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Comparison Study on Clinical and Neuropathological Characteristics of Hamsters Inoculated With Scrapie Strain 263K in Different Challenging Pathways[1]

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Objective To understand the infectious characteristics of a hamster-adapted scrapie strain 263K with five different routes of infection including intracerebral (*i.c.*), intraperitoneal (*i.p.*), intragastrical (*i.g.*), intracardiac and intramuscular (*i.m.*) approaches. **Methods** Hamsters were infected with crude- or fine-prepared brain extracts. The neuropathological changes, PrP^{Sc} deposits, and patterns of PK-resistant PrP were analyzed by HE stain, immunohistochemistry (IHC) assay and Western blot. Reactive gliosis and neuron loss were evaluated by glial fibrillary acidic protein (GFAP) and neuron specific enolase (NSE) specific IHC. **Results** The animals inoculated in *i.m.* and *i.p. ways* with crude PrP^{Sc} extracts showed clinical signs at the average incubation of 69.2±2.8 and 65.5± 3.9 days. Inoculation in *i.c.* and intracardiac ways with fine PrP^{Sc} extracts (0.00035 g) caused similar, but relative long incubation of around 90 days. Only one out of eight hamsters challenged in *i.g way* with low dosage (0.01 g) became ill after a much longer incubation (185 d), while all animals (4/4) with high dosage (0.04 g) developed clinical signs 105 days postinfection. The most remarkable spongiform degeneration and PrP^{Sc} deposits were found in brain stem among the five challenge groups generally. The number of GFAP-positive astrocytes increased distinctly in brain stems in all infection groups, while the number of NSE-positive cells decreased significantly in cerebrum, except *i.c.* group. The patterns of PK-resistant PrP in brains were basically identical among the five infection routes. **Conclusion** Typical TSE could be induced in hamsters by inoculating strain 263K in the five infection ways. The incubation periods in bioassays depend on infective dosage, administrating pathway and preparation of PrP^{Sc}. The neuropathological changes and PrP^{Sc} deposits seem to be related with regions and inoculating pathways.

Key words: Transmissible spongiform encephalopathy; Scrapie; Strain 263K; PrP^{Sc}; Bioassay; Neuropathology; Inoculating pathways

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INTRODUCTION

Prion diseases, also called transmission 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-Straussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI) in human beings, as well as scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, and encephalopathies in mink, cats, mule deer, elk, and several exotic ungulates. Although the agent responsible for TSE has not been completely characterized, three main theories on the nature of TSE agent were proposed, which are special virus that has unusual characteristics, virino that is a very small piece of DNA acting like a virus, and prion that is a malformed protein in the brain.

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]. The 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. Moreover, PrP^{C} is required for scrapie and other TSEs spread within the central nervous system (CNS)[3].

PrP^{C} is expressed extensively in most of mammalian tissues, while the expression level of PrP^{C} is diversified in each tissue. The expression level of PrP^{C} controls both the rate and the route of neuroinvasive infection, from peripheral entry portal to the CNS. In general, the expression level of PrP^{C} in the CNS is much higher than that in the peripheral tissues[4]. In the CNS, PrP^{C} is highly expressed in the upper cortical neurons in neocortex and Purkinje cell in the cerebellum[5,6]. In peripheral tissues of adult mouse and sheep, PrP^{C} is expressed in many systems, such as peripheral nervous system, lymphoreticular system, respiratory system, digestive system, urine system, skin system, blood system, *etc.*[4,7-9]. Wide distributions of PrP^{C} in various tissues may provide the possibility of prion transmission from peripheral tissues to central nerve system in mammalian hosts. In fact, three prominent possibilities of neuroinvasion pathways have been accepted, including peripheral nervous system (PNS) by axonal transport, lymphoreticular system (LRS) and hematogenous spread by blood cell[10].

Scrapie strain 263K has a very low pathogenicity for mice, as it originates as a mutant that is strongly selective on passage in hamsters[11]. Although it is not natural, 263K is the most widely used strain of agent in scrapie research because it produces very short incubation periods in golden hamsters and exceptionally high infectivity titers in clinically affected brain[12]. Several infection routes with strain 263K have been confirmed to be able to cause prion diseases in hamster, such as intracerebral (*i.c.*), intraspinal, intraocular, intraperitoneal (*i.p.*) and oral infection[10,13,14], among them central infection ways, such as *i.c.*, is the most effective transmission way. Recently, other pathological events in brain tissues such as neuronal cell damage, apoptosis and gliosis were described elsewhere[15-17]. However, there is still lack of direct evidences to show hamster's peripheral blood cells with detectable amounts of PrP^{C}, as well as blood transmission with scrapie strain 263K, although it is believed that blood system might act as a vehicle aiding the PrP^{Sc} transporting with endothelial cell in human and mouse[9]. Research on blood transmission of TSE agents in different animals will stress our knowledge on the pathogenesis of scrapie and other TSEs.

In this study, five different infection ways with strain 263K were administrated into hamsters, including *i.c.*, *i.p.*, intragastrically (*i.g.*), intracardiac and intramuscular (*i.m.*) ways. Neuropathological changes and PrP^{Sc} deposits, as well as astrocytosis and loss of

neuron in separate brain regions were comparatively analyzed among various infection pathways.

MATERIALS AND METHODS

Scrapie Sample Preparation

Scrapie strain 263K was prepared from infected hamster brain tissues, which had been confirmed by Western blot and immunohistochemistry[18]. For ***i.m.***, ***i.p.*** and ***i.g.*** infections, crudely prepared scrapie solution was performed from 10% brain homogenates in phosphate buffered saline (PBS). For ***i.c.*** and intracardiac injection, 10% brain homogenates were sonicated (400 W, 10 sec for 30 times) and tissue debris were removed by centrifugation at 20 000 g for 90 min at 4℃, referred as finely prepared scrapie solution, described elsewhere[19]. An aliquot of 100 μL crudely prepared scrapie sample was roughly equal to 0.01 g infected brain, while an aliquot of 100 μL finely prepared scrapie sample was roughly equal to 0.0075 g infected brain.

Hamster Inoculation

A total of thirty-six 2-week old female golden hamsters were challenged by scrapie strain 263K through various pathways. Besides four animals for ***i.c***. inoculation as positive control, eight hamsters were randomly selected for intracardiac, ***i.m.***, ***i.p.*** and ***i.g.*** inoculation. For ***i.c.*** infection, 5 μL fine scrapie sample was injected into parietal lobe at a depth of 4 to 5 mm. For intracardiac infection, 5 μL fine scrapie sample was injected into the left ventricle. For ***i.m.*** infection, 100 μL crude scrapie sample was injected into the right hindlimb. For ***i.p***. infection, 200 μL crude scrapie sample was injected into the peritoneum. For ***i.g.*** infection, 100 μL or 400 μL crude scrapie sample was filled into the stomach. All the samples were mixed with 1 mg/mL ampicillin at final concentration before challenge. Two hamsters were fed as negative control.

Necropsy

Necropsy was performed within 36 h after natural death or immediately after the animal was killed by ether and exsanguination. The brain was removed from the tested hamster for scrapie diagnosis as described elsewhere. Parts of the collected samples were stored at –70℃ until use, and the rests were fixed in 10% formalin at room temperature.

Histological and Immunohistochemical Procedures

Before histological processes, all the fixed tissues were immersed in 88% formic acid for at least 1 h for inactivation[20]. Paraffin sections (5 μm in thickness) were subjected to conventional staining with hematoxylin and eosin (HE). For PrP immunohistochemistry (IHC), the slices were treated with microwave irradiation (Galanz, WP1000L30-2) in distilled water for 30 minutes, followed by 3% hydrogen peroxide-methanol for 10 minutes and 0.1% trypsin for 20 minutes at room temperature. The slices were blocked with 0.1% normal horse serum for 20 minutes, and reacted with PrP-specific monoclonal antibody FH11 at dilution of 1:400 overnight at 4℃. For visualization of immunostaining, the slices were developed with an avidin-biotin labelling kit and AEC kit (Vector Laboratories, Inc.). The slices were counterstained slightly with hematoxylin, dehydrated, and mounted in glycerolvinyl alcohol.

Neuronal and Glial Cell Markers

The sections were first treated with microwave irradiation (Galanz, WP1000L30-2) in distilled water for 10 minutes, followed by 3% hydrogen peroxide-methanol for 10 minutes at room temperature and then with 0.1% normal horse serum for 20 minutes. After washed, the sections were incubated with the primary antibodies at 4℃ overnight. The rabbit polyclonal antibody to neuron specific enolase (NSE, Boster Technology Inc.) and the rabbit polyclonal antibody to glial fibrillary acidic protein (GFAP, Boster Technology Inc.) were used at a dilution of 1:200. Following incubation with the primary antibody, the sections were incubated with a horseradish peroxidase conjugated secondary IgG antibody (Santa Cruz) diluted at 1:500 for 2 h at room temperature, and finally stained with AEC kit (Vector Laboratories, Inc.). The stainings of NSE and GFAP were observed without counterstaining. The positive stains were counted and averaged within three fields of each section.

Prion Protein Purification

Brain homogenates were prepared and prion protein was purified referring a procedure described previously[21], with some modifications. 10% homogenates of 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). The tissue debris was removed by low speed centrifugation at 2 000 g for 10 minutes, and the supernatants were further centrifuged at 20 000 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.

Proteinase K Digestion and Western Blot Analysis

Prior to Western blot, the purified prion proteins were mixed with 200 μg/mL proteinase K at 37℃ for 1 hour. Digestion was terminated by addition of an equal volume of 2×sodium dodecylsulphate sample buffer (125 mmol/L Tris hydrochloric acid, 20% v/v glycerol, pH 6.8, 4% w/v sodium dodecylsulphate, 4% v/v 2-mercaptoethanol, 8mmol/L 4-(2-aminoethyl)-benzene sulfonyl fluoride, and 0.02% w/v bromophenol blue) and immediately transferred to a 100℃ heating block for 10 min. All samples were centrifuged at 14 000 rpm for 1 minute in a microfuge before electrophoresis on 15% polyacrylamide gels. The gels were electroblotted on to 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 washed in PBST for 30 minutes, blots were incubated with a horseradish peroxidase conjugated anti-IgG (Santa Cruz) in PBST for 2 hours, followed by development in DAB substrate.

RESULTS

Onset and Incubation of Scrapie in Bioassays Depended on Infective Dosage, Administrating Pathway and Preparation of PrP^{Sc}

A total of five different infection routes have been used in this study, including *i.c., i.p., i.m.,* intracardiac and *i.g.* ways. To define the potential ways of scrapie 263K infection, a larger dosage of infectious agents (0.01-0.02 g brain tissues) was administrated in the other peripheral ways, compared with *i.c*. and intracardiac routes (0.00035 g brain tissues). Except

TABLE 1

Summary of the Clinical and Neuropathological Features of Scrapie Strain 263K Infected Hamsters With Different Challenging Pathways

Pathway	Dosage (g/Case)	Mean Incubation (day)	No. of Ill/Inoculated Cases	Vacuolization			PrP^{Sc} Deposits			PrPres (Positive/Test Cases)
				Brain Stem	Cerebellum	Cerebrum	Brain Stem	Cerebellum	Cerebrum	
i.c.	0.00035	91.5±20.5	4/4	+++	++	+/-	+++	++	+	4/4
Intracardiac	0.00035	90.1±11.5	7/7	+++	++	+/-	+++	++	++	7/7
i.m.	0.01	69.2±2.8	8/8	+++	++	+/-	+++	++	++	8/8
i.p.	0.02	65.5±3.9	8/8	+	+/-	+/-	++	+	+	8/8
i.g.	0.01	185	1/8	+	+	+/-	++	+	++	1/8
i.g.	0.04	105	4/4	+	+	+/-	+	+	++	4/4

Note. Semi-quantitative representation of the main neuropathological and PrP^{Sc} deposit findings: +++ marked, ++moderate, +mild, +/- uncertain.

one hamster died immediately after intracardiac injection, the rest of them remained alive till the occurrence of illness. The main clinical manifestations included ataxia, tremor (especially of head and neck), hyper-reactivity to tactile and acoustic stimulation, abnormalities of posture, locomotor incoordination that progressed to recumbency, and loss of weight. Among them, ataxia was referred to as the commonest sign. The clinical abnormities aggravated progressively without intermittent recovery. There was little difference in clinical signs among the five individual groups.

The incubation period showed almost the same picture between the intraperitoneal and intramuscular attacks with crude PrP^{Sc} extracts, and between the intracerebral and intracardiac ways with fine PrP^{Sc} extracts (Table 1). Being similar to *i.c.* inoculation with strain 263K of 0.0005 g crudely prepared brain tissues (66.7±1.1 days)[18], animals inoculated in *i.m.* and *i.p. routes.* with crude PrP^{Sc} extracts started to show clinical signs around 60-80 days postinfection, with the average incubation of 69.2±2.8 and 65.5±3.9 days (Table 1). Inoculation in *i.c.* and intracardiac routes with fine PrP^{Sc} extracts also caused illness in all tested hamsters, but with a relative long incubation of around 90 days (Table 1). It seemed that preparations of PrP^{Sc} might influence the incubation time. Contrarily, the *i.g.*-administrated animals showed different characteristics both in mobility and incubation time. With the dosage of 0.01 g infectious brain tissues, only one out of eight hamsters showed typical clinical manifestations after a much longer incubation time (185 days post infection, Table 1), which was confirmed by PrP^{Sc} assays subsequently. The rest remained alive even more than 240 days post inoculation, which was roughly four times longer than the incubation time when challenged in *i.c.* route with crudely prepared brain tissues[18]. Western blot and immunohistochemistry (IHC) of the brain tissues did not reveal any detectable PrP^{Sc} (data not shown). However, when the administration dosage increased to 0.04 g, all the tested animals (4/4) developed typical clinical signs, and even if the incubation period became much shorter (105 days post infection, Table 1). It indicated that administration of scrapie agents through various pathways could induce typical TSE illness, if the dosage was large enough.

Neuropathological Changes Seemed to Be Region- and Inoculating Pathway-relative

To see the difference of neuropathological changes in various brain regions based on distinct administration ways, various brain regions including cerebrum, cerebellum and brain stem were screened. Generally, vacuole degenerations were predominantly identified in brain stems in all tested groups (Table 1, Fig. 1). Severe and extensive vacuolization was frequently identified in the animals challenged by *i.c.*, *i.m.* and intracardiac ways. In some areas, small vacuoles were fusing each other, making the brain tissues like spongiform. Compared with the changes in the *i.c.*, *i.m.* and intracardiac ways, the vacuolar degenerations were clearly less severe in the animals of *i.p.*, and *i.g.* groups, by distributing small vacuoles sporadically. Cerebellum was the second most severely involved region, especially in the hamsters of *i.c.*, *i.m.* and intracardiac inoculations, in which the vacuolization occurred mostly in the layer of Purkinje cells. Only a few vacuoles could be seen in the animals challenged by *i.p*, and *i.g.* inoculations. Extensive vacuolization seemed to be uncommon in cerebrum in all five tested groups.

Besides spongiform degeneration in brain tissues, some other neuropathological changes were repeatedly observed. Cell proliferation was found in half of the tested animals, mainly in the deep layer of the cerebral cortex (Fig. 1). Loss of Purkinje cells in cerebellum was commonly found in the hamsters with *i.c.*, *i.m.* and intracardiac challenges, however, rarely observed in those with *i.p.* infection (Fig. 1). None of the hamsters infected in *i.g.*

way showed obvious loss of Purkinje cells in cerebellum. Nucleus shrink was also commonly observed in almost all tested samples, often involving the areas of brain stem and cerebrum (Fig. 1).

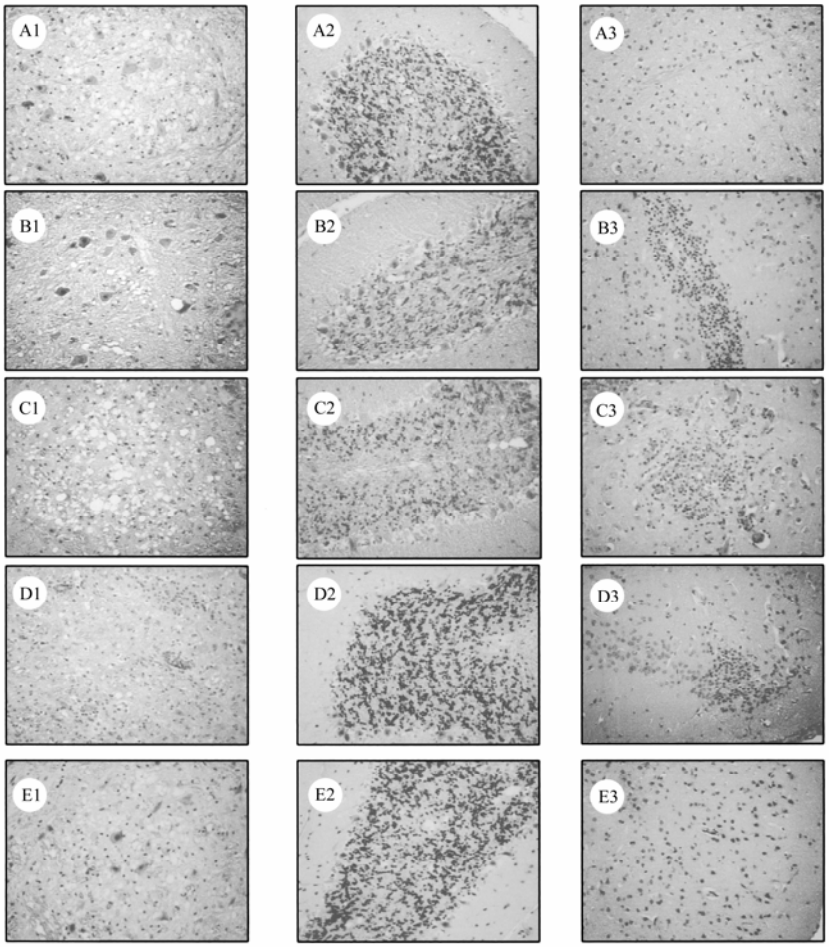


FIG. 1. Neuropathological assays of brain stem (1), cerebellum (2), cerebrum (3) collected from infected animals challenged in *i.c.*(A), intracardiac (B), *i.m.* (C), *i.p.* (D) and *i.g.* (E). (×320).

Severity and Extensity of PrP^{Sc} Deposits Were Coincident Well With Those of Spongiform Degeneration

To investigate the characteristics of PrP^{Sc} accumulation in the brain tissues with the five different infection routes, special IHC assays with PrP^{Sc} specific monoclonal antibody FH11 were done. In general, PrP^{Sc} immunoblots were widely distributed in the brain tissues of all infected hamsters, including brain stems (midbrain and pons), deep layer cerebral cortex, cerebellum, thalamencephalon and hippocampus, which corresponded well to the neuropathological findings (Fig. 2). Brain stem was the mostly affected region, in which widely distributed PrP^{Sc} blots were observed and accompanied with severe spongiform degeneration, especially in the *i.c.*, *i.m.* and intracardiac groups (Fig. 2, Table 1). Based on

extensively dispersive deposits of PrP^{Sc} in tissues, larger immuno-stained plaques were repeatedly observed surrounding the unequirotal vacuoles. In line with the spongiform changes, large plaques of PrP^{Sc} were unusual in *i.p.* and *i.g.* inoculated animals. Spot PrP^{Sc} blots were often found in the layer of granule cells, whereas more diffuse blots in the layers of molecules, Purkinje cells and medulla of cerebellum (Fig. 2，Table 1). The severity and patterns of PrP^{Sc} deposits in cerebellum did not reveal remarkable diversity among the five challenge groups in this study. PrP^{Sc} deposits distributed generally in each layer of cerebrum, but mainly in the layer III to V as described previously[18]. PrP^{Sc} was commonly deposited within neuron cytoplasma, while in some cases diffusely extracellular PrP^{Sc} deposits could be identified. Corresponding to the pathological vacuolization in HE staining, the severity of PrP^{Sc} deposits was less prominent in cerebrum. Diffusive and sporadic PrP^{Sc} blots were seen in thalamencephalon with various vacuoles (data not shown). Collectively, the severity and extensity of PrP^{Sc} deposits were coincident well with those of spongiform degeneration.

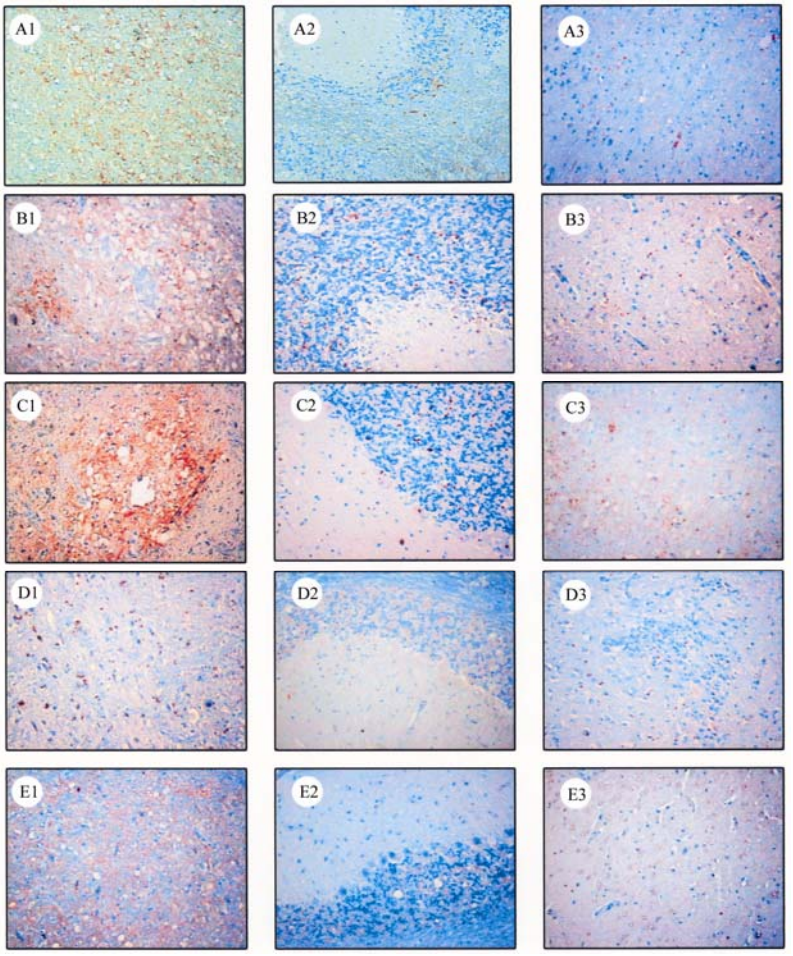
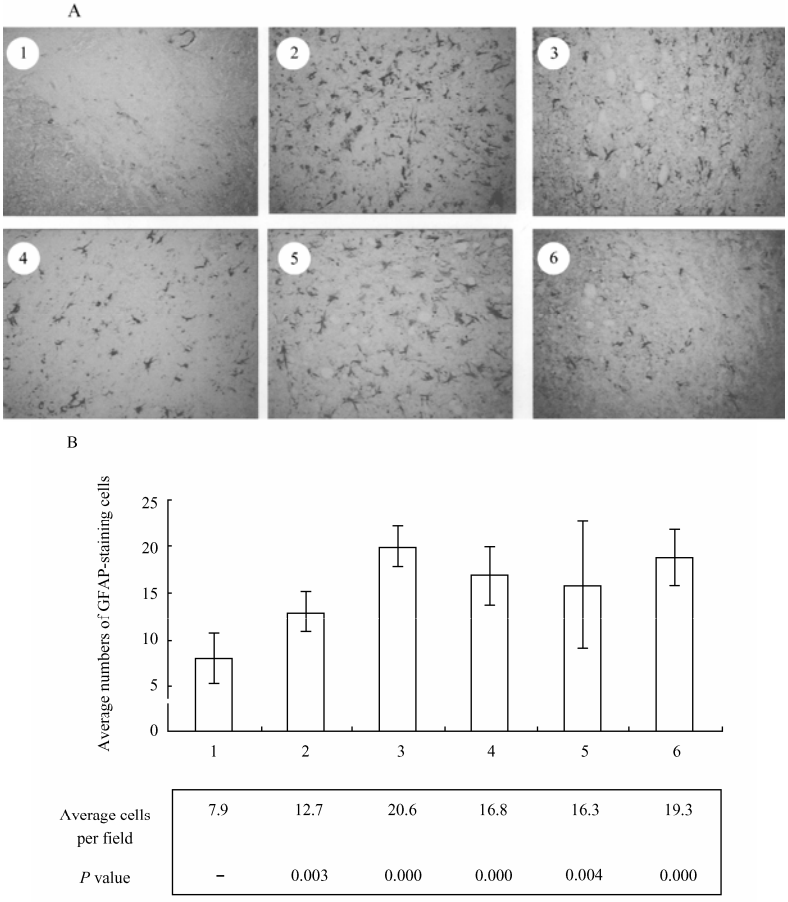


FIG. 2. PrP-specific IHC staining of brain stem (1), cerebellum (2), cerebrum (3) collected from infected animals challenged in *i.c.*(A), intracardiac (B), *i.m.* (C), *i.p.* (D), *i.g.* (E). (×320).

Astrogliosis in Brain Stem and Loss of Neuron in Cerebrum Were Common Features in Scrapie-infected Hamsters

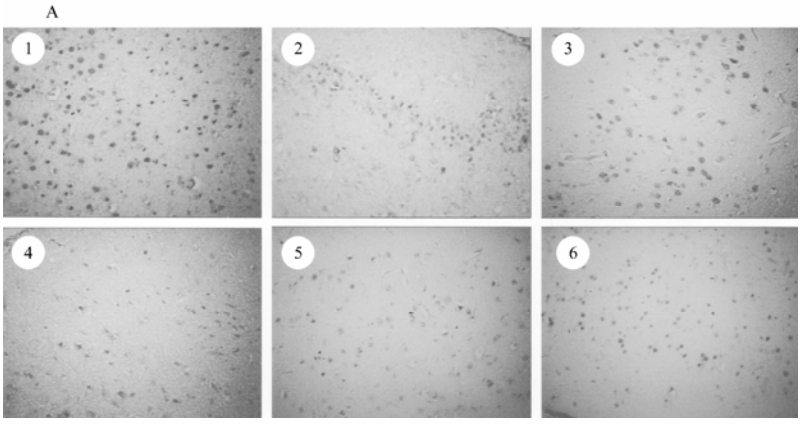
To analyze reactive gliosis during the pathogenesis, glial fibrillary acidic protein (GFAP) in various brain regions was evaluated. An increase in the number of GFAP-immunoblotting astrocytes, which had a large polygon cellular body and many various lengths of fibrils, was repeatedly observed in the scrapie-infected animals within all five infected groups, and compared with the controls (Fig. 3A). Statistic analyses of GFAP staining cells in brain stem per three randomly selected fields under light microscopy (×320) showed significant differences (P<0.01) when compared the average number of the positive staining cells of normal control with that of each five tested pathways (Fig. 3B). It seemed that the peripheral infection was likely to induce more astrocytes than direct intracerebral infection. Correlating well with the characteristics of severe spongiform degeneration and accumulation of PrP^{Sc}, GFAP-positive astrocytes increased distinctly in the region of brain stem as well.

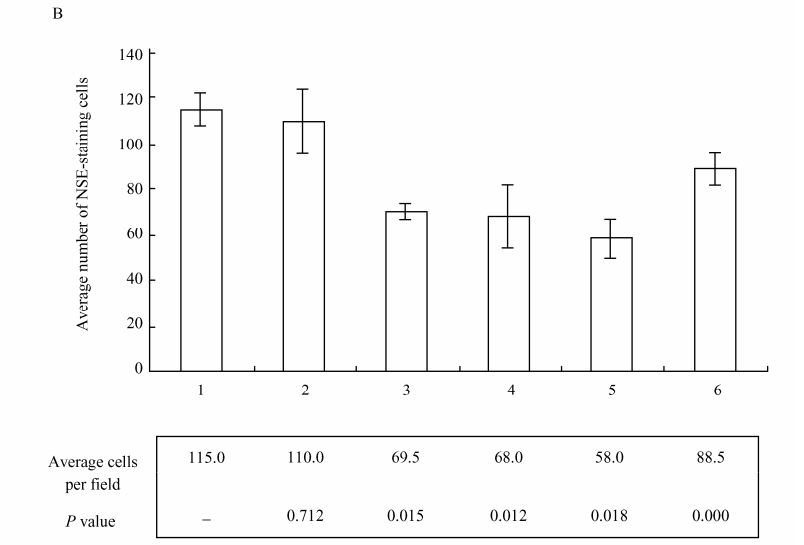


Average cells per field	7.9	12.7	20.6	16.8	16.3	19.3
P value	–	0.003	0.000	0.000	0.004	0.000

FIG. 3. Astrogliosis in brain stems of infected animals. A: GFAP-specific IHC analyses of the tissues of brain stems collected from normal control (1), as well as infected hamsters in *i.c.* (2), intracardiac (3), *i.m.* (4), *i.p.* (5) and *i.g.* (6) (×320). B: Quantitative evaluation of GFAP-positive cells from three randomly selected fields. The average cell number and statistic (*t* test) *P* values are shown under the graph.

Neuron specific enolase (NSE) was manifested as neuron loss in brain tissues. NSE positive attaining cells in cerebrum (layer III to V) of three animals from each group as well as two normal hamsters were counted for per three randomly selected fields under light microscopy (×320) (Fig. 4A). Except *i.c.* group, loss of neuron in the remaining four groups was significant ($P<0.05$) (Fig. 4B), that was approximately related to the distribution of nucleus shrink in scrapie-infected hamster cerebrums.





Average cells per field	115.0	110.0	69.5	68.0	58.0	88.5
P value	–	0.712	0.015	0.012	0.018	0.000

FIG. 4. Losses of neurons in cerebrums of infected animals. A: NSE-specific IHC analyses of cerebrum tissues collected from normal control (1), as well as infected hamsters *i.c.* (2), intracardiac (3), *i.m.* (4), *i.p.* (5) and *i.g.* (6) (×320). B: Quantitative evaluation of NSE-positive cells from three randomly selected fields. The average cell number and statistic (*t* test) *P* values are shown under the graph.

Patterns of PK-resistant PrP in Hamster Brains Were Basically Identical Among Five Infection Routes

To address the presence of PK-resistant PrP (PrP-res) in central nerve tissues as well as the potential difference in PrP patterns and PK-treatment, Western blot analyses were

carried out using PrP-specific monoclonal antibody 3F4. As expected, PrP-res in brains were detected from all ill animals which were separated around 22 to 27 KDa (Fig. 5). There was no detectable difference in either glycosylating patterns or electrophoresis positions among the five infecting pathways. The predominant isoform of PrP-res was of diglycosylated form, followed by the monoglycosylated and aglycosylated form as described previously[18], indicating again that the glycosylating patterns and electrophoresis positions of PrP-res were strain-dependent.

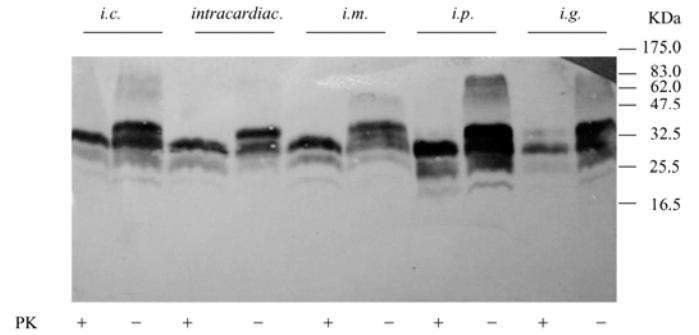


FIG. 5. Western blots of PK-treated (+) or untreated (-) brain samples from clinically affected hamsters challenged in *i.c.*, intracardiac, *i.m*, *i.p.*, and *i.g*. Protein molecular markers are shown in the right.

DISCUSSION

It has been reported that scrapie strain 263K could cause prion diseases in hamsters through various infection pathways, such as intracerebral (*i.c.*), intraspinal, intraocular, intraperitoneal (*i.p.*) and oral challenge[10,13,14]. Here we reported for the first time that strain 263K could induce typical spongiform encephalopathy in the inoculated hamsters through intracardiac and *i.m* pathways.

The incubation period of tested hamsters challenged with a scrapie strain depended mainly on the route of inoculation and the dose of agent, whereas some other factors, e.g. animal gender, ambient temperature (hibernation) and splenectomy have been described to influence the incubation time somehow[22]. It has been reported that the average incubation period changed along with per 10-fold dilution of brain infectivity[23]. Among the five infection ways, *i.g.* infection was least efficient that needed a larger dosage of infectious agent and a longer incubation time. Only one hamster (1/8) developed typical clinical signs after a longer incubation time (185 days post inoculation) at a low infection dosage, while all tested animals (4/4) became ill 105 days post infection at a high dosage,which clearly indicated a dosage-dependent effect. Prolonged incubation (roughly three incubation periods) after challenged at a low dosage of scrapie agent reminded us to re-evaluate the commonly used observing time (two incubation periods) for inactivation of prion infectivity. Although the mechanism of prolonged incubation in *i.g.* infection remains unclear, acidic environment in stomach and numerous proteases in gastro-intestinal tract might contribute to reducing the infectivity of TSE agents[1]. Animals infected in *i.p*. and *i.m.* ways with crudely prepared PrP^{Sc} revealed similar incubation times, which were approximately the same as that infected in *i.c.* pathway[24], though the dosage used in the latter was extremely low. Interestingly, animals infected in *i.c.* and intracardiac ways with the same dosage of finely prepared PrP^{Sc} revealed identical incubation times, indicating that blood transmission of scrapie agent was

as efficient as direct intracerebral injection at the used dosage. Although iatrogenic CJD (iCJD) through circular system had a much longer incubation time than that of iCJD through neurosurgical[25], the exact dosages in two different ways were hard to evaluate. Therefore, one may attribute it, at least partially, to the dosage used.

In our study, severe vacuolization was predominantly identified in brain stems, and secondarily in cerebellum, while mild pathological changes were observed in the other brain regions in all five infecting routes. These results were consistent with the early reports[26]. IHC confirmed the same distributions of PrP^{Sc} deposits in brain regions. Brain stem seemed to be a most frequently affected area in some prion diseases, which is usually used as diagnostic target for BSE and feline spongiform encephalopathy (FSE). Compared with nerval and blood infection pathways (*i.m.* and intracardiac), both vacuolization and PrP^{Sc} deposits in brain stem of the *i.g.* infected hamsters were significantly mild, whereas cerebrum seemed to be the most frequently affected area. Different distribution of neuropathological changes between neuronal infection and peripheral infection was described previously[14]. At present, there was evidence that enteric nervous system (ENS) and peripheral nervous system (PNS) were involved in the pathogenesis of orally communicated transmissible spongiform encephalopathies[10]. Different transport ways of PrP^{Sc} to central nervous system might result in different distributions of neuropathological changes in brain regions. It is interesting to note that *i.p.* challenge induced similar neuropathological distribution as *i.g.* one. Although the pathway of scrapie agent to brain in *i.p.* challenge remains to be defined, the possibility that *i.p.* challenge, besides the transmission in circular system, shares similar pathway like *i.g.* challenge to transport PrP^{Sc} *in vivo* could not be excluded.

Blood transmission with 263K could induce comparably severe lesions in brain stem as nerve infection. It seemed as efficient as nerve transmission to induce scrapie in hamsters. Recent study illustrated that PrP^{C} was undetectable in blood cells of hamster[9]. Therefore, it is reasonable to consider that PrP^{Sc} may only be transported but not replicated in blood. Early report showed that PrP^{Sc} was not detected at infection sites with an impaired blood-brain barrier, such as the area postrema or the choroid plexus. However, investigation of the integrity of the blood-brain barrier in a hamster model of scrapie (263K) during the clinical phase of the disease with magnetic resonance (MR) imaging in combination with gadolinium-diethylenetriaminepenta-acetic acid (Gd-DTPA) enhancement found that blood-brain barrier function was disrupted in the clinically-affected animal[27]. One thing to be emphasized is that the hamsters used in this study were young hamsters. Undergrown blood-brain barrier might contribute to transmission of scrapie agent from peripheral circulating system to central nerve tissues. An age-related bioassay will help to address the role of blood-brain barrier in TSE.

IHC of GFAP staining revealed a remarkable astrocytosis in brain stems compared with normal control. Although astrocytosis could be observed more or less in other brain regions, such as cerebellum, cerebral cortex, hippocampus and thalamencephalon (data not shown), there was no significant statistic difference compared with healthy animals in general. Doubly labeled immunohistochemistry for proliferating cell nuclear antigen (PCNA) and glial fibrillary acidic protein (GFAP) showed that the astrocytosis in scrapie-infected animals was, at least in part, due to actual replication of astrocytes in animals[15]. Reactive astrocytosis corresponding well to severe spongiform degeneration and PrP^{Sc} were accumulated in brain regions during 263K infections in our study as well as in other study, suggesting a ‘snowball effect’, namely astrocytosis might play an important role in amyloidosis, while amyloidosis might induce further astrogliosis[28]. Although nucleus shrink was also observed obviously in brain stem, loss of neuron was not predominant in

brain stem in all tested animals (data not shown). In contrast to the situation in brain stem, loss of neuron was observed in deep cerebral cortex layer of almost all the animals with peripheral infection ways except for those with intracerebral infection. It was reported that some other factors, e.g. acidic fibroblast growth factor (aFGF), might play roles in astrocytosis and in protection of neuron in scrapie-infected hamsters[17]. This might partially explain a slight loss of neuron compared with severe vacuole and PrP^{Sc} deposition in brain stem.

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