kuwata

差出人: Oonishi Yasuyuki [Oonishi.Yasuyuki@mp.medience.co.jp]
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宛先: kuwata@gifu-u.ac.jp
CC: Aida Yuu
件名: P092のサル静注試験:追加実験につきまして
添付ファイル: P092のサルPK試験(20130812).doc

岐阜大学　桑田先生

お世話になっております.
担当者が感染症になってしまい，追加実験ができずにおりました.
申し訳ございません.

以下の内容で水曜日投与で実施いたしたいと考えております.

前回のマレイン酸塩の静注実験では，12.5mg/mL の濃度液では
溶血は起こったものの，動物にショック症状は発現しなかった
ことから，12.5mg/mL を用いて投与液量を増やし，かつ投与速度を
落として雌 1 例に 100 mg/kg の用量で投与を行いたいと考えています.

投与用量：100 mg/kg
投与液濃度：12.5mg/mL
投与速度：0.5mL/分,
投与液量：8mL/kg, 3kg の動物で 24mL 投与
投与時間：48 分

モンキーチェアに固定できるのは，せいぜい 1 時間程度ですので
これ以上投与速度を遅くするのは難しいと考えています.

この条件で，血中濃度及び脳脊髄液濃度を測定し，どの程度，
脳脊髄液へ移行するか見てみたいと思います.

これでも十分な移行が見られない場合は，反復投与を行って，
脳脊髄液中濃度が上がるかどうかを確認したいと考えています.

以上，ご了承いただければ幸いでございます.
以上の内容をまとめたものを添付いたします.

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三菱化学メディエンス株式会社 創薬支援事業本部
試験研究センター　安全性研究部
大西　康之
〒314-0255
茨城県神栖市砂山 14-1
Oonishi.Yasuyuki@mp.medience.co.jp
TEL:0479-46-3461

P092 のサル PK 試験

試験番号 B130597 として実施

【被験物質】
マレイン酸

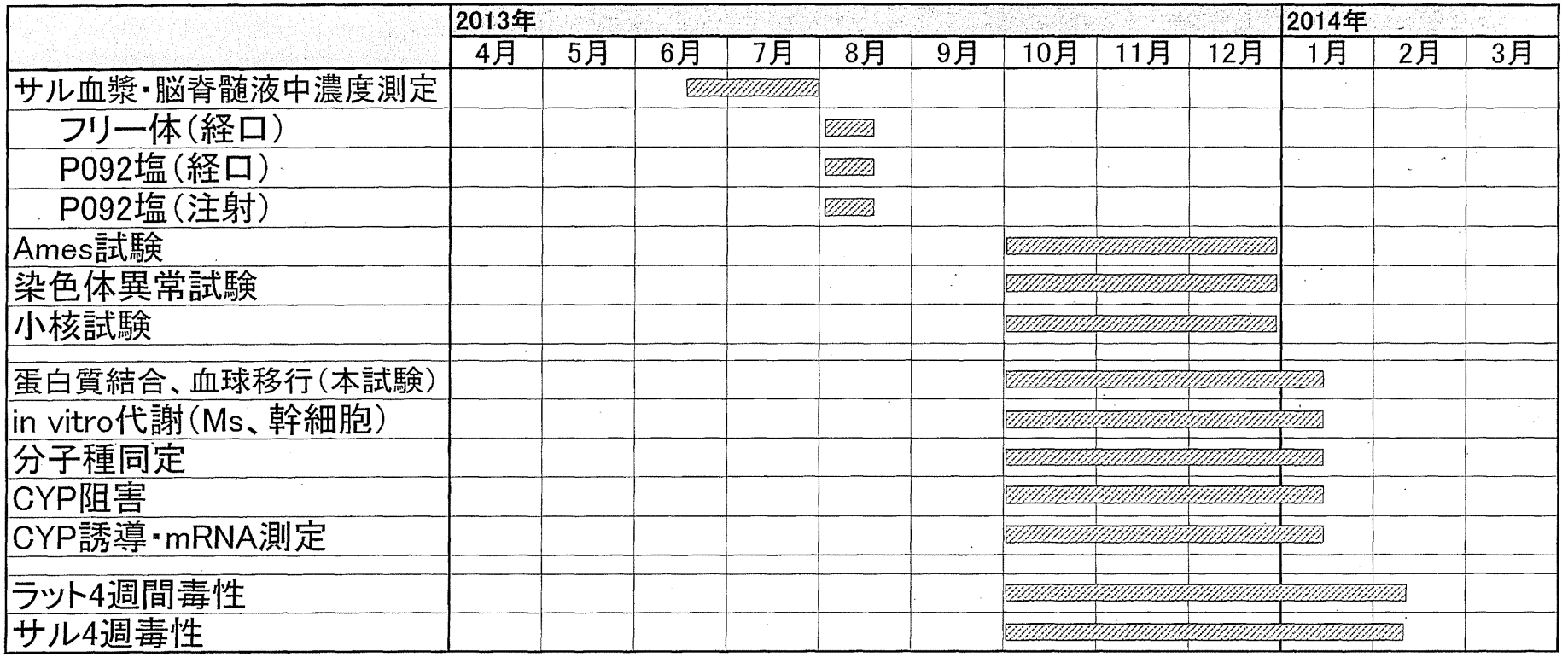
【静脈内投与試験】
動物数：雌 1 例（サル）
脳脊髄液中濃度：400nmol/L を目標（IC50: 200 nmol/L, MW: 約 500）
目標血中濃度を 1000 nmol/L（血中濃度）→500 ng/mL（血漿中濃度）

循環血量：65 mL/kg，3kg とすると，195 mL → 100mg/kg とすると
300mg/195mL=1.5mg/mL（血中に投与する被験物質量）

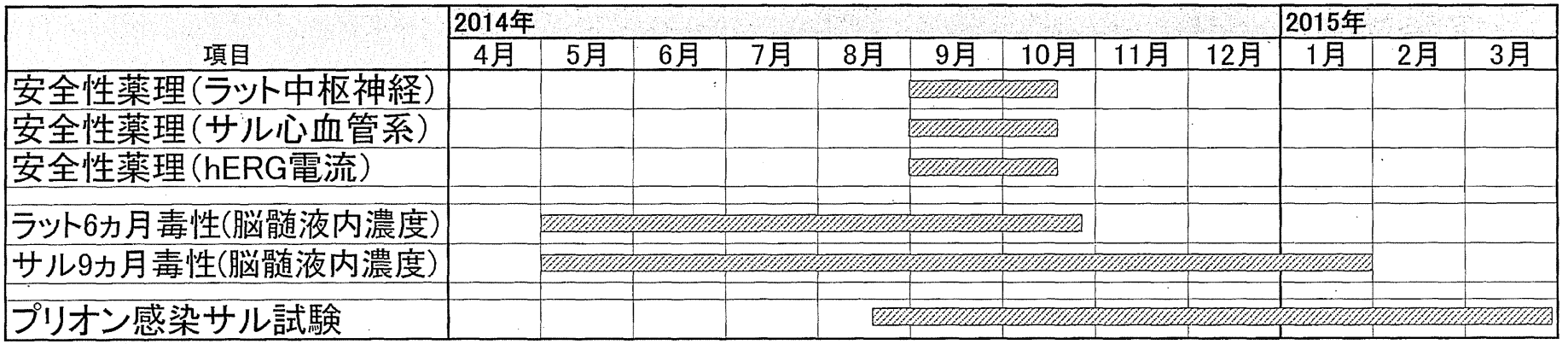
100mg/kg の投与用量を達成するために
①12.5mg/mL 液＝8mL/kg の投与液量→3kgBW とすると 24mL（total）
→0.5mL/min の速度とすると計 48 分

採血・測定ポイント：投与後 5 分,2,4,8,24 時間（計 5 ポイント）
脳脊髄液採取ポイント：投与後 2-4 時間，24 時間（計 2 ポイント）
測定検体数：血漿 5 検体，脳脊髄液 2 検体
剖検：投与後 24 時間に実施，採材
組織検査：小腸，大腸，肝臓，腎臓，脾臓

2013年度



2014年度



[II] 分担研究報告

厚生労働科学研究費補助金 難治性疾患等克服研究事業（難治性疾患克服研究事業）
プリオン病に対する低分子シャペロン治療薬の開発　分担研究報告書

プリオン病の自然歴調査と低分子シャペロン化合物による治験

研究分担者：水澤英洋　東京医科歯科大学大学院脳神経病態学(神経内科学)
研究代表者：桑田一夫　岐阜大学人獣感染防御研究センタープリオン研究部門
研究協力者：山田正仁　金沢大学大学院医薬保健研究域医学系脳老化･神経病態学(神経内科学)
研究協力者：堂浦克美　東北大学大学院医学系研究科プリオン蛋白分子解析学
研究協力者：坪井義夫　福岡大学医学部神経内科学
研究協力者：岩崎　靖　愛知医科大学加齢医科学研究所神経病理学
研究協力者：佐藤克也　長崎大学大学院医歯薬学総合研究科感染分子学
研究協力者：浜口　毅　金沢大学大学院医薬保健研究域医学系脳老化･神経病態学(神経内科学)
研究協力者：三條伸夫　東京医科歯科大学大学院脳神経病態学(神経内科学)

研究要旨　プリオン病はまだ発症機序が解明されておらず、いくつかの臨床試験は行われたものの有効な治療法もないため、わが国で開発されたプリオン蛋白の物理化学的解析に基づいた新規合成化合物 P092 の効果が期待されている。今年度は、P092 のファースト・イン・ヒューマンの治験を開始するため、プリオン病の患者登録ならびに自然歴調査を含む臨床研究体制として日本プリオン病コンソーシアム(Japanese Consortium of Prion Disease: JACOP)を構築した。そして、治験の開始に向けて、プロトコールの作成と患者登録・追跡調査を開始した。

A.研究目的

本研究の目的は、プリオン病という年間 100 万人に 1 例という頻度の希少疾患において、①低分子シャペロン化合物 P092 のヒトにおけるファースト・イン・ヒューマンの医師主導型治験を開始できる体制を構築する。そのための準備として、施設、および患者登録制度と各地域におけるフォローアップを含む臨床研究体制を構築する。そして、その体制を利用して、②我が国におけるプリオン病の各病型の自然歴を明らかにすることの 2 点である。

B. 研究方法

1) プリオン病治療薬による医師主導型治験体制の構築

わが国のプリオン病患者は毎年 150 名程度の発生があり、特定疾患としての医療券は約 500 件発行されている。本疾患は 100 万人に 1 名の発症と言われるように、きわめて希な疾患である。したがって、臨床試験を行うには全国規模で患者を組み入れなければ研究に必要な症例数を確保できない。我が国では 1999 年 4 月から厚生省の特定疾患「遅発性ウイルス感染症調査研究班」にサーベイランス委員会を組織し全国を網羅したプリオン病のサーベイランス体制を構築し、その後、厚生労働省の難治疾患克服研究事業「プリオン病のサーベイランス及び感染予防に関する調査研究班」、さらには「プリオン病のサーベイランスと感染予防に関する調査研究班」に引き継がれている。したがって、このプリオン病のサーベイランス委員会と協力することは、全国規模の臨床研究体制の構築にはきわめて有用である。今年度は、サーベイランス関連施設以外の都道府県の中核病院や国立病院機構の病院などに広く施設登録を依頼した。「プリオン病のサーベイランス及び感染予防に関する調査研究班」および「プリオン病および遅発性ウイルス感染症に関する調

査研究班」との緊密な連携により、臨床研究体制をより強固なものとする。

2) プリオン病臨床研究体制の活用

わが国では 1999 年 4 月より、プリオン病に関する全国レベルでのサーベイランス調査が続けられており、年 2 回のサーベイランス委員会で新たな症例が認定されている。これまでに 4281 件を調査し、本邦患者の約 90%に達すると思われる 2162 人（男：922 人、女：1,240 人）が認定されており、そのデータから、わが国では人口 100 万人対の罹患率は 1.01 人で欧米の罹患率と同等であることが判明している。わが国における現行の医療制度においては治療法のないプリオン病などの難治性疾患を特定の医療機関で長期にフォローすることは不可能であり、これまでにプリオン病患者の発症後の自然歴は把握できていない。プリオン病の代表的疾患である古典型孤発性クロイツフェルト・ヤコブ病は発症から 3〜6 ヶ月で無動性無言になることが知られているが、このような大まかなデータでは、将来治療薬試験を開始したとしても、臨床症状の推移を効果判定に用いることは困難である。

そこで、構築されたプリオン病臨床研究体制である日本プリオン病コンソーシアム(Japanese Consortium of Prion Disease: JACOP)を活用して、プロトコールと重症度分類(rating)による患者登録とフォローアップを行い、自然癒を解明する。

（倫理面への配慮）

疫学的・臨床研究に際しては、それぞれの疾患の患者や家族からインフォームドコンセントを得て行うと共に個人情報の守秘を計る。サーベイランスについては委員長の所属施設にて倫理審査を受け承認されている。

C. 研究結果

1) プリオン病臨床研究体制の構築

プリオン蛋白の物理化学的構造解析から開発された化合物GN8を修飾・発展させたP092がプリオン蛋白へ結合し、その構造変換を抑制することより、プリオン蛋白の異常化が抑制でき、治療薬として利用できる可能性が示されている。現在、実用的な容量設定やマウスやサルを用いた有効性、および安全性実験が進められている。P092のファースト・イン・ヒューマンの医師主導型治験をスムーズに行うために、臨床研究体制をあらかじめ構築しておく必要がある。

昨年度はJACOPを設立し、プリオン病のサーベイランスおよび感染予防に関する研究班とプリオン病および遅発性ウイルス感染症に関する調査研究班の臨床系班員を中心に参加を依頼し、11施設の登録がなされた。今年度は各都道府県の主要な病院、および国立病院機構の病院に依頼をし、登録施設は22となった。さらに30以上の施設で登録手続きが進んでおり、最終的には50施設以上が登録され、広く日本全国をカバーする予定である。JACOP事務局は東京医科歯科大学に設置し、その運営は9名の委員によって構成される運営委員会によって行われている。

2) プリオン病臨床研究体制の活用

プリオン病は、前述のようにきわめて希であり、かつ多くは発症後1年程度で死に至るきわめて進行の早い疾患である。一方、中には数年の経過を示す緩徐進行型も存在し、個々の病型の患者数はさらに稀少である。したがって、患者登録には全国に呼びかけて参加を求める必要がある。医師主導型治験のプロトコールはプリオン病の臨床的特徴を網羅し、第一相では緩徐進行型を対象とすることが望ましいと考えられるが、医薬品医療機器総合機構からもそのように求められていることより、まずは、緩徐進行型（P102L変異によるGSS、V180I変異による遺伝性CJD、MM2C型孤発性CJDなど）を主としたプリオン病各病型の自然歴を明らかにする。これらの自然歴は、医師主導型治験の際には、必要に応じてコントロールとして使えるように考慮して準備を行った。また、登録患者のリンパ球、脳脊髄髄液、血清などを保存できるようにサンプル保管室、培養細胞保管室内に、本研究目的の超低温槽、細胞保管容器を設置し、超低温槽に関しては、2011年3月の大震災の教訓より、2施設（本学、および東京都健康長寿医療センター）に同一検体を分散して保管できるように超低温槽を整備し、患者サンプルの保管

を開始した。今年度は4名のプリオン病患者が登録され、定期的なフォローが行われている。

自然歴データの観察項目として、サーベイランスと同一項目の経時的変化に加え、2013年に発表された評価尺度 (Thompsonら, Brain 136, 1116-1127, 2013)と、「統一多系統萎縮用評価尺度 (UMSARS; unified multiple system atrophy rating scale)についても考慮した。

D. 考察

国内外において、プリオン病の罹患率は人口100万人あたり年間1人であることが明らかになっているが、国内外を問わず、これまでにプリオン病における正確な自然歴を調査された報告はない。

我々は、現時点で参加施設を全国 50 施設以上とし、少なくとも各都道府県に1施設、さらにサーベイランス調査で明らかになっているプリオン病患者が多い地域には複数の医療機関の登録を進めている。今回の課題の治験薬の臨床試験のためにはもちろん、プリオン病の発症機序の理解のためにも、正確な自然歴調査は必要不可欠で有り、JACOP 構築の意義はきわめて大きい。

JACOP の構築により、治験に応用可能な自然歴調査のプロトコールを作成し、今年度は慢性進行型のプリオン病にも対応できるように重症度分類(rating)を評価項目に追加した。さらに調査体制、試料保存体制も充実させ、自然歴調査による患者登録を開始した。今後、治験開始準備と平行して、登録患者を増やし、定期的な追跡調査が始まっている。今後は、迅速かつスムーズにファースト・イン・ヒューマンの医師主導型治験へ移行できるよう更に準備を進めてゆく。

E. 結論

わが国におけるオールジャパンのプリオン病の研究体制である JACOP を構築し、現在 50 以上の施設が登録、あるいは登録準備中である。慢性進行型のプリオン病に対応できるようにプロトコールを改編した。今後は世界初のプリオン蛋白の物理化学的解析に基づき作製された新規化合物 P092 を用いた治験の準備を進めながら、自然歴調査を継続、JACOP 登録施設を強化してゆく。

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なし

F. 健康危険情報

なし。

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H. 知的財産権の出願・登録状況（予定を含む。）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

[III] 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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書籍

なし

[IV] 研究成果の刊行物・別刷

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FK506 reduces abnormal prion protein through the activation of autolysosomal degradation and prolongs survival in prion-infected mice

Takehiro Nakagaki,[1] Katsuya Satoh,[1] Daisuke Ishibashi,[1] Takayuki Fuse,[1] Kazunori Sano,[1] Yuji O. Kamatari,[2] Kazuo Kuwata,[2] Kazuto Shigematsu,[3] Yoshifumi Iwamaru,[4] Takato Takenouchi,[5] Hiroshi Kitani,[5] Noriyuki Nishida[1] and Ryuichiro Atarashi[1,6]*

[1]Department of Molecular Microbiology and Immunology; Nagasaki University Graduate School of Biomedical Sciences; Sakamoto, Nagasaki Japan; [2]Life Science Research Center; Gifu University; Yanagido, Gifu Japan; [3]Department of Pathology; Japanese Red-Cross Nagasaki Atomic Bomb Hospital; Nagasaki, Japan; [4]Prion Disease Research Center; National Institute of Animal Health; Tsukuba, Ibaraki Japan; [5]Animal Immune and Cell Biology Research Unit; National Institute of Agrobiological Sciences; Tsukuba, Ibaraki Japan; [6]Nagasaki University Research Centre for Genomic Instability and Carcinogenesis; Nagasaki, Japan

Keywords: FK506, prion, autophagy, therapy, microglia, degradation, tacrolimus

Abbreviations: TSE, transmissible spongiform encephalopathies; CJD, Creutzfeldt-Jakob disease; PRNP, prion protein; $PRNP^{C}$, normal prion protein; $PRNP^{Sc}$, abnormal prion protein; PPS, pentosan polysulfate; CNS, central nervous system; MTOR, mechanistic target of rapamycin; FKBP, FK506-binding protein; GSS, Gerstmann-Sträussler-Schenker syndrome; recPRNP, recombinant PRNP; SPR, surface plasmon resonance; MDC, monodansylcadaverine; AIF1/IBA1, allograft inflammatory factor 1 or ionized calcium-binding adapter molecule 1; GFAP, glial fibrillary acidic protein; UPS, ubiquitin-proteasome system

Prion diseases are fatal neurodegenerative disorders and no effective treatment has been established to date. In this study, we evaluated the effect of FK506 (tacrolimus), a macrolide that is known to be a mild immunosuppressant, on prion infection, using cell culture and animal models. We found that FK506 markedly reduced the abnormal form of prion protein ($PRNP^{Sc}$) in the cell cultures (N2a58 and MG20) infected with Fukuoka-1 prion. The levels of autophagy-related molecules such as LC3-II, ATG12–ATG5 and ATG7 were significantly increased in the FK506-treated cells, and resulted in the increased formation of autolysosomes. Upregulation of the autophagy-related molecules was also seen in the brains of FK506-treated mice and the accumulation of $PRNP^{Sc}$ was delayed. The survival periods in mice inoculated with Fukuoka-1 were significantly increased when FK506 was administered from day 20 post-inoculation. These findings provide evidence that FK506 could constitute a novel antiprion drug, capable of enhancing the degradation of $PRNP^{Sc}$ in addition to attenuation of microgliosis and neuroprotection.

Introduction

The transmissible spongiform encephalopathies (TSE), prion diseases, are fatal neurodegenerative disorders and include Creutzfeldt-Jakob disease (CJD) in humans. Histologically, TSEs are characterized by neuronal loss, maculation and activation of astrocytes and microglia, and the conformational conversion of normal prion protein ($PRNP^{C}$) to the abnormal form ($PRNP^{Sc}$) is central to the pathogenesis.[1,2] For this reason, drug discovery for the prion diseases has focused primarily on compounds capable of inhibiting the conversion of PRNP. A number of drugs, including pentosan polysulfate (PPS),[3] quinacrine,[4] anti-PRNP antibodies[5,6] and others,[7,8] have been proposed as potential anti-prion agents. Most act by binding to $PRNP^{C}$ and inhibiting the interaction between $PRNP^{C}$ and $PRNP^{Sc}$, resulting in a reduction of the conversion of $PRNP^{C}$ into $PRNP^{Sc}$. PPS has been shown to prolong the survival of infected animals, but only when the drug is administered into the brain directly because it cannot cross the blood brain barrier.[9] Anti-PRNP antibody treatments present the same problem. Quinacrine is strongly inhibitory in vitro but has little effect on patient survival.[10] Other small compounds that show anti-prion effect in vitro have been reported to possess prophylactic effect in vivo but failed to stop pathogenesis of the disease after onset. Put simply, an effective treatment for patients with prion diseases does not yet exist.[11] Furthermore, while suppression of the conversion from $PRNP^{C}$ to $PRNP^{Sc}$ by these anti-PRNP drugs remains important, alone it is not sufficient, and new therapeutics targeting areas other than the conversion process should be considered.[12]

One possible target for drug development is molecules which control protein-degradation pathways in cells. Neurodegenerative diseases are generally thought to be caused by the accumulation of misfolded, aggregate-prone proteins such as α-synuclein in Parkinson disease or dementia with Lewy bodies, and

*Correspondence to: Ryuichiro Atarashi; Email: atarashi@nagasaki-u.ac.jp
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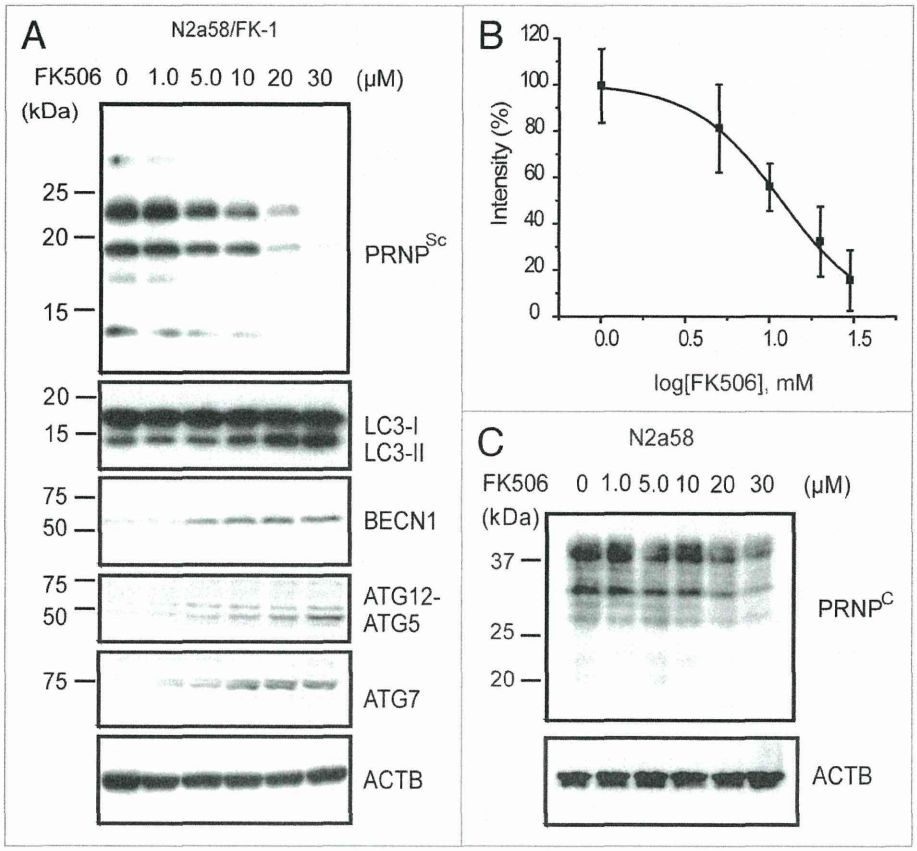

Figure 1. FK506 reduces $PRNP^{Sc}$ and activates autophagy in a prion-infected cell culture model. (**A**) The Fukuoka-1-infected cells were treated with different concentrations of FK506 for 48 h. The amount of $PRNP^{Sc}$ and autophagy-related molecules (LC3-I and II, BECN1, ATG12–ATG5, and ATG7) in cells were analyzed by western blotting. (**B**) The $PRNP^{Sc}$ band intensities are expressed as a percentage of those of the negative controls. The results in the graph are the mean ± SD of three independent experiments. The logistic regression curve that fits the data are shown. (**C**) The levels of $PRNP^{C}$ in N2a58 cells treated with different concentrations of FK506 for 48 h were analyzed by western blotting. The data are representative of three independent experiments.

polyglutamine-containing proteins in Huntington disease or spinocerebellar ataxia, and PRNP in prion diseases. The clearance of these aggregated proteins depends mainly upon autophagy, which is the major molecular mechanism for degrading cytoplasmic materials in the lysosome,[13,14] and over the past decade, many researchers have focused on the autophagy-lysosomal system as a quality control regulator of protein in neurodegenerative diseases.[13,14] Of note, the chemical-induced enhancement of autophagy has been reported to decrease aggregates in the CNS and retard the progression of neurological symptoms in several animal and cell culture models of neurodegenerative diseases, including TSE.[14–19] The autophagy-lysosomal system is regulated by several mechanisms, one example of which is mechanistic target of rapamycin (MTOR) signaling.[20]

FK506, also named tacrolimus, is well known as an immunosuppressive drug that is mainly used post-transplantation to decrease the activity of the recipient's immunity. It has been reported that FK506 binds to the immunophilin FKBP12 (FK506 binding protein), and creates a new complex. This FKBP12-FK506 complex is known to interact with and inhibit calcineurin, resulting in the suppression of both T-lymphocyte signal transduction and IL2 transcription.[21] Recent studies reveal neuroprotective effects in cerebral ischemia[22] and several neurodegenerative disorders,[23,24] including TSE.[25] On the other hand, FKBP39 has recently been reported to suppress autophagy in drosophila.[26] This result raises the possibility that FK506 could also influence the autophagy-lysosomal system in mammalian cells. In this study, we examined the effect of FK506 treatment on prion infection, autophagic pathways, and the relationship between them, using prion-infected cell cultures and animal models.

Results

FK506 decreases $PRNP^{Sc}$ in cell culture models. To investigate the effect of FK506 on the amount of $PRNP^{Sc}$, we treated neuroblastoma cells overexpressing $PRNP^{C}$ (N2a58 cells) and infected them with the mouse-adapted Gerstmann-Sträussler-Schenker syndrome (GSS) Fukuoka-1 strain (N2a58/Fukuoka-1), together with different concentrations of FK506 over 48 h, and found that the amount of $PRNP^{Sc}$ decreased in correlation with the concentration of FK506 (**Fig. 1A and B**). The effective concentration for 50% reduction of $PRNP^{Sc}$ (IC_{50}) over 48 h was 11.9 μM. We next tested whether FK506 reduces $PRNP^{Sc}$ in a microglial cell line overexpressing $PRNP^{C}$ (MG20 cells). The inhibitory effect on $PRNP^{Sc}$ accumulation was also observed for MG20 cells infected with Fukuoka-1 (**Fig. S1A**). Furthermore, we determined the effect of FK506 on $PRNP^{C}$ levels in uninfected cells. FK506-treated N2a58 (**Fig. 1C**) and MG20 (**Fig. S1B**) cells exhibited a mild reduction in $PRNP^{C}$ levels, suggesting that a decrease in $PRNP^{C}$ may also contribute to a reduction in $PRNP^{Sc}$ accumulation.

To examine whether FK506 directly binds to $PRNP^{C}$, we examined the affinity of FK506 to recombinant PRNP (recPRNP) using the surface plasmon resonance (SPR) method.[27] The positive control, GN8, that is known to bind to $PRNP^{C}$ and inhibit the conversion,[28] showed the typical binding signals in a dose-dependent manner, while the affinity of FK506 to recPRNP was very low (**Fig. S3**). Although most of the compounds reported to have therapeutic action in prion diseases bind to $PRNP^{C}$, SPR revealed that FK506 did not bind to recPRNP, indicating that the anti-prion effect of FK506 is indirect.

FK506 increases the levels of autophagy-related molecules and the formation of autolysosomes in prion-infected cell culture models. To investigate the effect of FK506 on the activation of autophagy, we first analyzed the levels in N2a58/Fukuoka-1 cells of several autophagy-related molecules: LC3-II, ATG7, ATG12–ATG5 complex and BECN1/Beclin 1.[29] An increase in all the molecules tested was observed following treatment with FK506 (**Fig. 1A**). Autolysosomes were evaluated by staining with an auto-fluorescent drug, monodansylcadaverine (MDC), which detects acidic compartments including autolysosomes.[30] Because autophagy is strongly induced by starvation, increased fluorescence was seen in the cells treated with Hank's Balanced Salt Solutions (HBSS), as the positive control (**Fig. 2A**). A similar increase in MDC signals was seen in the FK506-treated N2a58/Fukuoka-1 cells, which had twice as many vacuoles as the untreated cells (**Fig. 2A**). The increased signals were also observed for uninfected N2a58 cells treated with FK506 (**Fig. S2**).

To determine whether the degradation of $PRNP^{Sc}$ was due to autolysosomal activity, we treated the cells with NH_4Cl,[31] which is an effective inhibitor of lysosomal hydrolases that acts by increasing the pH inside lysosomes. The levels of LC3-II were observed in the following order: FK506 + NH_4Cl > NH_4Cl > FK506 > no treatment (**Fig. 2B**). These observations can be explained by the fact that LC3-II itself is also degraded by lysosomal hydrolases. The decreased levels of $PRNP^{Sc}$ by FK506 were recovered when the cells were added with NH_4Cl (**Fig. 2C**, lane 4). These results indicate that $PRNP^{Sc}$ is actively degraded by autolysosomes when cells are treated with FK506.

FK506 prolongs survival of prion-infected mice. To examine the effect of FK506 on the survival periods of prion-infected mice, CD-1 mice were inoculated intracerebrally with Fukuoka-1 strain. FK506 was intraperitoneally administered (1.0 or 0.1 mg/kg/day,) from 20 or 60 d post-inoculation (d.p.i.). In the untreated controls, symptoms appeared at 110 d.p.i. and the mice died around 120 d.p.i. (**Fig. 3A**; **Table 1**). While there was no significant difference in symptom onset or survival between the mice treated from 60 d.p.i and the control, those mice treated from either 20 d.p.i. survived until about 140 d.p.i (**Fig. 3A**; **Table 1**). Next, transgenic mice overexpressing Syrian hamster PRNP[32] [Tg(Sha *Prnp*)] were inoculated intracerebrally with hamster scrapie-prion strain 263K. FK506 (1.0 mg/kg/day, orally) was administered either from 14 d.p.i. or 28 d.p.i. The mice treated from 14 d.p.i. survived about 14 d longer than the mice receiving vehicle only ($p < 0.05$) (**Fig. S4**). These results indicate that FK506 treatment led to prolonged survival in prion-infected animal models, although the effect was influenced by the timing of administration.

FK506 upregulates autophagy and decreases $PRNP^{Sc}$ in the brains of mice. Some of the mice treated from 20 d.p.i. were sacrificed at 110 d.p.i. and their brains examined to determine the degree of accumulation of $PRNP^{Sc}$ and levels of autophagy-related molecules. The amount of $PRNP^{Sc}$ in the treated mice was considerably less than that in the control at 110 d.p.i. (**Fig. 3D and E**). In contrast, there was no significant difference between the brains of treated mice and those of the control by the terminal stage. Levels of LC3-II, ATG7 and ATG12–ATG5 complex were significantly increased in the brains of mice receiving FK506 (**Fig. 4**), supporting the view that FK506 decreased the accumulation of $PRNP^{Sc}$ via activation of autolysosomes in animal brains as well as in cells.

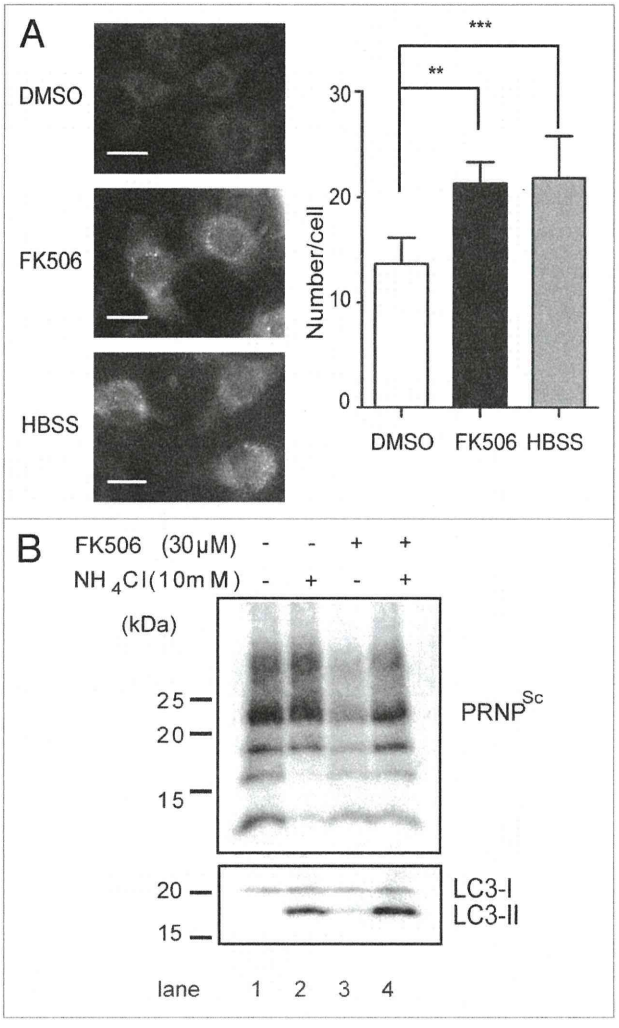

Figure 2. FK506 induces the formation of autolysosomes in prion-infected cells. (**A**) Autolysosomes in the cells treated with 10 μM of FK506 or DMSO for 24 h were visualized using 0.1 mM of monodansycadaverine (MDC) for 30 min (left three panels). Scale bars: 20 μm. As a positive control, cells were treated with HBSS for 30 min before MDC treatment. The right graph shows the average number of MDC-labeled autolysosomes in a single cell. Values represent the mean ± SD of three independent experiments. Statistical analysis was done using one-way ANOVA followed by the Tukey-Kramer test. **$p < 0.01$, ***$p < 0.001$ compared with the cells treated with DMSO. (**B**) To inhibit lysosomal activity, Fukuoka-1-infected cells were pretreated with 10 mM of NH_4Cl for 24 h (lanes 2 and 4) prior to addition of 30 μM of FK506. The amount of $PRNP^{Sc}$ and LC3-II in cells was analyzed by western blotting. The data are representative of three independent experiments.

FK506 partially suppresses pathological changes associated with prion diseases in Fukuoka-1-infected mouse brains

Next, we analyzed the degree of vacuolation, gliosis and $PRNP^{Sc}$ in the brains of Fukuoka-1-infected mice. We evaluated the degree of vacuolation by calculating the percentage of

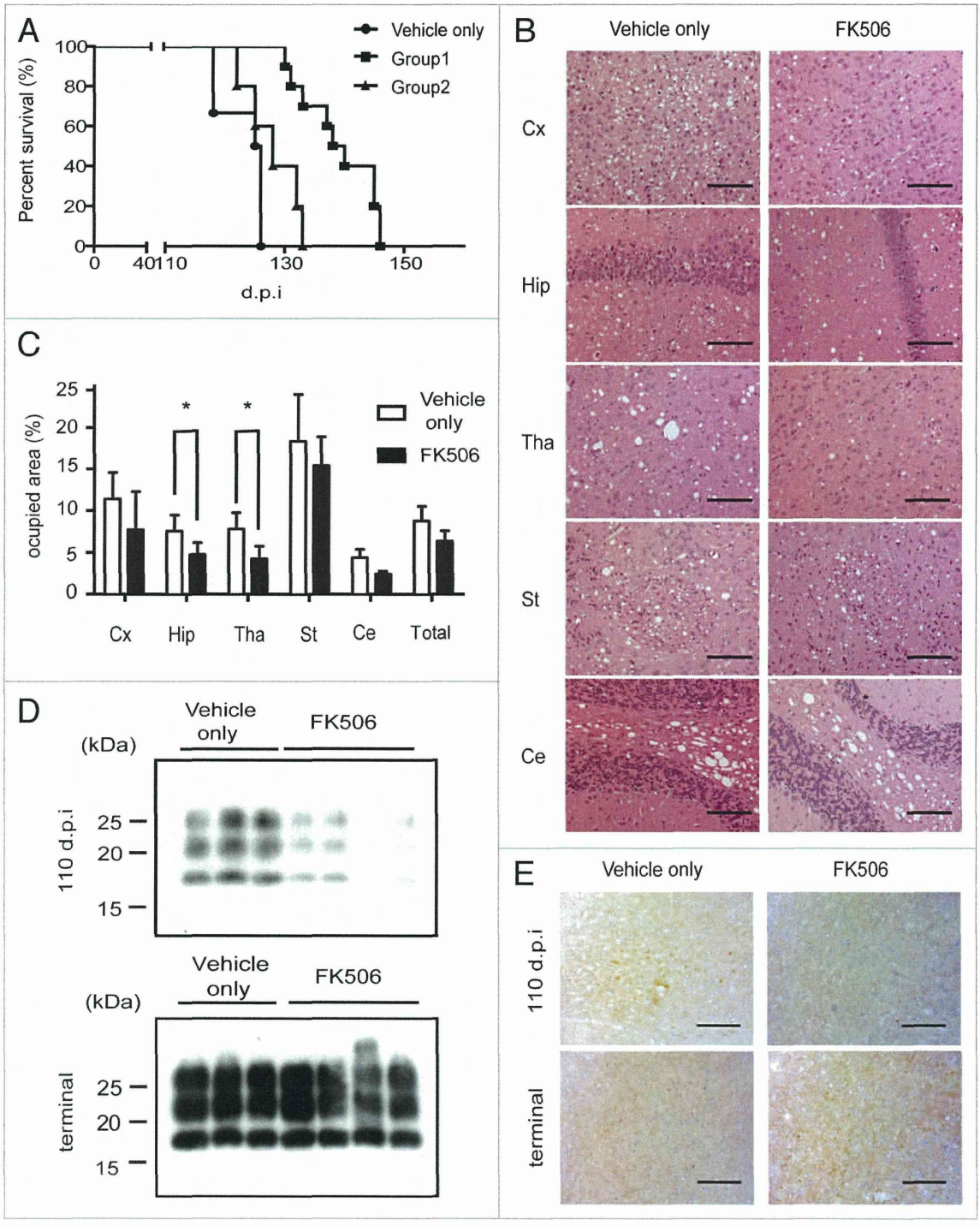

Figure 3. FK506 prolongs survival in Fukuoka-1-infected mice. (**A**) Survival curves in the Fukuoka-1-infected mice administered FK506 intraperitoneally. The control mice (n = 6, circle) and FK506-treated mice (group 1: from 20 d.p.i., n = 10, square; group 2: from 60 d.p.i., n = 5, triangle) were compared. Mice in group 1 survived significantly longer than those of the control group ($p < 0.01$, Logrank test). (**B**) Comparison of spongiform change at 110 d.p.i. between control animals (vehicle only) and group 1 mice. The brain sections of cortex (Cx), hippocampus (Hip), thalamus (Tha), striatum (St) and cerebellum (Ce) stained with hematoxylin and eosin are shown. Scale bars: 100 μm. (**C**) In 3 to 5 randomly selected areas of each tissue sample, individual vacuoles were measured and the total vacuolated area was expressed as a percentage of the entire surface of the specimen. Statistical significance was determined using the two-tailed Student's t-test. *$p < 0.05$ compared with the control. Error bars indicate SD (n = 3). (**D**) Accumulation of $PRNP^{Sc}$ in brain tissues at 110 d.p.i. (upper panel) and terminal stage (lower panel) from controls (vehicle only) or group 1 mice was analyzed by western blotting. (**E**) Immunohistochemical staining of $PRNP^{Sc}$ in thalamus samples of mice sacrificed at 110 d.p.i. and those sacrificed at the terminal stage, using SAF32 antibody. Scale bars: 100 μm. Data are representative of at least three mice.

Table 1. Survival periods of prion-infected mice administered FK506

Mouse	Strain	FK506 (mg/kg/day)	Start point (d.p.i)	Number	Mean ± SD (days)	p value
CD-1	Fukuoka-1	0		6	125.5 ± 1.6	
			20	10	139 ± 2.0	< 0.001
		1.0	60	5	128 ± 2.1	n.s
			20	5	141 ± 2.6	< 0.01
		0.1	60	5	123 ± 2.1	n.s

n.s, no significance

the vacuolated area in each section (**Fig. 3C and D**). Treatment with FK506 suppressed vacuolation in the hippocampus and thalamus. We also analyzed the degree of activation of microglia and astrocytes by immunohistochemical staining and western blotting. The expression of allograft inflammatory factor 1(AIF1/IBA1), which is an EF-hand protein and is reported to be upregulated in activated microglia,[33,34] was decreased in the treated mice (**Fig. S3A**). The area occupied by microglia tended to be decreased in the brains of treated mice, especially in the cortex (**Fig. S5B and S5C**). To analyze astrocytosis, we used an anti-glial fibrillary acidic protein (GFAP) antibody as a marker. A small suppression in the treated mice was observed, however, the difference did not reach statistical significance (**Fig. S6**). These results indicate that FK506 delays the accumulation of $PRNP^{Sc}$ in the brains of Fukuoka-1-infected mice and partially attenuates the activation of microglia and spongiform change at 110 d.p.i.

Discussion

In our experiments, accumulation of $PRNP^{Sc}$ was suppressed by FK506 treatment in both prion-infected cells and mice. Furthermore, increased amounts of autophagy-related molecules such as LC3-II, ATG12–ATG5 complex and ATG7 were detected and the formation of autolysosomes was significantly increased in FK506-treated prion-infected cells. These results support the notion that FK506 enhances the degradation of $PRNP^{Sc}$ via upregulation of autophagy, although the molecular basis remains to be elucidated.

There are two major pathways involved in the degradation of abnormally folded proteins in cells: the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal system. The UPS is involved in the targeted degradation of most short-lived proteins or proteins that fold improperly within the endoplasmic reticulum. Although inhibition of the UPS causes cytosolic $PRNP^{C}$ accumulation, it is unlikely that the degradation of $PRNP^{Sc}$ occurs mainly in the UPS,[35,36] as $PRNP^{Sc}$ aggregates are unable to enter the narrow channel into the proteasome. On the other hand, the UPS has been reported to be impaired in TSEs,[37,38] and it has been demonstrated that both $PRNP^{Sc}$ and aggregated β-sheet-rich recPRNP inhibit the activity of proteasomes. Thus, it is reasonable to postulate that autophagy is one of the major degradation pathways of $PRNP^{Sc}$.

Mukherjee et al. have recently reported that FK506 suppressed the function of calcineurin leading to the reduction of prion-induced neurodegeneration,[25] and concluded that FK506 treatment does not alter the extent of accumulation of $PRNP^{Sc}$, astrocytosis or microglial activation at the terminal stage of the disease. These findings are consistent with our observation that no significant difference was observed between the brains of FK506-treated mice and those of control animals at the terminal stage. However, in our study, both accumulation of $PRNP^{Sc}$ and microgliosis in the mice treated from 20 d.p.i. were suppressed at 110 d.p.i., when symptoms were already in evidence in the untreated controls. These observations indicate that both the rate of accumulation of $PRNP^{Sc}$ and microgliosis were decreased in the FK506-treated mice, and it is most likely that the delayed accumulation of $PRNP^{Sc}$ was primarily due to the increased degradation of $PRNP^{Sc}$ via activation of autophagy. Furthermore, our SPR experiments revealed that there was no direct interaction between recPRNP and FK506, providing further evidence that the principle anti-prion effect of FK506 is not due to inhibition of the conversion into $PRNP^{Sc}$.

The importance of autophagy in the clearance of $PRNP^{Sc}$ is also supported by the fact that certain chemicals such as trehalose,[17] lithium[18] and rapamycin[19] decrease the amount of $PRNP^{Sc}$ in prion-infected cultured cells or mice by inducing the upregulation of autophagy. In contrast, treatment with trehalose or lithium did not significantly prolong the survival of prion-infected mice, although these findings may be partially explained by the fact that the effective concentration of both drugs is extremely high (mM range), and therefore difficult to achieve in vivo.[17,18] Rapamycin did extend survival to a moderate extent only under certain experimental conditions.[19,25] In our study, the early administration at 20 d.p.i of FK506 prolonged the survival of Fukuoka-1-infected mice. In contrast, the late administration (60 d.p.i.) had little effect on survival duration. The difference in the protective effect afforded by FK506 as a consequence of administration time may be explained by the assumption that activation of autophagy can completely disrupt small aggregates of $PRNP^{Sc}$, but not large $PRNP^{Sc}$ aggregates. It is reasonable to postulate that the size of $PRNP^{Sc}$ aggregates has increased by the late stage. Moreover, autophagy may sometimes cause fragmentation of large aggregates of $PRNP^{Sc}$, resulting in enhancement of $PRNP^{Sc}$ formation. The above possibilities also explain why there was no difference in accumulated $PRNP^{Sc}$ at the terminal stage. However, further studies are required to investigate the molecular basis of these results and the optimum time for administration of FK506.

Increasing evidence suggests that inflammation processes such as gliosis are involved in the pathogenesis of prion diseases.[39]

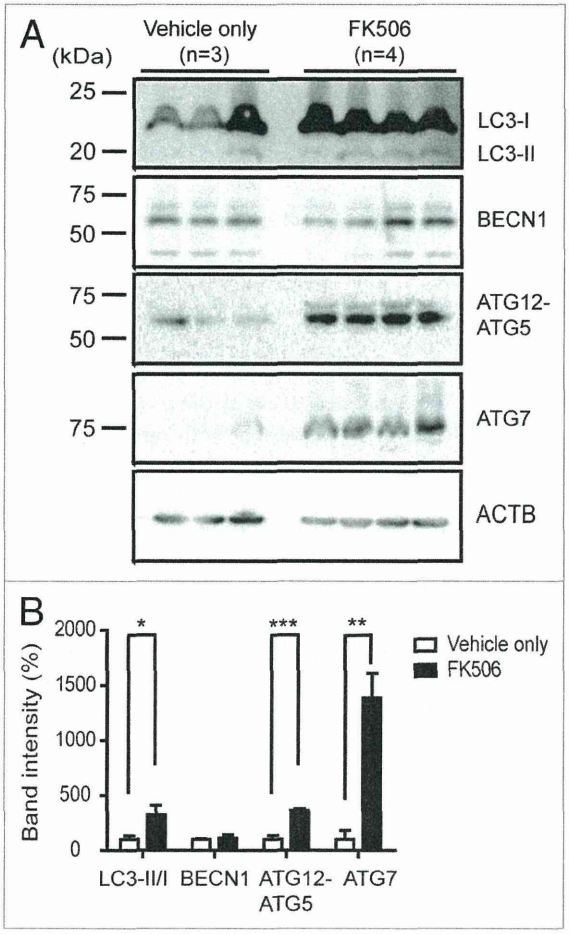

Figure 4. FK506 upregulates autophagy-related molecules in the brains of prion-infected mice. (**A**) The control mice (n = 3) and FK506-treated mice (group 1: from 20 d.p.i., n = 4) were compared at 110 d.p.i. The amounts of autophagy-related molecules (LC3, BECN1, ATG12–ATG5 and ATG7) and ACTB/β-actin in brain tissues were analyzed using western blotting. (**B**) Band intensities of autophagy-related molecules are expressed as a percentage of those of the control (vehicle only). Values represent the mean ± SD of three independent experiments. Statistical significance was determined using the two-tailed Student's t-test. *p < 0.05, ** p < 0.01, ***p < 0.001 compared with the control group.

Suppression of microglial activation by FK506 has also been reported in cerebral ischemia[22] and a tauopathy[23] mouse model. Microglia are thought to exert two opposing effects in prion diseases. Following exposure to $PRNP^{Sc}$, microglia produce neurotoxins that exacerbate neurodegeneration.[40,41] In contrast, it is reported elsewhere that activated microglia may afford protection to neurons through phagocytosis and digestion of aggregated $PRNP^{Sc}$.[42,43] It is possible that the balance between the two contrary effects of microglial activation may proceed in a time-dependent manner. When treatment was initiated at an early stage (20 d.p.i.) microglia appeared to play a deteriorative role, while at a late stage (60 d.p.i) they offered a protective role. Nevertheless, further studies are needed to determine the exact role of microgliosis in the pathogenesis of prion diseases, and to establish the contribution by FK506 to the suppression of microglial activation, with that of stimulating autophagy. FK506 immunosuppression is clearly mediated by the inhibition of calcineurin in T-cells,[21] but it remains to be determined whether suppression of microglial activation and activating autophagy depend on the inhibition of calcineurin. Thus, it would be worthwhile examining the effect of non-calcineurin inhibiting derivatives such as V-10,367 and GPI-1046.[44]

Inhibition of the peptidyl-prolyl isomerase activity of FKBPs may constitute another mechanism for the anti-prion effect of FK506, as a number of FKBPs are suspected to accelerate the aggregation of abnormal proteins.[24] However, it remains unknown whether this activity influences the production of $PRNP^{Sc}$.

FK506 has the advantage of having been widely used for many years in the clinical setting, in contrast to most of the other substances that have been proposed as therapeutic agents for TSEs. Of particular interest is its effect on Crohn disease, since carriers of the *ATG16L1* gene and certain other mutations which are thought to impair autophagy, have increased susceptibility to the disease.[45,46] Furthermore, oral administration of FK506 has clinical benefit because of its ease of administration and its low cost. Taken together, these characteristics raise the possibility that FK506 may become a valuable therapeutic agent for prion diseases. It would be of great value to additionally examine whether combination therapy using FK506 together with other drugs possessing different areas of action would be an even more beneficial strategy for the effective management of TSEs.

In conclusion, we have shown that FK506 treatment decreases the levels of $PRNP^{Sc}$ in prion-infected cell culture models and prolongs the survival of prion-infected mice, both of which are accompanied by upregulation of autophagy-related molecules. Our findings provide evidence that FK506, in addition to attenuation of microglial activation and neuroprotection, induces the activation of the autophagy-lysosomal system and facilitates the elimination of accumulated $PRNP^{Sc}$ via this mechanism.

Materials and Methods

Ethics statement. All animal experiments were permitted by the committee of the Nagasaki University in accordance with the Guidelines for Animal Experimentation of Nagasaki University and conformed to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Reagents. M-20 antibody (Santa Cruz Biotechnology, sc7694) is a goat polyclonal antibody recognizing the C-terminus of mouse PRNP. SAF32 antibody (SPI-BIO, A03202) is a mouse monoclonal antibody recognizing the octapeptide repeats in mouse PRNP. The anti-AIF1/IBA1 antibody to detect microglia (Wako Pure Chemical Industries, 016–20001 for western blotting, 019-19741 for immunohistochemistry) and the anti-GFAP antibody to detect astrocytes (DAKO, Z033429) are rabbit polyclonal antibodies. Antibodies to detect autophagy-related molecules were rabbit polyclonal antibodies: anti-ATG5 (Cell Signaling Technology, 2630), -ATG12 (2011), -BECN1 (3738), -LC3 (Medical and Biological Laboratories, PM036) and