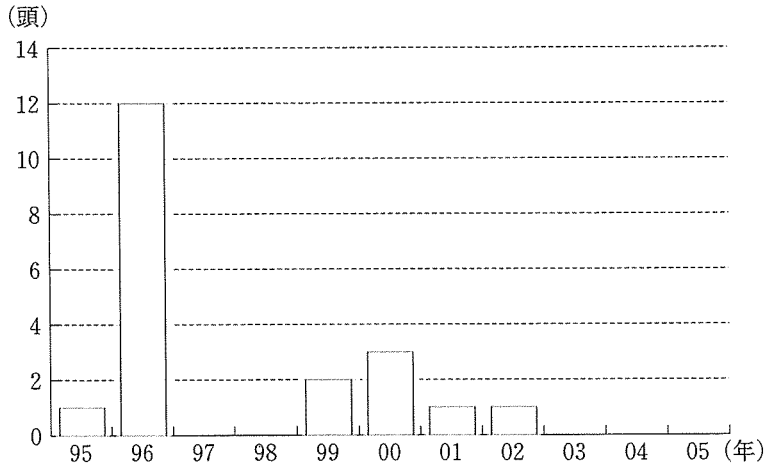

図2 我が国で摘発されたBSE感染牛の出生年の分布

他の欧州諸国では，2000年もしくは2001年以降，BSE感染牛の数が著しく増加している．この増加は能動的サーベイランスの導入により，BSE感染と診断されたウシの数が増加した結果である．例えば，2003年のフランスの137頭のBSE感染牛の内訳は，臨床症状からBSEが疑われたウシで13頭，死亡牛などの高リスクグループの能動的サーベイランスで87頭，と畜場でのスクリーニングで37頭である．この数字は，BSE汚染状況の正確な把握には，と畜場におけるスクリーニング，およびリスク牛の能動的サーベイランスが必要であることを示す格好の例である．欧州での発生も2003-4年頃から減少傾向に転じている．

一方，我が国やイスラエルでのBSEの発生，北米におけるBSEの発生が示すように，BSEの感染源は英国，欧州にとどまらず，世界各地に広がったと認識する必要がある．今後，国や地域のBSE汚染状況を明らかにするためには，能動的サーベイランスの実施が必要である．

b. 我が国でのBSE発生状況

図2に，我が国で摘発されたBSE感染牛の出生年を示した．これまでに20頭が摘発されているが，1996年生まれのウシで最も多い．英国におけるvCJDの発生を受けて，我が国では1996年に肉骨粉を牛の飼料に使用しない行政指導を行った．しかし，最近のBSEスクリーニング/サーベイランスでは1999年および2000年生まれのウシからBSE感染牛が計5頭摘発されている．したがって，この時期にもBSE汚染源が存在していたことになる．BSEの潜伏期は平均4-8年であり，1999-2000年生まれのウシはこれから好発年齢に達する．また，1997-98年生まれのウシではBSE感染牛が確認されていないが，この時期の汚染状況を判定するには，もう少しスクリーニング/サーベイランス結果の蓄積を待つ必要がある．

c. vCJDの発生状況

表3に英国におけるヒトプリオン病の発生状況とvCJD患者が確認された国を示した[7]．2005年8月までに英国では157人の患者が確認されているが，増加傾向は認められていない．英国以外ではフランスなどでvCJD患者が確認されている．2005年に，我が国でも，英国およびフランスに短期間滞在経験のある男性がvCJDに罹患していたことが判明した．

vCJDでは，異常型プリオン蛋白質(PrP^{Sc})が扁桃や盲腸の粘膜下リンパ濾胞などの末梢リンパ系組織に蓄積し，容易に検出できる[8,9]．一方，vCJD以外のヒトプリオン病の場合は，リンパ系組織からPrP^{Sc}は容易には検出されない．英国で盲腸摘出術あるいは扁桃除去術により摘出された盲腸あるいは扁桃を検索した結果，12,674検体中3検体がPrP^{Sc}陽性であった．この結果を英国の人口に外挿し，3,800人程度の人がvCJDの潜伏期にある可能性を指摘する報告もある[10]．

表3 イギリスのヒトプリオン病発生状況と，各国におけるvCJD発生状況

年	孤発性CJD	変異CJD	医原性CJD	遺伝性プリオン病
95	35	3	4	5
96	40	10	4	6
97	60	10	6	5
98	63	18	3	5
99	62	15	6	2
00	50	28	1	3
01	28	20	4	5
02	72	17	0	5
03	77	18	5	6
04	49	9	2	4
05	10	2(7)	0	1

括弧内の数字は存命の患者数.

国	vCJD患者数
フランス	13
アイルランド	2
イタリア	1
アメリカ	1
カナダ	1
オランダ	1
日　本	1
スペイン	1
ポルトガル	1

3. 新たな課題

a. 非定型BSE，非定型スクレイピーの存在

野外に存在するヒツジスクレイピーには様々な"株"が存在することが知られている．一方，BSEは一つの株と考えられてきた．しかし，2003年にイタリアで，これまでのBSEとはPrP^{Sc}の脳内蓄積部位，生化学性状(糖鎖型，分子量)が明らかに異なるBSE発症牛が報告された[11]．時を同じくして，日本[12]，フランス[13]でもPrP^{Sc}の生化学性状が従来型と異なるBSE感染牛が報告された．

イタリアの非定型BSE症例は，PrP^{Sc}の生化学性状，脳内のPrP^{Sc}の分布など多くの情報が得られている．典型的なBSEではPrP^{Sc}の糖鎖型は二糖鎖型が優勢であるのに対して非定型例では一糖鎖型が優勢である．典型的なBSEはPrP^{Sc}の蓄積は延髄閂部，視床下部，視床で認められる．延髄閂部でPrP^{Sc}の蓄積量が多いのは，経口ルートで侵入したプリオンが，迷走神経を経て背側迷走神経核に到達することを反映している．一方，イタリアの非定型BSEでは，視床で蓄積が最も多く，次いで嗅球，海馬などで多い．非定型BSE症例が感染によるものと仮定すると，PrP^{Sc}分布の違いは，プリオンが中枢神経系組織へ到達する経路が典型的なBSEとは異なる可能性を示唆している．

ヒツジスクレイピーでも，従来知られているスクレイピーとは病型が異なるものが発見されている．従来型のスクレイピーではPrP^{Sc}の蓄積は延髄閂部で最も高い．一方，新たに発見されたスクレイピーでは閂部におけるPrP^{Sc}の蓄積が低く，PrP^{Sc}の蛋白分解酵素抵抗性も弱い[14]．ノルウェーで初めて発見され，その後，ドイツやフランスでも見つかっている[15]．従来型のヒツジスクレイピーはPrPアミノ酸型171Q/Q(コドン171Q/Q)で多く発生し，171Q/Rで少なく，171R/Rを有するヒツジはスクレイピー抵抗性と考えられていた．しかし非定型ヒツジスクレイピーは171Q/Rのヒツジで発生が多く，171R/Rのヒツジでも感染が認められている[15]．

b. BSE病原体に感染した可能性のあるヤギの摘発

フランスでBSE病原体の性状と区別できないプリオンに感染したヤギが発見された[16]．このヤギの中枢神経系組織に蓄積したPrP^{Sc}はBSE感染牛およびBSE実験感染ヤギのものと類似していたが，スクレイピー感染ヤギのものとは異なっていた．また，このヤギの材料をマウスへ伝達した場合の神経病変分布とマウスの脳で産生されたPrP^{Sc}も，BSE実験感染綿山羊の材料をマウスに伝達した場合と区別できなかった．BSE病原体が飼料などを介してヤギに感染したのか，あるいはこのようなプリオン野外株がヤギに存在していたのかを結論づけることはできない．しかし，BSE病原体様のプリオン

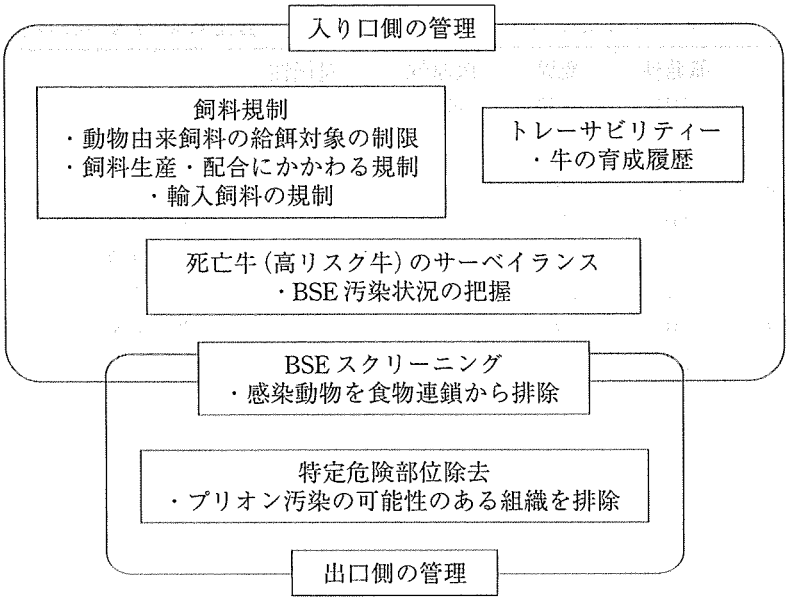

図3 我が国のBSE対策

が小反芻動物に存在したことから，小反芻動物由来の食肉などの安全性評価と管理措置を再検討する必要がある．

c. 輸血によるvCJDの伝播

これまでの動物実験における成績を総合すると，リンパ系装置にPrP^{Sc}が検出される場合は血液中にもプリオンが存在すると考えるのが妥当である．vCJDでは神経系以外にも，扁桃，盲腸の粘膜下リンパ濾胞などのリンパ系組織でもPrP^{Sc}が検出されることから，輸血によるvCJDの伝播が危惧されていたが，現実のものとなった．1例(PrPコドン129: M/M)は，献血の3年半後にvCJDを発症したヒト由来の赤血球を輸血された6年半後にvCJDを発症した[17]．もう1例(PrPコドン129: M/V)は，献血の18カ月後にvCJDを発症したヒト由来の赤血球を輸血された人で，5年後に腹大動脈瘤破裂により死亡した．この患者の脾臓からPrP^{Sc}が検出されたが，脳からは検出されなかった[18]．この患者が将来vCJDを発症したかは知ることができない．しかし，これらの事例から，潜伏期にあるvCJD感染者の血液中に輸血により伝播し得る量のプリオンが存在することは事実である．

4. BSE対策

ヒトに感染することが判明している動物プリオン病はBSEのみであるので，本稿では，BSEが動物からヒトに感染することを防止するための対策について述べる．

a. 出口側の管理

我が国のBSE対策の柱は，①食用に供されるウシのBSEスクリーニング，②特定危険部位の除去，③リスク牛(死亡牛)のBSEサーベイランス，④飼料規制，⑤トレーサビリティー，である(**図3**)．このうちBSEスクリーニングと特定危険部位の除去は，BSE感染牛を流通から排除するための措置，言い換えると出口側の管理措置であり，ウシからヒトへの直接的な感染阻止に主眼が置かれている．

我が国の食肉検査で実施されているBSE検査はBSE感染牛の排除が目的であり，スクリーニングとして位置づけられている．どのような方法にも検出限界があることから，BSEスクリーニングでは検出限界以下のプリオンしか蓄積していないウシは陰性と判定される．一方，BSE感染牛の組織に分布するプリオンの99%が，脳，脊柱，眼球，三叉神経節，回腸遠位部の特定危険部位に存在すると推計されているので，特定危険部位の除去は，食肉の安全性を保証する有効な方法と考えられる．しかし，除去方法や除去効率に問題があること，また，病末期のウシでは末梢神経など特定危険部位以外の

組織にもプリオンが存在することから[19]，特定危険部位の除去だけでも不十分である．現在，我が国では，BSEスクリーニングと特定危険部位の除去が双方の欠点を互いに補い，高度に食肉の安全性を保証している．

この管理措置は，BSEが国内に侵入したことに対応するための緊急措置であり，その実行に莫大なお金と労力を使用している．食肉の安全性を効率的に保証するためには，後述する入口側の管理，すなわち，BSEに感染していない動物を産生するための管理措置に力を注ぐ必要がある．

b. 入口側の管理

BSEは自然状態ではウシからウシへ感染する可能性が非常に低いことから，BSE汚染源の遮断を継続的に実施することで，BSEフリーのウシを飼育・生産することは可能である．このためには，リスク牛や綿山羊の能動的サーベイランスによる動物プリオン病の発生状況の把握，徹底した飼料規制によるBSE汚染源の遮断，およびトレーサビリティー制度によるウシの育成履歴の記録，などの入口側の管理措置が重要となる．

BSE低汚染地域では，臨床症状からBSEを疑うことは困難である．したがって，汚染状況の正確な把握にはリスク牛の能動的サーベイランスの継続が必須である．前述したBSEスクリーニングは汚染状況の正確な把握にも大きく貢献している．今後，最も重視しなければならないのは飼料規制の遵守である．国内でのリサイクル，飼料の配合，飼肥料の輸入，飼肥料の使用など，それぞれの現場で規制の遵守が強く求められる．BSEの根絶は飼料規制の有効性にかかっており，関係各位のモラルが今後のBSEコントロールを左右することになる．

おわりに

PrP^{Sc}のオリゴマーが感染因子プリオンの本体であることが確定的となり，難解な病原体の謎も徐々にひもとかれつつある[20]．しかし，プリオンおよびプリオン病を理解するためには，プリオン増殖の分子機構解明，神経変性機序の解明など，一層の基盤研究の進展が必要である．同時に，食・医療などの安全性を確保するためには，応用面の研究推進も必須である．我が国では，今後数年間はBSE感染牛が散発的に摘発されることが予想されることから，BSEスクリーニング/サーベイランス，特定危険部位の除去を継続する必要がある．一方，BSE清浄地に復帰するために，入口側の管理に力を注ぐ必要がある．BSEはウシからヒトへ感染が拡大し，更に輸血によりヒトからヒトへ感染が広がることも明らかとなった．医薬品原料などのプリオン汚染の評価，その他の汚染源の摘発のためには，高感度プリオン検出技術の開発が望まれる．また，vCJDを含めて，ヒトプリオン病の治療法はないので，その開発も重要な課題である．

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●特集　感染症病原体と細胞膜との関わり

異常型プリオン蛋白質の生合成と伝達

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Biosynthesis and Transmission of Abnormal Prion Protein

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Transmissible spongiform encephalopathies（TSEs），also called prion diseases, are fatal neurodegenerative diseases including scrapie in sheep and goats, bovine spongiform encephalopathy, and Creutzfeldt-Jakob disease（CJD）in humans. The causative agent of prion diseases, often called as prion, is composed mainly of pathogenic conformers（PrP^{Sc}）of a host protein called cellular prion protein（PrP^{C}）. The direct interaction of two PrP isoforms including PrP^{Sc}-dependent conversion of PrP^{C} is thought be a central event in pathogenesis of prion disease. Although the molecular mechanism of conversion is not yet fully understood, studies using neuronal cells persistently infected with prion have been disclosed many important aspects of the biosynthesis of PrP^{Sc}. For instance, the mature PrP^{C} expressed on the cell surface acts as a substrate for PrP^{Sc} formation, and a process that involves a conformational transformation takes place in subcellular compartments associated with the degradation pathway of PrP^{C}, including a sphingolipid-rich membrane microdomain, called a lipid raft and acidic compartments such as endosomes. Studies using in vitro conversion reactions have suggested that the conversion process is akin to autocatalytic polymerization and provided evidences on the binding domain being involved in the PrP^{C}-PrP^{Sc} interaction. Furthermore, in vitro conversion reaction using membrane-associated PrP^{C} and PrP^{Sc} revealed that insertion of PrP^{Sc} into the host membrane would be prerequisite to the induction of PrP^{C} conversion. More recently, it was reported that exosomes would involved in the transmission of prion to recipient cells. These recent lucid findings provide a new insight into the biosynthesis and transmission of prion. However, there are still many things remains to be elucidated for complete understanding of the biosynthesis of PrP^{Sc} and the full identity of prion itself.

Key words : prion protein／conversion／lipid raft／multivesicular bodies

1. はじめに

人のクロイツフェルト・ヤコブ病（CJD），羊のスクレイピーや牛のBSEに代表されるプリオン病は致死性神経変性性疾患であり，人での発生率はおおよそ100万人当り1人である．プリオン病は発生原因から，感染性，遺伝性，および孤発性の三種に分類される（Table 1）．動物のプリオン病は全て感染性プリオン病に分類される．感染因子“プリオン”に汚染された成長ホルモン，脳硬膜の使用による医原性CJD，BSEが食物連鎖により人に感染したと考えられている変異CJD，および，パプアニューギニアのフォア族で宗教的な食人儀式により伝播していたクールーは，感染性プリオン病に分類される．家族性CJD，GSS，FFIはPrP遺伝子の変異が原因の遺伝性プリオン病である．人プリオン病の80～85%を占める孤発性CJDは，プリオンの感染およびPrP遺伝子の変異と関連がなく，偶発的に正常型プリオン蛋白質（PrP^{C}）が異常型プリオン蛋白質（PrP^{Sc}）に変化することが病気の始まりと考えられる．感染性プリオン病ではPrP^{Sc}が外から宿主に侵入することが感染の第一段階である．感染性を有する蛋白質の増殖・

Table 1 Classification of Prion Diseases

動物のプリオン病	宿主、発生動物
スクレイピー	羊、山羊
慢性消耗病(Chronic wasting disease, CWD)	鹿、エルク
牛海綿状脳症(Bovine spongiform encephalopathy, BSE)	牛
伝達性ミンク脳症(Transmissible mink encephalopathy, TME)	ミンク
猫科動物の海綿状脳症(Feline spongiform encephalopathy, FSE)	家猫、ピューマ、チーター、オセロットなど
その他の反芻動物の海綿状脳症	クードゥー、エランド、ニアラ、オリックスなど

人のプリオン病	原因
クロイツフェルト・ヤコブ病(CJD)	
孤発性 CJD	孤発（不明）
家族性 CJD	遺伝
医原性 CJD	感染
変異 CJD	感染
ゲルストマン・ストライスラー症候群(GSS)	遺伝
家族性致死性不眠症(FFI)	遺伝
クールー	感染

Table 2 Possible role of PrP^C

リンパ球の活性化
シナプス伝達
神経細胞のシグナル伝達
概日周期の調節
抗酸化機構
Cu^{2+}の代謝
神経細胞死
細胞内 Ca^{2+}の恒常性

伝播機構は不明な点が多く残されているが，細胞膜系が関与することは明白である．本稿では，プリオンの増殖・伝播と膜系の関わりを示す最近の報告を含め，PrP^{Sc}の増殖，細胞間伝播機構について概説したい．

2. プリオン蛋白質（PrP）とプリオン増殖の概念

PrPは宿主遺伝子PrPにコードされる．遺伝子産物PrP^Cはグリコシルフォスファチジルイノシトール（GPI）により細胞膜に結合するGPI膜結合型糖蛋白質である．PrP^Cは多くの組織で発現しているが，特に中枢神経系組織で発現が高い．PrP^Cの生理機能に関して様々な報告があるが（Table 2），PrP欠損マウスは正常に発育・繁殖することから，生命維持に必須な機能は担っていないと考えられる．

プリオン病に罹患した動物の脳組織にはPrP^{Sc}が蓄積する．PrP^{Sc}もPrP^Cと同様に宿主遺伝子PrPの産物であるので，アミノ酸配列はPrP^Cと同じである．しかし，PrP^CとPrP^{Sc}は高次構造が異なり，その結果PrP^{Sc}は凝集体を形成するために，蛋白質分解酵素抵抗性や不溶性という生化学性状を示す．蛋白質分解酵素抵抗性とPrP^{Sc}は同義ではなく，PrP^{Sc}の一性状に過ぎない．プリオン病に罹患した動物の組織に存在するPrP，あるいはプリオンの感染性を担うPrP分子を示す言葉としてPrP^{Sc}が使用されることが多い．

PrP^{Sc}は感染因子“プリオン”の主要構成要素で，PrP^{Sc}オリゴマーが感染因子を形成すると考えられている．感染性プリオン病では，PrP^{Sc}オリゴマーが宿主に侵入することが感染であり，PrP^{Sc}オリゴマーが核（seed）となって鋳型のように働き，PrP^CをPrP^{Sc}に転換する．これが繰り返されてPrP^{Sc}オリゴマーが成長することが，プリオンの複製と見做すことができる（Fig. 1）．一方，孤発性および遺伝性プリオン病では“感染”とは無関係に，偶発的にPrP^{Sc}オリゴマーが形成されることが病気の始まりである．PrP^C（もしくは少なくとも一部がアンフォールドした遷移体PrP^U）とPrP^{Sc}オリゴマーは平衡状態にあるが，PrP^{Sc}のオリゴマーは不安定なので，PrP^{Sc}オリゴマーは容易には形成されない．しかし一度安定なPrP^{Sc}オリゴマーが形成されると，これが核となってPrP^CをPrP^{Sc}に転換してゆく（Fig. 1）．核形成以降のPrP^{Sc}増殖プロセスは感染性プリオン病と同じである．

3. 細胞におけるPrPの生合成

神経芽細胞にスクレイピー感染マウス脳乳剤を接種してクローニングを行うことで，スクレイピー持続感染細胞を得ることができる．PrP^CとPrP^{Sc}の生合成機構の解析には，こうして作出されたプリオン持続感染神経芽細胞を用いた細胞生物学的実験系が大きく貢献している（Fig. 2）．

PrP^Cは膜蛋白質であり，ER上で合成されたポリペ

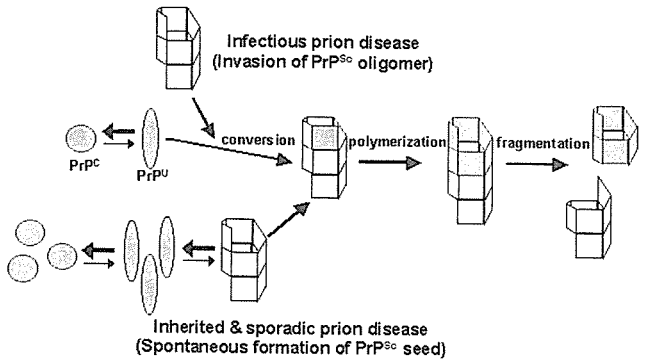

Fig. 1　Model for prion propagation.
Invasion of exogenous PrP^{Sc} oligomer (infection) causes infectious prion disease. Incoming exogenous PrP^{Sc} binds PrP^{C} (or its unfolded intermediate, PrP^{U}) and then PrP^{C} is converted into new PrP^{Sc}. Incoming PrP^{Sc} acts as a seed for the conversion process. On the other hand, spontaneous formation of PrP^{Sc} seed from PrP^{C} or PrP^{U} initiates inherited and sporadic prion diseases. Once stable PrP^{Sc} seed is generated, the following conversion process is the same as that in the infectious prion disease.

プチド鎖はすぐにER内腔に移行する．このときN末端のシグナル配列は除去される．ERでC末端の22-23アミノ酸の除去とGPIアンカー付加，分子内SS結合の形成，高マンノース型糖鎖の付加が起こる．一部のPrP^{C}，おそらく正しく折りたたまれなかったPrP^{C}はERを通過できずにER付随ユビキチンプロテアソーム系で分解される[1]．ゴルジ装置を経る過程で複合糖鎖へと修飾され成熟型PrP^{C}はGPIアンカー型膜蛋白として細胞表面に発現する．PrP^{C}は速やかに合成されるが半減期も短い（Table 3）．細胞膜上に発現した成熟型PrP^{C}はエンドサイトーシスにより細胞内に取り込まれる．クラスリン被覆小胞を介する経路と非クラスリン被覆小胞を介する経路があると考えられている[2~4]．細胞内に取り込まれたPrP^{C}はN末端のトリミングが起こり，111番目のアミノ酸近傍で切断され，PrP^{C}末端側の領域を含む約18 kDaのポリペプチドが生じる[5]．この切断はスフィンゴ脂質とコレステロールに富む細胞膜ドメイン（ラフトあるいはカベオラ）で起こり[3]，切断にはメタロプロテアーゼが関与するとの報告もある[6,7]．この分解中間産物はエンドドーソムと考えられる酸性コンパートメントで分解される[8~10]．細胞内に取り込まれたPrP^{C}の一部は再び細胞膜にリサイクルされる[8]．

PrP^{C}に比べPrP^{Sc}の生合成と分解は非常に遅い（Table 3）．PIPLC処理により細胞表面に発現するPrP^{C}を除去すると，プリオン持続感染細胞における

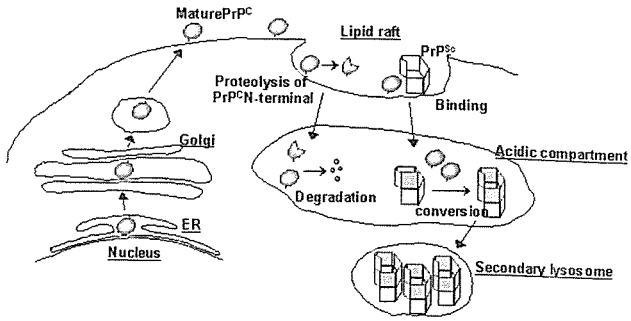

Fig. 2　Biosynthesis of prion protein in the cells.
PrP^{C} matures during the secretory pathway and expressed on the cell surface as GPI-anchoring protein. In the lipid raft and/or acidic compartments such as endosome, N-terminal part of PrP^{C} was partially proteolysed, and then degradated. Initial interaction between PrP^{C} and PrP^{Sc} is believed to take place in lipid raft, and the conversion of PrP^{C} to PrP^{Sc} occurs along a degradation pathway, possibly in lipid raft and/or acidic compartments. PrP^{Sc} is accumulated in the secondary lysosome.

PrP^{Sc}の産生が阻害されることから，細胞膜上に発現した成熟型PrP^{C}がPrP^{Sc}生成の基質となること，およびその生成は細胞膜を含めエンドサイトーシスの過程で起こることが示された[9,10]．ロバスタチン処理により細胞膜をコレステロール飢餓状態にするとPrP^{Sc}の産生が阻害される[3]．PrP^{Sc}は主に二次リソソームに蓄積すると考えられているが[11,12]，ラフトにもPrP^{Sc}が存在することから，PrP^{C}とPrP^{Sc}の会合などのPrP^{Sc}形成の初期段階は細胞膜上のマイクロドメインであるラフトで起こることが示唆されている[13,14]．一方，エンドソームやリソソームに作用する薬剤の幾つかでPrP^{Sc}生成阻害効果が認められることから[15]，PrP^{Sc}への転換はエンドソームのような酸性コンパートメントでも起こると考えられる．PrP^{Sc}の半減期は非常に長いが，硫酸多糖処理[16]や抗PrP抗体でプリオン感染細胞を処理した場合[17,18]，3日程度の処理でPrP^{Sc}は消失する．これらの処理はPrP^{C}の正常な代謝経路に影響してPrP^{Sc}生成の基質であるPrP^{C}がPrP^{Sc}に供給されるのを阻害すると考えられる[19]．つまり，基質であるPrP^{C}の供給を遮断するとPrP^{Sc}は増殖できず細胞内で分解される．

4.　無細胞実験系における蛋白質分解酵素抵抗性PrP分子（PrP-res）の形成

スクレイピー感染動物脳から精製したPrP^{Sc}と^{35}S標識PrP^{C}を特定条件下で反応させると，^{35}S標識PrP^{C}がproteinase K（PK）抵抗性の[^{35}S]PrP-resに変

Table 3 Characteristics of PrP^C and PrP^{Sc}

	PrP^C	PrP^{Sc}
凝集性	−	+
非イオン系界面活性剤に対する溶解性	易溶性	難溶性
蛋白質分解酵素感受性	感受性	抵抗性
細胞内局在	細胞膜表面	二次リソソーム
合成時間	<30分	6−15時間
半減期	5時間	>24時間
二次構造	αヘリックス：43% βシート：3%	αヘリックス：30% βシート：45%

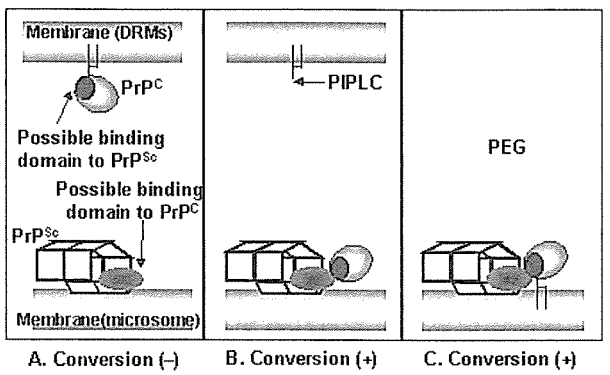

Fig. 3 Conversion of membrane associated PrP^C (an epitome of Baron et al., 2002[26]).
A. No conversion occurred when PrP^C (in DRMs) and PrP^{Sc} (in microsome) were inserted into the separate membranes. B. PrP^C could be converted if PrP^C was released from DRMs by PIPCL treatment. C. Conversion took place if PrP^C and PrP^{Sc} were inserted into the same membrane surface by PEG-induced fusion between DRMs and microsome.

換する[20]．この反応系（in vitro conversion）で産生された[^{35}S]PrP-resは脳内に存在するPrP^{Sc}と同様の生化学性状を示すが，感染性が付随しないことから，PrP^{Sc}と厳密に区別する意味でPrP-resと表記する．PrP-resへの転換はseedとなるPrP^{Sc}に依存しており，seedとなるPrP^{Sc}の変性に伴い転換活性は消失する[21]．プリオン伝達の種の壁を再現できること[22,23]，株特異的なPrP^{Sc}の生化学性状も再現できることなどから[24]，ある程度in vivoで起きている$PrP^C \rightarrow PrP^{Sc}$の転換機構を再現可能な試験系である．この反応系で，PrP^{Sc}とPrP^Cの結合の特異性・選択性が証明できたことから，PrP^CとPrP^{Sc}の特異的な結合がPrP^CからPrP^{Sc}への構造転換への第一段階であること，PrP^CがPrP^{Sc}のレセプターに成りうることが示唆された[25]．

in vitro conversionは可溶性のPrP^Cと精製PrP^{Sc}を使用しているが，細胞レベルあるいは神経組織を考えた場合，$PrP^C \rightarrow PrP^{Sc}$への転換反応は膜分画で起こると考えられることから，より生理的な条件に近い実験系を構築する必要があった．Baronらは，^{35}S-メチオニンでメタボリックラベルした細胞から調製したTriton-X不溶性画分（detergent-resistant membranes, DRMs）をラフトに存在するPrP^Cの供給源に，スクレイピー感染マウスから調製したミクロソーム画分を細胞膜に付随したPrP^{Sc}の供給源として，in vitro conversionを行った[26,27]．その結果，膜に付随するPrP^CとPrP^{Sc}同士を混合しただけではPrP^Cの転換は起こらず，PrP^Cを膜から遊離させると転換反応が起こった．さらに興味深いことに，膜に付随したPrP^CとPrP^{Sc}を混和し，ポリエチレングリコールでDRMsとミクロソーム画分を融合させると，PrP^CがPrP-resに転換した（Fig. 3）．PrP^CのC末端側で構成されるドメインがPrP^{Sc}との結合に関与することが抗体による結合阻害試験から示されているが，細胞膜に結合したPrP^CのC末端側は細胞膜による立体障害のために，異なる膜上にあるPrP^{Sc}と結合できない可能性がある．しかし同一膜上にPrP^CとPrP^{Sc}が存在することで，PrP^CとPrP^{Sc}の会合が可能となり，その後PrP^CがPrP-resへと構造転換するのかもしれない．この結果は，in vivoでは単純にPrP^CとPrP^{Sc}が特異的に結合することが転換反応の第一段階でなく，両者を有する膜同士の融合が第一段階であることを示唆するとともに，細胞間でのPrP^{Sc}の伝播を考える上で重要な知見である．

5. プリオンの細胞間の伝播

プリオンの体内侵入から神経組織への到達を考えた場合，プリオンは少なくとも数回，細胞から細胞へ移る必要がある．羊のスクレイピーの場合，自然状態では経口ルートで感染が成立する．経口ルートで取り込まれたプリオンは，パイエル氏板などの消化管附随リンパ装置（GALT）から体内に侵入してGALTの濾胞樹状細胞で増殖した後，末梢神経から内臓神経を経て脊髄腰部に到達する経路，および末梢神経から迷走神経を経て延髄に到達する経路で，中枢神経系組織へ到達する[28]．末梢神経から中枢神経系組織へ侵入する際は，一度シナプス間隙を通過しなければならない．PrP^{Sc}はどのようにしてシナプス間隙を通過して次の神経細胞へ移るのであろうか？

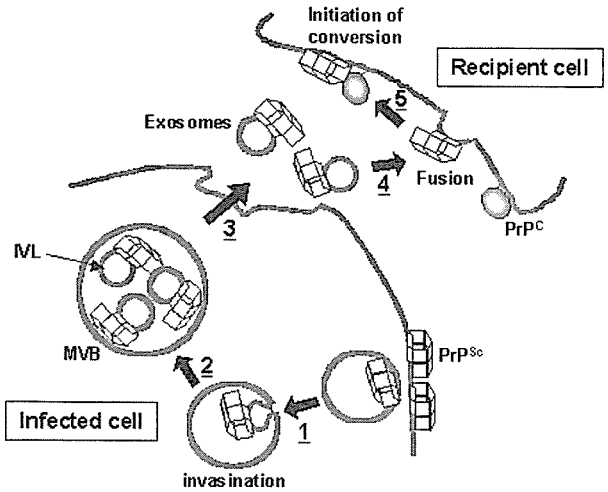

Fig. 4　Possible mechanisms of transmission of PrP^{Sc} from infected cell to uninfected cell (an epitome of Baron *et al.*, 2002[26]; Fevrier *et al.*, 2004[30], 2005[34]). PrP^{Sc} transits to endocytic compartments and invasination of endosome membrane forms IVLs bearing PrP^{Sc} (1) during MVBs formation (2). IVLs bearing PrP^{Sc} are secreted into extracellular milieu as exosomes (3). The exosomes would be fused to the membrane of recipient cells (4) and the fusion would allow the interaction between PrP^{C} and PrP^{Sc} on the membrane of recipient cell (5).

レシピエントとドナーの細胞を共培養によりプリオンが効率良く伝達すること[29]，精製PrP^{Sc}よりもPrP^{Sc}を含むミクロソーム分画が，細胞への感染効率が高いことなどが知られている．前述したBaronらの成績でも，PrP^{Sc}とPrP^{C}が同一膜上にあるとPrP^{C}の構造転換が起こることから，膜小胞がPrP^{Sc}の細胞間伝播に関与することが予想されていた．

最近Raposoらは，プリオン持続感染細胞から放出されたexosomesにPrP^{Sc}が存在し，実際にプリオンを非感染細胞に伝達できることを示した[30]．小胞を含むエンドソーム（multivesicular endosomes）はMVBs（multivesicular bodies, 多胞体），多胞体内腔の小胞はIVLs（intraluminal vesicules）と呼ばれる．MVBsは細胞膜から取り込まれた膜蛋白質やトランスゴルジからソートされた膜蛋白質を含み，ライソソームと融合して，MVBs中の蛋白質は分解される．しかし全てのMVBsがライソソームと融合するのではなく，網状赤血球ではIVLsが細胞外に放出される[31,32]．細胞外に放出されるIVLsをexosomesと呼ぶ．網状赤血球にはライソソームがないことからexosomesが老廃蛋白の処理を行うと考えられていた[32]．しかし，造血系細胞ではexosomesが様々な機能分子を含んでおり，免疫系の調節に関連することが示唆されている．例えばexosomesがMHCクラスII-ペプチド複合体を樹状細胞間で伝達する[33]．このようにexosomesが膜小胞による細胞間のコミュニケーションに関与することが明らかになりつつある．

IVLsはエンドソーム膜が内腔に陥入してできるので，膜蛋白質の細胞外ドメインはIVLsの外側に，内腔は細胞質という構造となる．IVLs表面に附随したPrP^{Sc}がexosomeとして細胞外に放出され，レシピエントとなる細胞に融合した場合，PrP^{Sc}とレシピエント細胞上のPrP^{C}が同一の膜表面に位置することになる．Baronらの成績と合わせて，PrP^{Sc}の細胞間伝播にexosomesによる細胞膜の伝達が関与する興味深いモデルを提供すると考えられる（Fig. 4）[34]．

6. おわりに

プリオン仮説が登場してから20年以上が経過した．ウイルス，細菌などの病原体は“一個の病原体”を電子顕微鏡で見ることが可能であるが，依然として“一個のプリオン”の形態は不明である．PrP^{Sc}が主要な病原体構成と考えられるが，未だ最終的な結論は得られていない．一個のプリオン粒子の構造を明らかにすることは，プリオンの謎を解く重要な鍵である．不可解な点が多い病原体ではあるが，PrP^{Sc}と感染価を指標にした実験結果から，本稿で紹介したように，プリオンの増殖に関して多くの知見が得られてきた．また，本稿では最近の総説を紹介するに留めるが，PrP^{C}の生合成や細胞内輸送に関しても細胞生物学の進展により多くの知見が集積している[35]．しかし，プリオン増殖の分子機構を紐解くにはほど遠い．ウイルスが細胞内で増殖する際に，宿主細胞の微小環境と因子を利用するのと同じく，プリオンの増殖にも宿主細胞の微小環境と因子が関与する．このような微小環境と因子を明らかにしていくことで，プリオン増殖の分子機構が構築できるだろう．

基礎生物学的にはプリオン増殖の分子機構の解明は一つのゴールである．一方，病気に目を向けると，プリオン病は稀な病気ではあるが，一度発症すると進行性に死に至る致死的な病気であるが，未だ有効な治療法はない．プリオン増殖の分子機構の理解により，プリオン病治療の標的が見えてくると思われる．PrP^{Sc}増殖を阻害する物質はプリオン病治療薬の候補となる．これまでに，プリオン持続感染細胞やin vitro conversionを利用して，硫酸デキストランや硫酸ペントサンなどの硫酸多糖体，ポルフィリン誘導体，キナクリンなどのアクリジン誘導体，クロルプロマジンなどのフェノチアジン誘導体，分枝ポリアミン，変異PrP，抗PrP抗体など，PrP^{Sc}増殖抑制活性を有する物質が数多く同定されている．幾つか

については，in vivo でも PrP^{Sc} の増殖を抑制することが報告されている．今後，プリオン病治療候補薬の作用機序の解明，プリオン増殖機構の解明が進み，より効果的な治療薬や治療法の開発へつながることを期待したい．

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Propagation of a protease-resistant form of prion protein in long-term cultured human glioblastoma cell line T98G

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Human prion diseases, such as Creutzfeldt–Jakob disease (CJD), a lethal, neurodegenerative condition, occur in sporadic, genetic and transmitted forms. CJD is associated with the conversion of normal cellular prion protein (PrP^C) into a protease-resistant isoform (PrP^{res}). The mechanism of the conversion has not been studied in human cell cultures, due to the lack of a model system. In this study, such a system has been developed by culturing cell lines. Human glioblastoma cell line T98G had no coding-region mutations of the prion protein gene, which was of the 129 M/V genotype, and expressed endogenous PrP^C constitutively. T98G cells produced a form of proteinase K (PK)-resistant prion protein fragment following long-term culture and high passage number; its deglycosylated form was approximately 18 kDa. The PK-treated PrP^{res} was detected by immunoblotting with the mAb 6H4, which recognizes residues 144–152, and a polyclonal anti-C-terminal antibody, but not by the mAb 3F4, which recognizes residues 109–112, or the anti-N-terminal mAb HUC2-13. These results suggest that PrP^C was converted into a proteinase-resistant form of PrP^{res} in T98G cells.

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INTRODUCTION

Fatal human prion diseases, including sporadic Creutzfeldt–Jakob disease (CJD), inherited prion diseases, iatrogenic CJD, kuru and variant CJD, are transmissible spongiform encephalopathies that are characterized by the formation and accumulation of an abnormal isoform of prion protein (PrP) in the brain (Prusiner, 2001). The PrP^{res} isoform is an insoluble aggregate that is resistant to proteinase K (PK) digestion. The conversion from cellular prion protein (PrP^C) into PrP^{res} could be a potential therapeutic target for prion diseases, but the mechanism of the conversion is unclear.

Several animal cell lines, including mouse neuroblastoma cells (Butler *et al.*, 1988; Race *et al.*, 1987), mouse hypothalamic neuronal cells (Nishida *et al.*, 2000; Schätzl *et al.*, 1997), mouse Schwann cells (Follet *et al.*, 2002) and rat pheochromocytoma cells (Rubenstein *et al.*, 1984), have been infected successfully with scrapie agents, and a human neuroblastoma cell line can also be infected with CJD agents (Ladogana *et al.*, 1995). These cells have been used to study the conversion mechanisms (Lehmann & Harris, 1997) and the subcellular localization (Naslavsky *et al.*, 1997; Vey *et al.*, 1996) of PrP^{res} and to evaluate therapeutic agents (Caughey & Raymond, 1993; Doh-Ura *et al.*, 2000). However, the efficiencies of infection and propagation of PrP^{res} are relatively low. The mouse cell line SMB was established from a scrapie-infected mouse brain (Clarke & Haig, 1970) and has been used to study the properties of PrP (Birkett *et al.*, 2001). Recently, stable cell lines were established from mouse peripheral neuroglial cells expressing ovine PrP and simian virus 40 T antigen. These cells were readily infectible by sheep PrP^{Sc}, a scrapie isoform of PrP (Archer *et al.*, 2004). However, there are currently no human cell lines that have been used to study the conversion mechanism from PrP^C into PrP^{res}.

PrP mRNA is expressed not only in neurons, but also in glia (Moser *et al.*, 1995) and PrP^{Sc} accumulates in the cytosol and cell-surface membrane of glial cells (van Keulen *et al.*, 1995). The role of glial cells in prion disease is not clear. Human glioblastoma T98G cells, like normal cells, become arrested in G_1 phase under stationary-phase conditions (Stein, 1979). In a previous study, we showed that T98G cells express PrP^C mRNA constitutively and produce a high level of endogenous PrP^C in G_1 phase (Kikuchi *et al.*, 2002). In the present study, we have investigated whether PrP^C is

converted into PrP^{res}, a marker for prion diseases, in cultured T98G cells under various conditions.

METHODS

Materials. A primer set for the human PrP coding sequence (CDS) (GenBank accession no. AL133396) [5′-CGAGGCAGAGCAGTCATT-3′, starting 18 nt before the ORF, and 5′-AGATGGTGAAAACGAGAAGAC-3′, ending 6 nt after the ORF (expected product size, 806 bp)] and an internal primer set (5′-GGCAGTGACTATGAGGACCGTTAC-3′ and 5′-GTAACGGTCCTCATAGTCACTGCC-3′, corresponding to nt 424–447 relative to the start site of the ORF) were synthesized chemically. Peptide *N*-glycosidase F (PNGase F) and *Bsa*AI were purchased from New England Biolabs and RPMI 1640 medium was purchased from Nissui Pharmaceutical. A BCA protein assay kit and SuperSignal West Femto Maximum Sensitivity substrate were from Pierce Biotechnology. Hybond-P PVDF membranes were purchased from Amersham Biosciences. Anti-human PrP mAb 3F4 was purchased from Signet Laboratories and 6H4 from Prionics AG. Fetal calf serum (FCS), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, HRP-conjugated goat anti-rabbit IgG, HRP-conjugated rabbit anti-chicken IgG, aprotinin, leupeptin, PMSF, 4-methylumbelliferyl-β-D-galactoside (4-MUG) and mouse IgG were purchased from Sigma. PK was purchased from Merck and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) from Roche Diagnostics. SuperScript II reverse transcriptase and random primers were purchased from Invitrogen. β-Galactosidase-conjugated goat anti-mouse IgG was purchased from American Qualex, DNase I from Takara, KOD-Plus-DNA polymerase from Toyobo and 1,4-diazabicyclo[2.2.2]octane (DABCO) from Nacalai Tesque.

Preparation of antibodies. The preparation of chicken mAb HUC2-13 (IgG) against human PrP peptide residues 25–49 was reported previously (Matsuda *et al.*, 1999). The preparation of rabbit polyclonal antibody HPC2 (IgG) against human PrP peptide residues 214–230 was also reported previously (Kikuchi *et al.*, 2002).

Cell culture. Human glioblastoma cell line T98G (JCRB9041) at nominal passage level 433 was provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). Human astrocytoma U373MG cells were kindly provided by Dr T. Kasahara (Kyoritsu College of Pharmacy, Tokyo, Japan). Cell cultures stored in liquid nitrogen were thawed as passage 0 (P0) and cultured at 37 °C in monolayers on a T75 plastic tissue-culture flask in RPMI 1640 medium supplemented with 10 % (v/v) heat-inactivated FCS, 60 μg kanamycin ml^{-1} and 10 mM HEPES/NaOH, pH 7·2. All cell lines were subcultivated routinely at a 1:5 or 1:10 split ratio once a week.

PCR direct sequencing and RFLP analysis. Extraction of total RNA from the cells and RT-PCR analysis were performed according to a published method (Kikuchi *et al.*, 2002) with slight modifications. Briefly, 5 μg total RNA was treated with DNase I for 15 min at room temperature. Random primers and SuperScript II reverse transcriptase were added to 20 μl (2·5 μg total RNA) and the mixture was incubated at 42 °C for 60 min to synthesize cDNA. Subsequently, 10 μl cDNA solution was subjected to PCR in a total volume of 50 μl, which included 0·2 mM dNTPs, 1 mM $MgSO_4$, 1 U KOD-Plus-DNA polymerase and 50 pmol sense and antisense primers. The amplification programme was as follows: denaturation at 94 °C for 20 s, annealing at 60 °C for 30 s and elongation at 68 °C for 60 s for 40 cycles. Final elongation was performed at 68 °C for 1 min. PCR was carried out in a GeneAmp PCR system 2400 (Applied Biosystems). PCR direct sequencing was performed with a CEQ 2000XL DNA Analysis system (Beckman Coulter) using the primer set for human PrP CDS and an internal primer. Codon 129 polymorphisms were detected by RFLP analysis; the PCR product (200 ng DNA) was digested with 5 U *Bsa*AI for 60 min at 37 °C; after incubation for 20 min at 80 °C, restriction fragments were separated by electrophoresis in 2 % agarose gels and visualized following ethidium bromide staining.

Preparation of whole-cell lysates. All cell lines were plated at $5·0 \times 10^5$ cells per 9 cm dish (55 cm^2) in 10 ml medium on day 0 (D0). The medium was changed every 4 days. At the indicated times, cells were washed twice with ice-cold PBS and scraped into lysis buffer [$1·8 \times 10^4$ cells μl^{-1}; 10 mM Tris/HCl (pH 7·5), 150 mM NaCl, 1 % sodium deoxycholate, 0·1 % SDS, 1 % NP-40, 10 mM NaF, 1 mM EDTA, 0·5 mM Na_3VO_3, 10 mM tetrasodium pyrophosphate] with protease inhibitor cocktail [0·06 trypsin inhibitor units (TIU) aprotinin ml^{-1}, 20 μM leupeptin and 1 mM PMSF]. After sonication, insoluble material was pelleted by centrifugation at 500 *g* for 15 min at 4 °C to yield whole-cell lysates. Protein concentration was determined by the BCA protein assay.

Subcellular fractionation. At the indicated times, cells were washed twice with ice-cold PBS and scraped into PBS/2·5 mM EDTA with the protease inhibitor cocktail. After sonication, insoluble material was pelleted by centrifugation at 500 *g* for 15 min at 4 °C to yield homogenates. The postnuclear fraction was centrifuged at 100 000 *g* for 60 min at 4 °C to obtain a cytosolic fraction and a membrane fraction. The membrane fraction was dissolved in PBS/2·5 mM EDTA with the protease inhibitor cocktail. Protein concentration was determined by the BCA protein assay.

Detergent solubility test. A detergent solubility test was carried out according to a described method (Capellari *et al.*, 2000) with slight modifications. Cells were washed twice with ice-cold PBS and scraped into PBS/2·5 mM EDTA with the protease inhibitor cocktail. After sonication, insoluble material was pelleted by centrifugation at 500 *g* for 15 min at 4 °C to yield homogenates. The postnuclear fraction was dissolved in 9 vols 0·5 % NP-40/0·5 % deoxycholate/PBS with the protease inhibitor cocktail and centrifuged at 100 000 *g* for 60 min at 4 °C to obtain a detergent-insoluble pellet fraction and a soluble supernatant fraction. The supernatant fraction was precipitated with 4 vols methanol for 16 h at −20 °C. Both fractions were resuspended in the same volume of lysis buffer.

Protease-resistant PrP assay. To generate material for the protease-resistant PrP assay, aliquots of the sample (50 μg protein) were precipitated with 4 vols methanol for 16 h at −20 °C to remove the protease inhibitor cocktail (Capellari *et al.*, 2000), centrifuged at 14 000 *g* for 15 min at 4 °C and the pellet was dissolved in 50 mM Tris/HCl (pH 7·2). Samples were treated with PK (at 10 μg ml^{-1} unless stated otherwise) at 37 °C for 30 min, according to a described method (Caughey *et al.*, 1999). After incubation, digestion was stopped by the addition of AEBSF to 4 mM. Samples were prepared with the protease inhibitor cocktail at a concentration that did not inhibit the activity of PK (Fig. 1a, lane 1).

Enzymic deglycosylation. For the removal of Asn-linked oligosaccharides, aliquots of whole-cell lysates were treated with PNGase F as follows (Kikuchi *et al.*, 2002): lysates (50 μg protein) were denatured by boiling for 10 min in 0·5 % SDS, 1 % 2-mercaptoethanol. After addition of NP-40 to 1 %, the lysates were incubated at 37 °C for 2 h with 0·77 IUB mU PNGase F in 50 mM phosphate buffer (pH 7·5).

Immunoblotting. Usually, 50 μg total protein (prepared from approximately $1·7 \times 10^5$ cells) was subjected to SDS gel electrophoresis. Briefly, aliquots of the samples were mixed with 2× electrophoresis sample buffer. After boiling for 10 min, the samples

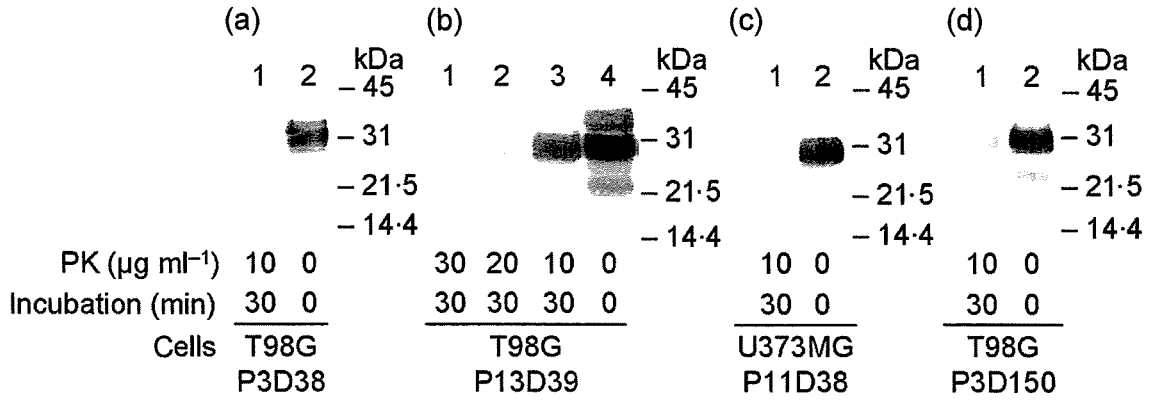

Fig. 1. Formation of a protease-resistant form of PrP in T98G cells is increased in a long-term incubation after repeated passages. T98G cells and U373MG cells were incubated under the following conditions with 10% FCS/RPMI 1640 and whole-cell, methanol-precipitated lysates (50 μg protein) were treated with PK (10 μg ml^{-1} unless stated otherwise) at for 30 min at 37 °C. (a) T98G cells were incubated for 38 days after 3 passages (P3D38); lysates were treated with PK (lane 1) or left undigested (lane 2). (b) T98G cells were incubated for 39 days after 13 passages (P13D39); lysates were treated with 10, 20 or 30 μg PK ml^{-1} (lanes 1–3) or left undigested (lane 4). (c) U373MG cells were incubated for 38 days after 11 passages (P11D38); lysates were treated with PK (lane 1) or left undigested (lane 2). (d) T98G cells were incubated for 150 days after 3 passages; lysates were treated with PK (lane 1) or left undigested (lane 2). PK-treated lysates were subjected to immunoblot with the 6H4 antibody as described in Methods.

were electrophoresed on 12·5% acrylamide gel and the proteins were transferred onto PVDF membranes. The membranes were blocked with 0·5% casein in PBS (casein/PBS) and incubated with anti-prion antibodies in casein/PBS. Immunoreactive bands were visualized with HRP-conjugated anti-IgG and SuperSignal West Femto Maximum Sensitivity substrate, according to the manufacturer's instructions (Pierce Biotechnology).

Indirect immunofluorescence staining. T98G cell monolayers grown on a 15 mm glass coverslip (Matsunami) in a 9 cm dish (55 cm^2) were maintained in 10 ml medium. At the indicated times, cells were washed twice with ice-cold PBS and then fixed with 3·7% formaldehyde in PBS for 30 min at 4 °C. The fixed cells were washed twice with PBS and then treated with 0·2% Triton X-100 in PBS for 15 min at room temperature. The cells were blocked with 10% normal goat serum in PBS (NGS/PBS) for 60 min and incubated with antibody (100 ng ml^{-1}) for 16 h at 4 °C. After extensive washing with 0·05% Tween 20/PBS, cells were treated with Alexa 594 goat anti-mouse IgG (H+L) conjugate (5 μg ml^{-1}) (Molecular Probes) in NGS/PBS for 1 h at 4 °C, washed with 0·05% Tween 20/PBS and mounted with 2·5% DABCO/90% glycerin/PBS. The stained cells were observed and photographed with the aid of a fluorescence microscope (Olympus).

Competitive ELISA. ELISA was carried out according to a method described previously (Kikuchi *et al.*, 1991). For a dilution buffer, casein/PBS was used throughout the present study. Briefly, the wells were coated with 100 ng recombinant bovine PrP (rBoPrP) (Takekida *et al.*, 2002) in PBS and left at 4 °C overnight. Appropriately diluted standard rBoPrP solutions or samples were added to the antigen-coated wells and incubated at room temperature for 60 min, in a total volume of 50 μl, with 6H4 antibody (460 pg). The wells were washed, incubated with β-galactosidase-conjugated goat anti-mouse IgG for 60 min, washed again and then incubated with 4-MUG as a substrate at 37 °C for 60 min. Enzyme activity was determined by fluorescence intensity measurements.

RESULTS

Production of protease-resistant isoform of PrP in T98G cells

We analysed whole-cell lysates of long-term cultured T98G cells by immunoblotting with anti-PrP antibodies. When we cultured the cells for 38 days after 3 passages [passage 3, day 38 (P3D38)], the lysates revealed two bands (35 and 31 kDa) that reacted with mouse anti-human PrP mAb 6H4 (Fig. 1a, lane 2) and were destroyed completely after digestion with PK (Fig. 1a, lane 1). When lysates from cells that were cultured for 39 days after 13 passages [passage 13, day 39 (P13D39)] were digested with PK (10, 20 or 30 μg ml^{-1}), the 35 kDa band, but not the 31 kDa band, was diminished (Fig. 1b), indicating the presence of PrP^{res}. We then attempted to detect PrP^{res} formation in long-term cultures of another human glial cell line, U373MG, an astrocytoma line that expresses consistently high levels of PrP^{C} mRNA (Satoh *et al.*, 1998). The lysates from P11D38 U373MG cells exhibited the 31 kDa band that reacted with the 6H4 antibody and disappeared after digestion with PK (Fig. 1c). Lysates from P3D150 T98G cells showed a faint 31 kDa band after PK treatment (Fig. 1d). In contrast, P13D39 T98G cells had produced highly PK-resistant PrP. These data indicated that PrP^{res} propagation in T98G cells required not only long-term culture, but also a high passage number.

Examination of phenotypic variants of PrP^{res}

We first asked whether an inherited or a sporadic CJD-like form of PrP^{res} was propagated in T98G cells. Inherited prion

diseases are determined by mutations in the 762 bp CDS of the prion protein gene (*PRNP*) (Kovács *et al.*, 2002). We performed PCR direct sequencing of the *PRNP* mRNA that was expressed in short- and long-term cultured T98G cells and found no mutations other than the presence of both adenine and guanine at the first position of codon 129 (the basis of the common M129V polymorphism) (data not shown). When digested by *Bsa*AI, the 806 bp PCR product from the M129V haplotype (Fig. 2a, lane 1) yielded products of 402 and 404 bp and also undigested wild-type product (Fig. 2a, lane 2), which we confirmed by RFLP analysis. These results indicated that T98G cells were heterozygotes, having both methionine and valine at codon 129 of *PRNP* with no coding-region mutation.

Next, to estimate the size of the deglycosylated PrP^{res}, we treated the lysates from P40D40 T98G cells with PK and/or PNGase F. PNGase F yields a full-length (25 kDa) and an N-terminally truncated (18 kDa) form of PrP^{C} (Kikuchi *et al.*, 2002). As shown in Fig. 2b, PNGase F treatment reduced the glycosylated 35 and 31 kDa bands (lane 4) to 25 and 18 kDa (lane 3), representing the deglycosylated full-length and N-terminally truncated forms. An additional PNGase F treatment changed fully glycosylated (31 kDa) and partially glycosylated (23 kDa) forms of PrP^{res}, detectable after digestion with PK (lane 2), to an unglycosylated form of 18 kDa (lane 1). These results established that the size of the deglycosylated PK-resistant fragment in T98G cells was approximately 18 kDa.

Confirming heterogeneity of PrP^{res} by immunoblotting with sets of anti-PrP antibodies

To further investigate the heterogeneity of PrP^{res} from long-term cultured T98G cells, we determined the antigenicity of PrP^{res}. By immunoblotting with sets of antibodies to PrP (Kikuchi *et al.*, 2002), we detected a full-length PrP (35 kDa) in lysates from P40D40 T98G cells that reacted with the anti-N terminus PrP antibody HUC2-13 (Fig. 3a, lane 2), as well as with the 6H4 antibody (Fig. 3c, lane 2). Following PK treatment of the lysates, the 31 kDa band was still detected by 6H4 antibody (Fig. 3c, lane 1), but not by HUC2-13 antibody (Fig. 3a, lane 1), indicating that PK treatment had cleaved the N terminus of PrP^{res}. The 31 kDa band was also detected by the anti-C terminus PrP antibody HPC2 (Fig. 3d, lane 1). HPC2 antibody, which reacts strongly with the deglycosylated form of PrP^{C}, but weakly with the glycosylated form (Kikuchi *et al.*, 2002), also recognized the N-terminally truncated form of PrP^{res}. Surprisingly, the 3F4 antibody, which recognizes residues 109–112, failed to detect the N-terminally truncated form of PrP^{res} (Fig. 3b), such as is seen with the HUC2-13 antibody (Fig. 3a). These experiments showed that the N-terminally truncated form of PrP^{res} in T98G cells lacks the epitope that is recognized by the 3F4 antibody.

Subcellular localization and detergent solubility of PrP^{res} in T98G cells

To determine the subcellular localization of PrP^{res}, we studied the distribution of PrP in P40D40 T98G cells

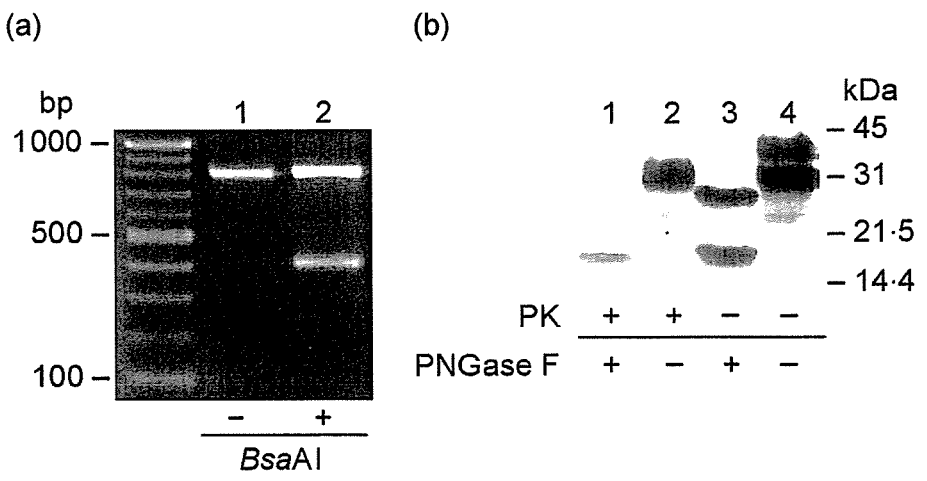

Fig. 2. Molecular analysis of PrP^{res} in T98G cells. (a) Detection of polymorphism at codon 129 on PrP mRNA in T98G cells. T98G cells were incubated with 10% FCS/RPMI 1640 for 5 days after 43 passages (P43D5) and total RNA was prepared, reverse-transcribed and PCR-amplified as described in Methods and digested with *Bsa*AI (lane 2) or left undigested (lane 1). A DNA size marker (100 bp ladder) is shown on the left. (b) Analysis of deglycosylated forms of PrP in T98G cells. T98G cells were incubated with 10% FCS/RPMI 1640 for 40 days after 40 passages (P40D40); whole-cell, methanol-precipitated lysates were treated with PK (lanes 1 and 2) or left undigested (lanes 3 and 4). All lysates were incubated with (lanes 1 and 3) or without (lanes 2 and 4) PNGase F for 120 min. PK-treated lysates were subjected to immunoblot with the 6H4 antibody as described in Methods.

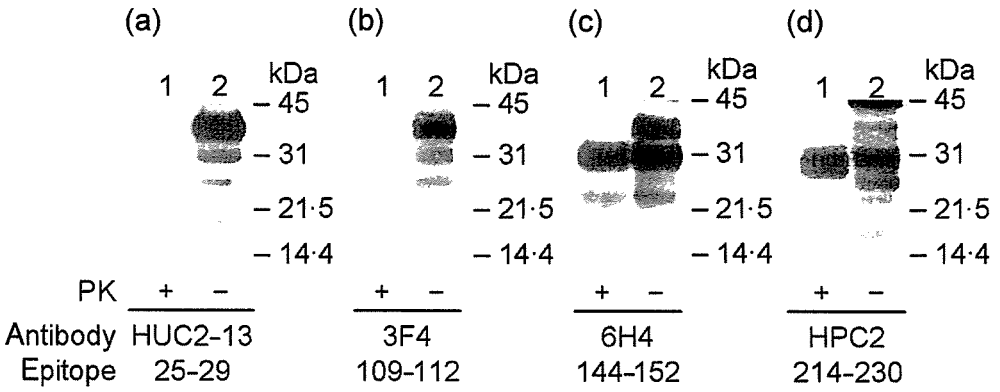

Fig. 3. Immunoblot analysis using anti-PrP antibodies for the protease-resistant form of PrP in T98G cells. T98G cells were incubated with 10% FCS/RPMI 1640 for 40 days after 40 passages (P40D40); whole-cell, methanol-precipitated lysates were treated with PK (lane 1) or left undigested (lane 2). PK-treated lysates were subjected to immunoblot with the HUC2-13 (a), 3F4 (b), 6H4 (c) or HPC2 (d) antibodies as described in Methods. Epitope recognition sites located within PrP are shown as amino acid numbers.

by indirect immunofluorescence staining. Immunoreactive PrP with 6H4 antibody was observed on the cell surface as a bright fluorescent signal (Fig. 4a), whereas little signal was observed with mouse IgG, a control antibody purified from normal mouse serum (data not shown). We next prepared membrane and cytosolic fractions from homogenates of P40D40 T98G cells and measured the amount of PrP by competitive ELISA using the 6H4 antibody. PrP was recovered predominantly in the membrane fraction (Table 1). As shown in Fig. 4b, the distribution of PrP^{res} in P40D40 T98G cells (left panel) was similar to that of PrP^{C} in P3D36 T98G cells (right panel); PrP^{res} was detected in the membrane fraction (left panel, lane 3), as well as in homogenates (left panel, lane 1), but no PrP was detected in the cytosolic fraction (left panel, lanes 5 and 6). These data indicated that most PrP^{res} was in the membrane fraction, probably on the plasma membrane. To test the detergent solubility of PrP, the homogenates of P40D40 T98G cells were centrifuged in non-ionic detergents. A large proportion of immunoreactive PrP was found in the supernatant fraction (Fig. 4c, lane 3), but no PrP was detected in the pellet fraction (Fig. 4c, lane 2). These

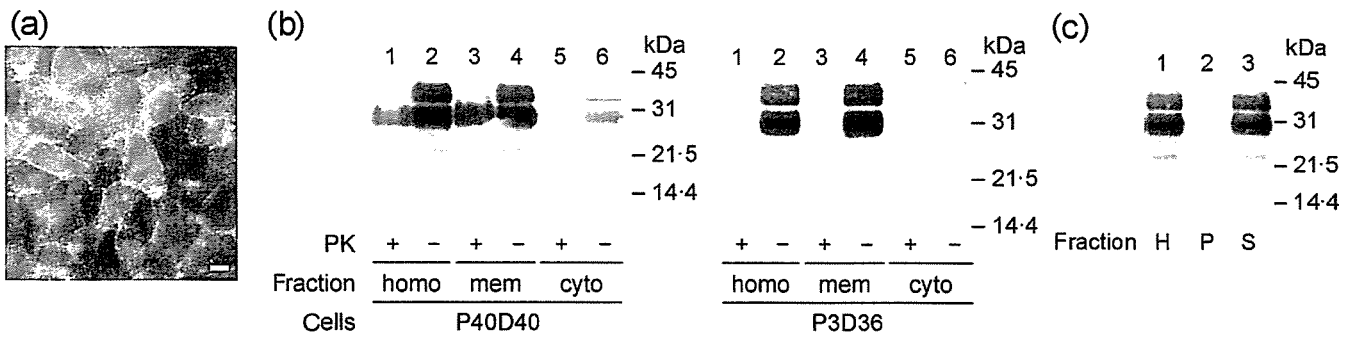

Fig. 4. Subcellular localization and detergent solubility of PrP^{res} in long-term cultured T98G cells. T98G cells were incubated with 10% FCS/RPMI 1640 in the long-term incubation after repeated passages. (a) T98G cells for 40 days after 40 passages (P40D40) on a 15 mm glass coverslip were subjected to indirect immunofluorescence staining with the 6H4 antibody as described in Methods. Bar, 10 μm. (b) T98G cells for 40 days after 40 passages (P40D40, left panel) and for 36 days after 3 passages (P3D36, right panel) were scraped into PBS/2·5 mM EDTA and sonicated. Homogenates (homo) were separated into a membrane fraction (mem) and a cytosolic fraction (cyto). Methanol-precipitated lysates were treated with PK (lanes 1, 3 and 5) or left undigested (lanes 2, 4 and 6). PK-treated samples were subjected to immunoblotting with the 6H4 antibody as described in Methods. (c) T98G cells for 40 days after 40 passages (P40D40) were scraped into PBS/2·5 mM EDTA and sonicated. Homogenates (H) of 50 μg protein were centrifuged as described in Methods to obtain a non-ionic detergent-insoluble pellet (P) and a soluble supernatant fraction (S). Homogenates, pellet and supernatant fractions (50 μg protein each) were subjected to immunoblot with the 6H4 antibody as described in Methods.

Table 1. Subcellular localization of PrP in long-term cultured T98G cells

The amount of PrP is expressed as recombinant bovine PrP equivalents per 10^7 cells. Values are means ± SEM ($n=4$).

Sample	PrP content	
	pmol	%
Homogenate	263·4±20·9	100·0
Membrane fraction	228·9±17·5	86·9
Cytosolic fraction	9·9±0·5	3·8

experiments indicated that PrP^{res} in T98G cells was non-ionic detergent-soluble.

DISCUSSION

The mechanism of the conversion of PrP has not been studied in human cell cultures, due to the lack of a model system. In the present study, we developed such a system by culturing human glioblastoma T98G cells, which express endogenous PrP^{C} constitutively. After reaching a high passage number, long-term cultured T98G cells converted PrP^{C} into PrP^{res}.

Direct sequencing of amplified *PRNP* mRNA and RFLP analysis indicated that the T98G cells were heterozygotes at codon 129 (129M/V) and that no new coding mutations were present in cells that had been subjected to long-term cultures. The deglycosylated form of PK-treated PrP^{res} in T98G cells migrated at approximately 18 kDa. In human prion diseases, two major types of PrP^{res} can be identified, based on electrophoretic migration; the relative molecular mass of the unglycosylated form is approximately 21 kDa (described as type 1) or 19 kDa (described as type 2) (Parchi *et al.*, 1997). Accordingly, PrP^{res} in T98G cells is similar to the previously described MV2 phenotypic variant (Parchi *et al.*, 1999a). However, the size of the deglycosylated PK-resistant fragment in T98G cells was smaller than that of the corresponding fragments observed in type 2 PrP^{res}. Most importantly, the 3F4 antibody, which is a well-characterized antibody known to target residues 109–112 as its epitope (Kascsak *et al.*, 1987; Matsunaga *et al.*, 2001), did not react with PK-digested PrP^{res} in T98G cells, suggesting that the N-terminal PrP region up to residue 109 might be absent in PK-treated PrP^{res} in T98G cells. Human PrP^{res} peptide is divided into three regions that are defined by their PK-cleavage patterns: an N-terminal region (residues 23–73) that is invariably PK-sensitive, a C-terminal region (residues 103–231) that is invariably PK-resistant and a variably digested region (residues 74–102), where the major cleavage sites are at G82 in type 1 and at S97 in type 2 (Parchi *et al.*, 2000). The 3F4 antibody was used to type PrP^{res} (Parchi *et al.*, 2000). Therefore, there are striking differences in the antigenicity, which reflect the PK-cleavage patterns, between type 2 PrP^{res} in sporadic CJD brain and in T98G cells. It is unlikely, but not impossible, that PK treatment generated conformational changes in the mid-region of PrP^{res} that interfered with epitope recognition by the 3F4 antibody. Further studies are needed to classify the type of PrP^{res} in lysates from long-term cultured T98G cells.

So far, human PrP^{Sc} has been analysed on immunoblots with the 3F4 antibody. Our finding may explain why previous studies have failed to detect PrP^{res} in cultured cells. Interestingly, an N-terminally truncated 18 kDa fragment of PrP (designated C1) in normal and sporadic CJD brains has similar properties except that it is PK-sensitive; it is recognized by the anti-C terminus antibody, but not by the 3F4 antibody, is cleaved around residue 111 and is associated with cell membranes (Chen *et al.*, 1995). PrP^{C} from human brain homogenates ($n=6$) originally displayed a partial PK resistance (20 μg ml^{-1} for 10 min) and has been detected by the antibody that recognizes residues 145–163, but not by the 3F4 antibody (Buschmann *et al.*, 1998). Taking the data from the various studies of PrP immunoreactivity into consideration, we believe that it would be better to incorporate an additional antibody that recognizes the C terminus of PrP into the standardly used protease resistance-dependent PrP^{Sc} assay.

Among the sets of antibodies used in this study, the anti-N-terminal portion antibodies (HUC2-13 and 3F4) reacted strongly with the fully glycosylated form and moderately with the partially glycosylated form. In contrast, the antibodies against the C-terminal portion of PrP (6H4 and HPC) reacted moderately with the fully glycosylated form and strongly with the partially glycosylated form. It is possible that PK digestion induces a conformational change of digested PrP and enhances its immunoreactivity to the anti-C-terminal antibodies. Recently, it has been reported that the amino acid motif Tyr-Tyr-Arg (YYR), located in a β-sheet, is exposed in PrP^{Sc}, whilst it is cryptic in PrP^{C}, and that antibodies recognize YYR in PrP^{Sc}, but not in PrP^{C} (Paramithiotis *et al.*, 2003). Another paper has reported that PK digestion enhances immunoreactivity to the anti-PrP antibody that recognizes the epitope YYR, located in a β-sheet (Brun *et al.*, 2004). These reports suggest that conformation of the C-terminal portion of PrP^{Sc} is essential for immunoreactivity of anti-YYR antibodies. The 6H4 antibody also recognizes residues 144–152 of PrP, including a YYR motif that is located in an α-helix, not in a β-sheet (Korth *et al.*, 1997). Further study is needed to clarify the immunoreactivity of anti-C-terminal PrP antibodies.

It has been proposed that PrP^{C} is converted into PrP^{res} either on the cell surface or in endocytic cellular compartments. PrP^{C} is a surface protein that contains a glycosylphosphatidylinositol anchor (Stahl *et al.*, 1987). A portion of PrP^{Sc} is also localized on the cell surface of scrapie-infected mouse neuroblastoma ScN2a cells (Naslavsky *et al.*, 1997; Vey *et al.*, 1996), although it is also found in lysosomes (Taraboulos *et al.*, 1990). Subcellular localization of PrP^{res} in long-term cultured T98G cells was similar to that of PrP^{Sc}-infected cells, being present on the cell surface.

PrP^{Sc} in ScN2a cells is sedimented by centrifugation in non-ionic detergents (Caughey *et al.*, 1991). Mutant PrP in stably transfected Chinese hamster ovary cells, which express murine homologues associated with human inherited prion diseases, is also non-ionic detergent-insoluble (Lehmann & Harris, 1996). However, the PrP^{res} in T98G cells is detergent-soluble. PrP^{res} in the human neuroblastoma cell line M-17 BE(2)C carrying the familial subtype CJD, the glutamic acid to lysine substitution at codon 200 (E200K), is also partially non-ionic detergent-insoluble (Capellari *et al.*, 2000). The present study indicates that not all PrP^{res} is non-ionic detergent-insoluble.

Many cultured cells that express PrP^{res} mutants carrying substitutions of inherited prion disease show considerably less protease resistance (up to 3·3 $\mu g\ ml^{-1}$ for 10 min), compared with PrP^{res} mutants isolated from the human brain (Capellari *et al.*, 2000; Harris, 2001). In contrast, the PrP^{res} in T98G cells displayed a high resistance to digestion with PK (10 $\mu g\ ml^{-1}$ for 30 min), but was less resistant than PrP^{res} in brain homogenates of sporadic CJD (up to 100 $\mu g\ ml^{-1}$ for 24 h). Sporadic CJD is typically characterized by widespread spongiform degeneration with loss of neurons, gliosis and formation of amyloid plaques (Parchi *et al.*, 1999a). It has recently been reported that six cases of sporadic fatal insomnia, a prion disease mimicking fatal familial insomnia, had no coding-region mutation of *PRNP* with the 129 M/M genotype and an approximately 19 kDa deglycosylated PrP^{res}, the same as that of type 2 (Mastrianni *et al.*, 1999; Parchi *et al.*, 1999b). Familial progressive subcortical gliosis may also be a prion disease, characterized by astrogliosis at the cortex–white matter junction (Petersen *et al.*, 1995). All patients from two families with that disease showed no coding-region mutation of *PRNP*, the 129 M/M genotype and the 18·1–19·3 kDa form of deglycosylated PrP^{res} (Petersen *et al.*, 1995). T98G cells were grown out of human glioblastoma multiforma tumour tissue of a 61-year-old Caucasian man (Stein, 1979). We consider it possible that he also had a sporadic form of prion disease.

Conversion from PrP^{C} into PrP^{res} is an important process, because most prion diseases are characterized by presence of PrP^{res}. Some knowledge of the conversion mechanism is based on studies of scrapie-infected cells. Recently, it has been reported that several conditions can induce the formation of PrP^{res} in cultured cells. Proteasome inhibitors cause accumulation of the unglycosylated form of PrP^{res} in treated cells (Lehmann & Harris, 1997; Ma & Lindquist, 1999; Yedidia *et al.*, 2001). PrP that misfolds during maturation in the endoplasmic reticulum is delivered to the cytosol for degradation by proteasomes (Béranger *et al.*, 2002; Ma & Lindquist, 2001; Yedidia *et al.*, 2001). It has been hypothesized the conversion into PrP^{res} might occur when the number of PrP molecules exceeds the capacity of the cell to degrade them (Ma & Lindquist, 2002). Another study showed that manganese-treated mouse astrocytes express the glycosylated form of PrP^{res} (Brown *et al.*, 2000). Here, we report for the first time the conversion of PrP^{C} into PrP^{res} in the widely used human glioblastoma cell line T98G; a large number of passages and prolonged incubation under routine cell-culture conditions are required. *In vitro*-generated PrP^{res} is reportedly not sufficient for the production of infectivity (Caughey *et al.*, 2001; Hill *et al.*, 1999) and further study is needed to clarify the infectivity of PrP^{res} in T98G cells (indeed, caution should be taken with T98G cells in the laboratory). Infectivity assays of PrP^{res} in T98G cells are now in progress in transgenic mice.

In conclusion, T98G cells should be a useful model for studying the mechanisms of PrP^{C} conversion into PrP^{res}.

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