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Treatment of Scrapie Pathogen 263K With Tetracycline Partially Abolishes Protease-resistant Activity *in vitro* and Reduces Infectivity *in vivo*[1]

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Objective To study the possible effect of tetracycline on protease-resistant activity *in vitro* and infectivity *in vivo* of a scrapie strain 263K. **Methods** Scrapie pathogens were incubated with tetracycline at different concentrations for various periods of time and protease-resistant PrP signals were evaluated with proteinase K-treatment and Western blots. The preparations treated with tetracycline were intracerebrally inoculated into golden hamsters and typical TSE manifestations were noted. PrP^{Sc} in brain tissues of the infected animals was detected by PrP specific Western blot assays. **Results** Protease-resistant PrP was significantly reduced in or removed from the preparations treated with tetracycline in a dose-dependant manner. Compared with the control group after incubated for 53.75±0.50 days, the preparations treated with 5 mmol/L and 20 mmol/L tetracycline prolonged the incubation time of 61.5±1.73 and 59.5±0.58 days (P<0.05). **Conclusion** Treatment of scrapie pathogen 263K with tetracycline reduces or removes its protease-resistant activity *in vitro*.

Key words: Scrapie; Prion; Tetracycline; Protease-resistant activity; Animal experiment

INTRODUCTION

Prion diseases, also known as transmissible spongiform encephalopathies (TSE), are fatal neurodegenerative disorders that have attracted great attention not only for their unique biological features but also for their impact on public health. This group of diseases includes kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Straüssler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI) in human beings, as well as scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattles, and encephalopathies in mink, cats, mule deer, elk, and several exotic ungulates. Prions are devoid of nucleic acid and seem to be composed exclusively of a modified isoform of the prion protein (PrP) designated PrP^{Sc}[1]. Normal cellular PrP (PrP^{C}) is converted into PrP^{Sc} through a process whereby a portion of its α-helical and coil structure is refolded into β-sheet[2]. This structural transition is accompanied with profound changes in the physicochemical properties of PrP[3], such as insolubility, protease resistance and accumulation in the brain in the form of amorphous aggregates and amyloid fibrils[4]. PrP^{Sc} is responsible for neuronal degenerative and glial activation and is critical for disease transmissibility by converting PrP^{C} into PrP^{Sc}[5]. PrP^{Sc} represents a primary target for therapeutic strategies.

It has been found that tetracycline binds to synthetic PrP peptides, hinders assembly of these peptides into amyloid fibrils, reverts the protease resistance of PrP^{Sc} extracted from brain tissue of patients with sporadic CJD, and prevents neuronal death and astrocyte proliferation induced by PrP peptides *in vitro*[6]. A study group has even proved that tetracycline reverses protease-resistance of PrP^{Sc} and reduces prion infectivity[7]. Here we report the

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effect of tetracycline on proteinase resistant activity *in vitro* and infectivity *in vivo* of a scrapie strain 263K.

MATERIALS AND METHODS

Preparation of PrP^{Sc} Extracts

Brain homogenates from hamsters infected with scrapie strain 263K were prepared and prion protein was purified as described previously[8]. Ten percent of homogenates from the tested brain samples were prepared in lysis buffer (100 mmol/L NaCl, 10 mmol/L EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mmol/L Tris, pH 7.5). Tissue debris were removed by low speed centrifugation at 2000×g for 10 minutes, and the supernatants were further centrifuged at 20000×g for 90 minutes at 4℃. The pellets were resuspended in 40 μL deionized and distilled water (dd water) and stored at –70℃ until use.

Co-incubation of PrP^{Sc} With Tetracycline

PrP^{Sc} extract aliquots were mixed with an equal volume of tetracycline hydrochloride solutions containing 10 mmol/L Tris-HCl and 1 mmol/L $CaCl_2$ to give final concentrations of 0.1 mmol/L, 1 mmol/L, 5 mmol/L, and 20 mmol/L, respectively. Sample aliquots were incubated at 37℃ for 10 min, 12 h, 24 h, 48 h, 72 h, and 96 h, respectively. For analysis of protease resistant activity, tetracycline-treated preparations were digested with 200 μg/mL proteinase K at 37℃ for 1 h. Digestion was terminated by addition of an equal volume of 5 × sodium dodecylsulphate sample buffer (125 mmol/L Tris hydrochloric acid, 50% (v/v) glycerol, pH 6.8, 10% (w/v) sodium dodecylsulphate, 10% (v/v) 2-mercaptoethanol, 20 mmol/L 4-(2-aminoethyl)-benzene sulfonyl fluoride, and 1% (w/v) bromophenol blue, and was immediately transferred to a 100℃ heating block for 10 min.

Experimental Scrapie

Eighteen 2-week old female golden hamsters were subjected to bioassays and six hamsters were randomly allocated to three groups. 10^{-2} dilutions of brain homogenates from scrapie 263K-infected hamsters at the terminal stage of disease were mixed with the same volume of tetracycline hydrochloride to give the final concentration of 5 mmol/L and 20 mmol/L, separately. After incubation at 37℃ for 24 h, 10 μL individual preparations was injected into the right cerebral hemisphere of hamsters. The animals were observed twice a week to monitor the onset and progression of clinical manifestations. As described previously[9-10], ataxia was used as the sign of onset of experimental scrapie in hamsters. All animals in the three groups were killed at the final stage of disease and their brain homogenates were detected by Western blot as described below.

Western Blot Analysis

All samples were centrifuged at 14 000 rpm for 1 minute in a microfuge before electrophoresis on 15% polyacrylamide gels. The gels were electroblotted onto nitrocellulose membrane and blocked for 2 hours or overnight in 5% (w/v) defatted milk powder in PBS containing 0.05% (v/v) Tween-20 (PBST). The blotting membranes were incubated with a PrP-specific monoclonal antibody 3F4 (Dako) diluted at 1:1000 in PBST for 2 hours at 37℃. After washing with PBST for 30 minutes, members were incubated with a horseradish peroxidase conjugated anti-IgG (Santa Cruz) in PBST for 2 hours, followed by development in DAB substrate.

RESULTS

Treatment of Tetracycline Remarkably Abolished Protease-resistance of PrP^{Sc} in vitro

To observe the effects of tetracycline on PK-resistance of PrP^{Sc}, various amounts of tetracycline were mixed with PrP^{Sc} extracts from scrapie 263K at 37℃ for 10 min. Western blot assays showed that the amount of protease-resistant PrP started to reduce in the preparation of 0.1 mmol/L tetracycline in a dose-dependant manner, and dropped down to 50.59% and 29.17% in the preparations of 5 mmol/L and 20 mmol/L tetracycline, respectively, compared with that of the preparation without tetracycline (Fig. 1).

To ascertain the time-related characteristics of tetracycline on PK-resistance of PrP^{Sc}, various preparations with different amounts of tetracycline were incubated for 10 min, 12 h, 24 h, 48 h, 72 h, and 96 h, respectively, and were subsequently employed for PK-treated Western blot assays. Compared with the freshly prepared PrP^{Sc}, the preparation having undergone all treatment procedures except for tetracycline treatment showed relatively lower portions of PK-resistant PrP signals, but remained almost unchanged from 10 min to 96 h incubation at 37℃ (Fig. 2). PrP^{Sc} signals in all preparations treated with tetracycline started to become weak after incubation for 10 min, but remained quite stable within 48 h. Only in the preparations with a higher concentration of tetracycline (5 and 20 mmol/L) PK-resistant PrP signals reduced significantly after 72 and 96 h incubation (Fig. 2), indicating that the influence of tetracycline on PK-resistance of PrP^{Sc} occurred immediately after mixture. Prolonging

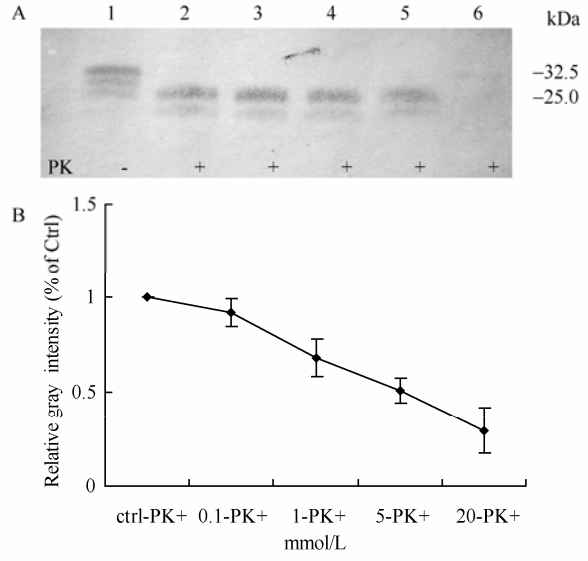

FIG. 1. Protease resistance of PrP^{Sc} after treatment with tetracycline (A) and quantification of protease resistance signals of PrP^{Sc} in various preparations (B). Lane 1: positive control before protease K digestion; lane 2: positive control after protease K digestion; lane 3: 0.1 mmol/L; lane 4: 1 mmol/L; lane 5: 5 mmol/L and lane 6: 20 mmol/L. +: after PK digestion; -: before PK digestion.

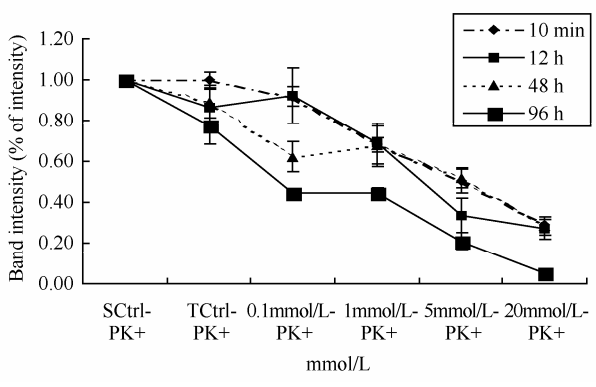

FIG. 2. Time-related reduction of protease resistance of PrP^{Sc} after treatment with tetracycline.

treatment time might strengthen the effect of the preparations with a higher concentration of tetracycline, but had very limited effect of the preparations with a lower tetracycline concentration.

Treatment of Tetracycline Prolonged Incubation Time of Scrapie

To examine whether the physicochemical changes of PrP^{Sc} induced by tetracycline are associated with a decrease in prion infectivity, the brain homogenates of scrapie strain 263K-infected hamsters were treated with 5 and 20 mmol/L tetracycline and intracerebrally inoculated into two-week old hamsters. All the hamsters developed ataxia, tremor (especially of head and neck), hyperreactivity to tactile and acoustic stimulation, abnormal posture, and locomotor incoordination that progressed to recumbency, and weight loss. No difference in clinical manifestations among various groups was noticed. The mean incubation time of scrapie in the hamsters receiving the preparations treated with 5 and 20 mmol/L tetracycline (61.5±1.73 and 59.5±0.58 days, respectively) was longer than that (53.75±0.50 days) in the control group (0 mmol/L tetracycline) ($P < 0.01$ and $P < 0.05$, Table 1). However, no significant difference in incubation times was found between groups treated with 5 and 20 mmol/L tetracycline.

To detect PrP^{Sc} in the brain tissues of infected hamsters, all animals were killed at the terminal stage

TABLE 1

Clinical Features and Identification of PrP^{Sc} in Brain Tissues of Hamsters Inoculated With Scrapie Preparations Treated With Tetracycline

Group With Tetracycline	Clinical Sign (Ataxia)	Mean Incubation (Days)	Western Blot (PrP^{Sc})
0	6/6	53.75±0.50	6/6
5 mmol/L	6/6	61.50±0.58*	6/6
20 mmol/L	6/6	59.50±1.73**	6/6

Note. *, **: $P<0.01$, $P<0.05$ *vs* control group.

of disease and PrP^{Sc} was exacted from the brain stem and analysed by Western blot. Protease-resistant PrP^{Sc} existed in all experimental hamsters, in which the amount of PrP^{Sc} had no obvious difference among these three groups (Fig. 1). Meanwhile, the glycosylating patterns of PrP^{Sc} in these groups were coincident, the diglycosylated form of PrP^{Sc} was predominant followed by monoglycosylated and nonglycosylated PrP^{Sc}. These data suggested that treatment of PrP^{Sc} from scrapie 263K with intracerebral inoculation of tetracycline could delay the occurrence of the disease, but had no effect on the molecular characteristics of newly formed PrP^{Sc}.

DISCUSSION

Tetracycline shows structural analogies with other potential anti-prion compounds, such as Congo red, tetrapyrroles, and polyamines[11-13]. Unfortunately, these compounds are not suitable for clinical therapy because of incapability of crossing the blood-brain barrier and/or severe toxicity. All these molecules contain an extended hydrophobic core formed by aromatic moieties with a large number of hydrophilic substituents conferring an amphiphilic property. It is conceivable that these characteristics enable strong interactions between such compounds and lipophilic domains of PrP^{Sc} and are important structural features of the anti-prion activity[13-15].

Tetracycline has been reported to be capable of reverting abnormal physicochemical properties and abolishing neurotoxicity of PrP peptides *in vitro*[6]. Forloni and his co-workers found that incubation with tetracyclines decreases protease-resistance of PrP^{Sc} exacted from the brain of vCJD and BSE in a dose-dependent manner[7], which was verified in our study. The protease resistant activity of PrP^{Sc} is widely used as a diagnostic hallmark of TSE and correlates with the infectivity of various TSE pathogens in experimental tests. Our previous study revealed that some physical and chemical agents which destroyed protease resistance of PrP^{Sc} from scrapie strain 263K *in vitro*, reduced or eliminated its infectivity when challenged into hamster brains[16]. Meanwhile, the conditions removing protease resistances are much less stringent than those for removing infectivity of PrP^{Sc}. Changes in protease resistance of PrP^{Sc} may occur immediately when PrP^{Sc} is mixed with tetracycline and become predominant when reaction time is prolonged, even in the preparations with a lower dosage of tetracycline. However, analyses of the intensity of the total PrP signals before PK treatment did not reveal remarkable changes in the preparations with or without tetracycline, even after 96 h incubation (data not shown), indicating that tetracycline does not influence the peptide structure of PrP. Loss of protease resistance of PrP^{Sc} after treatment with tetracycline may be due to its influence on its secondary or tertiary structures. A possible conformational change was induced by interaction between PrP^{Sc} and tetracycline triggers proteolysis on pathological PrP proteins. Such features have been seen in situations treated with other chemicals, *e.g.* Congo red and GdnHCl[13, 17-18]. The reduction of protease resistance of PrP^{Sc} in this study seemed to start immediately after treatment with tetracycline, implying a fast and strong binding activity of such compounds with amphophilic characteristics of the potential lipophilic domain of PrP^{Sc}. In fact, it has been known that both PrP^{Sc} aggregates and oligomeric-monmeric forms of PrP peptides bind to tetracycline and are highly amyloidogenic and neurotoxic[7,19].

This study also showed that mixture of scrapie 263K agents and tetracycline before intracerebral inoculation delayed occurrence of clinical signs and prolonged incubation time of scrapier. Although all hamsters receiving tetracycline-treated scrapie 263K-infected brain homogenates developed scrapie, there was a significant difference in mean incubation time of scrapie between tetracycline-treated groups and control group, demonstrating that administration of tetracycline to scrapie 263K delays occurrence of encephalopathies in the challenged hamsters. The challenge dosage of scrapie 263K in this study was high, whose LD_{50} was previously evaluated as high as 8.2-log10 LD_{50} i.c. units /0.001 g[20], which may explain the phenomenon that experimental scrapie

occurred in all hamsters receiving 263K. Similar results were obtained in a previous study, in which the incubation time of scapie was significantly prolonged[7], suggesting that the animals can survive if they receive lower dosages of TSE agents pre-treated with tetracycline. It is reasonable to speculate that tetracycline is effective enough to reduce or remove the infectivity of prion in natural states, because only very low prion levels can initiate the acquired form of prion diseases, such as iatrogenic CJD.

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