D-Leucine Suppresses Prion Formation in Prion-Infected Culture Cells

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Abstract

Prion disease is an infectious and fatal disease. The pathogen consists of an abnormal form of the prion protein; designated PrPSc. PrPSc is insoluble, highly resistant to digestion by proteases and all disinfectants. In contrast, the cellular form of prion protein PrPC is easily soluble and digested by proteases. Direct interaction between PrPSc and PrPC is believed to induce the propagation of prions; however, the molecular mechanisms underlying this step have not been clarified. Despite efforts to find molecules that can specifically interact with the prion protein conformer and thereby inhibit prion production through disruption of PrPSc and PrPC interaction, and thus may disturb prion production by inhibiting the above interaction, there is no efficient way to date and data regarding this issue are limited. Our findings provide novel insight into possible therapeutic approaches for treating prion disease.

Keywords

Prion, Prion protein, D-amino acid, Propagation, Prion disease

Introduction

Prion diseases are lethal neurodegenerative disorders involving the misfolding of the host encoded cellular prion protein, PrPC to PrPSc [1]. PrPSc is detergent soluble and proteinase K (PK)-sensitive, whereas PrPC is detergent insoluble and partially PK-resistant [2]. These differences in biophysical properties are thought to be due to different conformations of the two isoforms. The former molecule is highly α-helical, while PrPSc contains a large proportion of β-sheet structures [3,4]. Although PrPSc is thought to a pathogenic agent of disease, the molecular mechanism underlying PrPC to PrPSc conversion remains elusive.

Genetic types of the disease in humans comprise familial CJD (fCJD), fatal familial insomnia (fFFI) and variably protease-sensitive prionopathy (VPSPr) [5], the most recently identified prionopathy [6]. A rapidly expanding group of prion diseases as well as the frontotemporal dementias and some forms of amyotrophic lateral sclerosis (ALS) [7,8]. Several molecules such as quinacrine [9], antibodies [10] or pentosan sulfate [11] have demonstrated the ability to eliminate PrPSc, however, these agents have shown no or modest treatment efficacy in clinical trials [12]. Meanwhile, recent evidence has shown that significant concentrations of D-amino acids exist naturally even in the central nervous system and have demonstrated important physiological functions [13-15]. Furthermore, they have been exhibited to strongly prevent biofilm formation by bacteria and can also destroy existing biofilms due to their ability to breakdown amyloid fibril [16,17].

Based on this background, we have examined whether D-amino acids are capable of destroying PrPSc amyloid using a cell culture system.

Materials and Methods

Cell culture

Mouse neuroblastoma 2a (N2a) cells and the cells infected with the mouse adapted prion strain 22L were maintained at 37°C in 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM)(Sigma-Aldrich) [18]. D- and L- amino acids were purchased from Wako Pure Chemical Industries, Ltd. D-amino acid-dependent PrPSc-degrading efficacy was measured by western blot analysis. Following incubation for a limited period of time with the specified concentration of D- or L-amino acids, the cell lysates were incubated with proteinase K (1μg/ml) for one hour at 37°C, at which time digestion was stopped with the addition of PMSF at a final concentration of 1mM. Finally, 1% (w/v) of SDS was added and the proteins were methanol precipitated at -80°C for at least two hours. After centrifugation at 1,800xg for 30 minutes, the protein pellets were subjected to SDS- PAGE and western blotting using a PVDF membrane (Millipore). The membranes were incubated with the monoclonal primary antibody SAF83 (SPI Bio) followed by horseradish peroxidase-conjugated anti-mouse IgG as the secondary antibody (GE Healthcare). The membranes were subsequently developed with ECL-prime (GE Healthcare) and analyzed using a Versa Doc 5000MP (Bio-Rad).

Results and Discussion

In this study, the D-amino acid anti-prion amyloid-breaking activity was examined using an assay to determine the PrPSc levels in ScN2a cells infected with scrapie strain 22L. As indicated in Figure 1a, without PK treatment, PrP was detected as a broad band (Figure...
1a, panel b, lane 1). In contrast, in the cells treated with PK, PrP<sub>Sc</sub> was detected as three bands exhibiting the modification of zero, one or two sugar residues, respectively (Figure 1a, panel a, lane 1). Meanwhile, N2a cells that were not infected by prions did not show resistance to PK and clearly disappeared after the application of proteases (Figure 1a, panel a, lane 2).

1mM of D-amino acids was added to ScN2a cell cultures directly. 

Figure 1: (A) D-leucine has prion amyloid breaking activity (Panels a, c, e): PrP<sub>Sc</sub> levels in N2a (lane 2) or ScN2a (lanes 1, 3-7) cells were measured by PK treatment and western blotting after 24 hours incubation in the presence of D-amino acids (1.0mM). The amino acids added in each lane are as follows: lane 3: Met, lane 4: Tyr, lane 5: Ala, lane 6: Phe, lane 7: Lys, lane 8: Arg, lane 9: Asn, lane 10: Cys, lane 11: Pro, lane 12: His, lane 13: Asp, lane 14: Glu, lane 15: Ser, lane 16: Thr, lane 17: Leu, lane 18: Val, lane 19: Ile, lane 20: Gin; (Panels b, d, f): N2a or ScN2a cells were cultured for 24 hours in the presence of D-leucine at various concentrations and detected by western blotting without PK treatment. (Panel g): Densitometric measurement of PrP<sub>Sc</sub> bands of panels a, c and e.

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and the cells were incubated at 37°C for 24 hours. After the incubation, cells were treated with (Figure 1a, panel a, lanes 3-7, panels c and e) or without (Figure 1a, panel b, lanes 3-7, panels d and f) proteinase K (PK). As shown in Figure 1a, D-leucine specifically reduced the amount of PrPSc, indicating the suppression of prion formation (Figure 1a, panel e, lane 17, panel g, lane 17) and this suppressive activity was increased in a dose-dependent manner (Figure 2, panel a). We also examined the amyloid-breakdown activity of L-amino acids under the same conditions as Figure 1a, however, there was no discernible effects (Figure 1b, Figure 2, panel b). Incidentally, D-tryptophan exhibited cytotoxicity and resulted in cell death after incubation, therefore further experiments using D-tryptophan were omitted (data not shown). The anti-prion activity of D-leucine peaked at 48 hours of incubation, subsequently becoming inactivated at 72 hours, (Figure 3, panel a) suggesting that D-leucine was metabolized. This effect was not seen with L-leucine incubation (Figure 3, panel b).

Despite an intensive search for therapeutic compounds for prion disease, none have significantly increased the survival of patients. Their effectiveness is often limited by their restricted access across the blood-brain-barrier (BBB), and no potential candidate has been identified to date that is effective after an infection has been established in the central nervous system [19,20].

Notably, all amino acids, except for glycine, are able to alter their conformation into two isometric L- and D-forms, as they have the potential to form two stereoisomers around a central carbon atom. D-amino acids were once thought to be an unusual state that exists only in the cell walls of bacteria. However, the development of
experimental procedures has revealed that D-amino acids are present widely in organisms, even in mammals, including humans [13]. Based on these points of view, we have examined if D-amino acids possess the ability to breakdown the PrP-amyloid by using prion-infected ScN2a cells as a measure of proteinase K resistance. Consequently, the present findings demonstrated that D-leucine has a strong specific anti-prion activity, which subsequently increases the sensitivity of ScN2a cells to proteinase K, resulting in the clearance of the pathogenic form of prion protein PrPSc.

Figure 2: Dose-dependency of the anti-prion activity of D-leucine.

ScN2a cells were treated for 24 hours with various concentrations of D-leucine (panel a) or L-leucine (panel b). Levels of PrPSc were analyzed after PK digestion of cellular lysates by immunoblotting.

Figure 3: D-leucine reduces levels of protease resistant PrPSc in a time-dependent manner.

ScN2a cells were treated with D-leucine (panel a) or L-leucine (panel b) with various incubation times. Levels of PrPSc were analyzed after PK digestion of cellular lysates by immunoblotting.

effect [21,22], they exist naturally in dietary proteins and in foods [23], indicating that they are probably harmless. It remains to be established how decreased resistance to proteolysis results in reduced levels of PrPSc in ScN2a cells by D-leucine and the in vivo anti-prion activity of D-leucine remains to be determined using mice inoculated with infectious prion. Based on our finding, D-leucine, together with previous findings, would be an interesting prominent candidate for the therapeutic approach of prion disease.

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References