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LRRK2 is expressed in B-2 but not in B-1 B cells, and downregulated by cellular activation

Makoto Kubo [a], Yoshiko Kamiya [a], Ryuichi Nagashima [a], Tatsunori Maekawa [a], Koji Eshima [b], Sadahiro Azuma [c], Etsuro Ohta [a], Fumiya Obata [a,d,*]

[a] Division of Clinical Immunology, Graduate School of Medical Sciences, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan
[b] Department of Immunology, Kitasato University School of Medicine, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan
[c] Department of Laboratory Animal Science, Kitasato University School of Medicine, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan
[d] R & D Center for Cell Design, Institute for Regenerative Medicine and Cell Design, Kitasato University School of Allied Health Sciences, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan

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ABSTRACT

LRRK2, the causal molecule of familial Parkinson's disease, is expressed strongly by one of the B cell subsets, B-2 cells, but not by the other subset, B-1 cells, in the mouse peritoneal cavity, spleen, and peripheral blood. Bone marrow pre-B cells or T cells exhibited little LRRK2 expression. LRRK2 expression was dramatically downregulated upon activation of B-2 cells with various types of stimulation. These results suggest that LRRK2, whose true function has not yet been clarified, may play some important role(s) in the development and function of B cells, particularly the maintenance of B-2 cells in a resting status.

1. Introduction

Leucine-rich repeat kinase 2 (LRRK2) is the causal molecule of autosomal dominant familial Parkinson's disease, PARK8, which was originally defined in a study of a large Japanese family, the Sagamihara family (Funayama et al., 2002, 2005; Paisan-Ruiz et al., 2004; Zimprich et al., 2004). LRRK2 is a large complex protein with an approximate molecular mass of 260 kDa and contains multiple domains including the LRR (leucine-rich repeat), ROC (Ras of complex), COR (C-terminal ROC), kinase, and WD40 domains (Meylan and Tschopp, 2005; Paisan-Ruiz et al., 2004; Zimprich et al., 2004). The binding of GTP to the ROC domain, as well as dimer formation, is known to increase the kinase activity of LRRK2 (Deng et al., 2008; Ito et al., 2007; Smith et al., 2006). Although it has been reported that LRRK2 phosphorylates itself (autophosphorylation), and its potential interactors include moesin, eukaryotic initiation factor 4E-binding protein (4E-BP), β-tubulin, and mitogen-activated kinase kinase (MKK) 3, 6, and 7 (Gandhi et al., 2008; Hsu et al., 2010; Imai et al., 2008; Jaleel et al., 2007; West et al., 2007), the true substrate and true function of LRRK2 remain unknown.

Analyses of LRRK2 expression in human and rodent brain have demonstrated that LRRK2 is expressed in various regions including the substantia nigra, putamen, cortex, and cerebellum (Higashi et al., 2007a,b; Melrose et al., 2006; Paisan-Ruiz et al., 2004; Simon-Sanchez et al., 2006; Westerlund et al., 2008; Zimprich et al., 2004). It is also known that LRRK2 expression is much higher in the spleen, lung, kidney, and testis than in other organs, including the brain (Biskup et al., 2007; Larsen and Madsen, 2009; Maekawa et al., 2010; Westerlund et al., 2008). In particular, we have recently reported that B cells are the major LRRK2-expressing cell population in mouse spleen (Maekawa et al., 2010). Macrophages express LRRK2 weakly, but T cells show no expression. These results suggest a possibly novel function of LRRK2 in the immune system, especially in B cells.

B cells are the effectors of humoral immunity, and are classified into two subsets, B-1 B cells and B-2 B cells (or simply B-1 cells and B-2 cells, respectively) according to differences in developmental lineage and function (Berland and Wortis, 2002). B-1 cells develop primarily during the fetal stage as well as in the perinatal phase, whereas B-2 cells are produced from bone marrow during postnatal life (Hardy and Hayakawa, 2001; Herzenberg, 2000). B-1 cells are defined by their expression of the pan-T cell surface glycoprotein CD5, and have a $CD43^{+}$, $CD23^{-}$, $CD45R^{lo}$, immunoglobulin (Ig) M^{hi}, and IgD^{lo} immunophenotype (Berland and Wortis, 2002; Hardy and Hayakawa, 2001). They constitute a substantial fraction of B cells in the peritoneal and pleural cavities, and 5–10% of those in the spleen, but are absent from lymph nodes

* Corresponding author. Division of Immunology, School of Allied Health Sciences, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan. Tel.: +81 42 778 8215; fax: +81 42 778 8075.
E-mail address: obata@ahs.kitasato-u.ac.jp (F. Obata).

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(Hayakawa et al., 1983). In contrast, B-2 cells are conventional B cells characterized as $CD5^{-}$, $CD43^{-}$, $CD23^{+}$, $CD45R^{hi}$, IgM^{lo}, and IgD^{hi} (Berland and Wortis, 2002; Hardy and Hayakawa, 2001). They constitute a major fraction of B cells in spleen and lymph nodes (Berland and Wortis, 2002). B-1 cells spontaneously, in the absence of antigen stimulation, secrete IgM that is reactive with bacterial carbohydrate antigens and autoantigens (Berland and Wortis, 2002; Hayakawa et al., 1984). In contrast, B-2 cells produce IgM and IgG that are reactive with huge variety of foreign antigens only after antigen stimulation (Baumgarth et al., 1999). In keeping with this, typical B-1 Ig genes have fewer N insertions than those of most B-2 cells, and do not contain somatic mutations (Berland and Wortis, 2002; Hardy, 2006; Herzenberg, 2000).

In the present study, as one approach for elucidating the possible role of LRRK2 in B cells, we investigated whether LRRK2 expression differs between B-1 and B-2 cell subsets in various immune tissues. Because our previous Western blotting analysis using polyclonal anti-LRRK2 antibody revealed the need for careful discrimination of protein bands other than the full-length LRRK2, we quantified LRRK2 mRNA expressed by each of the B cell subsets. We found that LRRK2 mRNA was expressed at a much higher level in B-2 cells than in B-1 cells in the peritoneal cavity, spleen and peripheral blood. Furthermore, the expression of LRRK2 in B-2 cells was dramatically down-regulated by various types of stimulation. These results suggest that LRRK2 may have a functional role that is strongly restricted to resting B-2 cells, and not to B-1 or activated B-2 cells.

2. Materials and methods

2.1. Animals

C57BL/6 and alymphoplasia (aly/aly) mice were purchased from CLEA Japan, Inc. (Tokyo Japan). Female mice at 7–16 weeks of age were used for this study. Mice were cared for and handled in accordance with the guidelines of the Animal Experimentation and Ethics Committee of Kitasato University.

2.2. Lymphocyte purification and immunofluorescence staining

Whole lymphocytes were obtained by spleen disruption, blood collection, peritoneal cavity washout, and bone marrow washout. The cell suspensions were depleted of erythrocytes by hypotonic lysis at 4 °C, except for peripheral blood, from which erythrocytes were depleted by density gradient centrifugation using Lymphosepar II™ (IBL, Gunma, Japan). Lymphocyte subsets were purified on a magnetic activated cell-sorting (MACS™) separation column using magnetic microbeads coated with anti-CD5, -CD19, -CD43, and -CD90.2 monoclonal antibodies (MAbs) (Miltenyi Biotec, Gladbach, Germany). After MACS separation, cell subpopulations were stained for flow-cytometric analysis using combinations of the following fluorochrome-conjugated MAbs: CD3 (fluorescein isothiocyanate: FITC), CD5 (FITC), CD19 (spectral red: SPRD), CD23 (phycoerythrin: PE), CD43 (PE), CD45R/B220 (PE), and CD90.2 (PE). All of these antibodies were purchased from Beckman Coulter (Miami, FL). The stained cells were analyzed with an EPICS XL flow cytometer and EXPO32™ software (Beckman Coulter).

2.3. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from each of the separated cell subsets using TRIzol™ Reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized using a ThermoScript RT-PCR System (Invitrogen). Murine LRRK2 mRNA expression was assessed using the following combination of PCR primers: 5′-TCTGGCTGGAACCCTGCTAT-3′ and 5′-AACTGGCCATCTTCATCTCC-3′ (product size: 155 bp). As the internal control, murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified with the following combination of primers: 5′-GAGGCCGGTGCTGAGTATGTCGTG-3′ and 5′-TCGGCAGAAGGGGCGGAGAT-3′ (product size: 116 bp). The qRT-PCR was performed in 25-μl volumes of triplicated reaction mixture containing 1 μl of cDNA, 12.5 μl of SYBR Green I Dye (Applied Biosystems, Foster City, CA), and primer pairs using a 7500 Real-time PCR System (Applied Biosystems).

2.4. B cell stimulation

MACS-sorted B cells (2×10^5) were cultured in 0.2 ml of RPMI 1640 medium containing 5% heat-inactivated fetal bovine serum, 10 mM HEPES (pH 7.25), 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin in 96-well U-bottom microtiter plates. They were stimulated with either a mixture of phorbol 12-myristate 13-acetate (PMA) (Sigma) and ionomycin (Sigma), lipopolysaccharide (LPS) (Sigma, St. Louis, MO), or $F(ab')_2$ fragments of goat anti-mouse IgM (anti-IgM) antibody (Beckman Caulter) for 24 h.

3. Results

3.1. Isolation of B cell subsets from immune tissues

B cells are classified into B-1 and B-2 cell subsets according to their process of development, phenotype, and function. To compare the expression of LRRK2 mRNA in B-1 and B-2 cells, we separated the two subsets from the peritoneal cavity of a C57BL/6 mouse, the original site from which B-1 and B-2 cells had originally been defined. B-1 cells were isolated with anti-CD5 antibody-conjugated magnetic microbeads after depletion of T cells using anti-CD90.2 antibody-conjugated magnetic microbeads (Fig. 1). Because the B-1 cell-depleted fraction still contained a substantial number of $CD5^{+}$ cells, instead of using it as the source of B-2 cells, we isolated B-2 cells as the CD43-negative fraction using anti-CD43 antibody-conjugated magnetic microbeads according to Hein et al. (Hein et al., 1998), as CD43 is expressed on a wide variety of leukocytes including T cells, granulocytes, macrophages, plasmacytes, and B-1 cells, but not on resting B-2 cells.

Flow-cytometric analysis indicated that the cells in the B-1 and B-2 fractions prepared from the peritoneal cavity had each of the characteristic phenotypes, i.e., almost all cells in the B-1 fractions were positive for CD19 (98.4%), CD5 (96.0%), and CD43 (94.1%), but few of them were positive for CD23 (9.3%) and $CD45R^{hi}$ (24.0%); almost all cells in the B-2 fractions were positive for CD19 (98.2%), $CD45R^{hi}$ (96.0%), and CD23 (84.5%), but few were positive for CD5 (1.4%) and

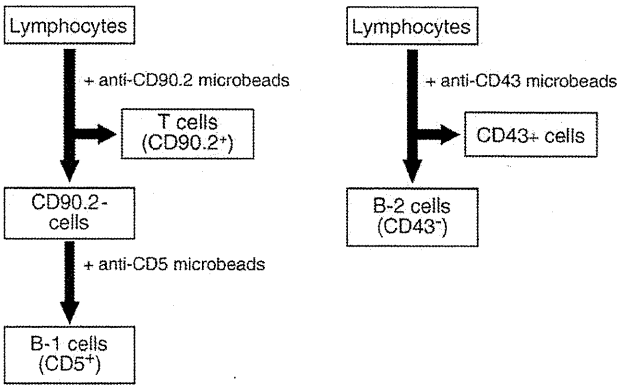

Fig. 1. Schematic representation of the isolation procedures for B-1 and B-2 cells.

Table 1
Flow-cytometric analysis of cell subsets separated from C57BL/6 and aly/aly mice.

Strain	Tissue	Subsets	B-1/B-2 ratio[a]	CD19$^+$	CD5$^+$	CD43$^+$	CD23$^+$	CD45R^{hi}	CD90.2$^+$	CD3$^+$
C57BL/6	Peritoneal cavity	B-1 cell	1:16	98.4%	96.0%	94.1%	9.3%	24.0%	2.9%	ND
		B-2 cell		98.2%	1.4%	14.8%	84.5%	96.0%	ND	0.3%
		T cell		21.5%	80.7%	ND	ND	ND	63.7%	85.2%
	Spleen	B-1 cell	1:120	81.0%	17.5%	20.5%	48.8%	76.2%	12.5%	ND
		B-2 cell		94.7%	1.3%	2.2%	87.4%	99.2%	ND	0.6%
		T cell		8.4%	91.9%	ND	ND	ND	95.4%	90.2%
	Peripheral blood	B-1 cell	1:20	ND	64.9%	ND	ND	60.9%	ND	ND
		B-2 cell		87.1%	6.6%	1.9%	90.9%	92.5%	ND	1.4%
		T cell		9.5%	90.8%	ND	ND	ND	91.0%	90.2%
	Bone marrow	Pre-B cell		73.4%	2.9%	5.7%	63.6%	54.9%	ND	0.7%
		T cell		35.1%	39.8%	ND	ND	ND	53.8%	41.3%
aly/aly	Peritoneal cavity	B-1 cell	1:7	96.8%	86.7%	84.4%	ND	ND	ND	ND
		B-2 cell		98.4%	5.2%	1.7%	12.3%	83.0%	ND	ND
		T cell		34.6%	96.0%	ND	ND	ND	33.3%	94.0%
	Spleen	B-1 cell	1:16	87.1%	13.2%	24.1%	43.8%	87.8%	ND	ND
		B-2 cell		90.9%	2.2%	2.4%	71.3%	91.2%	ND	0.6%
		T cell		5.2%	93.5%	ND	ND	ND	96.2%	94.4%

ND: Not determined.
[a] Estimated from the B-1 and B-2 cell numbers obtained.

CD43 (14.8%) (Table 1, Fig. 2). The B-2 cells prepared from spleen and peripheral blood exhibited the phenotypes identical to those of B-2 cells prepared from the peritoneal cavity, whereas the B-1 fraction prepared from these tissues was less pure than that prepared from the peritoneal cavity, possibly due to a markedly low B-1/B-2 ratio (Table 1, Fig. 2). We also prepared CD43-negative B cells from bone marrow (pre-B cells) that exhibited a phenotype, except for CD23, similar to that of B-2 cells. The bone marrow CD5-positive B cells were too few to analyze.

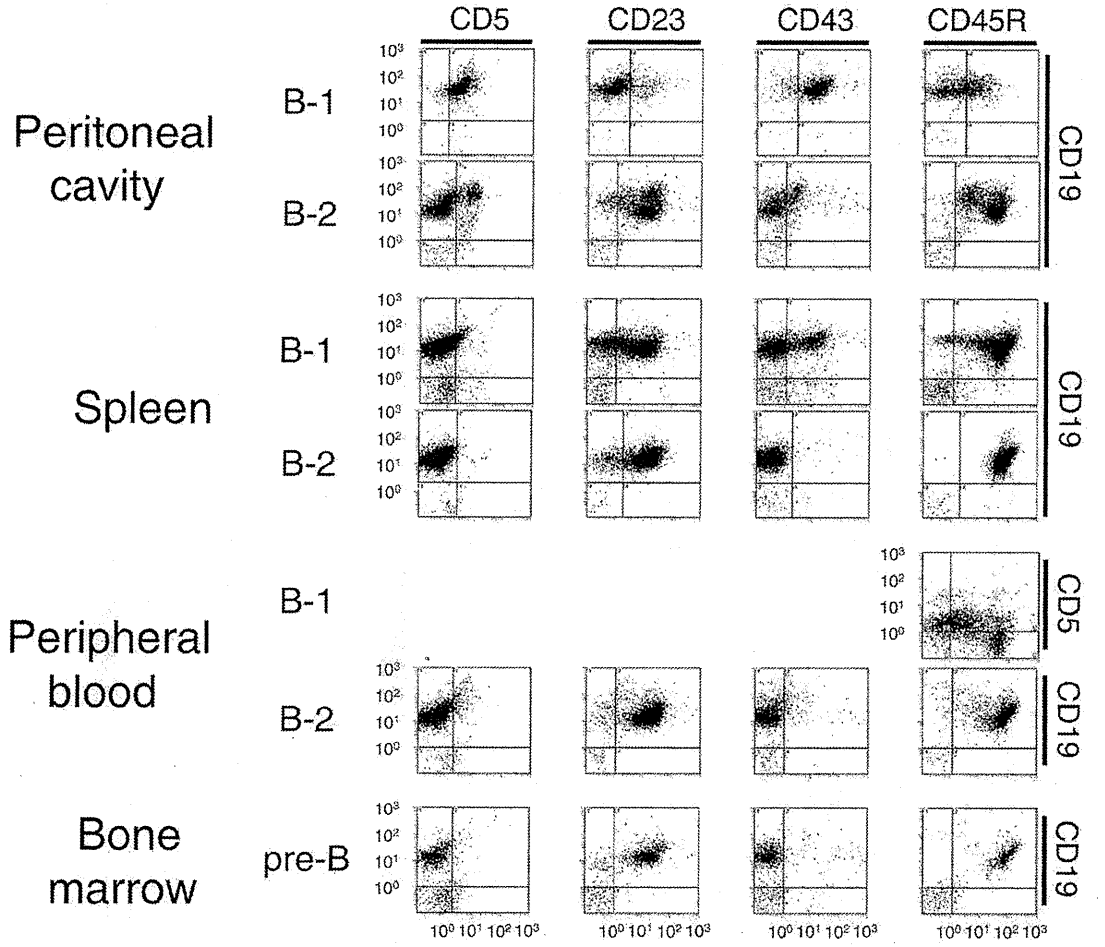

Fig. 2. Flow-cytometric characterization of isolated B cell subsets from spleen, peripheral blood, peritoneal cavity, and bone marrow. Each B cell subset was obtained by magnetic activated cell-sorting (MACS) as described in Materials and methods. B cell subsets from each tissue were characterized by flow-cytometric analysis using immunofluorescent antibody staining for CD5, CD19, CD23, CD43, and CD45R. Data for one of three independent experiments are shown.

3.2. LRRK2 mRNA expression in B-1 and B-2 cell subsets

LRRK2 mRNA expression in B-1 and B-2 cells as well as T cells isolated from the peritoneal cavity, spleen, and peripheral blood of the C57BL/6 mouse was investigated by qRT-PCR. This revealed that the B-2 cells expressed a much higher level of LRRK2 mRNA than the B-1 cells in all three tissues (6.7-, 3.8-, and 12.8-fold for the peritoneal cavity, spleen, and peripheral blood, respectively) (Fig. 3A). These results suggested that LRRK2 may have a more active function in B-2 cells than in B-1 cells. In contrast to the B-2 cells, the bone marrow pre-B cells expressed a much lower level of LRRK2 mRNA, suggesting that LRRK2 had a differentiation-related expression profile. In accordance with our previous study of spleen T cells (Maekawa et al., 2010), T cells isolated from either of these tissues expressed little or no LRRK2 mRNA.

The alymphoidplasia (aly) mouse strain has a naturally occurring point mutation in the NF-kB inducing kinase (NIK) gene that results in defective development of lymph nodes and Payer's patches (Miyawaki et al., 1994). Because the aly mouse has been reported to have an increased B-1/B-2 ratio, we investigated whether this strain exhibits a LRRK2 expression profile among B cell subsets different from that of the C57BL/6 mouse. As reported previously (Fagarasan et al., 2000; Shinkura et al., 1999), the B-1/B-2 ratio in the aly mouse was markedly high in both the peritoneal cavity and spleen (2.3- and 7.5-fold, respectively, in comparison with the C57BL/6 mouse, Table 1). Nevertheless, B-1 and B-2 cells prepared from peritoneal cavity and spleen of the aly mouse exhibited CD profiles similar to those of equivalent cells prepared from the corresponding tissues of the C57BL/6 mouse (Table 1). The qRT-PCR indicated that in the aly mouse, as was the case in the C57BL/6 mouse, the B-2 cells expressed a higher level of LRRK2 mRNA than B-1 cells in both the peritoneal cavity and spleen (Fig. 3B). These results suggest that LRRK2 expression by B-2 cells is not regulated by the NF-κB pathway.

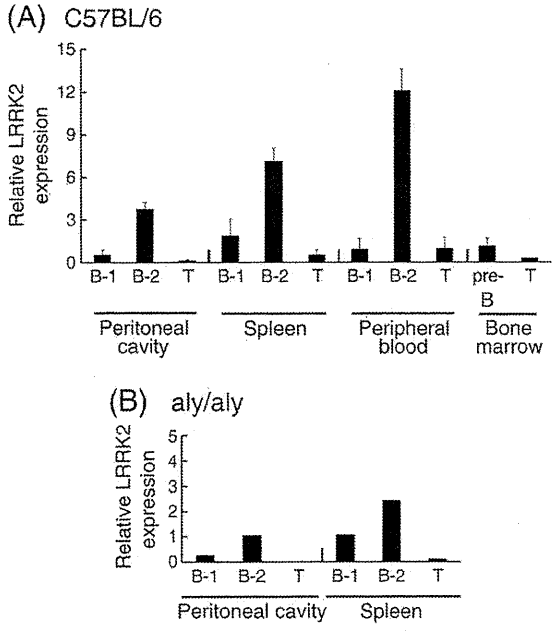

Fig. 3. Expression of LRRK2 mRNA in B cell subsets. B-1, B-2, and T cells were purified from each tissue of the C57BL/6 mouse (A) and the aly mouse (B) by MACS, as described in Materials and methods. RNA was isolated from each of the pooled cell populations from 10 mice. The amount of LRRK2 mRNA was measured by qRT-PCR and normalized relative to the expression of GAPDH. The average of three independent experiments is shown.

3.3. Downregulation of LRRK2 expression by activation of B-2 cells

As described above, we isolated B-2 cells as the CD43-negative B cell fraction. CD43 (sialophorin) is expressed at high levels on the surface of wide variety of leukocytes, including B-1 cells and activated B-2 cells, but not on resting B-2 cells. Based on speculation that the difference in LRRK2 expression between B-1 and B-2 cells is related to the activation status of B cells, we next investigated whether LRRK2 expression by B-2 cells is influenced by activation. Splenic B-2 cells from the C57BL/6 mouse were activated with either PMA plus ionomycin or LPS for 24 h. It was found that LRRK2 expression by B-2 cells was almost completely downregulated by activation (Fig. 4A). The LRRK2 downregulation was independent of the NF-κB pathway mediated by NIK, as LRRK2 mRNA expression by B-2 cells from the aly mouse was also downregulated by activation with anti-IgM or LPS (Fig. 4B). Our results suggest that LRRK2 in B-2 cells may have an important functional role in maintaining B-2 cells at the resting stage.

4. Discussion

In the present study, we found for the first time that LRRK2, a complex kinase that had been originally identified as the causative molecule of familial Parkinson's disease, is expressed differentially between the two B cell subsets, B-1 and B-2. B-2 cells (conventional B cells) isolated from the peritoneal cavity, spleen and peripheral blood expressed LRRK2 mRNA at a much higher level than B-1 cells. Although the true physiological function of LRRK2 has yet to be clarified, it is possible that it plays some important roles related to the characteristic development, phenotypes, and/or functions of B-2 cells.

CD43 (sialophorin), a major sialoglycoprotein expressed by a wide variety of blood cells, is one of the surface markers that can discriminate B-2 cells from B-1 cells, the former being negative and the latter positive (Wells et al., 1994). This antigen is also a marker of B-cell activation, as activation of B-2 cells has been reported to upregulate CD43 (Gulley et al., 1988; Rosenstein et al., 1999). These facts led us to speculate that LRRK2 expression might be negatively regulated in an activated state. Upon activation with PMA plus ionomycin or LPS, expression of LRRK2 by splenic B-2 cells was dramatically downregulated. It is possible that LRRK2 may play some important role in maintaining the status of B-2 cells so that they remain receptive to stimulation and signal transduction, such as those operating in immunoglobulin production, antigen presentation, and cell proliferation. In this case, downregulation of LRRK2 after activation would prevent B cells from becoming overactivated, and being induced to

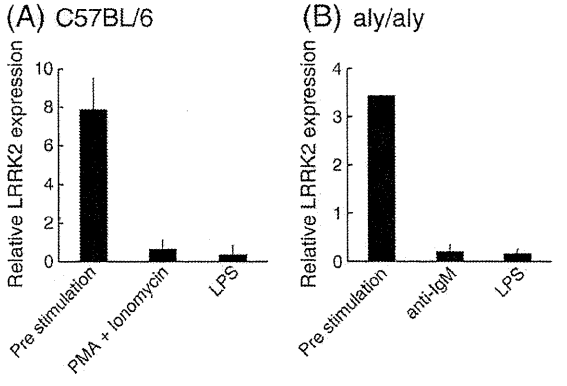

Fig. 4. Downregulation of LRRK2 expression in B-2 cells upon stimulation. (A) Splenic B-2 cells from the C57BL/6 mouse were stimulated in triplicate with either phorbol 12-myristate 13-acetate (PMA) at 300 ng/ml plus ionomycin at 600 ng/ml, or with lipopolysaccharide at 25 μg/ml (LPS), for 24 h. (B) Splenic B-2 cells from the aly mouse were stimulated with either $F(ab')_2$ fragments of either goat anti-mouse IgM at 15 μg/ml (anti-IgM), or with LPS, for 24 h. RNAs were isolated from pre-stimulated and stimulated cells, and the amount of LRRK2 mRNA was measured by qRT-PCR. Expression of LRRK2 normalized relative to GAPDH is shown.

undergo apoptosis. On the other hand, LRRK2 expression in B-1 cells may be sustained at a low level by spontaneous activation, as B-1 cells are known to have constitutively activated extracellular signal-regulated kinase and secrete immunoglobulin spontaneously in the absence of exogenous antigenic stimulation (Berland and Wortis, 2002; Dasu et al., 2009; Durand et al., 2009; Holodick et al., 2009). The bone marrow pre-B cells, although CD43-negative, expressed only a very low level of LRRK2. This is in accordance with their positivity for the activation markers, interleukin-7 receptor and CD25, thereby indicating a degree of activated status in the process of B cell development (Hardy and Hayakawa, 2001).

The NIK-mutant aly mouse, having an abnormally high B-1/B-2 ratio (Fagarasan et al., 2000; Shinkura et al., 1999), exhibited features of LRRK2 expression that were similar to those of the C57BL/6 mouse, i.e., higher expression in B-2 than in B-1 cells and downregulation after activation. These facts suggest that expression of the LRRK2 gene may not be regulated by the NIK–NF-κB pathway. Because three different forms of stimulation – PMA plus ionomycin, LPS, and anti-IgM antibody – similarly downregulated the expression of LRRK2, transcription factors functioning downstream of, and in common with these types of stimulation, such as p38 and c-Jun N-terminal kinase, could be possible regulators of LRRK2 gene expression. MKK6, an upstream kinase of p38, reportedly increases the intracellular level of LRRK2 (Hsu et al., 2010).

The molecular mechanism whereby mutant LRRK2 causes neurodegeneration in familial Parkinson's disease has not been conclusively clarified. The difficulty comes from the fact that the true function of LRRK2 is not known, although its kinase activity has been demonstrated using potential interactors such as moesin, 4E-BP, β-tubulin, and MKK3, 6, and 7 (Gandhi et al., 2008; Hsu et al., 2010; Imai et al., 2008; Jaleel et al., 2007). Investigation of the functional role of LRRK2 in B-2 cells from an immunological viewpoint could provide valuable clues, different from those obtained by neurological analysis, to clarifying its true biological function and its pathogenetic role in Parkinson's disease.

In patients with Parkinson's disease, increased serum levels of anti-melanin antibody and IgG deposition have been found in the substantia nigra (Double et al., 2009; Orr et al., 2005). It is possible that LRRK2 may contribute directly or indirectly to progression of the disease by regulating the humoral immune responses of B-2 cells. An interesting issue to be clarified is whether or not B-2 cells expressing mutant LRRK2 exhibit a normal immune response. It has been reported that B-lymphoblastoid cell lines carrying LRRK2 mutations show impaired growth (Tan et al., 2008). On the other hand, invasion of $CD8^+$ and $CD4^+$ T cells in the brain and alterations of the peripheral T-cell population (increased $CD8^+$ and decreased $CD4^+$ $CD25^+$) reportedly observed in Parkinson's disease patients indicate the contribution of a cellular immune response to progression of the disease (Baba et al., 2005; Brochard et al., 2009). Thus, it is apparent that Parkinson's disease is not only a lesion of neurons *per se* but also has an immune inflammatory character. LRRK2 is a candidate molecule that could play a role in the process of both neural dysfunction and immune inflammation in Parkinson's disease.

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Prevention of intracellular degradation of I2020T mutant LRRK2 restores its protectivity against apoptosis

Etsuro Ohta [a], Makoto Kubo [a], Fumiya Obata [a,b,*]

[a] Division of Clinical Immunology, Graduate School of Medical Sciences, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan
[b] R & D Center for Cell Design, Institute for Regenerative Medicine and Cell Design, Kitasato University School of Allied Health Sciences, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan

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ABSTRACT

Leucine-rich repeat kinase 2 (*LRRK2*) is the causal gene for autosomal dominant familial Parkinson's disease. We have previously reported a novel molecular feature characteristic to I2020T mutant LRRK2: higher susceptibility to post-translational degradation than the wild-type LRRK2. In the present study, we demonstrated that the protective effect of I2020T LRRK2 against hydrogen peroxide-induced apoptosis was impaired in comparison with the wild-type molecule. When the intracellular level of the protein had been allowed to recover by treatment with proteolysis inhibitors, the protective effect of I2020T LRRK2 against apoptosis was increased. We further confirmed that a decrease in the intracellular protein level of WT LRRK2 by knocking down resulted in a reduction of protectivity against apoptosis. These results suggest that higher susceptibility of I2020T mutant LRRK2 to intracellular degradation than the wild-type molecule may be one of the mechanisms involved in the neurodegeneration associated with this LRRK2 mutation.

Introduction

Parkinson's disease (PD) is a movement disorder caused by degeneration of dopaminergic neurons. *Leucine-rich repeat kinase 2* (*LRRK2*) is the gene responsible for autosomal dominant PD, PARK8, which we originally defined by linkage analysis of a Japanese family (Sagamihara family) [1–4]. LRRK2 belongs to the receptor-interacting protein (RIP) family, which has LRR (leucine-rich repeat), ROC (Ras of complex), COR (C-terminal ROC), kinase, and WD40 domains [5]. The Sagamihara family patients have the I2020T mutation in the kinase domain [4,6]. Up to now, a total of 23 LRRK2 mutations in various domains have been reported worldwide [2–4,7]. Patients with LRRK2 mutations exhibit clinical features indistinguishable from those of patients with sporadic PD, and LRRK2 is postulated to be a key molecule in the etiology of the disease. However, its true physiological function or the mechanism of neurodegeneration resulting from the mutation has not been conclusively clarified.

Accumulated data suggest that hyper-kinase activity reported for mutant LRRK2 molecules, particularly G2019S LRRK2, may be one possible mechanism for the pathogenesis induced by this molecule [8–13]. It has also been postulated that autophosphorylation of LRRK2 stabilize the kinase-active dimer and exacerbates the pathogenesis [14]. In the case of I2020T mutation, however, there is a degree of controversy; some studies have reported augmented kinase activity [9,15,16], whereas other studies of this mutation have demonstrated unchanged or impaired phosphorylation activity [11,17,18]. Thus, at least in the case of I2020T mutation, there is no consensus on the mechanism responsible for neurodegeneration.

In the previous study, we demonstrated that I2020T LRRK2 is more susceptible to post-translational degradation than the wild-type LRRK2 and G2019S LRRK2, indicating a novel molecular feature characteristic to I2020T LRRK2 [19]. In the present study, we investigated whether the high degradation rate of I2020T LRRK2 is related to the pathogenesis associated with this mutant molecule. We found that the wild-type LRRK2 exhibited a protective effect against apoptosis whereas I2020T mutant LRRK2 had impaired protectivity. Prevention of the intracellular degradation of I2020T LRRK2 markedly increased its protective effect against apoptosis. Finally, we investigated the relationship between the intracellular protein level of LRRK2 and its protectivity against apoptosis employing a LRRK2-knockdown experiment.

Materials and methods

Transfection of LRRK2. The mammalian expression cDNA construct of wild-type (WT) and I2020T mutant *LRRK2* cDNA with a V5 tag at the C-terminus was described previously [19]. Sequence

* Corresponding author. Address: Division of Immunology, School of Allied Health Sciences, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan. Fax: +81 42 778 8075.
E-mail address: obata@ahs.kitasato-u.ac.jp (F. Obata).

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analysis proved that T6059>C (I2020T) at exon 41 was the only difference between the WT and the I2020T *LRRK2* cDNA construct throughout the whole plasmid. HEK293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma) supplemented with 10% FCS and antibiotics. SH-SY5Y cells were cultured in DMEM nutrient mixture F-12 HAM (Sigma) supplemented with 10% FCS, and antibiotics. Transfection of the *LRRK2* cDNA plasmid was performed using Lipofectamine™ 2000 (Invitrogen) for HEK293 cells, and FuGENE® HD Transfection Reagent (Roche) for SH-SY5Y cells in accordance with the manufacturers' protocols. SH-SY5Y clones stably and uniformly expressing WT or I2020T LRRK2 have been described previously [19].

Western analysis. LRRK2-transfected cells were suspended in cell lysis buffer [Tris–HCl-buffered saline (pH 7.6) containing 1% digitonin, 1 mM phenylmethylsulfonyl fluoride, and 1 tablet of Complete mini protease inhibitor cocktail® (Roche)]. Cell lysates were obtained by centrifugation and subjected to Western analysis using horseradish peroxidase (HRP)-labeled antibody against the V5 tag (Invitrogen) for LRRK2 expression and HRP-labeled antibody against β-actin (Abcam) as an internal control.

Prevention of intracellular degradation of LRRK2. After 24 h of transfection with WT and I2020T *LRRK2* cDNA, HEK293 cells were treated with a cocktail of three proteolysis inhibitors, 1 μM MG-132 (Calbiochem) and 1 μM lactacystin (Sigma), both of which are proteasome inhibitors, and with 200 nM chloroquine (Sigma), a lysosome inhibitor. After 24 h of treatment, the cells were harvested and their lysates were analyzed by Western blotting, as described above. The stably LRRK2-expressing SH-SY5Y clones were also treated with the proteolysis inhibitors for 24 h and analyzed in the same manner.

Hydrogen peroxide (H_2O_2)-induced apoptosis. Apoptosis was induced by treatment of LRRK2-transfected cells with various concentrations (1–6 mM) of H_2O_2 for 50 min at 37 °C. In some experiments, the cells were treated with a cocktail of the proteolysis inhibitors MG-132, lactacystin and chloroquine for 24 h before addition of H_2O_2. Percentage of apoptotic cells was measured using an Annexin V-PE apoptosis Kit I™ (BD Biosciences) and an EPICS XL™ Flow Cytometer (Beckman Coulter) in accordance with the manufacturer's protocol. Apoptotic cells were also assessed by Western analysis of the lysates of transfected cells using an antibody against caspase-9 (Cell Signaling). For cell viability analysis, LRRK2-transfected cells were treated with 0.5 mM H_2O_2 for 30 min at 37 °C, and subjected to assay using a Cell Counting Kit-8™ (Dojindo) in accordance with the manufacturer's protocol.

Knockdown of transfected LRRK2. HEK293 cells were transfected with WT LRRK2 cDNA together with 25mer of Stealth™ RNAi for LRRK2 (5′-GAGCUGCUCCUUUGAAGAUACUAAA-3′; Invitrogen) or with an RNAi-control with the scrambled sequence. The effectiveness of knockdown of transfected LRRK2 was confirmed by Western analysis using anti-V5 antibody. After 24 h of co-transfection, the cells were treated with various concentrations (0.05–3 mM) of H_2O_2 for 30 min to induce apoptosis, and cell viability was analyzed.

Results

H_2O_2-induced apoptosis in LRRK2-transfected cells

To elucidate the physiological function of LRRK2 in the maintenance of cell viability, H_2O_2-induced apoptosis in LRRK2-transfected HEK293 cells was analyzed using annexin V staining. Among WT LRRK2-transfected cells treated with H_2O_2, the percentage of apoptotic cells was significantly lower than among untransfected cells, which expressed only endogenous LRRK2 molecules (Fig. 1A). In contrast, the percentage of apoptotic cells among I2020T mutant LRRK2-transfected HEK293 cells was significantly higher than that among WT LRRK2-transfected cells, and not significantly different

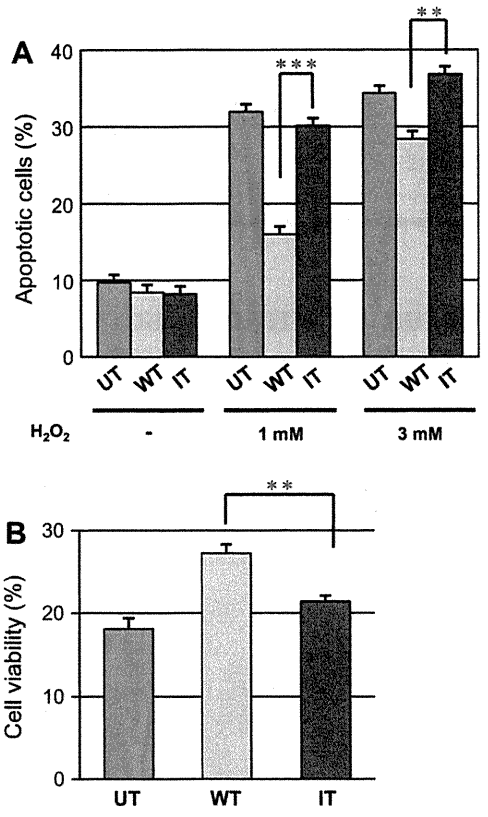

Fig. 1. H_2O_2-induced apoptosis in LRRK2-transfected cells. (A) WT and I2020T (IT) LRRK2-transfected HEK293 cells were treated with 1 or 3 mM H_2O_2 for 50 min. The percentage of cells showing apoptosis was measured by annexin V staining. (B) WT and I2020T (IT) LRRK2-transfected HEK293 cells were treated with 0.5 mM H_2O_2 for 30 min and the cell viability was measured. UT: Untransfected HEK293 cells. Stars represent statistical comparisons by one-way ANOVA ($n = 3$); $^{**}p < 0.005$. $^{***}p < 0.0005$.

from the situation in untransfected cells. Similar results were obtained for the LRRK2-transfected neuroblastoma cell line SH-SY5Y, although to a less marked extent due to the low transfection efficiency, and for SH-SY5Y clones stably and uniformly expressing WT or I2020T LRRK2 (Supplementary Fig. 1A and B). Consistently, the viability of I2020T LRRK2-transfected HEK293 cells was significantly lower than that of the WT LRRK2-transfected cells (Fig. 1B). These results suggest that WT LRRK2, but not I2020T mutant LRRK2, exerts a protective effect against H_2O_2-induced apoptosis.

Apoptosis of LRRK2-transfected cells after treatment with proteolysis inhibitors

In the previous study, we demonstrated that the I2020T mutant LRRK2 is more susceptible to post-translational degradation than the WT LRRK2 [19]. To investigate whether prevention of degradation of the mutant LRRK2 influences its ability to protect against H_2O_2-induced apoptosis, WT- and I2020T LRRK2-transfected HEK293 cells were treated with a cocktail of proteolysis inhibitors, MG-132 (a proteasome inhibitor), lactacystin (a proteasome inhibitor), and chloroquine (a lysosome inhibitor). As reported, treatment with this inhibitor cocktail increased the I2020T LRRK2 protein to a level similar to that of WT LRRK2 (Fig. 2A). Possibly because of the apoptosis-promoting effect of the protease inhibitors [20,21], the treatment significantly increased the percentage of annexin V-positive apoptotic cells among WT LRRK2-transfected cells, although the percentage was still lower than that among

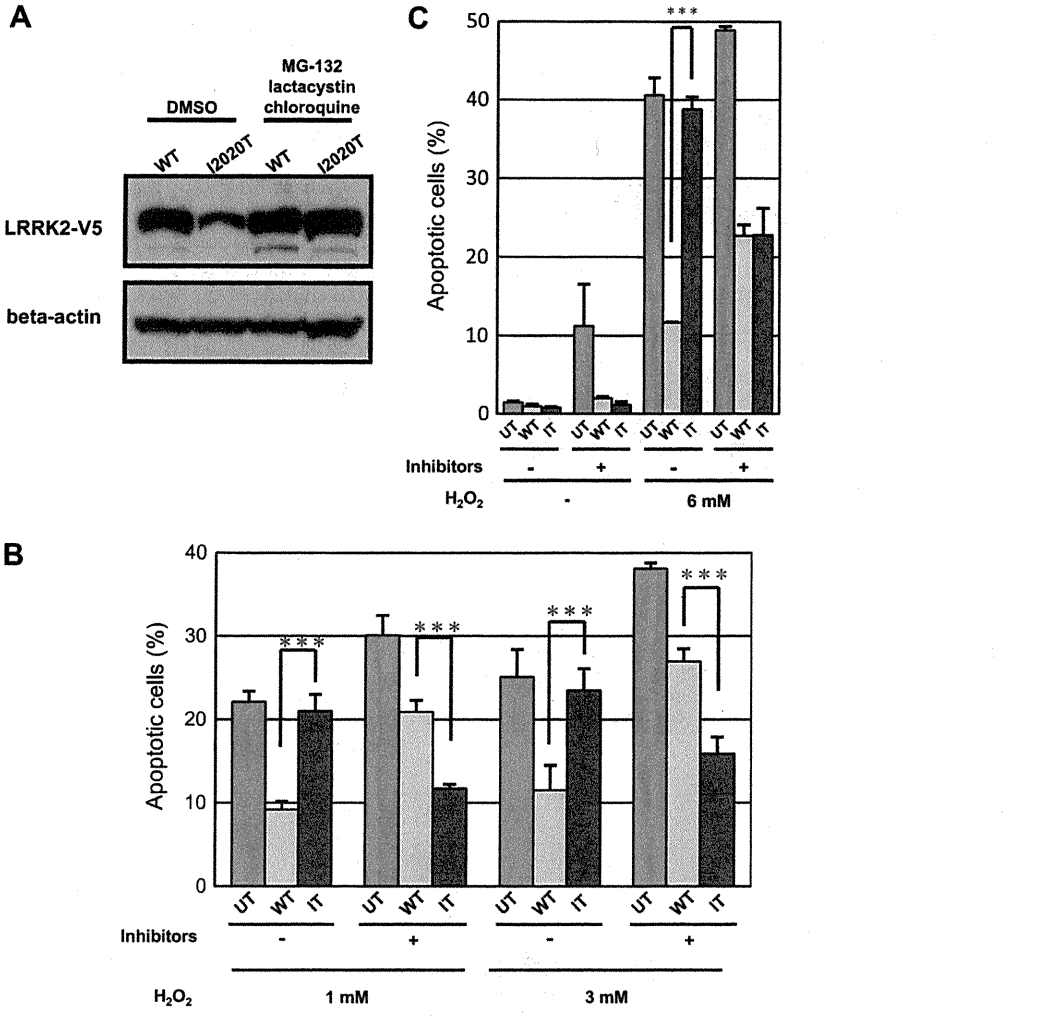

Fig. 2. Effect of proteolysis inhibitors on apoptosis in LRRK2-transfected cells. (A) HEK293 cells were transfected with WT or I2020T LRRK2 cDNA and treated with a cocktail of three proteolysis inhibitors (1 μM MG-132, 1 μM lactacystin, and 200 nM chloroquine) for 24 h. The LRRK2 level in the lysates was then analyzed by Western blotting with an antibody against V5 tag. (B) WT and I2020T (IT) LRRK2-transfected HEK293 cells were treated for 24 h with a cocktail of proteolysis inhibitors, and apoptosis was induced with 1 or 3 mM H_2O_2 for 50 min. UT: Untransfected HEK293 cells. (C) SH-SY5Y clones stably and uniformly expressing WT and I2020T LRRK2 (IT) were treated for 24 h with a cocktail of proteolysis inhibitors, and apoptosis was induced with 6 mM H_2O_2 for 4 h. UT: Untransfected SH-SY5Y cells. The percentage of apoptotic cells was measured by annexin V staining. Stars represent statistical comparisons by one-way ANOVA ($n = 3$); $^{***}p < 0.0005$.

untransfected cells subjected to the same treatment (Fig. 2B). Nevertheless, the same treatment of I2020T LRRK2-transfected cells markedly decreased the percentage of apoptotic cells to a level even lower than that among WT LRRK2-transfected cells.

Next, the effect of proteolysis inhibitors on apoptosis was analyzed using SH-SY5Y clones that over-expressed the WT and I2020T LRRK2 molecules stably and uniformly. Treatment with the proteolysis inhibitors increased the percentage of apoptotic cells among the WT LRRK2-expressing clones, although the percentage was still lower than that among the control cells (Fig. 2C). On the other hand, in I2020T LRRK2-expressing clones, the same treatment, which would otherwise have impaired the ability to protect against apoptosis, dramatically reduced the percentage of apoptotic cells to a level similar to that among the WT LRRK2-expressing clones. These results indicated that the ability of I2020T LRRK2 to protect against apoptosis could be restored by preventing its intracellular degradation.

Apoptosis was also analyzed by activation of caspase-9. The molecular ratio of activated relative to inactive caspase-9 in H_2O_2-treated cells was higher in I2020T LRRK2-transfected HEK293 cells than in WT LRRK2-transfected cells (Fig. 3). Although treatment with the proteolysis inhibitors increased the molecular ratio of activated caspase-9 in both WT- and I2020T LRRK2-transfected cells, this treatment reduced the ratio of activated caspase-9 in the I2020T LRRK2-transfected cells to a level lower than that in the WT LRRK2-transfected cells. These results, in terms of both annexin V staining and caspase-9 activation, indicated that the ability of I2020T LRRK2 to protect cells against apoptosis can be increased by preventing its degradation.

Influence of LRRK2-knockdown on protectivity against apoptosis

Finally, the relationship between the intracellular protein level of LRRK2 and protectivity against apoptosis was investigated in a knockdown experiment. Transfection of *LRRK2*-specific RNAi together with WT *LRRK2* cDNA into HEK293 reduced the protein level of transfected WT LRRK2 to 18% in comparison with the use of an RNAi-control (Fig. 4A). As described above, transfection of WT *LRRK2* cDNA into HEK293 markedly improved the viability of H_2O_2-treated cells (Fig. 4B). This protectivity of WT LRRK2 against

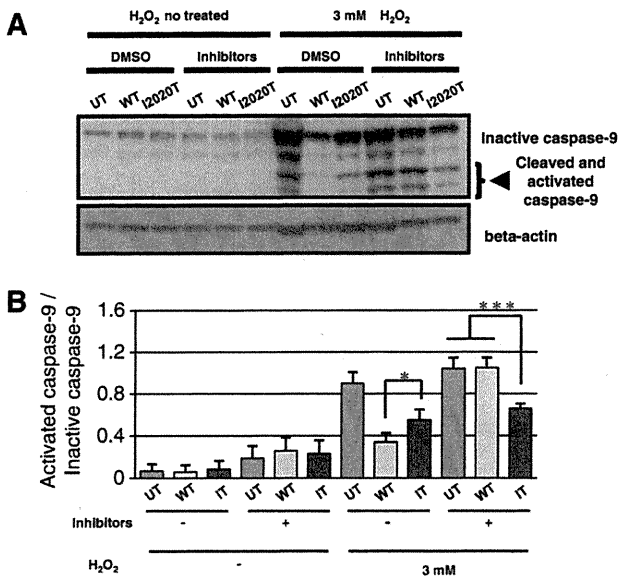

Fig. 3. Caspase-9 activation in LRRK2-transfected cells. (A) WT- and I2020T LRRK2-transfected HEK293 cells were treated with a cocktail of proteolysis inhibitors (MG-132, lactacystin and chloroquine), and apoptosis was induced with 3 mM H_2O_2 for 50 min. The level of cleaved and activated caspase-9 in the lysates was analyzed by Western blotting. UT: Untransfected HEK293 cells. (B) Graphical representation of the molecular ratio of activated caspase-9 relative to inactive caspase-9. Stars represent statistical comparisons by one-way ANOVA ($n = 3$); $^{*}p < 0.05$. $^{***}p < 0.0005$.

apoptosis was significantly abrogated by co-transfection of the LRRK2-specific RNAi but not the RNAi-control. These results indicate that the ability of LRRK2 to protect cells from H_2O_2-induced apoptosis is related with its intracellular protein level.

Discussion

We have previously reported a novel molecular feature characteristic to I2020T LRRK2: that it is more susceptible to post-translational degradation than the wild-type LRRK2 and G2019S mutant LRRK2 [19]. In the present study, we found that the increased intracellular protein level achieved by preventing degradation of I2020T LRRK2 restore its protectivity against apoptosis. Indeed the protease inhibitors used in this study have been reported to show various additional cellular effects, e.g., promotion of apoptosis (MG-132, lactacystin, and chloroquine) and activation of the CMV promoter (lactacystin) [20–25]. However, such effects, if any, would have appeared in both WT- and I2020T LRRK2-transfected cells in a similar manner. Therefore, an increased amount of I2020T LRRK2 after treatment with proteolysis inhibitors would be the most plausible explanation for the increased protective effect against apoptosis. The notion that the intracellular protein level of LRRK2 determines its protective effect against apoptosis is further supported by the fact that knockdown of transfected WT LRRK2 impaired its cell-protective effect. Similarly, the apparently opposite effects in WT- and I2020T LRRK2-transfected cells after proteolysis treatment would have been due to the fact that, in the latter case, the extent of the increased protective effect might have overcome the toxic effects of the inhibitors. When its degra-

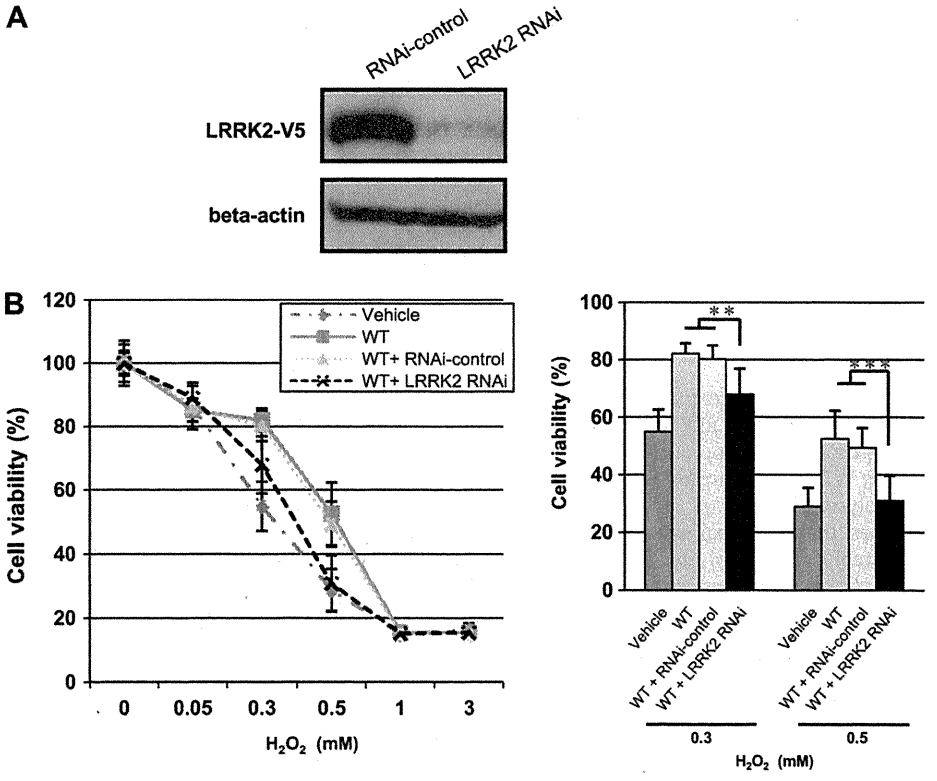

Fig. 4. Effect of a decrease in LRRK2 protein level on protectivity against apoptosis. HEK293 cells were transfected with WT *LRRK2* cDNA together with the LRRK2-specific RNAi or with the RNAi-control having the scrambled sequence. (A) After 24 h of co-transfection, the protein level of transfected WT LRRK2 was analyzed by Western blotting using anti-V5 antibody. (B) The cells after 24 h co-transfection were treated with 0.05, 03, 0.5, 1, or 3 mM H_2O_2 for 30 min to induce apoptosis, and cell viability was measured. Dash-dotted line (–·–·): vehicle, solid line (—): WT *LRRK2* cDNA, dashed line (– –): WT *LRRK2* + LRRK2 RNAi, dotted line (·····): WT *LRRK2* + RNAi-control. The results of treatment with 0.3 and 0.5 mM H_2O_2 are also represented by bar graph. Stars represent statistical comparisons by one-way ANOVA ($n = 6$); $^{**}p < 0.005$. $^{***}p < 0.0005$.

dation was prevented, the I2020T LRRK2 expressed by HEK293 exhibited an even stronger protective effect against apoptosis, in terms of both annexin V and caspase-9 analysis, than WT LRRK2, suggesting that I2020T LRRK2 might have a higher intrinsic potential than WT LRRK2 to activate a yet unknown apoptosis–protection pathway.

Although in the present study we found that the WT LRRK2 had a protective effect against H_2O_2-induced apoptosis and that knockdown of the WT LRRK2 abrogated this effect, there has been some controversy as to whether LRRK2 is protective or toxic for cells [12,15,26–28]. Under our experimental conditions, we did not observe any increase of apoptosis in WT LRRK2-transfected cells without H_2O_2 treatment. However, we could not exclude the possibility that over-expressed WT LRRK2 exerts a cytotoxic effect on cells in a steady state, whereas it functions as a maintenance or protective molecule when cells are exposed to oxidative stress. Interestingly, loss of the LRRK2-orthologue in *Drosophila* has been reported to induce an increase in susceptibility to oxidative stress and a lower survival rate, being consistent with the results of our LRRK2-knockdown experiments [29].

Although hyper-kinase activity of mutant LRRK2 molecules, particularly G2019S LRRK2, has been reported to be one possible mechanism for the pathogenesis induced by this molecule [8–13,15], there is controversy in the case of I2020T mutation. Some studies have reported augmented kinase activity [9,15,16], whereas other studies of this mutation have demonstrated unchanged or impaired kinase activity [11,17,18]. The results presented here suggest a new neurodegenerative mechanism induced by I2020T LRRK2, i.e., higher susceptibility to degradation gives rise to insufficiency of functional molecules to protect neurons from apoptosis. Several reports have revealed that insufficiency of gene products can cause dominant hereditary neurodegeneration, e.g., progranulin in frontotemporal lobar degeneration linked to chromosome 17 [30], transforming growth factor beta 2 and neurotrophin receptor trkB/C in the mouse PD model [31,32], and p73 in the mouse Alzheimer's disease model [33]. In addition, because LRRK2 has been reported to form dimers [9,34], any postulated molecular instability leading to degradation of I2020T LRRK2 may influence the stability and/or function of not only the I2020T/I2020T-homodimer but also the WT/I2020T-heterodimer, as is the case for GTP cyclohydrolase I in DYT5 dystonia [35,36] and KIT (mast/stem cell growth factor receptor) in piebaldism [37,38]. Finally, as in the case of I2020T LRRK2, the G2019S mutant LRRK2 exhibited impaired protectivity against H_2O_2-induced apoptosis (data not shown). As we reported previously, the G2019S LRRK2 does not differ from WT LRRK2 in susceptibility to degradation [19]. It cannot be excluded that each type of LRRK2 mutation affects a different molecular aspect of LRRK2, i.e., kinase activity, dimer formation, or susceptibility to degradation, all of which finally lead to neurodegeneration through a common and/or an independent pathway.

Conclusion

The intracellular protein level of LRRK2 determines protectivity against H_2O_2-induced apoptosis. The protective effect of I2020T mutant LRRK2 against apoptosis can be restored by preventing its intracellular degradation. Our results suggest a new etiology of neurodegeneration in PD caused by the LRRK2 mutation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.043.

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Age-dependent and cell-population-restricted LRRK2 expression in normal mouse spleen

Tatsunori Maekawa, Makoto Kubo, Ikue Yokoyama, Etsuro Ohta, Fumiya Obata *

Division of Clinical Immunology, Graduate School of Medical Sciences, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan

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ABSTRACT

Leucine-rich repeat kinase 2 (LRRK2) is the causal molecule of familial Parkinson's disease (PD), but its true physiological function remains unknown. In the normal mouse, LRRK2 is expressed in kidney, spleen, and lung at much higher levels than in brain, suggesting that LRRK2 may play an important role in these organs. Analysis of age-related changes in LRRK2 expression demonstrated that expression in kidney, lung, and various brain regions was constant throughout adult life. On the other hand, expression of both LRRK2 mRNA and protein decreased markedly in spleen in an age-dependent manner. Analysis of purified spleen cells indicated that B lymphocytes were the major population expressing LRRK2, and that T lymphocytes showed no expression. Consistently, the B lymphocyte surface marker CD19 exhibited an age-dependent decrease of mRNA expression in spleen. These results suggest a possibly novel function of LRRK2 in the immune system, especially in B lymphocytes.

Introduction

Leucine-rich repeat kinase 2 (LRRK2) is the causal molecule of autosomal-dominant familial Parkinson's disease (PD), PARK8, which was originally defined in a study of a large Japanese PD family, the Sagamihara family [1–4]. LRRK2 is a large complex protein with an approximate molecular mass of 260 kDa and contains several domains including the LRR (leucine-rich repeat), ROC (Ras of complex), COR (C-terminal ROC), RIP (receptor interacting protein) kinase, and WD40 domains [3–5]. Although the kinase activity toward candidate substrate molecules as well as regulation of the activity by the ROC domain have been studied extensively, the true physiological role of LRRK2 or the mechanism of neurodegeneration resulting from its mutation remains undisclosed.

Analyses of LRRK2 mRNA expression in human, mouse, and rat brain have demonstrated that LRRK2 is expressed in various regions including the substantia nigra, putamen, striatum, amygdala, hippocampus, cortex, and cerebellum [3,4,6–12]. In other organs such as kidney, lung, spleen, and lymph node, expression of LRRK2 mRNA has been reported to be far higher than in brain [6,13,14]. A similar tissue distribution has been reported for LRRK2 protein expression [11,12,15–18]. In contrast to mRNA analysis that uses nucleotide probes and primers specific to the LRRK2 sequence, however, the results of LRRK2 protein expression studies using commercial polyclonal anti-LRRK2 antibodies require careful interpretation because some of the antibodies have been reported to react with proteins around 260 kDa in size other than LRRK2 [13]. With regard to age-related change, LRRK2 mRNA expression in mouse brain, lung, heart, and liver has been reported to increase from embryonic day 11 to birth [13]. After birth, LRRK2 protein in mouse brain has been shown to increase until postnatal day 60 [18]. However, no later adulthood age-related changes in the expression of mouse LRRK2 mRNA or protein have been analyzed in either the brain or other organs, although rat striatum LRRK2 mRNA has been reported to increase until postnatal day 29, remaining constant thereafter until 24 months of age [6].

In the present study, we analyzed the organ/tissue distribution and age-related changes in the expression of mouse LRRK2 at both the mRNA and protein levels, using in the latter case an antibody of validated specificity. We found that LRRK2 was expressed in kidney, lung, and spleen at a level much higher than in any region of the brain. Levels of LRRK2 expression in brain and lung did not change during adulthood. By contrast, in spleen, a marked and age-dependent decrease of LRRK2 expression was found. This finding was explained by an age-dependent decrease of B-lymphocytes, the major LRRK2-expressing cell population, in the spleen.

Materials and methods

Animals. C57BL/6J (B6) mice aged 6–110 weeks were housed in a light- and temperature-controlled room with water and food ad libitum. Organs were removed after euthanasia with carbon dioxide. All procedures had been approved by the Animal Experimentation and Ethics Committee of Kitasato University.

* Corresponding author. Fax: +81 42 778 8075.
E-mail address: obata@ahs.kitasato-u.ac.jp (F. Obata).

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Reverse transcription-polymerase chain reaction (RT-PCR). Organs were homogenized in TRIzol Reagent (Invitrogen) and total RNA was isolated in accordance with the manufacturer's instructions. cDNA synthesis was performed by a ThermoScript RT-PCR System (Invitrogen). Quantitative (real-time) PCR was performed using SYBR Green PCR Master Mix and a 7500 Real Time PCR System (Applied Biosystems). PCR primers used were as follows: mouse *LRRK2* forward 5′-TCTGGCTGGAACCCTGCTAT-3′ and reverse 5′- AACTGGC CATCTTCATCTCC-3′, mouse *CD19* forward 5′-AGCGAATGACTGACCC CGCC-3′ and reverse 5′- CCAGGCCCATGCTCAGCGTT-3′, mouse *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* forward 5′- GAGGC CGGTGCTGAGTATGTCGTG-3′ and reverse 5′- TCGGCAGAAGGGGCG GAGAT-3′. The threshold cycle (C_t) value of *LRRK2* was normalized by the C_t value of the *GAPDH* gene.

Western blotting. Tissues were homogenized in digitonin buffer [1% digitonin, Tris-buffered saline (pH 7.6), 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail tablet (Roche)], and rotated at 4 °C for 1 h. Tissue lysates obtained by centrifugation were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using a 5–20% gradient e-PAGEL (ATTO), and blotted onto polyvinylidene fluoride membranes. The membranes were blocked in 2% skim milk or 2% ECL Advance Blocking Agent (GE Healthcare) in phosphate-buffered saline (PBS)-Tween 20 overnight at 4 °C. The membranes were probed with a rabbit polyclonal antibody against LRRK2 (AT106, Alexis) for 45 min at room temperature. After incubation with horseradish peroxidase (HRP)-labeled donkey anti-rabbit IgG (BioLegend) secondary antibody, bands were visualized using an ECL or ECL Advance Western Blotting Detection Kit (GE Healthcare). HRP-labeled monoclonal antibody against beta actin (Abcam) was used as a control.

Purification of spleen cell subpopulations. Spleens were cut into small pieces, filtered through nylon mesh to disperse single cells, and treated with hypotonic solution to lyse erythrocytes. T lymphocytes, B lymphocytes, and macrophages were separated using magnetic beads conjugated with antibodies against the cell surface markers, CD3, CD19, and CD11b, respectively, and LS Column Adaptor (Miltenyi Biotec). Cell population purity was confirmed by flow cytometry to be 92.5% for CD3, 96.8% for CD19, and 88.5% for CD11b.

Results

Expression of LRRK2 mRNA in adult mouse organs

To examine the expression level of mouse LRRK2 mRNA, quantitative PCR of various organs from C57BL/6J mice aged 20, 50, and 98 weeks was performed. This revealed that expression of LRRK2 mRNA in kidney, spleen, lung, and testis was high in mice at these ages, whereas expression in each of the brain regions examined (cortex, cerebellum, midbrain, medulla, and olfactory bulb), as well as in liver and heart, was low at the same ages (Fig. 1). Notably, the level of LRRK2 mRNA in the spleen of mice aged 98 weeks was much lower than that at 20 and 50 weeks, suggesting an age-related change in the mRNA level.

Relationship between expression of LRRK2 mRNA and aging

To further investigate the age-dependency of LRRK2 mRNA expression, spleen, lung, cortex, midbrain, and cerebellum of mice aged 6, 18, 34, 70, and 110 weeks were analyzed. In accordance with the results shown in Fig. 1, quantitative PCR analysis of the spleen indicated that expression of LRRK2 mRNA decreased markedly in an age-dependent manner (Fig. 2). The splenic level of LRRK2 mRNA at 70 and 110 weeks was about one fifth and one eighth of that at 6 weeks, respectively. On the other hand, the levels of LRRK2 mRNA in lung, cortex, midbrain, and cerebellum remained almost constant as aging progressed.

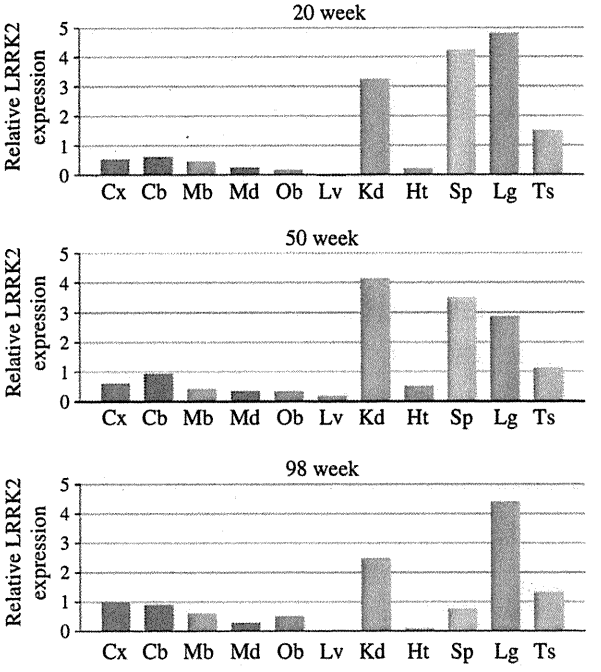

Fig. 1. Expression of LRRK2 mRNA in various organs of adult mouse. RNA was isolated from various organs of C57BL/6J mice aged 20, 50, and 98 weeks and subjected to quantitative PCR. Relative expression of *LRRK2* normalized by *GAPDH* is shown.

Expression of LRRK2 protein in adult mouse organs

AT106 is one of two commercial polyclonal antibodies that have been proved to recognize the endogenous mouse LRRK2 molecule by using knockout mice as a negative control [13]. As reported, Western analysis using this antibody identified LRRK2 proteins of about 260 kDa just below the nonspecific band (Supplementary Fig. 1A). Western analysis of lysates of various tissues from mice aged 50 weeks revealed that the levels of LRRK2 protein in these organs were consistent with the corresponding mRNA levels in each organ at the same age, as shown in Fig. 1, i.e., high in kidney, spleen, lung, and testis, but low in various brain regions (cortex, cerebellum, midbrain, medulla, and olfactory bulb), liver, and heart (Supplementary Fig. 1B).

Age-related changes in the splenic level of LRRK2 protein

To investigate age-related changes in the expression of LRRK2 protein in the spleen, tissue lysates of spleen and lung from mice aged 6, 18, 34, 70, and 110 weeks were subjected to Western analysis using AT106 antibody. Consistent with the results of mRNA analysis, the splenic level of LRRK2 protein decreased markedly with aging (Fig. 3). The level of LRRK2 protein at 70 and 110 weeks was about one third and one twelfth of that at 6 weeks, respectively. Thus, LRRK2 protein expression in the spleen was found to exhibit an age-dependent decrease like that of the mRNA.

LRRK2 expression in spleen cell subpopulations

Because the spleen contains various populations of immune cells, we next purified each population with magnetic bead-conju-

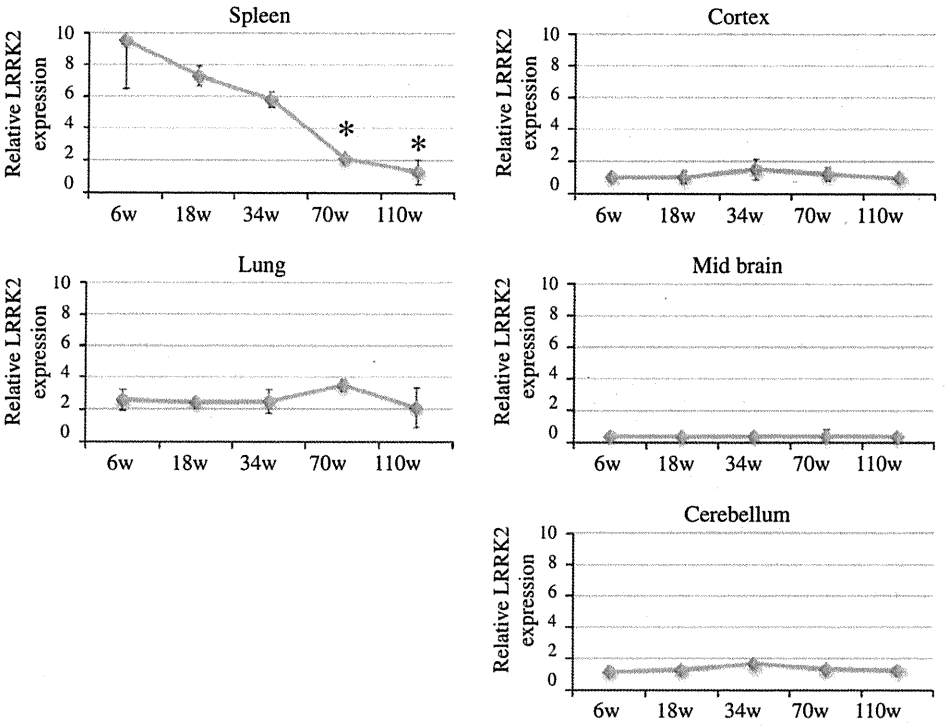

Fig. 2. Relationship between expression of LRRK2 mRNA and aging. RNA was isolated from spleen, lung, cortex, midbrain, and cerebellum of mice aged 6, 18, 34, 70, and 110 weeks, and subjected to quantitative PCR. Relative *LRRK2* expression normalized by *GAPDH* is shown. Stars represent statistical comparisons by one-way ANOVA ($n = 3$); * $p < 0.05$.

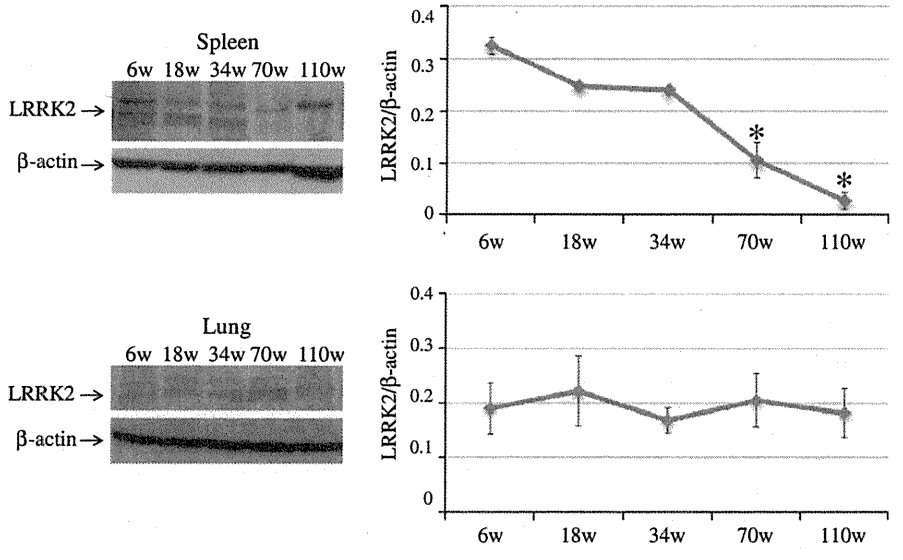

Fig. 3. Age-related change of the LRRK2 protein level in spleen. Tissue lysates were prepared from spleen and lung of mice aged 6, 18, 34, 70, and 110 weeks and subjected to Western analysis using AT106. LRRK2 protein levels normalized by β-actin are shown. Stars represent statistical comparisons by one-way ANOVA ($n = 3$); * $p < 0.05$.

gated antibodies directed against cell surface markers, i.e., CD3 (T lymphocytes), CD19 (B lymphocytes), and CD11b (macrophages), and investigated the expression of LRRK2 in each population. Western analysis with AT106 demonstrated that B lymphocytes were the major LRRK2-expressing cell population in the spleen (Fig. 4A). Macrophages expressed the LRRK2 protein weakly, but T lymphocytes showed no expression. Finally, we found that expression of the mRNA for the B lymphocyte marker CD19 exhibited an age-dependent decrease (Fig. 4B). These results suggested that the age-dependent decrease of LRRK2 expression in the spleen can be explained by a decrease in the number of splenic B lymphocytes.

Discussion

LRRK2 was originally identified as the causal molecule of autosomal-dominant familial PD, PARK8 [1–4]. Although the kinase activity has been demonstrated using several candidate

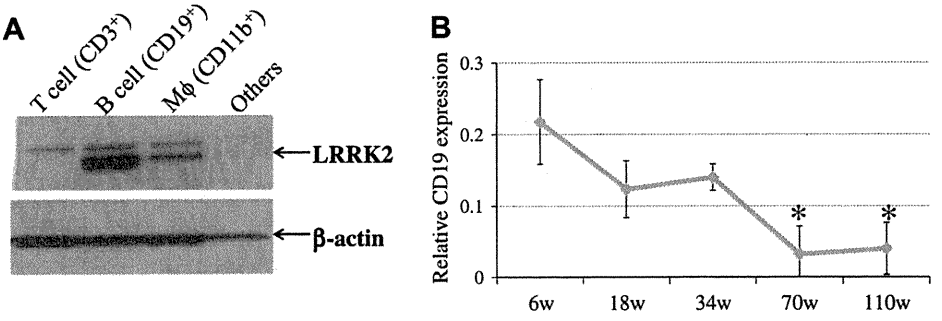

Fig. 4. LRRK2 expression in spleen cell populations. (A) Western analysis of LRRK2 expression in spleen cell populations. T lymphocytes, B lymphocytes, and macrophages were purified with magnetic bead-conjugated antibodies directed against each cell surface marker. Lysates of these cells and other residual spleen cells were subjected to Western analysis using the anti-LRRK2 antibody AT106. (B) Age-related change in the expression of *CD19* mRNA in spleen. RNA was isolated from the spleen of mice at various ages and subjected to quantitative PCR. Relative *CD19* mRNA expression normalized by *GAPDH* is shown. Stars represent statistical comparisons by one-way ANOVA ($n = 3$); $^{*}p < 0.05$.

substrates [19–23], the true substrate or the true physiological function of LRRK2 is still unknown. Analysis of the organ/tissue distribution of LRRK2 would provide valuable clues as to its function. In early studies, LRRK2 was reportedly expressed ubiquitously, but subsequently, differences in its expression level among organs and tissues became apparent [6,13,14,18]. In the present study, we analyzed the expression of LRRK2 at both the mRNA and protein levels in various organs, and found that LRRK2 expression in kidney, lung, and spleen was much higher than that in brain, being consistent with previous reports [13,14,18]. It is suggested, therefore, that LRRK2 may play some important roles in these organs. Histochemical analysis with AT106 and two additional anti-LRRK2 antibodies revealed immunoreactivity in bronchioles of the lung and proximal renal tubules of the kidney, further suggesting some specific function in these areas (data not shown).

In the present study, we found for the first time that among cells in the spleen, B lymphocytes were the major population expressing LRRK2, and that T lymphocytes did not express LRRK2. Although human B-lymphoblastoid cell lines have been shown to express LRRK2 strongly [16], it has been unclear whether human T lymphocytes express LRRK2. The age-dependent decrease of LRRK2 expression in the mouse spleen can be explained by a decrease in the number of B lymphocytes, as indicated by the age-dependent decline of the cell surface marker CD19, although there is some controversy regarding the age-dependent decrease of B lymphocytes in mouse spleen [24–27]. It can be postulated that LRRK2 may play a specific role related to B lymphocytes, but not to T lymphocytes, e.g., immunoglobulin production, antigen presentation, or other forms of B lymphocyte-specific signal transduction. Human B-lymphoblastoid cell lines expressing the R1441C mutant LRRK2 have been reported to have a lower growth rate than those expressing wild-type LRRK2 [28], suggesting a role of LRRK2 in cell growth or the cell cycle.

In PD patients, analysis of whom originally led to the discovery of LRRK2, increased serum levels of anti-melanin antibody and IgG deposition in the substantia nigra have been found [29,30]. In addition, accumulating evidence indicates that microglia, the brain equivalent of macrophages known to express LRRK2, play crucial roles in the pathogenesis of PD [31–33]. Indeed, LRRK2 expressed in neurons may serve as a key molecule in neurodegeneration, but LRRK2 may also contribute to the progression of PD as a form of immune-response-related molecule, for example directly or indirectly regulating the humoral immune responses of B lymphocytes and the production of inflammatory mediators by macrophages and microglia. Investigations to clarify the functional role of LRRK2 in the immune system are currently in progress.

Conclusions

Expression of LRRK2 decreased markedly in mouse spleen in an age-dependent manner. This finding was explained by an age-dependent decrease of B-lymphocytes, the major LRRK2-expressing cell population in the spleen. T lymphocytes showed no LRRK2-expression.These results suggest a possibly novel function of LRRK2 in the immune system, especially in B lymphocytes.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.041.

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Forum Minireview

Advanced Research on Dopamine Signaling to Develop Drugs for the Treatment of Mental Disorders: Regulation of Dopaminergic Neural Transmission by Tyrosine Hydroxylase Protein at Nerve Terminals

Chiho Sumi-Ichinose[1,*], Hiroshi Ichinose[2], Kazuhisa Ikemoto[1], Takahide Nomura[1], and Kazunao Kondo[1]

[1]*Department of Pharmacology, School of Medicine, Fujita Health University, Toyoake, Aichi 470-1192, Japan*
[2]*Department of Life Science, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226-8501, Japan*

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Abstract. 5*R*-L-Erythro-5,6,7,8-tetrahydrobiopterin (BH_4) is an essential cofactor for tyrosine hydroxylase (TH). Recently, a type of dopa-responsive dystonia (DRD) (DYT5, Segawa's disease) was revealed to be caused by dominant mutations of the gene encoding GTP cyclohydrolase I (GCHI), which is the rate-limiting enzyme of BH_4 biosynthesis. In order to probe the role of BH_4 in vivo, we established BH_4-depleted mice by disrupting the 6-pyruvoyltetrahydropterin synthase (PTS) gene ($Pts^{-/-}$) and rescued them by introducing human PTS cDNA under the control of the human dopamine β-hydroxylase (DBH) promoter ($Pts^{-/-}$-DPS). The $Pts^{-/-}$-DPS mice developed hyperphenylalaninemia. Interestingly, tyrosine hydroxylase protein was dramatically reduced in the dopaminergic nerve terminals of these mice, and they developed abnormal posture and motor disturbance. We propose that the biochemical and pathologic changes of $Pts^{-/-}$-DPS mice are caused by mechanisms common to human DRD, and understanding these mechanisms could give us insight into other movement disorders.

Keywords: tetrahydrobiopterin, dystonia, tyrosine hydroxylase, striatum, dopamine

1. Introduction

5*R*-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) is an essential cofactor for tyrosine hydroxylase (TH), tryptophan hydroxylase (TPH), and phenylalanine hydroxylase (PAH) (1). TH and TPH are rate-limiting enzymes for catecholamine and serotonin production, respectively, and PAH converts phenylalanine to tyrosine. Catecholamine and serotonin are important neurotransmitters that control various human behaviors, including movement, emotion, and reward. Recently, BH_4 was also noted as a key factor for nitric oxide (NO) production by stabilization of all three types of nitric oxide synthase (nNOS, eNOS, and iNOS) (2). NO is synthesized in various tissues and plays many different physiological roles. It acts as a signaling molecule coupling with glutamate receptors in the central nervous system (3), a mediator in the immune system (4), and an important vasodilator in the cardiovascular system (5). Insufficient levels of BH_4 lead to an uncoupling of the NOS reaction that could generate cytotoxic radicals (6).

BH_4 is synthesized from GTP by three enzymes, GTP cyclohydrolase I (GCHI), which is the rate-limiting enzyme; 6-pyruvoyltetrahydropterin synthase (PTS); and sepiapterin reductase (SPR). 4a-Hydroxy-BH_4 produced by the hydroxylation reaction with aromatic amino acids is partially reduced to BH_4 by recycling enzymes such as pterin 4a-carbinoamine dehydratase (PCD) and dihydropteridine reductase (DHPR) (Fig. 1).

There are two types of genetic disorders related to abnormal BH_4 metabolism. One is atypical hyperphenylalaninemia, which is caused by defects in genes encoding BH_4 biosynthetic or recycling enzymes (7). Hyperphenylalaninemia is caused by depletion of BH_4 as a

*Corresponding author. csichino@fujita-hu.ac.jp
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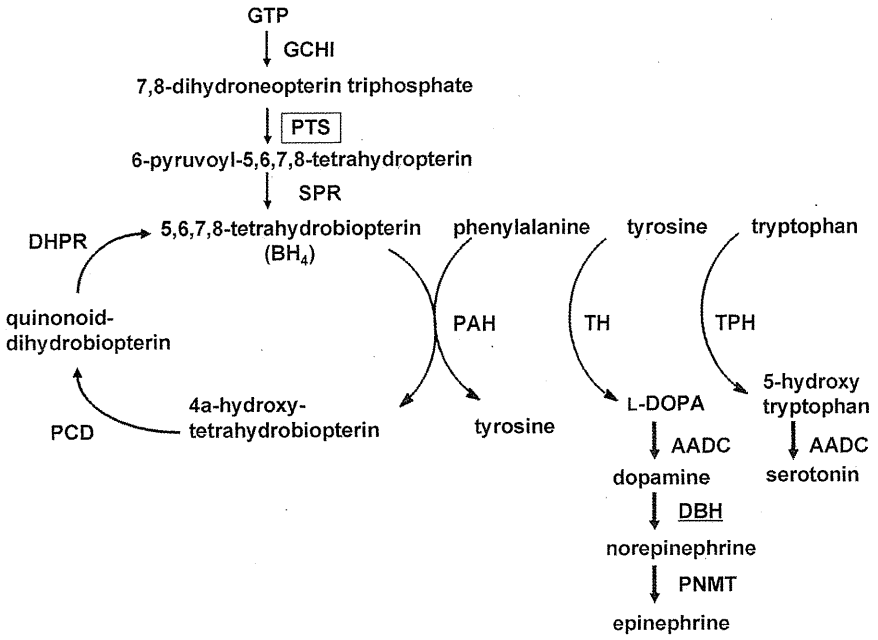

Fig. 1. Biosynthetic pathway of tetrahydrobiopterin. Abbreviations: GTP, guanosine triphosphate; GCHI, GTP cyclohydrolase I; PTS, 6-pyruvoyltetrahydropterin synthase; SPR, sepiapterin reductase; PCD, pterin-4a-carbinolamine dehydratase; DHPR, dihydropteridine reductase; PAH, phenylalanine hydroxylase; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; AADC, aromatic L-amino acid decarboxylase; DBH, dopamine β-hydroxylase; PNMT, phenylethanolamine *N*-methyltransferase. PTS, the gene we disrupted is boxed. DBH, the promoter of the gene we used for establishment of the transgenic mice is underlined.

cofactor for PAH. The other is DYT5, or dopa responsive dystonia (DRD, Segawa's disease), which is caused by an autosomal dominant defect in the GCHI gene (8, 9). DYT5 is a childhood onset dystonia with daily fluctuations, which can be well controlled by small amounts of L-DOPA. Aged DRD patients sometimes display symptoms associated with parkinsonism. Previous reports of two autopsied cases found a marked reduction of TH immunoreactivity in the striatum despite a lack of significant changes in aromatic L-amino acid decarboxylase, dopamine transporter, and vesicular monoamine transporter 2 (10). These findings strongly suggested that TH protein reduction selectively occurs in the nerve terminals of the brains of DYT5 patients and raised the question of whether BH_4 controls TH protein concentrations in the nerve terminals. Therefore, we established BH_4-deficient mice to study the mechanisms and molecular basis of dystonia. Initially, we planned to disrupt the GCH1 gene in mice as a model for DYT5; however, we encountered some technical difficulties in the construction process of the targeting vector. Thus, we decided to disrupt the PTS gene, which catalyzes the second step of BH_4 biosynthesis. We chose this gene because over 50% of human BH_4-deficient patients suffer from PTS deficiency (11).

2. The amount of TH protein in dopaminergic nerve terminals is strictly regulated by intracellular concentrations of BH_4

We disrupted the mouse PTS gene by employing gene targeting (12). PTS-null ($Pts^{-/-}$) mice were born according to Mendelian ratios, but they died within 48 h. We analyzed the brain homogenates of the newborns, and biochemical profiles of $Pts^{-/-}$ mice were very similar to human patients with atypical phenylalaninemia. The content of neopterin, which was originated from dihydroneopterin triphosphate, in the brain homogenate of $Pts^{-/-}$ mice was 17.6 times higher than that of wild-type ($Pts^{+/+}$) mice. The total content of biopterin, which was the sum of BH_4 and L-erythro-7,8-dihydrobiopterin, was reduced to 6.3% of the content in $Pts^{+/+}$ mice. The levels of brain dopamine, norepinephrine, and serotonin of $Pts^{-/-}$ mice were 14.6%, 6.3%, and 7.8% of those in *Pts* mice, respectively (Table 1). In addition, phenylalanine content in the brains of $Pts^{-/-}$ mice was 2-times higher than that of $Pts^{+/+}$ mice. The most interesting finding, however, was that TH activity in the brain homogenates of $Pts^{-/-}$ mice was only 7.0% of that in $Pts^{+/+}$ mice. The activity of TPH, which needs BH_4 as a cofactor, was not significantly changed. There was also no change in the activity of aromatic L-amino acid decarboxylase (AADC), which co-localizes with TH and TPH in dopaminergic and serotonergic neurons. Nitric oxide synthase activity in the brain homogenates of $Pts^{-/-}$ mice was significantly reduced to 72% of the activity in $Pts^{+/+}$ mice. Western