Biochemical and Biophysical Research Communications 473 (2016) 35–41

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Priming with ceramide-1 phosphate promotes the therapeutic effect of mesenchymal stem/stromal cells on pulmonary artery hypertension

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ARTICLE INFO

Article history:
Received 29 February 2016
Accepted 9 March 2016
Available online 15 March 2016

Keywords:
Ceramide-1 phosphate
Mesenchymal stem cell
Priming
Pulmonary artery hypertension

ABSTRACT

Some molecules enriched in damaged organs can contribute to tissue repair by stimulating the mobilization of stem cells. These so-called "priming" factors include bioactive lipids, complement components, and cationic peptides. However, their therapeutic significance remains to be determined. Here, we show that priming of mesenchymal stromal/stem cells (MSCs) with ceramide-1 phosphate (C1P), a bioactive lipid, enhances their therapeutic efficacy in pulmonary artery hypertension (PAH). Human bone marrow (BM)-derived MSCs treated with 100 or 200 μM C1P showed improved migration activity in Transwell assays compared with non-primed MSCs and concomitantly activated $MAPK^{p42/44}$ and AKT signaling cascades. Although C1P priming had little effect on cell surface marker phenotypes and the multipotency of MSCs, it potentiated their proliferative, colony-forming unit-fibroblast, and anti-inflammatory activities. In a monocrotaline-induced PAH animal model, a single administration of human MSCs primed with C1P significantly attenuated the PAH-related increase in right ventricular systolic pressure, right ventricular hypertrophy, and thickness of α-smooth muscle actin-positive cells around the vessel wall. Thus, this study shows that C1P priming increases the effects of MSC therapy by enhancing the migratory, self-renewal, and anti-inflammatory activity of MSCs and that MSC therapy optimized with priming protocols might be a promising option for the treatment of PAH patients.

1. Introduction

Stem cells (SCs) deposited in adult tissues function as guardians of the homeostasis of tissues/organs by undergoing self-renewal/maintenance of their own pool and by giving rise to differentiated cells that regenerate damaged tissues. In addition, adult SCs are also mobilized into peripheral blood during several models of organ injury in an attempt to enrich and regenerate damaged tissues (e.g., heart infarct, stroke) [1,2]. A variety of adult stem/progenitor cells, such as mesenchymal stem/stromal cells (MSCs) and multipotent adult SCs that have been isolated from bone marrow (BM), adipose, mobilized peripheral blood, dental pulp, and umbilical cord blood (UCB), have been used in clinical trials to regenerate damaged organs [3]. However, the therapeutic outcomes of adult SCs are limited, mainly due to poor engraftment and survival of the transplanted cells under *in vivo* conditions [4]. Thus, understanding of the precise mechanism underlying the migration and engraftment of SCs during tissue repair may overcome the significant challenges facing therapeutic strategies that

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http://dx.doi.org/10.1016/j.bbrc.2016.03.046
0006-291X/

List of abbreviations

BM	Bone marrow
C1P	Ceramide-1-phosphate
CFU-F	Colony-forming unit-fibroblast
CM	Conditioned medium
$_{desArg}$C3a	C3 cleavage fragments
LPS	Lipopolysaccharide
HSPC	Hematopoietic stem/progenitor cell
MCT	Monocrotaline
MSCs	Mesenchymal stem/stromal cells
PAH	Pulmonary artery hypertension
RVSP	Right ventricular systolic pressure
RV	Right ventricle
S1P	Sphingosine-1-phosphate
SCs	Stem cells
SDF-1	Stromal derived factor-1
sMAC	Soluble membrane attack complex
TNF-α	Tumor necrosis factor-α
UCB	Umbilical cord blood

use adult SCs.

Evidence is accumulating that a number of chemokines and growth factors, which are both secreted within damaged tissues, may play a crucial role in directing adult SCs to injured sites [5–8]. Of these, stromal derived factor-1 (SDF-1) and its receptor CXCR4 are pivotal for homing and engraftment of adult SCs [5]. Interestingly, the responsiveness of SCs to an SDF-1 gradient can be enhanced *in vitro* by some molecules enriched in damaged tissues. This phenomenon, known as "priming", has been well established in the process of hematopoietic stem/progenitor cell (HSPC) mobilization and engraftment [9]. Mechanistically, priming molecules stimulate the incorporation of CXCR4 receptor kinase into membrane lipid rafts, which allow physiologically lower doses of SDF-1 to become "biologically significant" in SC trafficking. Known priming factors include bioactive lipids, such as sphingosine-1-phosphate (S1P) [10] and ceramide-1-phosphate (C1P) [11,12], as well as complement C3 cleavage fragments (C3a and $_{desArg}$C3a) [13,14], soluble membrane attack complex (sMAC) C5b–9 [11], and cationic peptides, such as cathelicidin (LL-37) and β2-defensin released from activated granulocytes [15,16].

Recently, we showed that priming of human UCB-derived MSCs with S1P promotes their therapeutic effects on pulmonary artery hypertension (PAH), a rare disease characterized by the sustained elevation of pulmonary artery pressure and pulmonary vascular resistance that ultimately leads to right heart failure and death [17]. In the present study, we additionally show the role of C1P in priming human BM-derived MSCs in *in vitro* cell culture and *in vivo* conditions.

2. Materials and methods

2.1. Culture of human MSCs

Human BM-derived MSCs purchased from Lonza (Basel, Switzerland) were cultured following the manufacturer's instructions. Cells expanded for fewer than seven passages were used to ensure multipotency. The expression of surface proteins was analyzed by staining 5.0×10^5 MSCs with the indicated antibodies and then analyzing them using a BD FACS Canto II flow cytometer (BD Biosciences, Mountain View, CA). The following fluorophore-conjugated anti-human surface marker antibodies were purchased from BD Pharmingen (San Diego, CA): CD105 (APC-conjugated, clone 266), CD29 (PE, clone MAR4), CD45 (FITC, clone GoH3), CD34 (FITC, clone GoH3), and CD49f (FITC, clone GoH3).

2.2. In vitro characterization of MSCs

Cell proliferation, colony-forming unit-fibroblast (CFU-F), multipotency (*in vitro* differentiation into osteogenic, chondrogenic, or adipogenic lineages), cell migration, and *in vitro* anti-inflammation assays of MSCs were performed as previously described [17]. Before functional analysis, MSCs were treated with 100 or 200 μM C1P from bovine brain (Sigma–Aldrich, St Louis, MO) for 24 h.

2.3. Western blot

MSCs were starved for 12 h in DMEM containing 0.5% BSA at 37 °C, stimulated with 100 μM C1P for 5, 10, or 30 min, and then lysed on ice in RIPA lysis buffer containing protease and phosphatase inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA). Cell extracts (30 μg) were separated using 12% SDS-PAGE gels and analyzed to determine the phosphorylation of MAPKp42/44 and AKT (Ser473) (Cell Signaling Technology, Danvers, MA). Equal loading was confirmed using monoclonal or polyclonal antibodies against total MAPKp44/42 and total AKT (Cell Signaling Technology).

2.4. Reverse transcriptase and real-time quantitative PCR

Preparation of total RNA, reverse transcription, and quantification of the indicated transcripts were performed using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA), TaqMan Reverse Transcription Reagents (Applied Biosystems), and real-time quantitative PCR with the PikoReal™ Real-Time PCR System (Thermo Scientific) with iQ™ SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA), as described previously [18].

2.5. PAH animal model

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Ulsan College of Medicine (IACUC-2013-12-110). To induce PAH, male specific pathogen-free Lewis rats (8 weeks old, 250–280 g) were subcutaneously injected with monocrotaline (MCT; 60 mg/kg; Sigma). Rats in the control (CTL) group were injected with the same volume of PBS. Two weeks after MCT or PBS injection, unprimed MSCs (PAH + MSC group) or MSCs primed with 100 μM C1P for 3 h (PAH + C1P-MSC group) were injected into mice via the tail vein at a density of 2.5×10^5 cells in 200 μl PBS. For a vehicle control, PBS without cells (PAH group) was also injected. Two weeks after MSC injection, the therapeutic outcomes of the MSCs were examined via measurement of right ventricular systolic pressure (RVSP), right ventricular hypertrophy, and histological analysis, as described previously [17].

2.6. Statistical analysis

Data were analyzed using a non-parametric Mann–Whitney test or one-way ANOVA with the Bonferroni post-hoc test to detect statistically significant differences. We used GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA) to perform all analyses, and statistical significance was defined as $p < 0.05$.