Neuroscience Letters 581 (2014) 98–102

Contents lists available at ScienceDirect

Neuroscience Letters

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

Adipose stem cells-conditioned medium blocks 6-hydroxydopamine-induced neurotoxicity via the IGF-1/PI3K/AKT pathway

Xianjun Wang[a], Zhenyu Zhao[a], Jian Gong[a], Shengnian Zhou[b], Hongjun Peng[c,*], Adam Shatara[d], Timmy Z. Zhu[d], Rebecca Meltzer[d], Yansheng Du[d], Huiying Gu[d,**]

[a] Department of Neurology, Linyi People's Hospital, No. 49 Yizhou Road, Linyi, Shandong 276000, P.R. China
[b] Department of Neurology, Qilu Hospital of Shandong University and Bain Science Research Institute, Shandong University, No. 107 Wenhuaxi Road, Jinan, Shandong 250012, P.R. China
[c] Department of Pediatrics, Jinling Hospital, Nanjing University School of Medicine, Nanjing 210002, P.R. China
[d] Department of Neurology, Indiana University School of Medicine, Indianapolis, IN 46202, USA

HIGHLIGHTS

- IGF-1 antibody reduces neutoprotective activity against 6-OHDA of ASC-CM.
- Addition of IGF-1 into inactivated ASC-CM blocks 6-OHDA-induced neuronal death.
- ASC-CM provides neuroprotection against 6-OHDA-induced neurotoxicity through the phosphoinositide 3-kinase (PI3K)/AKT pathway.

ARTICLE INFO

Article history:
Received 21 May 2014
Received in revised form 31 July 2014
Accepted 18 August 2014
Available online 23 August 2014

Keywords:
Neuronal cell death
ASC-CM
6-hydroxydopamine
IGF-1
AKT

ABSTRACT

Previous studies suggest that the delivery of neurotrophic factors secreted from adipose stromal cells (ASC) protect the brain from 6-hydroxydopamine (6-OHDA)-induced neurotoxicity. However, it remains unclear which secreted neurotrophic factor has an important role in protecting 6-OHDA-treated neurons. Through the use of antibodies in this study, we demonstrated that specific neutralization of IGF-1 activity in ASC conditioned media (ASC-CM) significantly blocks ASC-CM-induced neuroprotection against 6-OHDA neurotoxicity. Consistently, this neuroprotection was mostly attributed to the activation of the AKT-mediated signaling pathway. In contrast, brain derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) in ASC-CM did not play a role in ASC-CM-induced neuroprotection against 6-OHDA.

1. Introduction

6-Hydroxydopamine (6-OHDA), an oxidative metabolite of dopamine, is a neurotoxin that has been widely used to generate experimental models for Parkinson's disease (PD) research [3]. Recently, several studies suggested that 6-OHDA toxicity is not exclusively mediated by specific uptake systems as this neurotoxin affects both catecholaminergic and non-catecholaminergic neuronal cells; this includes cerebellar granule neurons (CGN) [1,6,24]. CGN is a well-established in vitro neuronal culture model that has been widely used to study 6-OHDA neurotoxicity [6,12,19].

Adipose stromal cells (ASC) from mice, rats, non-human primates and humans were found to be able to differentiate into neural-like cells and glial cells both in vivo and in vitro [2,10,17,25,26,30,34]. However, there is no clear evidence to show that human ASC truly differentiate in vivo into functional neurons or form connections with neurons [16]. It is highly possible that functional improvements attributed to ASC may be due to trophic support of host cells by growth factors released from ASC [27,32,36]. ASC were reported to secrete many bioactive-level growth factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), brain derived neurotrophic factor

* Corresponding author.
** Corresponding author at: Department of Neurology, Indiana University School of Medicine, 975 West Walnut Street, Indianapolis, IN 46202, USA.
Tel.: +1 317 278 3340; fax: +1 317 274 3587.
E-mail addresses: hjpeng1975@hotmail.com (H. Peng), huiygu@iupui.edu (H. Gu).

http://dx.doi.org/10.1016/j.neulet.2014.08.033
0304-3940/

(BDNF), glial-derived neurotrophic factor (GDNF) and insulin-like growth factor-1 (IGF-1) [4,22,31,32,35]. These factors have been recently reported to be involved in neuroprotection against a variety of neuronal injury stimuli [14,29,31,32,36]. It was reported that the transplant of ASC protected neurons against 6-OHDA-induced neurotoxicity in rats possibly due to survival factors secreted by ASC [20]. Our follow-up study demonstrated that cell-free ASC-conditioned medium (ASC-CM) could directly protect both cerebellar granule neurons (CGN) and rostral mesencephalic neurons (RMN) against 6-OHDA neurotoxicity in vitro [13]. In previous studies of PD, both BDNF and GDNF were heavily investigated [23] and suggested to contribute neuroprotective effects against 6-OHDA neurotoxicity. However in this study, we demonstrated that IGF-1, not the proposed BDNF and GDNF, in ASC-CM played a key role in protecting CGN against 6-OHDA neurotoxicity in vitro. Additionally, as IGF-1 acted in other neuronal injury models [11], ASC-CM also protected CGN from 6-OHDA-induced neuronal death by activating the phosphatidylinositol 3-kinase-AKT signaling pathway (PI3K/AKT).

2. Materials and methods

2.1. Collection of ASC-CM

Isolation of human ASC was approved by the Indiana University Institutional Review Board and performed as described previously [13]. The lipoaspirate was digested in 1 mg/ml Collagenase Type I solution (Worthington Biochemical, Lakewood, NJ, USA) under gentle agitation for 1 h at 37 °C, filtered with 500 and 250 μm Nitex filters (Sefar American Inc., Kansas City, MO, USA), and then centrifuged at 200 × *g* for 5 min to separate the stromal cell fraction (pellet) from adipocytes. The pellet containing ASC was treated with red blood cell lysis buffer for 5 min at 37 °C and then pelleted at 300 × *g* for 5 min. The cell pellets were resuspended in Endothelial Growth Medium 2-MicroVascular (EGM2-MV; Lonza, Walkersville, MD, USA). ASC were plated in an uncoated T75 tissue culture flask at a density of 4×10^6 cells per cm^2 and incubated in a humidified chamber at 37 °C in an atmosphere of 5% CO_2. After overnight culture, the medium was replaced with fresh EGM2-MV (Lonza, Walkersville, MD, USA). Confluent ASC were washed three times with Basal Medium Eagle (BME) and cultured for another 24 h in serum-free BME. The conditioned media (ASC-CM) was then collected [13].

2.2. CGN cultures and assessment of neuronal viability

All animal protocols were approved by the Indiana University Animal Care and Use Committee. CGN were prepared from 8-day-old Sprague–Dawley rat pups (Harlan) as previously described [8,13]. Freshly dissected cerebella were dissociated and the cells were seeded at a density of $1.2–1.5 \times 10^6$cells/ml on poly-L-lysine coated dishes in BME supplemented with 10% fetal bovine serum (FBS), 25 mM KCl, and gentamicin (0.1 mg/ml). Cytosine arabinoside (10 μM) was added to the culture medium 24 h after initial plating. All experiments utilized CGN after 7–8 days in vitro (DIV). After replacing 50% ASC-CM for 2 h, CGN were exposed to 6-OHDA (70 μM) for an additional 24 h. After incubating with 10 μg/ml fluorescein diacetate (FDA, Fisher Scientific, Hampton, NH) for 5 min, viable CGN were quantified by counting fluorescein-positive (green) cells that result from the de-esterification of living cells [7]. Values were expressed as a percent of control cultures.

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, Sigma, St. Louis, MO, USA) assay was also performed to evaluate CGN's viability. Briefly, MTT was added to CGN at a final concentration of 0.25 mg/ml and incubated for 1 h at 37 °C to allow the conversion of MTT into purple formazan crystals. Thereafter, the incubation medium was removed and the cells were lysed with an equal volume of 100% DMSO and quantified using a microplate reader (Molecular Device, Sunnyvale, CA, USA) [21].

To neutralize the activity of individual growth factors, 1 μg/μl of each monoclonal inactivating antibody against IGF-1 (R&D System, Minneapolis, MN, USA), BDNF (Sigma, St. Louis, MO, USA), GDNF (Abcam, Cambridge, MA, USA), VEGF (R&D System, Minneapolis, MN, USA), or HGF (R&D System, Minneapolis, MN, USA) was incubated with ASC-CM individually overnight at 4 °C before examining ASC-CM-induced neuroprotection [36]. For the signaling pathway study, 10 μM LY294002 (a P13Kinase inhibitor, Tocris Bioscience, Bristol, UK) was used to pretreat CGN for 2 hr before neurons were subject to ASC-CM treatment.

2.3. Quantification of IGF-1 by ELISA

IGF-1 level in ASC-CM was measured using Human IGF-1 Quantikine ELISA kits (R&D system, Minneapolis, MN, USA), an enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions [33].

2.4. Depletion of IGF-1 from ASC-CM by immunoprecipitation (IP)

Anti-IGF-1 antibody (Abcam, Cambridge, MA, USA) was coupled to NHS-activated Sepharose 4 Fast Flow agarose (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) overnight at 4 °C following the manufacturer's instructions. After washing the mixture, the coupled antibody was incubated with ASC-CM at room temperature for 2 h and removed by centrifugation. ASC-CM depleted of IGF-1 was then ready to use.

2.5. Determination of phosphorylated AKT (p-AKT) and total AKT levels by Western blot

CGN were incubated with ASC-CM for 4 h and subjected to Western blot analysis for p-AKT and total AKT determination. Western blot analyses for p-AKT and total AKT determination were performed by using antibodies of AKT and the Ser473 phospho-AKT (Cell Signaling Technology, Danvers, MA, USA). Proteins were extracted from cells as previously described [32]. Densitometry of the bands and the ratio between p-AKT/AKT were determined.

2.6. Statistical analysis

Data are presented as a mean ± SD, with the number of determinations (*n*) representing separate experiments carried out independently. Data was evaluated at a 0.05 level of significance with one-way ANOVA with post hoc testing by Fisher's protected least significant differences procedure.

3. Results

3.1. Effects of antibodies of IGF-1, BDNF, GDNF, VEGF and HGF on ASC-CM-induced neuroprotection against 6-OHDA toxicity

To determine the contributions of secreted growth factors in ASC-CM, we first focused on the neuroprotective roles of IGF-1, BDNF, GDNF, VEFG and HGF; five possible neuroprotective factors that were identified in ASC-CM [14,29,31,32,35,36]. As performed in previous studies [18,36], we neutralized the activity of each factor individually in ASC-CM by using the inactivating antibody specific to each factor and then measuring the neuroprotective efficacy of each treated ASC-CM (50% replacement) in CGN. CGN represent a relatively homogenous population of neurons that contain <5% glia and are a useful model to investigate neuronal