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Heparin enhances the cell-protein misfolding cyclic amplification efficiency of variant Creutzfeldt–Jakob disease

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ABSTRACT

Highly sensitive *in vitro* screening tests are required to prevent the iatrogenic spread of variant Creutzfeldt–Jakob disease (vCJD). Protein misfolding cyclic amplification (PMCA) is a candidate for such a test, but the sensitivity of this method is insufficient. Polyanions were reported to enhance PMCA efficiency, but their effects on vCJD are unclear. We developed a cell-PMCA of vCJD, wherein cell lysate containing exogenously expressed human PrP was used as substrates, to investigate the effects of various sulfated polysaccharides on amplification efficiency. PrP^{res} amounts after cell-PMCA were analyzed by western blotting. Heparin, dermatan sulfate, and dextran sulfate (average molecular weight [MW] 1400 kDa) enhanced efficiency, but dextran sulfate (average MW 8 kDa) and a heparin pentasaccharide analog had no effect. Pentosan polysulfate inhibited cell-PMCA reaction. The amplification efficiency of cell-PMCA of vCJD increased to >100-fold per round with heparin. The enhancing effects of heparin on cell-PMCA were seed dependent: it was high for vCJD, low for sporadic Creutzfeldt–Jakob disease, and low to negligible for hamster-adapted scrapie-derived 263 K. In multi-round PMCA, signals were detected at earlier rounds with heparin than without heparin, and PrP^{Sc} in 10^{-10} diluted vCJD brain was detected by the sixth round. Heparin-assisted cell-PMCA of vCJD represents a significant step toward detecting very minute amounts of PrP^{Sc} in the body fluids of asymptomatic vCJD patients.

There is growing concern that variant Creutzfeldt–Jakob disease (vCJD) may be iatrogenically transmitted from human to human [13]. For the prevention of the iatrogenic spread of vCJD, highly sensitive *in vitro* screening tests that can detect very minute amounts of the disease-associated isoform of the prion protein (PrP^{Sc}) are required. Protein misfolding cyclic amplification (PMCA) is currently one of the most sensitive methods of detecting PrP^{Sc}, because it generates large quantities of a partially protease-resistant isoform of prion protein (PrP^{res}) from materials containing PrP^{Sc} through repeated cycles of sonication and incubation [3,18,19]. PMCA was able to detect PrP^{Sc} from the brain tissue of vCJD patients [14,20]; however, human tissue was used as substrate; Moreover, amplification efficiency of vCJD was considerably lower than that of 263 K, scrapie-derived hamster-adapted strain. Various additives enhance PMCA or *in vitro* conversion efficiency of animal prion diseases [16,21,22]. However, the effect of polyanions on PMCA depends on prion strains [6], and the effect is ill defined for vCJD. In this study, we developed cell-PMCA [4] of vCJD, because cell lysate used as substrate is inexpensive, readily available, and free from ethical matters, and studied whether various sulfated polysaccharides were able to increase the PMCA efficiency of vCJD.

Human brain tissue of a vCJD patient (No. 96-07) was obtained from the brain bank of the UK National CJD Surveillance Unit. Human brain tissue from a sporadic Creutzfeldt–Jakob disease (sCJD) patient (H3) was obtained at autopsy after informed consent was received for its use for research purposes [15]. Golden hamster brain infected with 263 K was kindly gifted by Prof. Doh-ura (Tohoku University). This study was performed with the approval of the local committee on ethics.

Low-molecular-weight (LMW) heparin sodium salt, with an average molecular weight (MW) of 4–6 kDa, was purchased from LKT Laboratories (Paul, MN, USA). High-molecular-weight (HMW) heparin sodium salt, with an average MW of 13–17 kDa, was from EMD Chemicals (Gibbstown, NJ, USA). Fondaparinux sodium was

Abbreviations: BSE, bovine spongiform encephalopathy; HMW, high molecular weight; LMW, low molecular weight; MW, molecular weight; PMCA, protein misfolding cyclic amplification; PPS, pentosan polysulfate; PrP, prion protein; sCJD, sporadic Creutzfeldt–Jakob disease; vCJD, variant Creutzfeldt–Jakob disease.

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from GlaxoSmithKline (Tokyo, Japan). Dermatan sulfate 1 sodium salt, dextran sulfate sodium salt, with average MW of 1400 kDa and dextran sulfate sodium salt, with average MW of 8 kDa were from MP Biomedicals (Solon, OH., USA). PPS was kindly gifted by Prof. Doh-ura (Tohoku University) [7]. Each polysaccharide, except Fondaparinux, was dissolved in water to a final concentration of 100 mg ml^{-1} and stored at 4 °C.

Human brain tissue derived from the sCJD or vCJD patients and golden hamster brain infected with 263 K were homogenized in 9 volumes (w/v) of homogenization buffer A (phosphate buffered saline containing protease inhibitors cocktail [Roche Diagnostics, Tokyo, Japan]). cDNAs for chimeric PrP between mouse and human, with methionine at codon 129 (129M), or between mouse and golden hamster were constructed as described previously [11]. Amino acid sequences encoded by these cDNAs are identical to human or hamster PrP except for the N-terminal signal sequences, which are derived from mouse PrP. cDNAs were subcloned into pIRESneo3 (Takara, Kyoto, Japan). Transient expression of hamster PrP was carried out as follows: 1.6 mg of polyethylenimine "Max" (nominally MW 40,000; Polysciences Inc., Warrington, PA, USA) was dissolved in 25 ml of Opti-MEM I (Invitrogen^{TM}, Life Technologies Corp., Carlsbad, CA, USA) and incubated at room temperature for 10 min. Thereafter, 0.4 mg of expression vector for hamster PrP was dissolved in 2 ml of Opti-MEM I, mixed with polyethylenimine solution, incubated at room temperature for 10 min, and transferred into a culture flask containing 4×10^8 FreeStyle 293F cells (Invitrogen^{TM}). The cells were collected 72 h after transfection, washed twice in phosphate buffered saline, resuspended in 4 volumes of PMCA buffer A (to a final concentration of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [pH 7.6], 80 mM NaCl, 4 mM ethylenediaminetetraacetic acid, and 1% NP-40 [Calbiochem^{TM}]), and sonicated using Sonifier 250 (Branson Japan, Tokyo, Japan). To prepare cells that stably expressed human PrP, FreeStyle 293F cells were first adapted to adherent culture by adding 10% (v/v) fetal bovine serum (Invitrogen^{TM}) to Dulbecco's modified Eagle medium GlutaMAX (Invitrogen^{TM}). The cells were then transfected with the expression vector for human PrP with Lipofectamine 2000 (Invitrogen^{TM}), according to the manufacture's instruction. On the day after the transfection, the cells were suspended with trypsin, spread onto 96-well plates at a concentration of less than 40 cells per well, and cultured with 200 μg ml^{-1} geneticin (Invitrogen^{TM}). Cells expressing the highest levels of human PrP were selected by western blot analysis, adapted to suspension culture, and maintained in FreeStyle expression medium supplemented with geneticin. Cell lysates were prepared as described above. Human and hamster PrP^C concentrations in 20% (w/v) cell lysates were greater than the hamster PrP^C concentration in 10% hamster brain homogenate (Fig. S1). Normal hamster brain was homogenized in 4 volumes of 2× PMCA buffer B (2× phosphate buffered saline, 8 mM ethylenediaminetetraacetic acid, 2% Triton X-100, and 2× protease inhibitors cocktail) and diluted by half with water just before use. Cell lysates or brain homogenates were stored at −80 °C in small aliquots.

The vCJD brain homogenate, 263 K-infected brain homogenate, and substrates were thawed, briefly centrifuged, mixed, placed in a 100 μl thin-walled PCR tube with a rod and a screw cap (Sarstedt, Numbrecht, Germany), and subjected to PMCA (in a total volume of 100 μl). sCJD brain homogenate was similarly prepared except that centrifugation was omitted. Sulfated polysaccharides were added at the indicated concentrations. One round of PMCA consisted of 48 cycles of sonication (5 sets of 5-s pulses at 1-s intervals; a power selector was set at "strong") and incubation (37 °C for 1 h). PMCA was performed with a fully automatic cross-ultrasonic protein-activating apparatus (ELESTEIN 070-GOT; Elekon Science Corp., Chiba, Japan). During PMCA, samples were continuously rotated at 2 rpm. For multi-round PMCA, PMCA products were diluted 1:3 in fresh substrate before the next round of PMCA.

Before and after PMCA samples were digested with 50 μg ml^{-1} proteinase K at 37 °C for 60 min. The samples were then subjected to western blotting as previously described [12] with the following modifications: the primary and secondary antibodies were diluted in wash buffer (25 mM Tris–HCl [pH 8.25], 150 mM NaCl, 0.1% Tween 20) and 0.5% (w/v) skimmed milk, and the membranes were washed in wash buffer.

Cell-PMCA was performed to investigate the effects of various sulfated polysaccharides on amplification efficiency of vCJD. We amplified 10^{-4} dilution of vCJD brain and compared signal intensities of PrP^{res} after PMCA with and without additives by western blotting (Fig. 1A–E). PrP^{res} amounts after amplification sometimes varied considerably (for example, see "no additives" in Fig. 1E). The

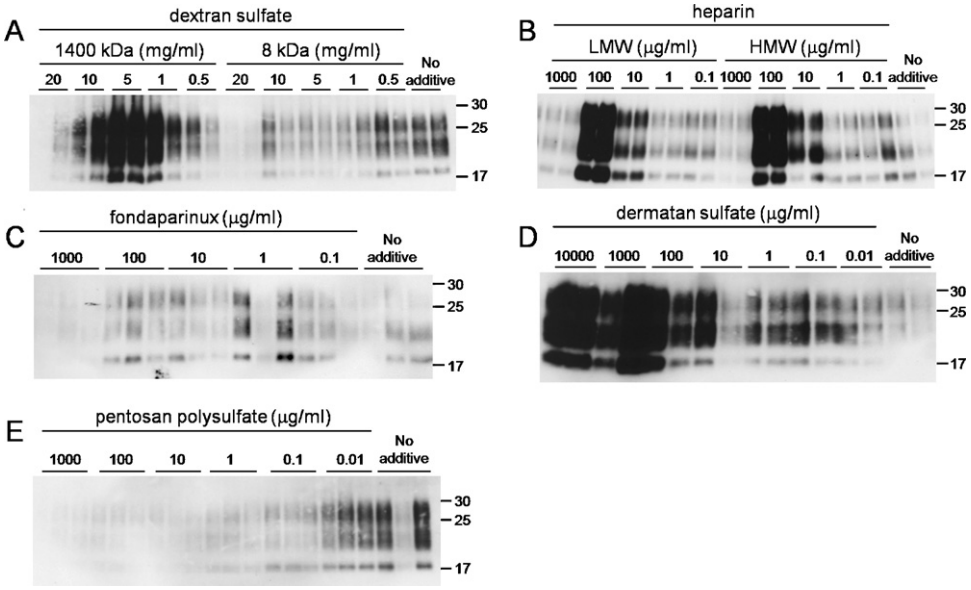

Fig. 1. Western blotting of cell-PMCA products with and without additives. Western blotting of PMCA products with and without various concentrations of sulfated polysaccharides (A–E) is shown. Molecular weight at the top of panel A indicates the mean molecular weight of dextran sulfate. LMW indicates low molecular weight. HMW indicates high molecular weight. No additive indicates that PMCA was performed without sulfated polysaccharides. Positions of molecular weight markers in kDa are indicated at the right of each panel.