryostat. The sections were completely dehydrated for LCM using a graded series of ethanol followed by xylene. Cerebellar Purkinje cells were selectively dissected from surrounding cerebellar tissue using a PixCell II LCM system (Arcturus Engineering, Sunnyvale, CA, USA). A total of 200 Purkinje cells from each genotype were collected to extract total RNA. Total RNA was extracted using a PicoPure RNA Isolation Kit (Arcturus Engineering) then treated with DNase I. The concentration of total RNA was determined using a RiboGreen RNA Quantitation Kit (Molecular Probe, Eugene, OR, USA).

RNA Amplification and gene array analyses

RNA extracted from Purkinje cells was amplified by modified linear amplification methods using a RiboAmp RNA Amplification Kit (Arcturus Engineering). Briefly, 1.0 ng of total RNA extracted from Purkinje cells was reverse transcribed by *in vitro* transcription according to the manufacturer's recommended protocol.

cDNA gene array analyses were carried out using Panorama Mouse Apoptosis Gene Arrays (Sigma-Genosys, Woodland, TX, USA). The arrays consist of duplicated spots of 243 mouse apoptosis-related genes on a pair of nylon membranes (8×12 cm). ^{33}P-labeled cDNA was generated from total RNA of each genotype by reverse transcription. The cDNA was hybridized to the membrane and then exposed to Kodak BioMax MR (Kodak, Rochester, NY, USA).

The film was scanned on a flat bed scanner and then the image was saved in a 16 bit grayscale tagged image file format. The optical density of the spots was obtained using ImaGene V.4.2 software (BioDiscovery Inc., Marina del Rey, CA, USA). The spot optical density was corrected by background subtraction and normalized by the mean ratio of the genomic DNA signal. Genes with significantly different expression levels were identified by ANOVA (Kerr *et al*., 2000) using GeneSight V.2.1 software (BioDiscovery Inc.). All experiments were performed in duplicate.

Immunohistochemistry

Wild type and leaner mice (n=3 respectively) were perfused with 4% paraformaldehyde transcardially and brains were removed for either routine paraffin embedding process or frozen sections. Sections were quenched, blocked then incubated with either cathepsin D antibody (1:100, Abcam, Cambridge, USA) or LC3B antibody (1:1,000, Novus Biological, Littleton, CO, USA) at 4, overnight. Then the sections were incubated with corresponding secondary and tertiary antibodies. The immunoreactive signal was detected by using diamino-benzidine and hydrogen peroxide as substrates. Stained sections were further counterstained with methyl green. Negative control staining was carried out by omitting incubation with primary antibodies. All sections were processed under identical staining conditions to compare staining intensity.

Western blot analysis

Total protein was extracted from whole cerebella of wild type and leaner mice (n=3 respectively) using T-PER protein extraction reagent (Pierce, Rockford, IL, USA). The protein concentration of each extract was BCA reagent (Pierce). Protein extracts from each cerebellum were loaded onto 10% SDS polyacrylamide gel and then transferred to PVDF membrane electrophoretically. The membrane was blocked with 5% skim milk and then incubated with cathepsin D antibody (1:1,000, Abcam) or b-actin (1:10,000, Sigma, St. Louis, USA) for an internal control. Immunoreactive bands were detected by a chemiluminescent reaction (Pierce) and the images were captured using Molecular Imaging System (Kodak). Densitometric analysis was performed to determined the optical density of the immunoreactive bands using NIH Image (Nahm *et al*., 2002). The values used for statistical analysis were calculated from the ratio between housekeeping protein to target protein. Statistical analysis was performed on each cathepsin D bands using Student's *t*-test (P=0.05). Results are reported as mean±SEM.

Results and Discussion

Purkinje cells are the only output neurons in the cerebellum, thus loss or dysfunction of Purkinje cells result in severe impairment of cerebellar function (Ito, 1984). The leaner mouse exhibits extreme ataxia, which is thought to be caused by loss of granule and Purkinje cells in the cerebellum. It

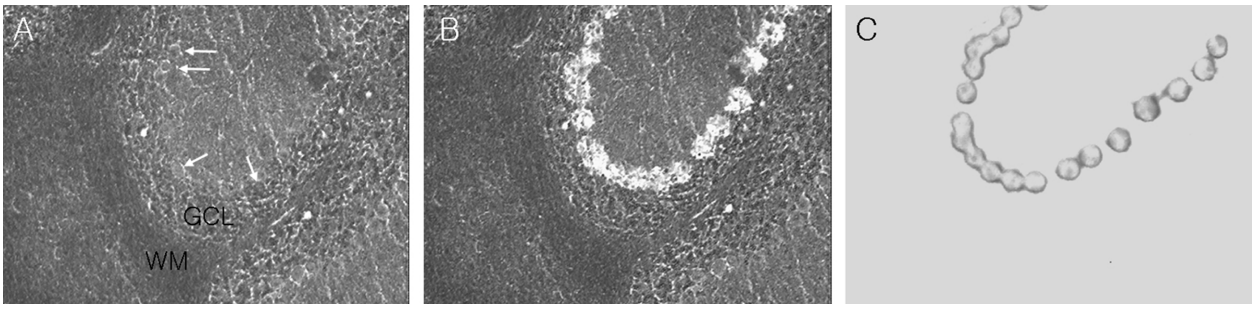

Figure 1. Photomicrographs show dissections of cerebellar Purkinje cells (white arrows) using laser capture microdissection. Pictures show before (A) and after (B) laser capture microdissection on an unstained cerebellar section and procured Purkinje cells on the transfer film (C). GCL, granule cell layer; WM, white matter.

has been known that leaner granule cells undergo apoptosis (Lau *et al*., 2004), however the mode of Purkinje cells has not been identified. Since Purkinje cells play critical roles in motor coordination and possibly some higher cognitive function (Lemon and Edgley, 2010), elucidating the mode of Purkinje cell death is quite important in understanding ataxic phenotype in the leaner mouse.

In the present study, we employed LCM and gene array techniques to investigate the mode of Purkinje cell death in the leaner cerebellum. By employing LCM, it was possible to prepare Purkinje cell-enriched samples, thus increase the chance of detecting Purkinje cell specific changes that take place in the leaner mouse (Figure 1).

Statistical analysis on the gene array results revealed 9 up-regulated genes that are potentially involved in Purkinje cell degeneration, while no down-regulated genes were identified (Table 1) Those up-regulated genes were classified into six different categories based on their function in cell death pathway. Some of the genes that are identified, such as DAD-1 and Bak, are mainly related to apoptotic cell death pathway (Hong *et al*., 2000; Krajewska *et al*., 2002).

One of the most interesting finding from the gene array results is an increase expression of cathepsin D. Cathepsin D is a lysosomal protease that plays important roles in during autophagic cell death (Levine and Yuan, 2005). Based on the increased cathepsin D expression in gene array analysis, we further investigated protein expression of cathepsin D using Western blot analysis and immunohistochemistry. Western blot analysis using protein extract from the whole cerebellum revealed procathepsin D near 50-60 kDa and cathepsin D heavy chain smaller than 30 kDa. Densitometric analysis on the immunoblots showed statistically significant increase of processed cathepsin D heavy chain expression in the leaner cerebellum (Figure 2). In addition, immunohistochemical staining for cathepsin D showed higher immunoreactivity in leaner Purkinje cells (Figure 3), indicating that autophagic process takes place in the leaner Purkinje cells. Moreover, we were able to verify that the results obtained from gene array further represent changes in the protein expression.

Autophagy is a process of cytoplasmic degeneration. During the process, cytoplasmic organelles are sequestered within double-membrane vesicles (autophagosomes) and these vesicles are digested by lysosomal proteases (Klionsky, 2007). One of the key proteins that contribute in assembling autophagosomal membrane is microtubule-associated protein

Table 1. Up-regulated genes in leaner Purkinje cells*

Group	Gene Name	Gene Description	Accession #	Ratio
ARF	Dap1	death-associated protein 1	AI196645	1.5
ARF	Ctsd	cathepsin D	NM_009983	1.7
ARF	MT-2	metallothionein 2	K02236	2.5
ARF	DAD-1	defender against cell death 1	NM_010015	3.6
MIT	Bak	Bcl2 homologous antagonist	NM_007523	1.3
STP	14-3-3 eta	tyrosine 3-monooxygenase	NM_011738	1.6
STP	TRANK	peroxiredoxin 4	NM_016764	2.4
CCR	PCNA	proliferating cell nuclear antigen	NM_011045	1.4
TNS	TALL-1	B-cell activating factor (Baff)	AF119383	2.0

*Genes identified by confidence analysis (P<0.05). ARF, apoptosis-related factors; MIT, mitochondrial associated genes; STP, signal transduction genes; CCR, cell cycle regulators; TNS, tumor necrosis factor superfamily.

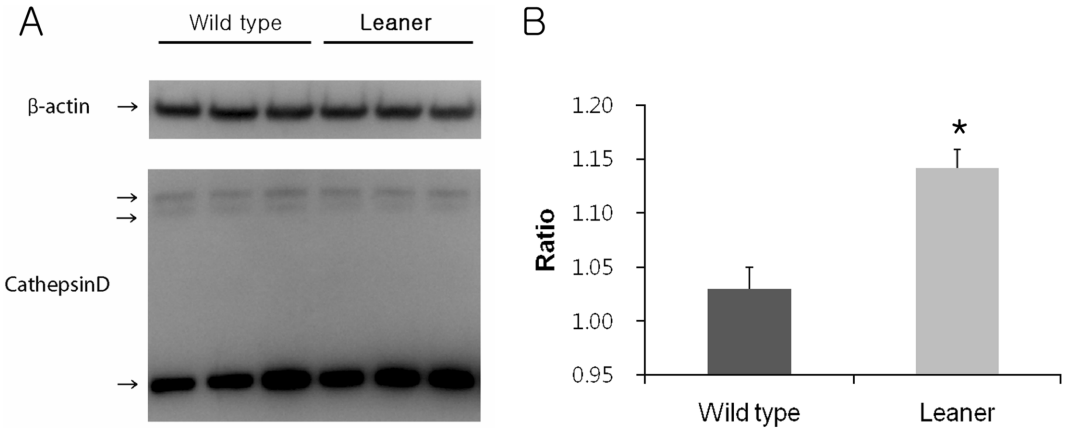

Figure 2. Western blot analysis of cathepsin D expression in wild type and leaner mice (A). Note that higher cathepsin D expression near 30 kDa in the leaner cerebellum. Densitometric analysis of cathepsin D normalized by b-actin. Leaner mice showed a significant increase in cathepsin D expression (B). Data are presented as mean±SEM. *P<0.05.

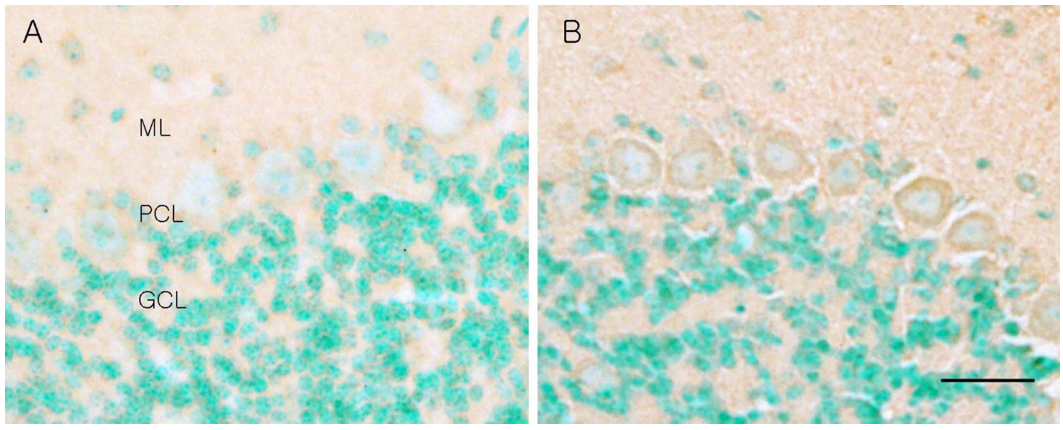

Figure 3. Photomicrographs of cathepsin D immunohistochemistry of wild type (A) and leaner mouse (B). Note that the leaner cerebellum shows increased cathepsin D immunoreactivity in the Purkinje cell bodies. ML, molecular layer; PCL, Purkinje cell layer; GCL, granule cell layer. Scale bar=20 μm.

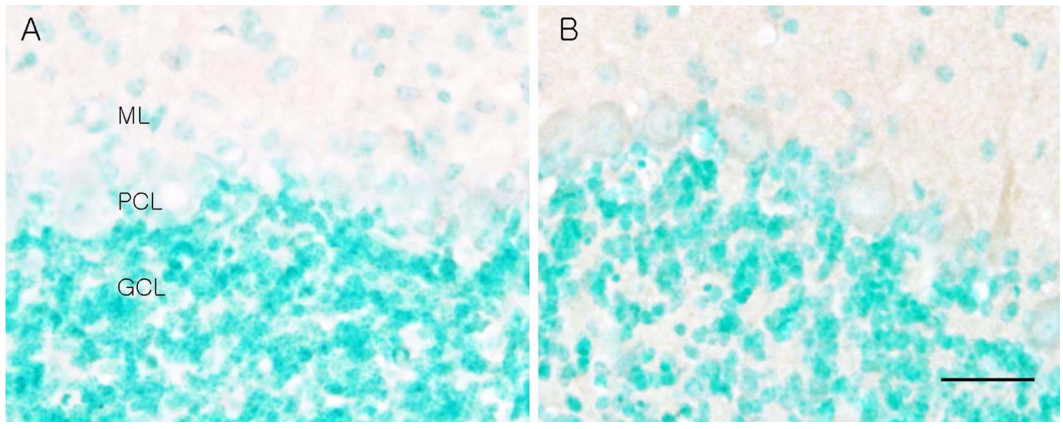

Figure 4. Photomicrographs of LC3 immunohistochemistry of wild type (A) and leaner mouse (B). Note that the leaner cerebellum shows increased LC3 immunoreactivity in the Purkinje cell bodies. ML, molecular layer; PCL, Purkinje cell layer; GCL, granule cell layer. Scale bar=20 μm.

1 light chain 3 (MAP1 LC3), which is commonly known as LC3 (Mizushima, 2004). Detection of LC3 is a key marker for distinguishing autophagic cell death to other types of cell deaths in mammalian cell, thus we carried out immunohistochemical staining for LC3 to determine whether leaner Purkinje cells undergo autophagic cell death. LC3 immuno-

histochemical staining on the leaner cerebellum revealed a large number of Purkinje cells that are positive to LC3 antibody compared to wild type Purkinje cells (Figure 4). Based on these findings, we determined that the majority of leaner Purkinje cells undergo autophagic cell death.

There are several mouse models that exhibit extensive Purkinje cell loss, including Nagoya rolling, lurcher and Purkinje cell degeneration (pcd) mice (Mullen *et al*., 1976; Zuo *et al*., 1997; Mori *et al*., 2000). Much attempts have been made to elucidate the mode of Purkinje cell death in various mouse models. Chakrabarti *et al*. (2009) showed that Purkinje cells in pcd mice take autophagic cell death pathway. The Purkinje cell death in lurcher mice was initially found to be autophagy (Selimi *et al*., 2003) but a recent study by Nishiyama *et al*. (2010) reported that lurcher Purkinje cells are degenerated through necrotic cell death. These findings suggest that Purkinje cells may undertake different degeneration modes based on the environment they are situated.

Although the genetic mutation of leaner mice has been identified, the direct cause of leaner Purkinje cell degeneration is still unknown. It is likely that the calcium channel mutation has adverse effects on normal function and survival of leaner Purkinje cells (Dove *et al*., 2000). Recently, deregulation of cellular calcium homeostasis has been found to be one of the inducer of autophagy (Vicencio *et al*., 2010). Thus altered calcium homeostasis in the leaner Purkinje cell might be the potential cause of autophagic cell death. Further investigation on the relationships between calcium homeostasis and initiation of cell death in leaner Purkinje cells are necessary to fully understand the autophagic processes.

Acknowledgment

This work was supported by the Korea Research Foundation Grant funded by the Korean government (MOEHRD, Basic Research Promotion Fund, KRF-313-2007-2-E00537).

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