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Tailor-made purified human platelet lysate concentrated in neurotrophins for treatment of Parkinson's disease

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ABSTRACT

Human platelet lysates (PLs), which contain multiple neurotrophins, have been proposed for treating neurodegenerative disorders, including Parkinson's disease (PD). However, current PLs suspended in plasma have high protein content and contain fibrinogen/fibrin and, following activation, also proteolytic and thrombogenic enzymes. Upon brain administration, such PLs may saturate the cerebrospinal fluid and exert neurotoxicity. We assessed whether purified PLs, concentrated in neurotrophins, protected dopaminergic neurons in PD models. Platelet concentrates were collected by apheresis and centrifuged to eliminate plasma and recover the platelets. Platelets were lysed by freeze-thaw cycles, and the 10-fold concentrated platelet pellet lysates (PPLs) were heat-treated (at 56 °C for 30 min). The heat-treated PPLs were low in total proteins, depleted in both plasma and platelet fibrinogen, and devoid of thrombogenic and proteolytic activities. They exerted very high neuroprotective activity when non-oncogenic, Lund human mesencephalic (LUHMES) cells that had differentiated into dopaminergic neurons were exposed to the MPP^+ neurotoxin. Heat treatment improved the neuroprotection and inactivated the neurotoxic blood-borne hepatitis C virus. PPL did not induce inflammation in BV2 microglial cells and inhibited COX-2 expression upon lipopolysaccharide exposure. Intranasal administration in mice revealed (a) diffusion of neurotrophins in the striatum and cortex, and (b) MPTP intoxication neuroprotection in the substantia nigra and striatum and the absence of neuroinflammation. These dedicated heat-treated PPLs can be a safe and valuable candidate for a therapeutic strategy for PD.

1. Introduction

Parkinson's disease (PD) is the second most frequent neurodegenerative disorder worldwide, affecting approximately 1% of individuals older than 60 years, and up to 4% in the oldest age groups [1]. The loss of dopamine production in the striatum, as a result of progressive neuronal degeneration in the substantia nigra pars compacta, is the primary physiological landmark of PD. Individuals affected by PD have symptoms encompassing bradykinesia, stiffness, and rest tremors of the limbs. Axial signs include postural instability, gait disorders, dysarthria, and dysphagia. Affordable and smart "disease-modifying strategy" treatments that provide neuroprotective and/or neuroregenerative benefits urgently need to be developed to reduce the emotional burdens of patients and caregivers, and limit increasing societal and economic impacts associated with a prolonged life expectancy. Given their physiological role in activating and modulating neuronal signaling pathways, neurotrophic growth factors represent a very promising, recently explored, disease-modifying biotherapy [2]. Recombinant (r-) platelet-derived growth factor (PDGF), brain-derived neurotrophic factor (BDNF), transforming growth factor (TGF)-β, basic-fibroblast

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growth factor (bFGF), and vascular endothelial growth factor (VEGF), administered *via* intracerebroventricular (ICV) or systemic routes, provide physiological functional benefits in several neurological pathologies, and enhance the survival of dopaminergic neurons in PD cellular and animal models [3,4]. However, recent randomized clinical studies using these single recombinant growth factors drugs did not improve conditions of patients with neurodegenerative conditions [5–7], suggesting that therapeutic approaches combining multiple, complementary neurotrophic growth factors should be evaluated to treat the physiologically complex pathological events associated with PD.

Human platelet lysate (PL or HPL) biomaterials play increasingly recognized roles in the fields of cell therapy and biotherapy in tissue engineering and regenerative medicine [8]. Standard PL biomaterials used in most clinical applications, e.g., for healing skin ulcers, bone defects, and joints, contain plasma and platelet proteomes, and have a high total protein content approaching 50–60 g/l [9]. Recently, a human PL preparation, suspended in plasma, was found to provide neuroprotective effects in PD and Alzheimer's disease models [10,11]. However, we are much concerned that administering plasma and heavy loads of proteins to the brain, over a long-term treatment period expected to be required for treating PD, may affect brain tissue homeostasis and lead to protein overload of the cerebrospinal fluid (CSF). Neuroinflammation and thrombogenicity are other serious risks to consider if plasma zymogens and coagulation factors are activated, and proteases, like thrombins, are generated during PL preparation to release the growth factors. Finally a major clinical concern with brain administration of standard PLs is a risk of neuro-infection by adventitious or endogenous blood-borne viruses, most particularly neurotoxic pathogens like the hepatitis C virus (HCV).

To address these numerous issues, we bio-designed and characterized a novel low-volume, low-protein content, heat-inactivated PL intended for brain administration, which is depleted of fibrinogen as well as proteolytic and thrombogenic factors, and enriched in neurotrophic growth factors. We evaluated whether such a PL can exert strong neuroprotective activity in *in vitro* and *in vivo* models of PD and should be carefully considered for further clinical development.

2. Materials and methods

2.1. Blood product preparation

2.1.1. Platelet concentrate (PC) collection

The Institutional Review Board of Taipei Medical University approved the study (no. 201504054). Twenty-eight non-leuko-reduced PCs were collected at the Taiwan FDA-licensed Taipei Blood Center (Taiwan Blood Services Foundation, Guandu, Taiwan) from volunteer healthy donors who provided written informed consent. PCs were obtained by apheresis technology using MCS+ cell separators (Haemonetics, Braintree, MA, USA) using validated procedures from the supplier. Donations were screened for non-reactivity against blood-borne viruses (HIV, HBV, HCV) following mandatory Taiwan FDA regulations. Platelets and other blood cell counts were determined for each donation using ABC Vet (ABX Diagnostics, Montpellier, France) [12]. PCs were kept on a platelet agitator at 22 ± 2 °C until processing on the same day or within 8 days of collection.

2.1.2. Preparation of PPLs and PLs

Platelet pellet lysates (PPLs) were prepared under aseptic conditions (Graphical abstract) by centrifugation (at 3000 × *g* for 30 min at 22 ± 2 °C) of PCs (200–250 mL) to pelletize the platelets. The plasma supernatant was carefully removed, and the surface of the platelet pellet was gently washed by ca. 2–2.5 mL of sterile phosphate-buffered saline (PBS), concentrated to 1/10th of the initial volume of PC, subjected to three freeze-thaw cycles (−80/+30 ± 1 °C), and clarified by centrifugation (at 4500 × *g* for 30 min at 22 ± 2 °C). Heat treatment was performed in a dry bath at 56 °C for 30 min. Controlled experiments were done using 37, 45, and 65 ± 1 °C for 30 min. After heat treatment, samples were immediately cooled down for at least 5 min on ice, then centrifuged (at 10,000 × *g* for 15 min at 4 ± 2 °C) to remove any precipitate. Apheresis PCs suspended in plasma were used to prepare a standard PL by three freeze-thaw cycles (−80/30 °C ± 1 °C) and were centrifuged (at 4500 × *g* for 30 min at 22 ± 2 °C) to remove cell debris. PPL and PL supernatants were stored frozen at −80 °C until use.

2.2. Cell culture

2.2.1. Lund human mesencephalic (LUHMES) cell model

2.2.1.1. Cells maintenance and differentiation. LUHMES cells were obtained from Dr. Leist (University of Konstanz, Konstanz, Germany) and cultured [13] as described in Fig. 1A. Briefly, undifferentiated LUHMES cells were propagated using Nunc™ (Thermo Fisher Scientific, Waltham, MA, USA) plastic cell culture flasks and multi-well plates that were pre-coated with 50 μg/mL poly-L-ornithine and 1 μg/mL fibronectin (Sigma-Aldrich, St. Louis, MO, USA) in water for 3 h at 37 °C. After removing the coating solution, culture flasks were washed with sterile distilled water and air-dried. Cells were grown at 37 °C in a humidified 95% air, 5% CO_2 atmosphere. The proliferation medium was Advanced Dulbecco's modified Eagle medium/Ham's F-12 (DMEM/F-12) (Thermo Fisher Scientific) containing N-2 supplement (Thermo Fisher Scientific), 2 mM L-glutamine (Thermo Fisher Scientific), and 40 ng/mL r-bFGF (R&D Systems, Minneapolis, MN, USA). After reaching approximately 80% confluence, cells were dissociated with 0.025% trypsin (Thermo Fisher Scientific) and passaged at 3 × 10^6 cells/flask. To induce differentiation into neuronal cells, 2 × 10^6 LUHMES cells were seeded and grown in a T75 flask in proliferation medium for 24 h, then in Advanced DMEM/F12 containing N-2 supplement, 2 mM L-glutamine (Thermo Fisher Scientific), 1 mM N^6,2′-O-dibutyryladenosine 3′,5'-cyclic monophosphate sodium salt (dibutyryl cAMP; Sigma-Aldrich), 1 μg/mL tetracycline (Sigma-Aldrich), and 2 ng/mL recombinant human GDNF (R&D Systems). After 2 days of culture in differentiation condition, LUHMES cells were cultured in a 24-well plate for further experiments at day 5.

2.2.1.2. Neurotoxic stimulation and viability assay. Once-differentiated (day 5) cells were exposed to various concentrations of PPL/heat-treated PPL (HPPL) (0.025%–15%; v/v) for 1 h followed by 30 μM of the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+; Sigma-Aldrich). Cell viability was assessed after 48 h by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) or flow-cytometry. The MTT reagent was added to the cell culture medium at a final concentration of 0.5 mg/mL. After a 1-h incubation, the medium was removed, and purple crystals present in viable cells were lysed in DMSO under vigorous shaking for 10 min. Aliquots were transferred to a 96-well plate to detect the absorbance at 570 nm (with 690 nm as the background value). Viability was also assessed using flow cytometry by staining with 0.5 μg/mL propidium iodide. In total, 10^4 cells were measured using a FACSCanto™ II cytometer (BD Biosciences, Franklin Lakes, NJ, USA) equipped with FACSDiva software. Each condition was evaluated in duplicate using two different cell culture plates, each of which contained controls. Data are expressed as the percent viability compared to control conditions in which LUHMES cells were not exposed to MPP^+.