F．健康危険情報

なし

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Teruya K, Doh-ura K. A thioflavin derivative facilitates cross-linking of abnormal PrP but not normal PrP. Asia-Oceania Symposium on Prion Diseases, Sapporo, July 24-25, 2010

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堂浦克美：ヤコブ病の克服研究　第4回プリオン病の市民講座　食と医療の安全、東京、2010年11月23日

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濃度 Cu（Ⅱ）イオンによって可逆的に制御される. 第 33 回日本分子生物学会・第 83 回日本生化学会合同大会、神戸、2010 年 12 月 7 日-10 日

H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
平成２２年度　分担研究報告書

セルロース誘導体の最適化に関する研究

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研究要旨
これまでに47種のメチルセルロース関連化合物を化学合成し、プリオン病発症抑制効果や抗プリオン活性を調べた。プリオン感染細胞N167株, Sc84株を用いたin vitro実験により、中分子量化合物であるGG-23MCのみに異常型プリオン発現阻害活性が認められた。また、プリオン感染マウスを用いた試験（実験中:112日目）により、23MC 投与マウスは112日目現在、全匹生存中であり発症抑制効果が認められている。更に、単糖では例外的にメチル化単糖に高い発症抑制効果が認められた。活性を改善する必要が残されているものの、これら化合物の発見はプリオン病予防薬開発の可能性を一歩前進させたと言える。

A．研究目的

これまでに研究代表者らにより、市販のセルロース誘導体にプリオン病の発症抑制効果があることが見いだされている。しかしながら、不均一反応で調製される市販のセルロース誘導体は化学構造的に不均一であるため、例えば市販のメチルセルロースのメチル基の置換状態はグルコース内の３つの水酸基での置換状態の違い、分子鎖に沿ったメチル基の置換位置の偏り、分子鎖間でのメチル基置換度の違いが生じ、化学構造-活性相関を詳細に検討することは不可能である。そこで、本研究では化学構造の明確なメチルセルロース誘導体を有機合成し、その化学構造がプリオン病発症抑制効果や抗プリオン活性に与える影響を詳細に検討することとした。最終的に、発症抑制効果や抗プリオン活性を与えるメチルセルロースの部分構造を明らかにし、より効果の高いセルロース誘導体関連化合物を開発することを目的とする。

B．研究方法

セルロース、セロビオース、グルコース、メチルグルコシドを原料とし、位置特異的な保護基の導入、メチル基の導入、グリコシル化、保護基の除去などの工程を経て、47種類のメチルセルロース系化合物を化学合成した。

次いで、プリオン持続感染細胞 N167 を用いてそれら化合物の異常型プリオン蛋白産生抑制効果を検討した。
さらに、プリオン感染マウスを用い、感染後、脳室内に化合物を 14 日間投与し潜伏期間により効果評価を行った。
(倫理面の配慮)
動物実験は施設の動物実験委員会の許可を受け、動物実験指針を遵守して行った。

C．研究結果

メチルセルロースに関連する化合物 47 種類を化学合成し、研究代表者の研究施設にてプリオン持続感染細胞を用いた in vitro の異常型プリオン蛋白産生抑制能を調べた。その結果、GG-23MC (DPw = 20.3, DPn = 9.2) のみに高濃度でわずかに効果が認められた。

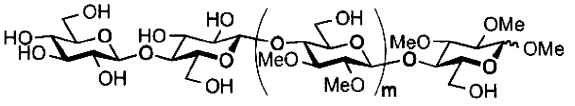

図　GG-23MC の化学構造式

次いで、47 種の化合物のうち、サンプル量が十分に確保出来た化合物 11 種類をプリオン感染マウスを用いて発症抑制効果を評価した。現在実験中であるが、マウスの生存日数から化合物の効果を比較すると、23MC (DS = 0.88, DP = 40) の効果が最も高いという結果が得られている。加えて、メチル化単糖が高分子の 23MC に次いで高い効果を示した。

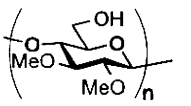

図　23MC の化学構造式

D．考察

これまで、in vitro で抗プリオン活性が確認されたセルロース誘導体は存在せず、GG-23MC は極めて興味深い化合物である。また、プリオン脳内感染マウスを用いた発症抑制効果の評価では、メチル化単糖が高分子の 23MC に次いで高い効果を示したことから、その作用機序を解明することは低分子のプリオン病予防薬を開発する上で重要な知見である。これまでに位置特異的置換あるいはブロック的置換メチルセルロース誘導体、さらに位置特異的かつブロック的置換メチルセルロース誘導体、グルコース、セロビオース誘導体の抗プリオン活性や発症抑制効果を体系的に研究した例はなかったが、無水グルコース単位の水酸基へのメチル基の導入位置の違いにより、それら化合物の抗プリオン活性や発症抑制効果は明らかに異なった。本年度得られた知見は、更に高い効果を持つメチルセルロース系化合物の開発に繋がると考える。

E．結論

化学構造の明確な位置特異的置換、あるいは位置特異的かつブロック的置換メチルセルロース誘導体および関連低分子化合物、合計 47 種を化学合成した。その結果、プリオン感染細胞を用いた in vitro 実験により GG-23MC

が、またプリオン感染マウスを用いたin vivo実験により、高分子化合物では23MC構造、低分子化合物ではメチル化単糖が効果的であることがわかった。

F．健康危機情報

なし

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H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

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厚生労働科学研究費補助金（難治性疾患克服研究事業）
平成２２年度　分担研究報告書

セルロース誘導体の修飾による最適化に関する研究

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研究協力者：村瀬　勝俊　　名糖産業株式会社
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研究要旨
従来のセルロース誘導体（ＣＥ）よりも発症抑制効果が高いＣＥの修飾体を選抜するとともに、低分子化ＣＥ修飾体を検討して、最も効果のあるＣＥの化学的特徴を明らかにしようとした。その結果、側鎖の脂肪鎖修飾やコレステロール修飾が効果を上げること、また還元末端の還元化が効果を上げることが示された。

Ａ．研究目的
これまでの研究で体内（皮下、腹腔内、静脈内、脳室内）への投与に比べ、経口投与ではセルロース誘導体（ＣＥ）の予防効果が低下するため、各種ＣＥ修飾体を作製し、経口投与での効果をプリオン感染マウスで検討することを計画していた。しかし、経口投与では用いる化合物が相当量必要であり、実験結果を得るまでに３年以上を要するなどの困難がある。そこで、まず従来の皮下投与でより効果の高いＣＥ修飾体を選抜するとともに、低分子化ＣＥ修飾体を検討して、最も効果のあるＣＥ修飾体の化学的特徴を明らかにしようとした。

Ｂ．研究方法
メチルセルロースを基本構造として、還元末端が非還元型、還元型、酸化型の修飾、メチル基以外の側鎖修飾としてヒドロキシプロビル基、ジエチルアミノエチル基、カルボキシメチル基、プロピル基、ステアリングリシジルエーテル基の導入を行った化合物を合成した。また、ヒドロキシプロピルメチルセルロースを基本構造として、未修飾 OH 基をコレステロールあるいは硫酸基で修飾した化合物を合成した。さらに、６糖セルビオースより還元末端が非還元型と還元型のメチルセルビオースを合成した。
これらの化合物をプリオン感染マウスに同量投与を行い、発症予防効果を検討した。
（倫理面の配慮）
動物実験は施設の動物実験委員会の許可を受け、動物実験指針を遵守して

行った。

C．研究結果

最も効果が高かったのはステアリングリシジルエーテル基を導入したメチルセルロースであった。また、ヒドロキシプロピルメチルセルロースを基本構造としたものでは、コレステロール基を導入したものであった。疎水性基の導入がＣＥの効果を向上させると考えられたが、疎水性が強いプロピル基で置換したものは置換度が高くなるに従って効果が低下する傾向にあった。また、ジエチルアミノエチル基やカルボキシメチル基を導入して、プラスあるいはマイナスのチャージを持たせた化合物は、未修飾化合物やプロピル基を導入した化合物よりも効果があった。一方、還元末端は非還元型が効果は高く、酸化型はやや効果は低下した。また、分子サイズが小さい６糖セルビオースでも非還元型が効果は高かった。

D．考察

効果が高いＣＥの特徴としては、化合物の物性として親水性であると同時に、側鎖や還元末端が分解を受けにくい構造であることが明らかとなった。動物に投与したＣＥが長期間にわたり泡沫状貪食細胞内に残ることが、ＣＥの驚異的な予防効果と関係があるのであれば、マクロファージに取り込まれやすく、長期間にわたり分解されにくい構造であることが予防効果の向上と関係するはずであり、今回の研究結果と矛盾はしない。今後より詳細に、部位特異的な側鎖修飾と治療予防効果の関係を明らかにすることで、一層効果が高いＣＥ化合物を創製できる可能性がある。一方、毒性症状との関係については、今後検討する必要がある。また腸管からマクロファージへの取り込みを高める工夫としてマンノース化などを検討しているが、その修飾により予防治療効果や毒性がどのような影響を受けるのかも検討する必要がある。

E．結論

予防治療効果が高いＣＥ化合物の特徴として、親水性であると同時に、側鎖や還元末端が分解を受けにくい構造であることが明らかとなった。

F．健康危険情報

なし

G．研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

研究成果の刊行に関する一覧表

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雑誌

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Anti-prion activity of protein-bound polysaccharide K in prion-infected cells and animals

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ABSTRACT

Protein-bound polysaccharide K (PSK) is a clinical immunotherapeutic agent that exhibits various biological activities, including anti-tumor and anti-microbial effects. In the present study, we report on the anti-prion activity of PSK. It inhibited the formation of protease-resistant abnormal prion protein in prion-infected cells without any apparent alterations in either the normal prion protein turnover or the autophagic function in the cells. Its anti-prion activity was predominantly composed of the high molecular weight component(s) of the protein portion of PSK. A single subcutaneous dose of PSK slightly but significantly prolonged the survival time of peritoneally prion-infected mice, but PSK-treated mice produced neutralizing antibodies against the anti-prion activity of PSK. These findings suggest that PSK is a new anti-prion substance that may be useful in elucidating the mechanism of prion replication, although the structure of the anti-prion component(s) of PSK requires further evaluation.

1. Introduction

Transmissible spongiform encephalopathies or prion diseases include Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome, and familial fatal insomnia in humans. All of these diseases are fatal and characterized by the accumulation of protease-resistant abnormal prion protein, prion, in the brain and lymphoid tissues. Although the precise mechanism of prion propagation remains uncertain, it is commonly assumed that protease-sensitive normal cellular prion protein (PrPc) is converted into protease-resistant abnormal prion protein (PrPres) by direct interaction of the two isoforms [1].

As the occurrences of variant CJD and iatrogenic forms of CJD increase, various efforts to find effective anti-prion remedies have been reported [2]. However, thus far, only a few of anti-prion compounds have been utilized in patients with prion diseases on a trial basis and have reportedly failed to halt the disease progression [3,4]. In these efforts, clinical drugs have been screened for anti-prion activity because they can quickly be brought onstream to be applied to patients with prion diseases [5,6]. We previously tested drugs clinically used in Japan and found one immunotherapeutic agent with anti-prion activity.

Here, we report on the anti-prion activity of the immunotherapeutic agent, protein-bound polysaccharide K (PSK), in prion infected cells and animals, and we discuss the mechanism of its prion-inhibitory activity. PSK is known to be effective in conjunction with chemotherapy and/or radiotherapy for some types of cancers through various immunological modulations [7], and also has anti-microbial activities [8], but this is the first time that its anti-prion activity has been revealed.

2. Materials and methods

2.1. Psk and other immunotherapeutic agents

PSK, a protein-bound polysaccharide preparation extracted from *Coriolus versicolor* with hot water, containing 30% protein and 70% sugar, was obtained from Kureha Co., Ltd. (Tokyo, Japan). The sugar portion is mainly composed of glucans with β-1,4 bonds in the main chain and β-1,3 or β-1,6 bonds in the side chain; the latter binds to the protein portion through O- or N-glycosidic bonds [9]. The protein predominant preparation of PSK (PSK-Pro; 87% protein and 13% sugar) and the sugar predominant preparation of PSK (PSK-Sug; 5% protein and 95% sugar) were also obtained from Kureha. They were prepared by chemical modification of PSK as described previously [10]. Other immunotherapeutic agents clinically utilized in Japan (Lentinan, Schizophyllan, Ubenimex, and Picibanil) were also obtained from their respective pharmaceutical companies. All samples were dissolved in sterile distilled water and stored at 4 °C until use.

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2.2. Cells and PrP analyses

We used mouse neuroblastoma cells that were either uninfected (N2a cells) or persistently infected with RML scrapie prion (ScN2a cells), 22L prion (N167 cells), or Fukuoka-1 prion (F3 cells), as described previously [11,12]. These cells were cultured in the presence or absence of test samples at 37 °C for 3 days, and confluently grown cells were lysed with lysis buffer. For analysis of PrPres, cell lysate was treated with 10 mg/mL proteinase K at 37 °C for 30 min, and PrPres was precipitated by centrifugation and suspended in a sample loading buffer. For the analysis of other proteins, cell lysate was used without protease treatments and mixed with a concentrated loading buffer. Immunoblotting analysis was performed using standard methods as previously described [11] with an anti-PrP monoclonal antibody (MAb) SAF83 (1:5000), anti-GAPDH MAb (1:5000), anti-β-actin MAb (1:1000), or anti-LC3 MAb (1:2000; nanoTools), followed by alkaline phosphatase-conjugated anti-mouse antibody (1:20,000; Promega). Immunoreactive signals were detected using CDP-Star detection reagent (GE Healthcare). Flow cytometric analysis and floatation assays were also performed to examine PrPc expression in the cells as described previously [11,13].

2.3. Fractionation of PSK constituents

Fractionation of PSK-Pro constituents by gel filtration method was performed in 10 mg of PSK-Pro dissolved in 1 mL of elution buffer (20 mM Tris–HCl, pH 7.5 containing 500 mM NaCl). After ultracentrifugation at 100,000g at 4 °C for 30 min, the supernatant was applied to an equilibrated column (1.6 cm diameter and 100 cm length) packed with Sephacryl S-200 HR (GE Healthcare) and eluted with elution buffer. Each fraction containing 6 mL eluate was collected.

2.4. Animal studies

Eight- to ten-week-old Tga20 mice overexpressing murine PrPc [14] were used to analyze the effectiveness of PSK and other immunotherapeutic agents *in vivo*. On the day following a single subcutaneous administration of the maximum tolerated dose of each drug or of vehicle alone, the mice were intraperitoneally infected with 100 μL of 1% (wt/vol) brain homogenate of RML prion. The duration from the infection to the terminal stage of the disease was measured as survival time.

To analyze the anti-PSK neutralizing factors in PSK-dosed mice, sera were collected from the mice 1 month after a single subcutaneous dose of 100 mg PSK or vehicle alone. After clarification of the sera by ammonium sulfate precipitation, IgG fractions were obtained using a protein G column (GE Healthcare). The animal experiments described here were performed with the approval of the Animal Experiment Ethical Committee of Tohoku University.

2.5. Statistical analysis

Data were evaluated using nonparametric Mann–Whitney or Kruskal–Wallis test in the subcutaneous PSK-dosing experiment or the oral multiple PSK-dosing experiment, respectively. Differences were considered significant for p values <0.05.

3. 3. Results

3.1. PSK effects in prion-infected cells

To analyze the inhibitory activity of PSK against PrPres formation in prion-infected cell models, we analyzed PrPres levels in three types of persistently prion-infected cell lines treated with PSK for 3 days. We found that PSK reduced the PrPres levels of all the cells in a dose-dependent manner (Fig. 1A). The 50% effective concentration value (EC_{50}) for PSK in ScN2a cells, N167 cells, and F3 cells was 7.68 μg/mL, 14.45 μg/mL, and 19.01 μg/mL, respectively. Four other clinically used immunotherapeutic agents (Lentinan, Schizophyllan, Ubenimex, and Picibanil) were also tested in ScN2a cells, which are the most sensitive to screening for anti-prion activities [11,12]. However, these four drugs did not exhibit the inhibitory activity against PrPres formation even when tested at maximum tolerated doses (data not shown).

To determine the efficacy of PSK, ScN2a cells were treated with 20 μg/mL PSK for 7 days, and subsequently the cells were cultured in the absence of PSK for a further 27 days. The results showed that PrPres levels did not recover to the detection limit of immunoblotting, suggesting irreversible inhibition of PrPres formation by PSK (Supplementary Fig. 1A). On the other hand, the postseeding 48 h treatment of ScN2a cells with 20 μg/mL PSK reduced the PrPres level to almost the same level (approximately 37% of the untreated ScN2a cells) as the 72 h treatment, while the postseeding 24 h treatment reduced only about 43% of the untreated ScN2a cells (Fig. 1B). This indicates that the inhibition of PrPres formation by PSK is dependent on the treatment duration to some extent. We also examined the direct effects of PSK on PrPres in the cell lysate. Incubation of ScN2a cell lysate with PSK before or after proteinase K treatment did not alter the PrPres levels (Supplementary Fig. 1B). This suggests that PSK does not either degrade PrPres directly or make PrPres protease-sensitive.

3.2. PSK influence on PrPc profiles and autophagy

Because alterations of the PrPc turnover in the cells cause modification of PrPres levels, we analyzed the PrPc levels in N2a cells treated with PSK. Total PrPc and cell surface PrPc levels were not apparently affected by PSK (Fig. 2A and B). Next, we examined the distribution of PrPc in the lipid raft microdomain by floating assay (Fig. 2C), and no significant difference was observed between PSK-treated and untreated cells. These results suggest that PSK does not modify the PrPc turnover.

Because it has been reported that the induction of cellular autophagy facilitates the clearance of aggregate-prone proteins [15], we analyzed the expression levels of LC3-II, an autophagosome formation marker, in PSK-treated ScN2a cells. As shown in Fig. 2D, LC3-II expression levels were not altered in the PSK-treated cells, while treatment of the cells with trehalose, which is known to induce cellular autophagy, caused both a decrease in PrPres levels and an increase in LC3-II expression levels, as reported previously [16]. This indicates that the PSK anti-prion activity is not related to autophagy induction.

3.3. PSK effects in prion-infected mice

We next addressed whether PSK could prolong the survival time of prion-infected mice. This was investigated by intraperitoneally inoculating Tga20 mice with RML prion strain, followed by a single subcutaneous administration of 100 mg PSK. PSK showed slight but significant prolongation of survival period in intraperitoneally infected mice in two independent experiments (128 ± 17.1 days in PSK ($n = 6$) versus 110 ± 3.8 days in control ($n = 6$) in experiment 1 ($p < 0.05$); 100 ± 6.1 days in PSK ($n = 5$) versus 93 ± 2.2 days in control ($n = 5$) in experiment 2 ($p < 0.05$)). However, when PSK was administered orally *ad libitum*, from intraperitoneal infection to disease terminal, mixed with feed at the following doses: 1%, 2%, and 4% with weight in the feed corresponding, respectively to *ca.* 1.5, *ca.* 3.0, and *ca.* 6.0 g/kg body weight/day, and no significant prolongation of survival period was observed

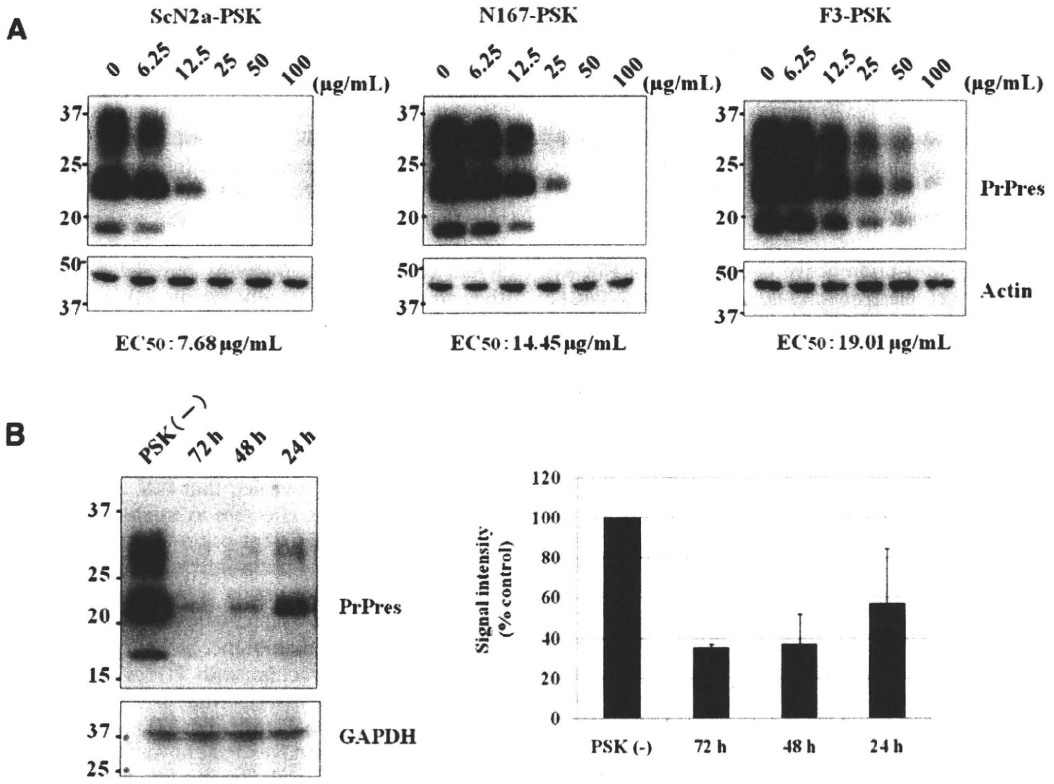

Fig. 1. Inhibitory effects of PSK on PrPres formation in various prion-infected cell models. (A) Each model of persistently prion-infected cells was treated with the indicated concentration of PSK for 3 days, and the PrPres levels were analyzed by immunoblotting. Molecular size markers on the left side of the immunoblots are shown in kilodaltons. (B) Incubation duration-dependent effects of PSK on the PrPres levels were examined by treating ScN2a cells with 20 μg/mL of PSK for 24, 48, or 72 h prior to the harvest.

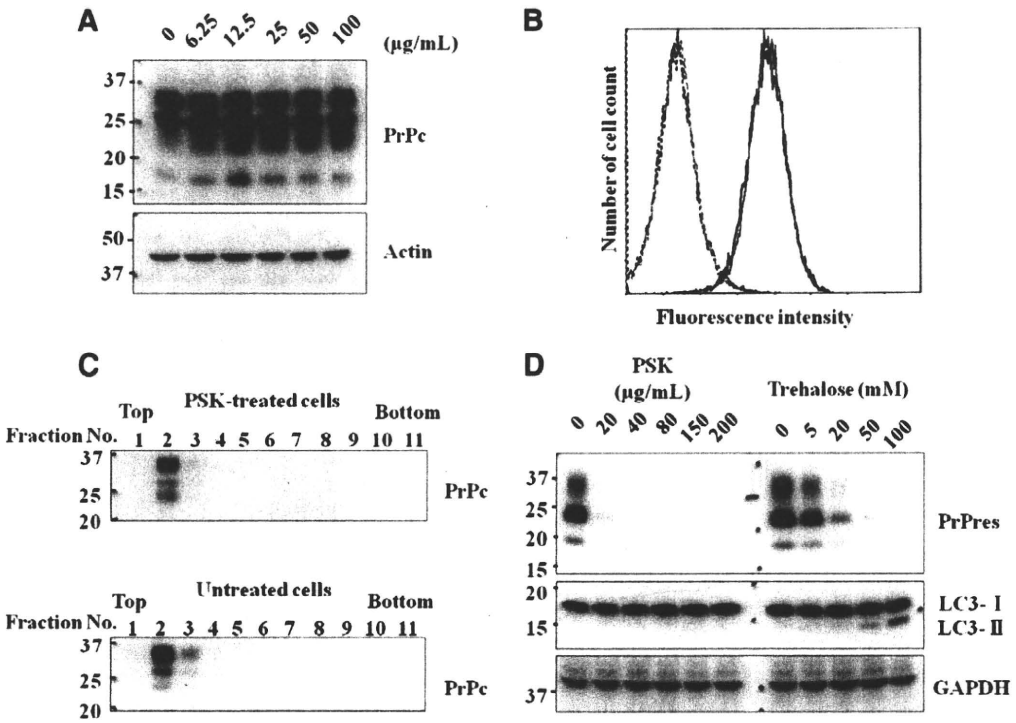

Fig. 2. No apparent effects of PSK on the turnover of PrPc and cellular autophagy. (A) Immunoblot analysis of total PrPc was performed in noninfected N2a cells treated with the indicated concentration of PSK for 3 days. (B) Flow cytometric analysis of PrPc on the cell surface was performed in noninfected N2a cells treated with 20 μg/mL of PSK for 3 days. Gray and black lines indicate PSK-treated cells and untreated cells, respectively. The broken line peaks on the left show their respective isotype controls. (C) Analysis of the distribution of PrPc in the lipid raft was performed by floatation assay in noninfected N2a cells treated with or without 20 μg/mL of PSK. (D) Involvement of autophagy induction was examined in ScN2a cells treated with the indicated concentration of PSK or trehalose for 3 days. Trehalose was used as a positive control of autophagy induction. Both LC3-II and PrPres levels were analyzed by immunoblotting.

(data not shown). These results suggest that PSK is ineffective in prolonging survival period in prion-infected mice when orally administered, as is normal in clinical situations.

We also similarly tested other immunotherapeutic agents in intraperitoneally prion-infected Tga20 mice. No significant prolongation of survival period was observed, even at the maximum tolerated doses (data not shown).

3.4. Anti-prion components in PSK

We tried to separate anti-prion active components in PSK by using polyacrylamide gel electrophoreses, such as SDS–PAGE, DOC–PAGE, and native PAGE, but PSK constituents were not successfully separated as distinct bands, even after PSK was dissolved in 8 M urea or 6 M guanidine hydrochloride (data not shown). However, the anti-prion activity of PSK was reduced when PSK was treated with a protease and/or a protein denaturant (Fig. 3A). Then, we tried to determine whether anti-prion components in PSK were in the PSK-Pro portion (sugar:protein = 13:87) or the PSK-Sug portion (sugar:protein = 95:5). As shown in Fig. 3B, the prominent anti-prion activity was observed in PSK-Pro. Its EC_{50} value was 3.14 μg/mL, although that of PSK-Sug was more than 50 μg/mL. To further study the active components of PSK-Pro, we analyzed PSK-Pro using Sephacryl S-200 HR gel filtration chromatography. PSK-Pro showed a single sharp peak around 150 kDa, estimated with silver staining after SDS–PAGE, which was eluted in Fraction-2 (Supplementary Fig. 1C, Fig. 3C). This high molecular weight fraction sample of PSK-Pro had strong inhibitory activity against the PrPres formation in ScN2a cells (Fig. 3D). The results indicate that a *ca.* 150 kDa-sized protein-related substance contains the main anti-prion component(s) of PSK.

3.5. Anti-PSK neutralizing antibody in PSK-injected mice

We speculated why the effect on survival periods in PSK-injected mice was not pronounced. Then, from the findings described above, we presumed that the mice injected with PSK could produce antibodies against PSK constituents that antagonize the anti-prion activity of PSK. To examine this inference, IgG fractions were obtained from the Tga20 mice 1 month after a single subcutaneous injection of 100 mg of PSK. After PSK was incubated with purified IgG fractions at 37 °C for 3 h, its anti-prion activity was tested in ScN2a cells. The IgG fractions purified from PSK-injected mice showed neutralizing activity against PSK, but those from untreated control mice did not (Fig. 4). This result suggests that the effects of PSK in mice might be antagonized by anti-PSK neutralizing antibodies produced in PSK-injected mice.

4. Discussion

In this study we revealed that PSK, a clinically used immunotherapeutic agent, is effective in inhibiting the PrPres formation in cell models of persistent prion infection as well as prolonging the survival period of prion-infected animals. The precise mechanism of the PrPres formation is still enigmatic, but it is assumed that PrPc molecules, which mainly localize in the lipid raft microdomain of the cell membrane, are altered to PrPres conformers through direct interaction with PrPres molecules [17–19]. Thus far, it has been demonstrated that lactoferrin [20] and some types of anti-PrP monoclonal antibodies [21] inhibit PrPres formation by affecting the PrPc turnover. PSK, however, neither altered the total or cell surface PrPc levels nor modified the localization of PrPc in the lipid raft microdomain. This suggests that the anti-prion

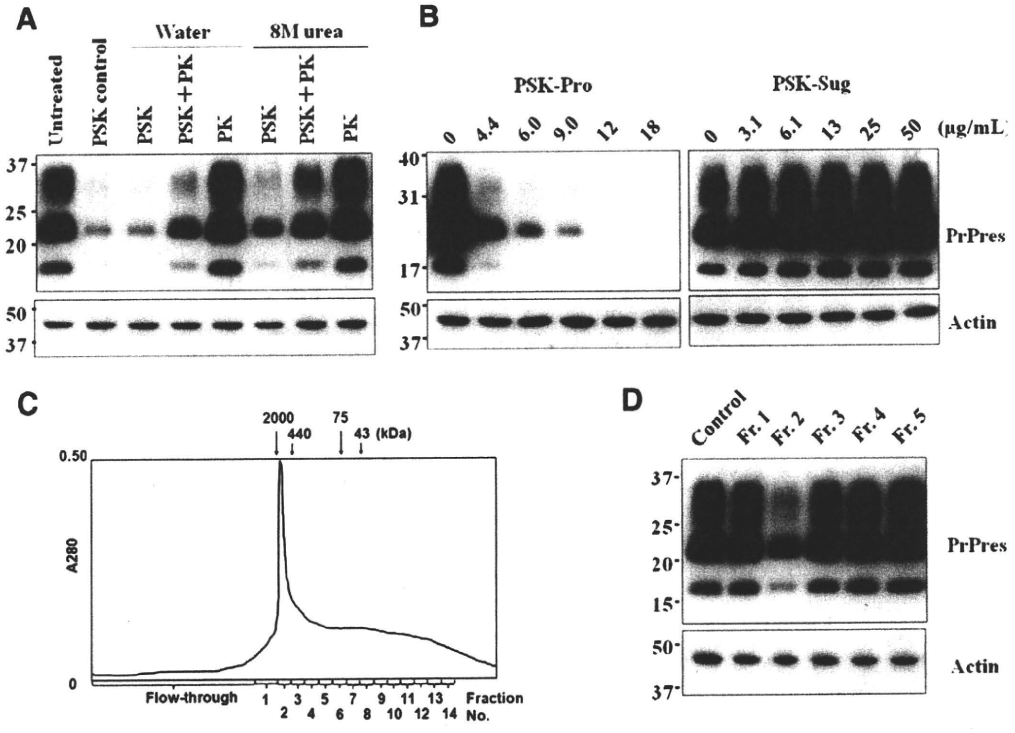

Fig. 3. Effects of PSK-Pro and PSK-Sug on the PrPres formation in ScN2a cells. (A) Immunoblot analysis of PrPres was performed in ScN2a cells treated for 3 days with 20 μg/mL of modified PSK prepared by protease digestion in the presence or absence of protein denaturant as followed. PSK in water or 8 M urea was digested with 700 μg/mL of proteinase K at 37 °C for 7 days and was dialyzed with water after inactivation of the protease by heating at 95 °C for 10 min. PSK control and untreated control in the immunoblot indicate the PrPres samples from the cells treated with freshly prepared 20 μg/mL PSK or vehicle only, respectively. (B) Immunoblot analysis of PrPres was performed in ScN2a cells treated with the indicated concentrations of PSK-Pro or PSK-Sug for 3 days. (C) Gel filtration chromatography of PSK-Pro was performed with Sephacryl S-200HR. Molecular size markers in kilodaltons are shown as arrows on the top of the chromatograph. (D) Anti-prion activities of fractionated PSK-Pro samples were examined by analyzing PrPres levels in ScN2a cells treated with the samples in a concentration of 3.75% in the culture medium for 3 days.

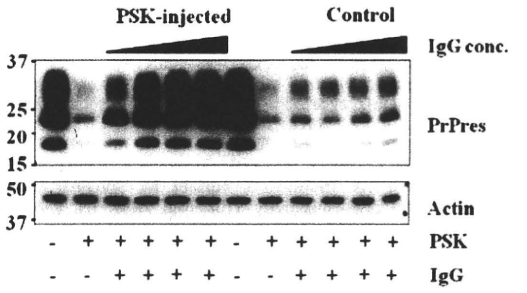

Fig. 4. Anti-PSK activity of IgG purified from PSK-injected mice. Anti-prion activity of PSK in ScN2a cells was neutralized by the addition of IgG purified from PSK-administered mice but not from vehicle-administered control mice. IgG fractions were purified from Tga20 mice subcutaneously administered 100 mg of PSK (PSK-injected) or only vehicle (control). ScN2a cells were treated with 20 μg/mL PSK alone or with a mixture of 20 μg/mL PSK and purified IgG fractions for 3 days.

activity of PSK is not attributable to any apparent alterations in the PrPc turnover of the cells.

On the other hand, cellular autophagy is known to facilitate the clearance of abnormally aggregated proteins [15]. Aguib et al. have reported that either lithium or trehalose enhance the clearance of PrPres molecules in prion-infected cells through the induction of cellular autophagy [16]. The present study, however, suggests that PSK does not enhance this clearance pathway of PrPres molecules, because PSK treatment of prion-infected cells did not induce autophagosome formation. Furthermore, the direct interaction of PrPres molecules with anti-prion compounds such as tetracycline and doxycycline [22] has also been reported to facilitate the degradation of PrPres molecules by protease digestion *in vitro*, but PSK treatment of ScN2a cell lysate before or after protease digestion did not influence the PrPres levels. This indicates that PSK does not have activities that change PrPres molecules into PK-sensitive conformers or proteolytically degrading PrPres molecules. Then, taken together with the findings of the present study in the cell models, it is likely that PSK inhibits the conversion of PrPc to PrPres directly or indirectly through, as yet, unrevealed cellular factors.

PSK is orally administered in combination with other chemotherapeutic agents to cancer patients to enhance their immunity to cancer. The anti-cancer activity of orally administered PSK has been confirmed using some animal models [23]. However, orally administered PSK did not prolong the survival periods of intraperitoneally prion-infected mice in the present study. This suggests that high molecular weight constituents of PSK, which were shown to be responsible for the anti-prion activity in prion-infected cells in the present study, are degraded through the process of absorption from the digestive tract. In fact, it has been reported that almost all of the metabolites of PSK in the urine, feces, and bile of animals orally administered PSK consist of low molecular weight components [24]. On the other hand, subcutaneously administered PSK was effective in prolonging the survival period of prion-infected mice, possibly because it might be absorbed into the circulation through surrounding lymph ducts and microvessels without degradation.

In addition to the discrepancy in the effects of PSK in prion-infected mice between clinically used oral dosing and subcutaneous dosing, three other lines of evidence in the present study suggest that the anti-prion activity of PSK is independent of the primary actions of PSK as an immune system boosting anti-cancer drug. The first is that the anti-prion activity of PSK was mainly present in the high molecular weight protein-related constituents, which are totally different from the main components, polysaccharides, responsible for the actions of not only PSK but also other immunotherapeutic agents, such as Lentinan and Schizophyllan, tested in the present study [23]. The second is that neutralizing IgG antibodies against the anti-prion activity of PSK were produced in the mice that were subcutaneously administered PSK. Although some reports have shown that anti-PSK polyclonal antibodies that can be used to analyze the pharmacokinetics of PSK are produced in immunized rabbits, it has never been reported that antibodies capable of neutralizing the anti-cancer effects of PSK were produced in the mice [25]. In addition, low affinity antibodies are more likely to be produced than high affinity neutralizing antibodies if the antigens are polysaccharides. The third is that other immunotherapeutic agents tested in this study showed no apparent effectiveness in both cell and animal models of prion infection. Therefore, the anti-prion activity of PSK is unlikely to be mediated by activation of innate immunity as previously reported in CpG oligonucleotide [26].

In conclusion, the effectiveness of PSK in both prion-infected cells and prion-infected mice was demonstrated in the present study. It was suggested that PSK exhibits the anti-prion activity through a different mechanism from those already known in its role as an immune system boosting anti-cancer drug. We could not test the effects of PSK-Pro and PSK-Sug in prion-infected animals because of limited test sample availability, but it is evident that PSK is a new type of anti-prion substance that may be useful for elucidating the mechanism of prion replication. However, the structure of anti-prion component(s) of PSK requires further evaluation.

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.2011.01.030.

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