(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

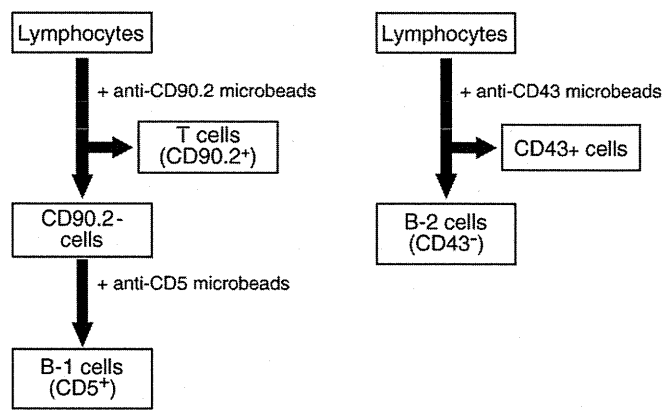

Fig. 1. Schematic representation of the isolation procedures for B-1 and B-2 cells.

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.

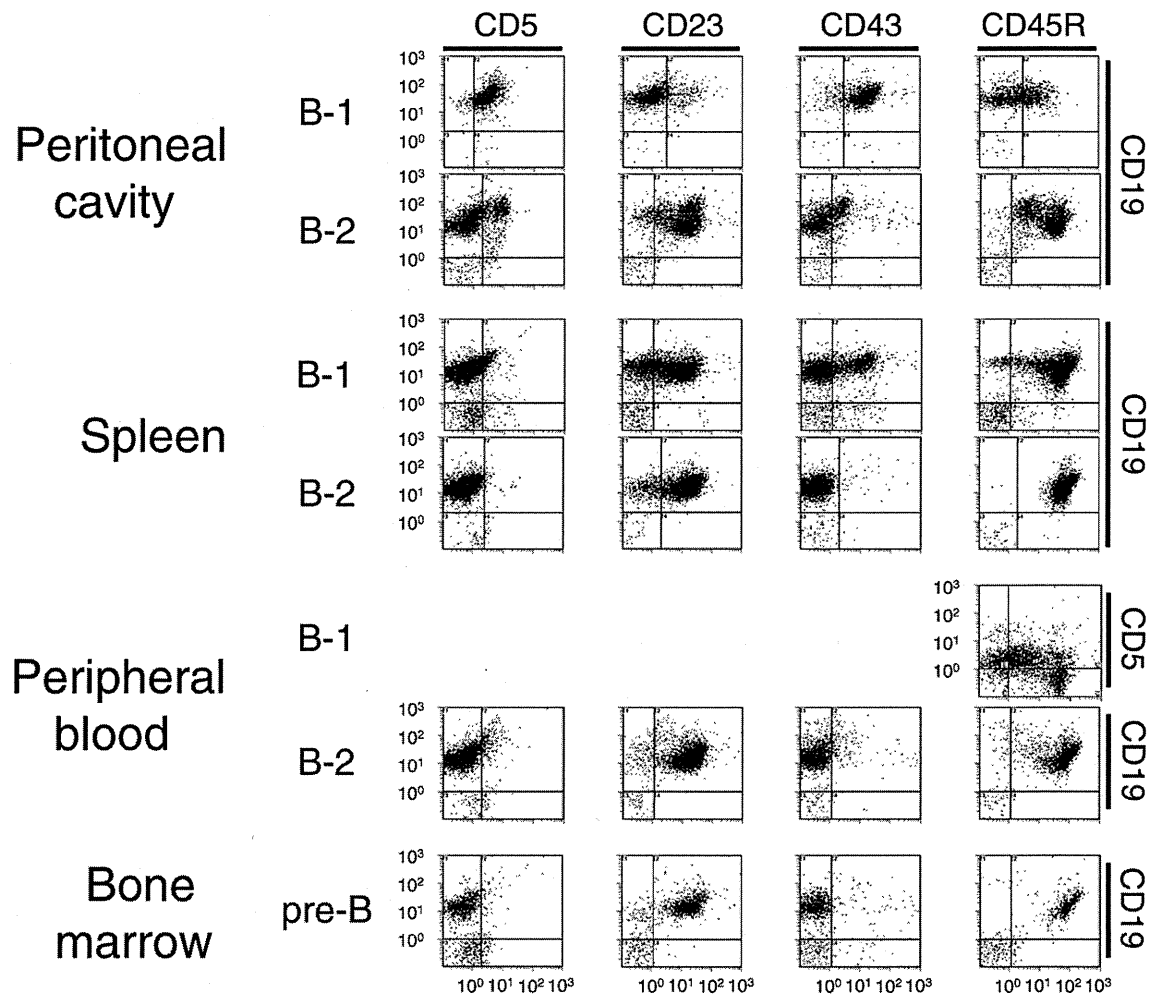

Fig. 2. Flow-cytometric characterization of isolated B cell subsets from spleen, peripheral blood, peritoneal cavity, and bone marrow. Each B cell subset was obtained by magnetic activated cell-sorting (MACS) as described in Materials and methods. B cell subsets from each tissue were characterized by 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.

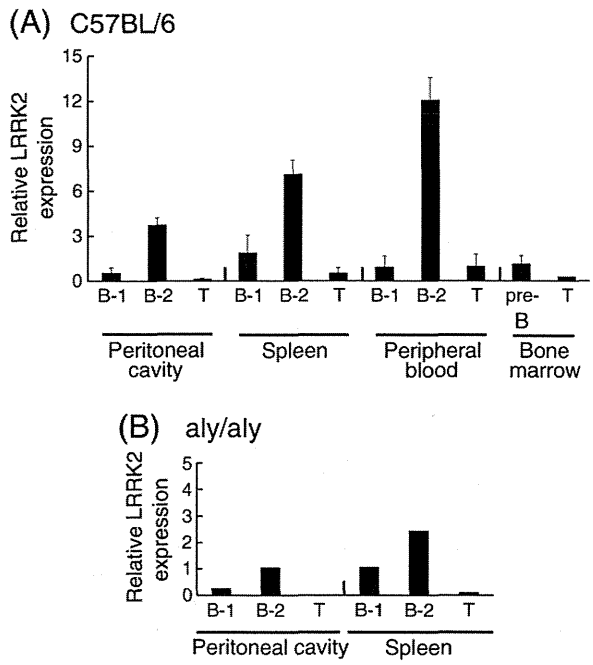

Fig. 3. Expression of LRRK2 mRNA in B cell subsets. B-1, B-2, and T cells were purified from each tissue of the C57BL/6 mouse (A) and the aly mouse (B) by MACS, as described in Materials and methods. RNA was isolated from each of the pooled cell populations from 10 mice. The amount of LRRK2 mRNA was 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

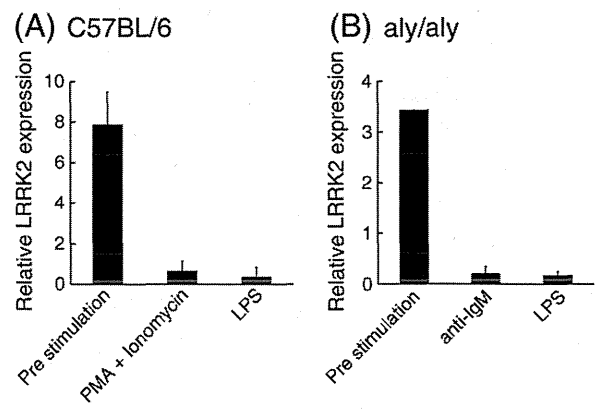

Fig. 4. Downregulation of LRRK2 expression in B-2 cells upon stimulation. (A) Splenic B-2 cells from the C57BL/6 mouse were stimulated in triplicate with either phorbol 12-myristate 13-acetate (PMA) at 300 ng/ml plus ionomycin at 600 ng/ml, or with lipopolysaccharide at 25 μg/ml (LPS), for 24 h. (B) 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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特集　脳深部刺激療法

Parkinson病に対する脳深部刺激の効果*

藤本健一**

Key Words：subthalamic nucleus, ventral intermediate nucleus, globus pallidus interna, pedunculopontine nucleus

はじめに

Parkinson病(PD)に対する脳深部刺激(DBS)療法が開始されて23年，わが国で保険適応となって10年が経過し，その効果に関する論文も多数発表されている．薬効の切れたoffでの症状がUPDRS(Unified Parkinson's Disease Rating Scale)で何％改善され，PD治療薬が1日当たりのL-dopa換算量(levodopa equivalent daily dose：LEDD)で何％減少したと，その効果を強調する報告が多い．その一方で，PDの患者から「知人がDBSの手術を受けたが，結果は期待はずれだった」という話を耳にすることも多い．この差はいったい何だろうか？PDは患者ごとに症状も異なれば進行の速さも異なる．DBSの実施時期も，電極の挿入部位もDBSに対する患者の期待度も異なる．このように背景が異なる事象を画一的に議論しても評価が一致しないのは当然である．病気の治療は患者・介護者の満足のため行う行為である．どのような症例にどのような治療法を選択するか，期待できる効果とリスクは何か，それを患者・介護者にどのように説明するか，治療満足度を高めるために主治医の果たす役割は大きい．本稿ではこれまでの報告と当施設での百余例のDBSの経験をもとに，PDに対するDBSの効果を論じる．DBSの治療満足度を高めるには，過去の経験から得られた事実を患者・介護者に正確に伝え，治療法の選択について十分に議論することが大切である．

脳深部刺激の効果と長期予後

DBSの標的はいくつかあるが，大きなインパクトを与えたのはLimousinらによる視床下核(subthalamic nucleus：STN)のDBSであった[1]．視床腹中間核(ventral intermediate nucleus：Vim)や淡蒼球内節(globus pallidus interna：GPi)は従来から凝固術の標的となっていたが，STNの凝固はバリズムを誘発する可能性がある．調節性のあるDBSの出現によって，STNはPD治療の新たな標的として注目を集めることになった．その後多くのSTN-DBSが実施され，現在ではその効果や副作用，長期予後についての評価がほぼ定まった．

まず基本的に，STN-DBSはPDのさまざまな症状のうち，L-dopaが有効な運動症状に対して効果を示す．長期臥床により筋肉が萎縮し関節が拘縮した症例にいくら大量のL-dopaを与えても歩けないのと同様に，このような症例にSTN-DBSを行っても歩けるようにはならない．PDでは最善の薬物療法を実施しても，5年後には約4割の

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患者がwearing-off現象を経験する．L-dopaを服薬すれば普通に動けるが，短時間で効果が切れて動けなくなる．このような症例でSTN-DBSは著しい効果を発揮する．すなわち，offの症状を改善してonの状態に近づけ，offでのUPDRS-II(ADLスコア)やUPDRS-III(運動スコア)を50％程度改善する[2]．その結果，1日のoff時間は約5時間短縮される[3)~5)]．薬物療法によるoff時間の短縮効果はせいぜい1日当たり1.5時間程度であるので，薬物療法と比較してSTN-DBSのoff時間短縮効果がいかに優れているかがわかる．

STN-DBSによってPD治療薬の必要量が減り，LEDDで50％以上の減量が可能である[2]．L-dopaの1回服薬量を減らすことによってdopa誘発性ジスキネジア(dopa induced dyskinesia：DID)を軽減することも可能で，DIDは70％近く軽減される[2]．さらにLEDDの減少に伴い，幻覚や妄想などの精神症状，消化器症状，起立性低血圧など，ドパミン補充に伴うさまざまな副作用を回避することができる．PD患者の中には副作用のため十分なドパミン補充(最善の薬物療法)が行えず，本来のonを体験できずに我慢を強いられている患者が存在する．STN-DBSの効果に関する従来の論文では，最善の薬物療法が行われている患者でのoff症状の改善効果が強調されているが，最善の薬物療法に耐えられない患者に対するDBSの効果はもっと注目されてよい．

DBSの長期予後に関して，治療開始5年後の効果が報告されている[6)7)]．Krackらは，平均55.0歳でLEDD 1,409±605mgの49例(5年フォローは42例)，Schüpbachらは，平均54.9歳でLEDD 1,468±811mgの37例(5年フォローは30例)に対する両側STN-DBS後5年間の経過を報告した．5年後の休薬状態(off)で，Krackらの報告[6]ではUPDRS-IIが49％低下，UPDRS-IIIが54％低下，DIDが58％低下，LEDDは63％低下，Schüpbachらの報告[7]ではUPDRS-IIが40％低下，UPDRS-IIIが54％低下，DIDが79％低下，LEDDが58％低下していた．

薬物療法と脳深部刺激療法の比較

最善の薬物療法を行った場合とDBSを行った場合とで，PDの症状はどの程度改善されるかを，患者を無作為割り付けして比較検討した研究がある．最初の研究はドイツとオーストリアの10施設で行われたもので，78ペア(156例)の進行期PD患者を対象として両側STN-DBSと最善の薬物療法とを比較した[3]．評価項目は6カ月後の生活の質と休薬状態(off)でのPDの重症度である．生活の質の評価にはPD患者への質問票であるPDQ-39(Parkinson's Disease Questionnaire-39)を，重症度の評価にはUPDRS-IIIを用いた．PDQ-39は78ペア中50ペアでSTN-DBS群の改善が優れていた($p=0.02$)．UPDRS-IIIは78ペア中55ペアでSTN-DBS群の改善が優れていた($p<0.001$)．重篤な有害事象はSTN-DBS群の13％，薬物療法群の4％に認められた($p<0.04$)．STN-DBS群のうち1例は脳内出血により死亡した．軽微なものも含めたすべての有害事象はSTN-DBS群の50％，薬物療法群の64％に認められ，薬物療法群で多かった($p=0.08$)．

DBSと最善の薬物療法の比較は米国の13施設でも行われた[4]．薬物療法で満足できる治療効果の得られなくなった進行期PD患者255例(70歳以上の高齢者25％を含む)を対象として，DBSと最善の薬物療法を継続した場合とを比較する研究である．対象患者を無作為に両側STN-DBS，両側GPi-DBS，薬物療法に割り付けることによって母集団のバイアスを排除した．治療開始6カ月後にDBS群では支障となるDIDを伴わないon時間が1日平均4.6時間増加したのに対して，薬物療法群では変化を認めなかった．運動機能もDBS群の方が薬物療法群と比べて有意に改善し($p<0.001$)，臨床的に意味のある運動機能の改善(UPDRS-IIIで5点以上改善)に達した患者の割合はDBS群の71％に対して薬物療法群は32％であった．DBS群は薬物療法群と比べてPDQ-39全般およびPDQ-39の8項目中7項目において有意な改善を示した($p<0.001$)．認知機能検査ではDBS群は薬物療法群と比較して情報処理能力の一部に軽度の低下を認めた．重篤な有害事象が1回以上発生したのはDBS群が49例，薬物療法群が15例で有意差を認めた($p<0.001$)．有害事象のうち39例は手術に起因するもので，脳出血による死亡が1例認められた．

同様の研究は英国においても実施された[5]．薬

物療法で満足できる症状コントロールのできなくなった366例の進行期PDを対象とした13施設での共同研究である．366例を手術群と薬物療法群それぞれ183例ずつに無作為に割り付けたが，実際に手術を受けたのは183例中178例(すべてDBS)であった．標的はSTNが174例，GPiが4例で，176例は両側に刺激電極を挿入した．1年後のPDQ-39に回答したのは162例であった．一方，183例の薬物療法群のうち12例は途中で気が変わって手術を受けるなどして，1年後に薬物療法を継続していてPDQ-39に回答したのは153例であった．153例中48例はアポモルフィンの持続皮下注射を受けていた．1年後のPDQ-39は手術群で5点改善したのに対して薬物療法群での改善は0.3点にとどまり統計的に有意差を認めた($p=0.001$)．PDQ-39の中で手術群と薬物療法群で差を認めたのは運動能力(−8.9, $p=0.0004$)，日常生活動作(−12.4, $p<0.0001$)，身体の不調(−7.5, $p=0.004$)の項目で，その他の項目では差を認めなかった．なお，手術群のうち36例(19%)は手術に関連した重篤な有害事象を経験し，手術時の脳内出血により1例が死亡した．

これらの無作為化対照試験に共通しているのは，DBSは最善の薬物療法に比べて運動症状やそれに伴う日常生活動作，患者満足度を改善したことである．通常の薬物療法のみならず，アポモルフィンの持続皮下注射を導入してもDBSには及ばなかった[5]．その一方で重篤な有害事象は明らかにDBS群に多く，その多くは手術に起因していた．各研究で手術に伴う脳内出血による死亡を1例ずつ認めた．DBSは成功すれば良好な結果が期待できる一方，手術に伴う危険を伴い，頻度は少ないが重篤な副作用を覚悟する必要があることが示された．

脳深部刺激のターゲット

PDに対するDBSでは，通常Vim, STN, GPiがターゲットとなる．最近は脚橋被蓋核(pedunculopontine nucleus：PPN)の刺激も研究されている．

PDに対するDBS治療の最初の標的はVimであった[8]．従来から行われていたVim凝固術の課程で，挿入した電極の先端を高頻度刺激するとPDの症状が軽快することからVim-DBSが試みられ，DBSが広まるきっかけとなった．Vim-DBSはPDにおける4〜5 Hzの安静時振戦や一定の肢位をとり続けると出現するre-emergent tremorに対して著効する[9][10]．筋強剛やDIDに対しても効果を示すが，動作緩慢やすくみ現象，姿勢反射障害などには無効である[11]．したがって，振戦が主症状のPD症例では良い適応となる．両側Vim核に凝固巣を作製すると意欲低下や物忘れが出現することがある．一方，DBSは凝固と異なり調節性があるため，両側治療でもこれらの副作用の危険が少ない．両側に振戦を認めて最初から両側Vim核の治療が必要なときはVim-DBSを選択するのが一般的である．なお，振戦型のPDは一般に進行が遅く，片側だけに症状を認める状態で数年から10年以上経過することも稀でない．このような症例では当面は対側Vim核だけの治療でよいため，あえてDBSを選択せず，従来のVim凝固術を選択することもある．

PDにおける振戦以外の症状に対してはSTNかGPiを標的とする．STN-DBSの方がGPi-DBSよりも動作緩慢や歩行障害に対する効果が勝り，刺激強度が低くて済み，LEDDを減らすことができることからSTN-DBSが選択されることが多い[12]．

GPi-DBSの利点はDID抑制効果が高いことで，これはL-dopa減量によらない直接効果と考えられている[10][13]．これに対してSTN-DBSによるDID抑制は，PD治療薬の減量に負うところが大きい．GPi-DBS開始3〜5年後の追跡調査では，振戦，筋強剛，DIDへの効果は持続するものの，動作緩慢，歩行障害，姿勢反射障害，日内変動などへの効果は減弱していた[13]．なお，GPiはSTNと比べて大きく，GPi内部での刺激部位によって効果を認める症状が異なる可能性がある．Bejjaniらは，GPiの腹側部の刺激は筋強剛やDIDに，背側部の刺激は動作緩慢や歩行障害に効果を示すとしている[14]．

STN-DBSとGPi-DBSを直接比較した無作為化対照試験は少ない．STN-DBSとGPi-DBS各10例ずつを対象としたパイロット試験によると，12カ月後の休薬時(off)のUPDRSはそれぞれ48%と39%改善した[15]．動作緩慢はGPiよりもSTNで改善率が高かったが，DIDの軽減率はそれぞれ62%と89%でGPiの方が高かった．L-dopaの減量はそ

表1　STN-DBSとGPi-DBSの比較

		STN-DBS	GPi-DBS	コメント
効果のある症状	振戦	◎	○	
	筋強剛	○	◎	
	動作緩慢	○	○	
	歩行障害	○	○	すくみがなければ有効
	姿勢反射障害	△	△	L-dopaが効けば有効
	すくみ足	△	△	L-dopaが効けば有効
	ジスキネジア	○	◎	GPiの方が直接的
長期予後		◎	?	GPiはエビデンスが少ない
刺激強度		弱	強	STN-DBSは電池寿命が長い
減薬効果		+	−	STN-DBSは約50％減薬可能
Moodへの影響		強？	弱？	Okunら[18]は有意差を認めず

れぞれ38％と3％でSTN-DBS群で顕著であった．

最近報告された米国の13施設での多施設共同研究では，299例を無作為にSTN-DBS群（147例）とGPi-DBS群（152例）に振り分けた[16]．24カ月後のoffでのUPDRS-IIIはSTNで10.7点，GPiで11.8点低下し，運動症状の改善効果に差を認めなかった．一方，LEDDはSTN-DBS群で408mg減少したのに対して，GPi-DBS群での減少は243mgにとどまり，両群間で有意差を認めた（$p=0.02$）．認知機能検査に関しては，STN群で視覚運動処理時間のみが有意に遅延したが，そのほかについては両群間で差を認めなかった．うつのスコアはSTN群で悪化，GPi群で改善した．刺激の平均強度はSTN群が3.16 V，GPi群が3.95 VとGPi群で有意に高く（$p<0.001$），刺激パルス幅の平均もSTN群が75.9μsec，GPi群が95.7μsecとGPi群で有意に長かった（$p=0.001$）．平均刺激頻度はSTN群が165 Hz，GPi群が168 Hzで，両群間で差を認めなかった．これは，GPi-DBSはSTN-DBSに比べて刺激装置の電池寿命が短く，頻回の交換が必要なことを意味する．また，この研究は治療開始後24カ月時点での比較であることに注意が必要である．GPi-DBS開始後3～5年後の追跡調査では，振戦，筋強剛，DIDへの効果は持続するものの，動作緩慢，歩行障害，姿勢反射障害，日内変動などへの効果減弱が指摘されている[13][17]．

GPiは比較的大きな神経核で，運動系と精神系は離れて存在する．一方，小さな神経核であるSTNは運動系と精神系が接近している．このためGPi-DBSはSTN-DBSに比べて精神系を刺激する可能性が低く，moodに対する影響の少ないことが期待される．Okunらは，45例のPD患者を無作為にGPi-DBS群とSTN-DBS群に割り付け，7カ月後にmoodと認知機能を調査した[18]．両群ともmoodは治療前と大きな差を認なかった．詳細な検討ではSTN-DBS群の「怒り」の項目のみがGPi-DBS群よりも増大した（$p=0.027$）．両群をまとめて治療前と比較すると，DBS後は緊張が解け幸せになる一方で，イライラして怒りやすく混乱する傾向を認めた．なお，GPi，STNとも刺激点を腹側に移動すると幸福さは失われ，活力も低下し，混乱状態に陥る傾向があった[18]．予想に反してGPi-DBSとSTN-DBSでmood変化に違いがなかったのは，この研究が片側治療のためかも知れない．この研究では同時にPDQ-39を用いてQOLの改善を調査しているが，QOLの改善はSTN-DBS群よりもGPi-DBS群の方が大きかった[19]．表1に両者の利点と欠点を示す．STNとGPiのどちらがより優れた標的であるかについては，まだ最終結論が得られていない．現状ではそれぞれの利点と欠点を踏まえ，患者に合わせた選択をすべきであろう．

すくみ足や姿勢反射障害は進行期PDにおけるADLの阻害要因である．しかし，薬物療法もSTN-DBSやGPi-DBSも，すくみ足や姿勢反射障害に対する治療効果は不十分である．動物実験では，PPNあるいはその近傍の細胞群が歩行や姿勢の制御に重要な役割を果たすことが知られている．そこでPPNをDBSの標的とすることが試みられた[20]．薬物療法で歩行や姿勢反射障害がコントロールできない6症例の進行期PD患者（年齢64.5±3.2歳，罹病期間12.1±3.0年）の両側STNとPPNに刺

激電極を挿入し，STNは130～185Hz，PPNは25Hzで刺激したところ，薬効の切れたoffのみならずonでの症状改善を認めた．とくにPPN-DBSにより歩行や姿勢反射障害が改善されたのは注目に値する．Ferrayaらは，薬物療法に反応せず，STN-DBSを実施してもすくみ現象が良くならかった6例に両側PPN-DBSを追加して術前と1年後を比較した．1例では著効したが，4例で軽度改善，1例で悪化し，すくみ現象への効果は一定しなかった[21]．STN-DBSでは130～180Hzの高頻度刺激が有用であるが，PPN-DBSでは15～25Hzの低頻度刺激が有効とされている．同じDBSでも刺激の意味が異なる可能性があり，そのメカニズムについてさらなる検討が必要である．

脳深部刺激の効果を認める症状，認めない症状

STN-DBSの長期予後を検討したKrackらによると，off時の運動機能で唯一構音障害のみが改善不十分であった[6]．彼らは，平均55.0歳の比較的若い患者に対してSTN-DBSを実施しているにもかかわらず，構音障害は改善しなかった．構音障害に関しては多くの報告でDBSの効果が及ばない症状としてとり上げられている[22]．その他のoff時の運動症状，すなわち振戦，筋強剛，動作緩慢，姿勢反射障害，歩行障害はSTN-DBS開始1年後にUPDRSで開始前の20～40％まで改善し，この効果は5年後でも20～60％に維持された[6]．一方，on時の症状ではDIDが5年後も著明に改善していたほか，筋強剛の改善が比較的優れており，1年後で58％，3年後で61％，5年後で78％に低下した．動作緩慢，姿勢反射障害，歩行障害の改善は不十分で，いずれも1～5年の間にSTN-DBS開始前より悪化した．構音障害やすくみ現象の悪化はより早く，1年後にはすでに開始前と同じかやや悪化していた[6]．まとめると，PDの運動症状のうちすくみ足や構音障害などの，いわゆるaxial symptomに対するSTN-DBSの効果は一時的あるいは限定的であった[22]．

Moreauらは，STN-DBS後時間経過とともに悪化するすくみ足に対して，STN-DBSで通常用いられる130～180Hz，3V前後の高頻度低電圧刺激よりも60Hz，4～5.5Vの低頻度高電圧刺激が有効であると主張している[23]．彼らは，初期には高頻度低電圧で刺激して振戦，筋強剛，動作緩慢を治療し，数年経過してすくみ足が出現したら低頻度高電圧刺激に切り替えることを推奨している．確かに60Hzでの低頻度高電圧刺激によりすくみ足が改善することがあるのは事実であるが，その結果，今度は振戦や筋強剛，動作緩慢が悪化することもあり，すべての症例にMoreauらの提唱する60Hzの低頻度高電圧刺激が合うわけではない．刺激のパラメータの問題はまだ未解決な点が多く，今後も詳細な検討が必要であろう．

脳深部刺激の効果に影響を与える要因

STN-DBS，GPi-DBSともL-dopaへの反応性が治療効果にもっとも影響を与える[2)10)]．DBS開始前L-dopaによってUPDRSの運動スコアがoffの状態から25～50％以上改善する場合，最良の治療効果が予測される．ただし，もともとL-dopaが効きにくい振戦は例外で，L-dopaの効果が乏しくてもSTN-DBS，GPi-DBSあるいはVim-DBSによって改善することが期待できる[10]．

DBSの効果と年齢については，手術時年齢がより若く，罹病期間が短い患者の方が運動症状の改善が著しい[24]．手術時年齢65歳未満の若年群53例（57.4±4.9歳）と65歳以上の高齢群34例（68.8±2.8歳）の比較によると，STN-DBS開始直後のUPDRS-IIIの改善は両群で同様に認められた．しかし，時間経過とともに薬の効いている状態（on）でのUPDRS-IIIやその中のaxial scoreで差を認めるようになり，若年群は高齢群よりも治療効果が持続した．Wearing-off現象やDIDは両群とも著明に軽減したが，日常生活動作やPDQ-39による生活の質は若年群で改善がより顕著であった[25]．Schüpbachらは，罹病期間が平均6.8年で55歳以下，offでのUPDRS-IIIのスコアが29±12の軽症から中等症のPD患者20人を無作為にSTN-DBS群（10人）と内服治療継続群（10人）に割り付けた．1年半後のPDQ-39は内服治療継続群では変化しなかったのに対して，STN-DBS群では24％改善した[26]．DBS開始前の重症度にも関係するので一概に年齢だけでは断言できないが，両側STN-DBSを行う年齢によって術後必要な薬が異なる．40歳代は薬物療法が不要となり，50歳代はドパミンア

ゴニストのみで済み，60歳を超えるとL-dopaが必要になることが多い．従来は進行期PD患者が手術対象とされたが，若いほど良い結果が得られるなら，より早期の手術導入を検討しようという意見がある[26]．しかし，薬物療法で治療可能な患者にリスクのある手術療法を勧めるべきかどうかについては議論がある．また，若年症例は運動症状に対する効果は著しいが，術後にドパミン調整異常症候群や衝動制御障害，pundingなどの問題行動を誘発する危険が高いので，その面での注意が必要である[27]．

STN-DBSを行っても言語や語義の流暢性低下以外に認知機能障害は起こらないとする研究が多いが，もともと認知機能障害のある症例にDBSを実施しても好ましい結果は得られない．薬物誘発性であることが明らかな幻覚や妄想はSTN-DBS後に治療薬の減量が期待できることから，治療効果に影響を与えることはない．ただし，Lewy小体型認知症に伴う幻覚や妄想のときは好ましい結果は得られない[10]．

PDにおけるうつの合併は多いが，STN-DBS後にうつが発現することもある．これはSTN-DBSの直接効果というよりも術後の治療薬の急激な変更に起因する可能性が指摘されている[28]．術前のうつ状態がSTN-DBSの治療効果に影響を与えることを示すエビデンスは明らかにされていない．術前のうつ状態はSTN-DBS後の自殺の危険因子にも該当しない[29]．病的賭博，買い物依存，過食症，性欲亢進などの，いわゆる衝動制御障害はSTN-DBS後に減薬することで改善する可能性もあるが，術後に出現することも知られている[27]．とくに術前から治療薬への依存症であるドパミン調整異常症候群に陥っていると，術後の減薬は困難である．STN-DBSを行うと意志決定速度が速くなることが確認されており，STN-DBSは衝動制御障害を起こしやすい状況を作り出す可能性がある[30]．衝動制御障害あるいは薬物依存の既往はSTN-DBS後の自殺の危険因子でもあるので，このような既往のある症例では注意が必要であろう[29]．

おわりに

脳深部刺激(DBS)療法によるParkinson病(PD)の運動症状に対する改善効果は著しい．それによりPD患者の社会への関与の仕方が変わり，家族内のダイナミックスにも大きな変化が生じる．DBSはPD患者のmoodにも変化を及ぼす可能性がある．また，DBS後の治療薬の減量は患者の精神状態に少なからぬ影響を与える．これらの急激な変化に上手に対応できないと，運動症状は良くなったのに生活の質(QOL)は最悪という状況を生み出すことになる[28]．術前から家族を含めた社会精神学的調整を行うことは，DBS後のQOLにとってきわめて重要である．一人暮らしがSTN-DBS後の自殺の危険因子であることからもわかるように[29]，家族のサポートは重要であり，DBSの効果に影響を与える重要な要因に含めるべきかもしれない．

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

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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.

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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.

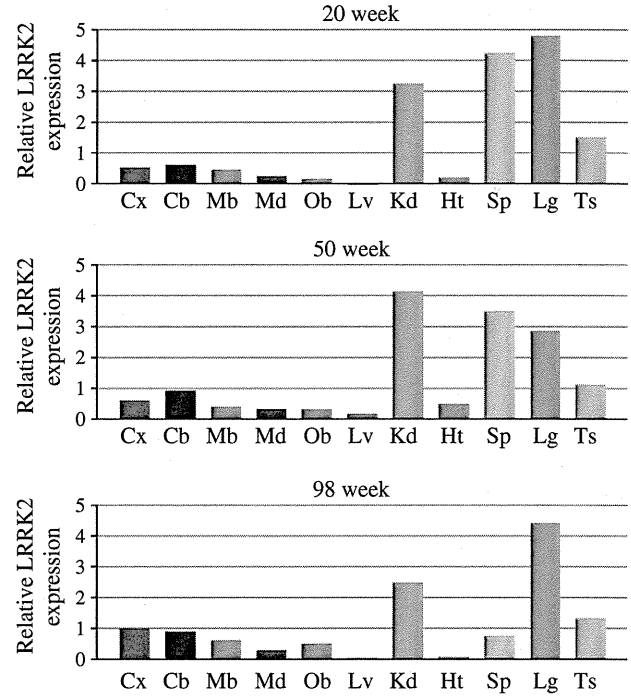

Fig. 1. Expression of LRRK2 mRNA in various organs of adult mouse. RNA was isolated from various organs of C57BL/6J mice aged 20, 50, and 98 weeks and subjected to quantitative PCR. Relative expression of *LRRK2* normalized by *GAPDH* is shown.

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-

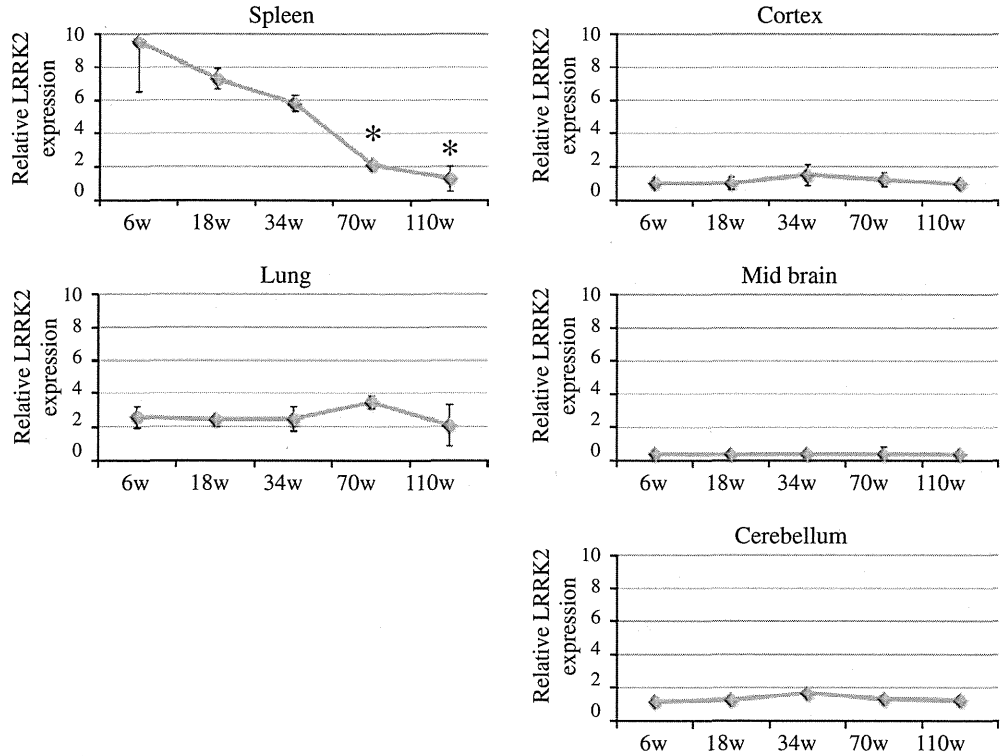

Fig. 2. Relationship between expression of LRRK2 mRNA and aging. RNA was isolated from spleen, lung, cortex, midbrain, and cerebellum of mice aged 6, 18, 34, 70, and 110 weeks, and subjected to quantitative PCR. Relative *LRRK2* expression normalized by *GAPDH* is shown. Stars represent statistical comparisons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

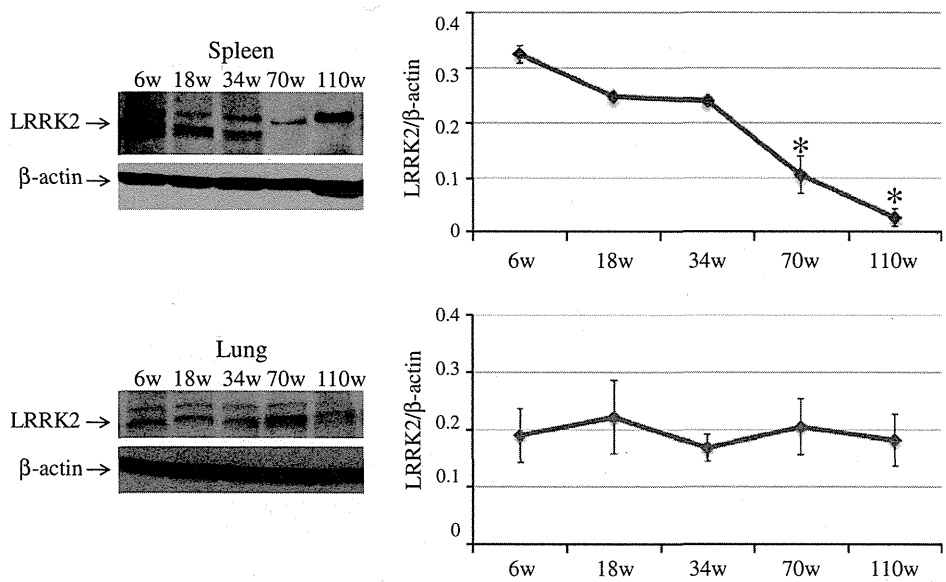

Fig. 3. Age-related change of the LRRK2 protein level in spleen. Tissue lysates were prepared from spleen and lung of mice aged 6, 18, 34, 70, and 110 weeks and subjected to Western analysis using AT106. LRRK2 protein levels normalized by β-actin are shown. Stars represent statistical comparisons by 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

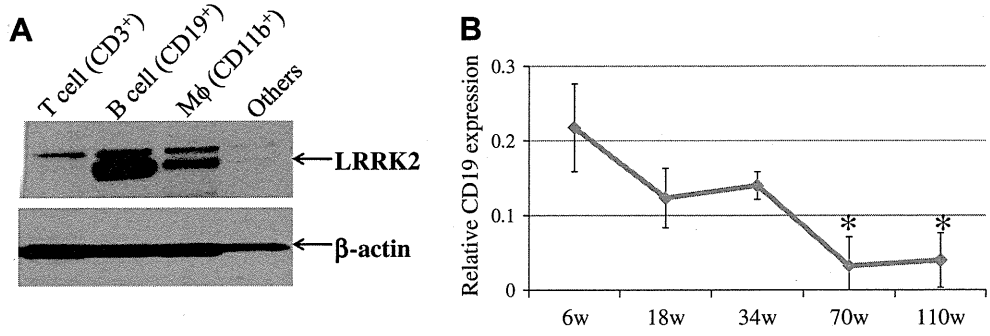

Fig. 4. LRRK2 expression in spleen cell populations. (A) Western analysis of LRRK2 expression in spleen cell populations. T lymphocytes, B lymphocytes, and macrophages were purified with magnetic bead-conjugated antibodies directed against each cell surface marker. Lysates of these cells and other residual 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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Prevention of intracellular degradation of I2020T mutant LRRK2 restores its protectivity against apoptosis

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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

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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

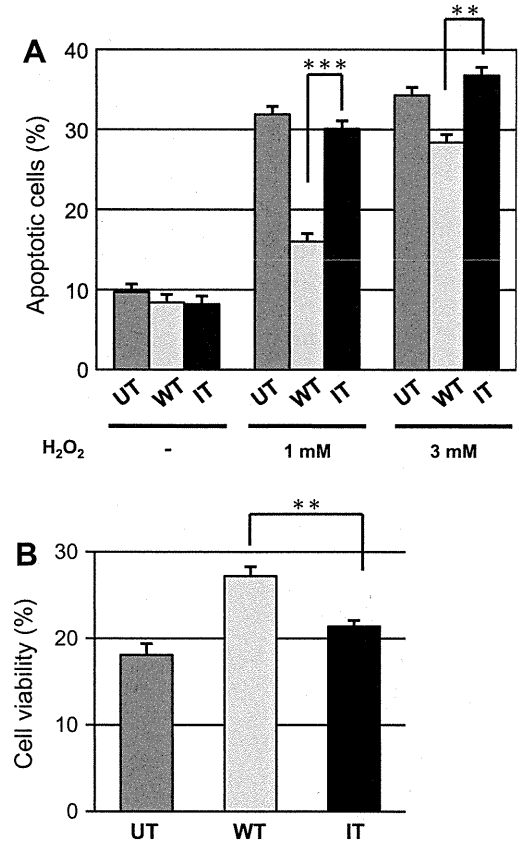

Fig. 1. H_2O_2-induced apoptosis in LRRK2-transfected cells. (A) WT and I2020T (IT) LRRK2-transfected HEK293 cells were treated with 1 or 3 mM H_2O_2 for 50 min. The percentage of cells showing apoptosis was measured by annexin V staining. (B) WT and I2020T (IT) LRRK2-transfected HEK293 cells were treated 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

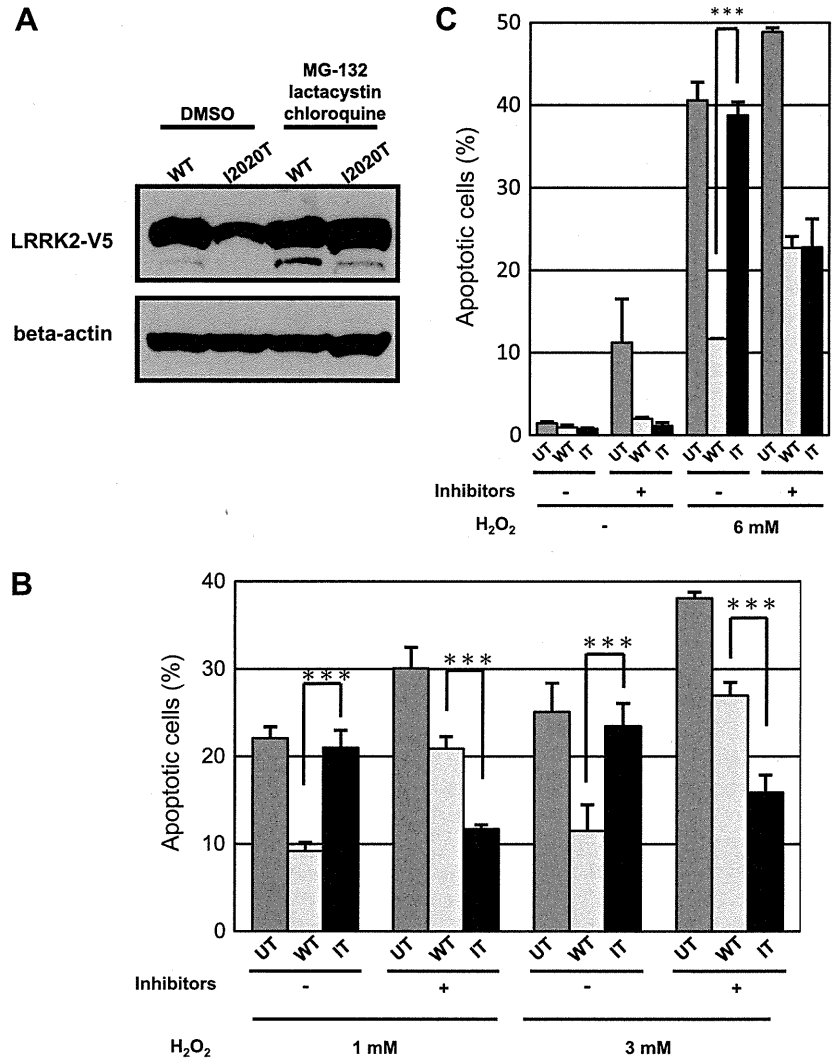

Fig. 2. Effect of proteolysis inhibitors on apoptosis in LRRK2-transfected cells. (A) HEK293 cells were transfected with WT or I2020T LRRK2 cDNA and treated with a cocktail of three proteolysis inhibitors (1 μM MG-132, 1 μM lactacystin, and 200 nM chloroquine) for 24 h. The LRRK2 level in the lysates 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