Neurobiology of Aging 35 (2014) 1582–1595

Contents lists available at ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Plasma rich in growth factors (PRGF-Endoret) reduces neuropathologic hallmarks and improves cognitive functions in an Alzheimer's disease mouse model

Eduardo Anitua [a], Consuelo Pascual [b,c], Desiree Antequera [b,c], Marta Bolos [b,c], Sabino Padilla [a], Gorka Orive [a,*], Eva Carro [b,c,**]

[a] Foundation Eduardo Anitua, Vitoria, Spain
[b] Neuroscience Group, Instituto de Investigacion Hospital 12 de Octubre (i+12), Madrid, Spain
[c] Biomedical Research Networking Center in Neurodegenerative Diseases (CIBERNED), Madrid, Spain

ARTICLE INFO

Article history:
Received 29 July 2013
Received in revised form 5 November 2013
Accepted 8 January 2014
Available online 17 January 2014

Keywords:
Alzheimer's disease
PRGF-Endoret
Intranasal administration
Aβ degradation
Cognition
Astrocytes
Transgenic mice
Cognitive impairment

ABSTRACT

Impaired growth factor function is thought to drive many of the alterations observed in Alzheimer's disease (AD) patients. Endogenous regenerative technology, PRGF (plasma rich in growth factor)-Endoret, is designed for the delivery of a complex pool of patient's own active morphogens that may stimulate tissue regeneration. We obtained and characterized PRGF-Endoret preparations from human blood. We used, as experimental approach in vivo, APP/PS1 mice, characterized by age-dependent brain amyloid-β (Aβ) accumulation. Intranasal administration of PRGF-Endoret to APP/PS1 mice resulted in an important decrease in brain Aβ deposition and tau phosphorylation. PRGF-Endoret-treated APP/PS1 mice also showed decreased astrocyte reactivity, and prevented protein synaptic loss. In vitro approaches demonstrated that PRGF-Endoret treatment modulated astrocyte activation, reducing inflammatory responses, and promoted Aβ degradation. Furthermore, PRGF-Endoret stimulated global improvements in anxiety, learning, and memory behaviors. Our findings show that PRGF-Endoret exerts multifunctional and complementary effects that result in the reversal of the broad range of cognitive deficits in AD, suggesting that PRGF-Endoret may hold promise as an innovative therapy in AD.

1. Introduction

Alzheimer's disease (AD) occurs generally late in life, affecting over 35 million individuals worldwide (Selkoe, 2012). This neurodegenerative disorder is characterized by deposits of amyloid-β (Aβ) in senile plaques, intracellular neurofibrillary tangles comprising hyperphosphorylated tau, synaptic dysfunction, and neuronal death, some or all of which are believed to cause the cognitive and behavioral deficits that typify this disease (Palop and Mucke, 2010; Querfurth and LaFerla, 2010; Selkoe, 2002, 2012; Walsh and Selkoe, 2004). It is now widely recognized that neuroinflammation is a prominent feature of AD brain, with inflammatory responses playing a significant role in modulating disease progression (McGeer and McGeer, 2010). Prolonged and widespread activation of astrocytes is apparent in AD brain, in which the severity of glial activation correlates with the extent of brain atrophy (Cagnin et al., 2001) and cognitive decline (Parachikova and Cotman, 2007). Important players in these processes are astrocytes, which, apart from the classical roles, function as regulators of synaptic activity (Christopherson et al., 2005; Nedergaard et al., 2003; Ullian et al., 2001).

While the controversy over the primary cause of the disease is still open, the impaired functions of growth factor cascades are thought to drive many of the alterations observed in AD patients. Indeed, levels of some neurotrophic factors are decreased in AD brains, including nerve growth factor (NGF), insulin-like growth factor (IGF-I), vascular endothelial growth factor (VEGF), and brain-derived neurotrophic factor (Houeland et al., 2010; Moloney et al., 2010; Nagahara et al., 2009; Steen et al., 2005). The therapeutic administration of high doses of these and other recombinant morphogens to primate or mouse models of AD resulted in enhanced clearance of Aβ, and reversal of cognitive dysfunctions (Capsoni et al., 2002; Carro et al., 2002; Nagahara et al., 2009; Spuch et al., 2010). Human platelets contain an enormous cocktail of

* Corresponding author at: Foundation Eduardo Anitua, Vitoria, Spain Jacinto Quincoces, 39, Vitoria (Álava) 01007, Spain. Tel.: +34 945 160 653; fax: +34 945 160 657.
** Alternate corresponding author at: Neuroscience Group, Instituto de Investigacion Hospital 12 de Octubre (i+12), Madrid 28041, Spain. Tel.: +34 913908765; fax: +34 913908544.
E-mail addresses: gorka.orive@fundacioneduardoanitua.org (G. Orive), carroeva@h12o.es (E. Carro).

0197-4580/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.neurobiolaging.2014.01.009

morphogens, proteins, and growth factors, which, when added to those present in human plasma create a combination of biologically balanced active mediators (Anitua et al., 2010; Italiano et al., 2008; Leslie, 2010). Advances in the field have enabled the development of standardized technologies that produce autologous plasma and platelet-based therapeutic formulations that can be easily prepared, handled, and used in the clinical setting. In particular, endogenous regenerative technology, plasma rich in growth factor (PRGF)-Endoret constitutes a biological system that provides a complex pool of a patient's own active mediators that may stimulate and accelerate tissue regeneration (Anitua et al., 2007, 2010; Leslie, 2010). PRGF-Endoret is autologous plasma rich in growth factors that has the European Community and the U.S. Food and Drug Administration approval to be clinically applied.

We hypothesized that the pool of morphogens and proteins from PRGF-Endoret would exert neuroprotective effects, resulting in the reversal of a broad range of AD-induced cognitive deficits. In this study, we found that intranasal administration of PRGF-Endoret to APP/PS1 mice for 4 weeks effectively reduced Aβ accumulation and tau hyperphosphorylation. More importantly, PRGF-Endoret treatment of APP/PS1 mice significantly alleviated astroglial activation and synaptic loss, and improved neurogenesis and cognitive deficits.

2. Methods

2.1. PRGF-Endoret preparations

Blood from healthy young male donors was obtained, after informed consent, through antecubital vein puncture. Endogenous regenerative technology (PRGF-Endoret), also known as plasma rich in growth factors, was obtained as described previously (Anitua et al., 2006). Briefly, blood from donor subjects was collected into 9 mL tubes with 3.8% (wt/vol) sodium citrate. Samples were centrifuged at 580*g* for 8 minutes at room temperature in a PRGF-Endoret system centrifuge (BTI Biotechnology Institute). The plasma fraction containing platelets but not buffy coat and erythrocytes was separated. Plasma fractions were incubated with 5% (wt/vol) calcium chloride (PRGF-Endoret activator; BTI Biotechnology Institute) for 1 hour at 37 °C in glass tubes. The released supernatants were collected by aspiration after centrifugation at 1000*g* for 20 minutes at 4 °C. Finally, platelet enriched plasma fractions were aliquoted and stored at −80 °C, until use. As shown in Table 1, growth factors (TGF-β1, PDGF, VEGF, HGF, EGF, IGF-1, and NGF) were measured in the supernatants using commercially available colorimetric sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc, Minneapolis, MN, USA).

2.2. Animals

Male double-transgenic APP/PS1 mice, a cross of the Tg2576 (over-expressing human AβPP695) and mutant PS1 (M146L) mice from our in-house colony (Instituto de Investigacion Hospital 12 de Octubre), were used. Age-matched mice not expressing the transgene were used as wild-type controls. Human PRGF-Endoret was delivered intranasally 3 times per week for 4 weeks, according to a modified procedure previously described (Capsoni et al., 2002). Mice were briefly anesthetized with isoforane, and PRGF-Endoret (total volume of 48 μL) was administered intranasally to APP/PS1 mice, 3 μL at a time, alternating the nostrils, with a lapse of 2 minutes between each administration, for a total of 16 times. In the control mice, saline (0.9% wt/vol) was administered. PRGF-Endoret was administered to 3 and 6-month-old APP/PS1 mice groups. From day 8 of the study, 50 mg/kg of BrdU was injected intraperitoneally to each mouse once a day for 7 days, and mice were sacrificed 28 days later. All animals were handled and cared for Council Directive 2010/63/UE of September 22, 2010.

Table 1
Concentrations of growth factors in the sample of human PRGF-Endoret used

Growth factors	Concentration (pg/mL)
Nerve growth factor (NGF)	81.65 ± 22.4
Vascular endothelial growth factor (VEGF)	130.46 ± 36.57
Insulin-like growth factor 1 (IGF-1)	95,220 ± 7.98
Platelet-derived growth factor (PDGF)	10.03 ± 2.96
Hepatocyte growth factor (HGF)	194.5 ± 23.22
Transforming growth factor (TGF)	13,350 ± 2.23

Data are mean ± SEM.
Key: SEM, standard error of the mean.

2.3. In vitro studies

2.3.1. Primary cell culture assays

Primary cortical astrocytes were obtained from Wistar rat on postnatal day 3, as previously described (Alvira-Botero et al., 2010). Cultures were kept at 37 °C in a humidified atmosphere containing 5% CO_2 for 7 days before experimentation. Cells cultures were then incubated in fresh medium with or without PRGF-Endoret, previously diluted at 7.5% and 10% in a sterile culture medium, alone or in combination with $A\beta_{42}$ (10 μM).

2.3.2. Aβ degradation

For immunocytochemistry, astrocytes (10^5 cells/well) were cultured on poly-L-lysine-coated glass slides, and treated with 7.5% and 10% PRGF-Endoret, alone or in combination with $A\beta_{42}$ (10 μM) for 6, 12, 24, and 48 hours, after which they were fixed in 4% paraformaldehyde for 1 hour. Then, cells were incubated with a mouse anti-Aβ (1:500, MBL, Nagoya, Japan), nuclei were stained with DAPI, and images were taken. For immunoassay, astrocytes (10^6 cells/well) in serum free medium were exposed to 7.5% and 10% PRGF-Endoret, and $A\beta_{42}$ (10 μM). At various time points, Aβ levels in cell culture supernatants and adherent cell homogenized fractions were determined as described previously.

2.4. Immunoassays

2.4.1. Synaptosome preparations

Hippocampus was lysed in homogenization buffer containing 320 mM sucrose, 4 mM HEPES (pH 7.4), 1 mM EGTA, 1 mM PMSF, and protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany), in a glass Dounce tissue grinder. The homogenate was centrifuged at 1000*g* for 10 minutes and the resulting supernatant was centrifuged at 12,000*g* for 15 minutes. After the second centrifugation, the pellet was re-suspended in homogenization buffer and centrifuged at 13000*g* for 15 minutes to obtain the final pellet containing the synaptosome-enriched fraction.

2.4.2. Western blotting

For western-blot analysis, samples were homogenized in lysis buffer and then in 2% sodium dodecyl sulphate-containing buffers, electrophoresed and blotted. The antibodies used for these experiments included: mouse anti-GFAP (1:500; Sigma), mouse anti-synaptophysin (1:2500; Chemicon), rabbit anti-synapsin (1:7000; Sigma), rabbit anti PSD-95 (1:1000; Abcam), mouse anti-SNAP-25 (1:1000; Abcam), mouse anti-β-actin (1:10,000; Sigma), mouse anti-Tau5 (1:500; Neuromarkers), mouse anti-Hpf-tau (1:1000, AT-8; Innogenetics), mouse anti-GSK3β (1:500; Santa Cruz Biotechnology), and rabbit anti-PIK3, rabbit anti-pPI3K, rabbit anti-Akt, rabbit anti-pAkt, rabbit anti-pSer-GSK3β, mouse anti-IκBα, rabbit