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Targeting the pathway of GSK-3β/nerve growth factor to attenuate post-infarction arrhythmias by preconditioned adipose-derived stem cells

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ABSTRACT

Adipose-derived stem cell (ADSC) transplantation is a promising new therapy to improve cardiac function after myocardial infarction. However, its low efficacy of transdifferentiation hampers its usefulness. Glycogen synthase kinase-3β (GSK-3β) signal has been shown to play a role in preconditioning-induced cardioprotection. We assessed whether *n*-butylidenephthalide (BP) primed ADSCs can attenuate arrhythmias by a GSK-3β-dependent pathway after myocardial infarction. Male Wistar rats after coronary ligation was randomly allocated to receive intramyocardial injection of vehicle, ADSCs, BP-preconditioned ADSCs, (BP + lithium)-preconditioned ADSCs, (BP + SB216763)-preconditioned ADSCs, and (BP + LY294002)-preconditioned ADSCs. ADSCs were primed for 16 h before implantation. After 4 weeks of implantation, ADSCs were retained in myocardium, reduced fibrosis and improved cardiac function. Sympathetic hyperinnervation was blunted after administering ADSCs, assessed by immunofluorescent analysis, and Western blotting and real-time quantitative RT-PCR of nerve growth factor. Arrhythmic scores during programmed stimulation in the ADSC-treated infarcted rats were significantly lower than vehicle. BP-preconditioned ADSCs had superior cardioprotection, greater ADSC engraftment and transdifferentiation, and antiarrhythmic effects compared with ADSCs alone. Simultaneously, BP increased the levels of phospho-Akt and down-regulated GSK-3β activity. The effects of BP against sympathetic hyperinnervation were blocked by LY294002, a PI3K inhibitor. Addition of either lithium or SB216763 did not have additional effects compared with BP alone. Compared with ADSC alone, BP-primed ADSC implantation improved stem cell engraftment and attenuated sympathetic hyperinnervation and arrhythmias through a PI3K/Akt/GSK-3β-dependent pathway, suggesting that a synergic action was achieved between BP pretreatment and ADSCs.

1. Introduction

Adipose-derived stem cell (ADSC) transplantation is a promising new therapy to improve cardiac function after myocardial infarction (MI) [1]. Most of stem cell replacement therapy experiments in MI focus on the hemodynamics effects. Despite the rapidity in which cellular therapy for MI has progressed from bedside to bench, many fundamental questions regarding the electrophysiological and arrhythmic consequences are unresolved. Earlier experimental studies suspected that mesenchymal stem cell therapy is proarrhythmic. Previous studies have shown that sprouting of sympathetic nerves in pig myocardium following transplantation of mesenchymal stem cells, which could increase the risk of arrhythmias [2]. However, this was not corroborated by other investigators, including Amado et al. [3], showing no increase in sudden death in pigs undergoing intramyocardial injection of mesenchymal stem cells compared to placebo. Gautam et al. [4] demonstrated that ADSCs reduced arrhythmia inducibility in infarcted rats. A

Abbreviations: α-SMA, α-smooth muscle cell actin; ADSC, adipose-derived stem cell; BP, *n*-butylidenephthalide; DHE, dihydroethidium; GSK-3β, glycogen synthase kinase-3β; hADSCs, human adipose-derived stem cells; MI, myocardial infarction; NGF, nerve growth factor; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; SB216763, (3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1Hpyrrole-2,5-dione.

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randomized, double-blind, placebo-controlled clinic trial revealed that intravenous adult human mesenchymal stem cells after acute MI lead to four-fold reduction of arrhythmias [5]. Therefore, whether cell therapy causes proarrhythmic risk is still uncertain.

Cardiac remodeling was associated with myocardial hypertrophy and ventricular arrhythmias following MI. Transmural MI interrupts efferent sympathetic nerves and denervates viable muscle distal to MI. Levels of nerve growth factor (NGF) expression within innervated tissues roughly correspond to innervation density [6]. We have demonstrated that NGF mRNA and protein levels in the myocardium in the border zone are increased in the infarcted rats during chronic stages of MI [7]. Increased sympathetic nerve activity plays an important role in generation of ventricular arrhythmia and sudden cardiac death [8].

Although immense progress has been made on the choice of optimizing transplantation conditions, little success has been achieved with respect to the strategies aiming to improve the efficiency of ADSC transplantation. Preconditioning of stem cells is an emerging strategy to curtail the massive death of cells after transplantation. Hypoxic preconditioning is known to be protective and has been shown to induce phosphorylation and activation of Akt. Akt activation has been shown to be associated with angiogenesis, myocyte renewal, and stem cell activation in myocardium [9]. Preconditioning signaling pathway involves an amplification loop leading to phosphoinositide 3-kinase (PI3K)/Akt activation and subsequent glycogen synthase kinase (GSK)-3β inhibition. Indeed, lithium, a GSK-3β inhibitor, has been shown to precondition mesenchymal stem cells [10]. GSK-3β is crucial to the regulation of nerve morphogenesis [11]. Very recently, we have shown that GSK-3 inhibition drastically prevents nerve extension by attenuating production of reactive oxygen species (ROS, 12).

Angelica sinensis, a traditional Chinese herbal medicine, has been shown to have a broad spectrum of biological activities, such as anti-inflammatory and regulation of the immune system including cardiovascular diseases [13]. The chloroform extract of Angelica sinensis contained six major compounds namely ferulic acid, senkyunolide I, senkyunolide H, coniferyl ferulate, *Z*/*E*-ligustilide and *n*-butylidenephthalide (BP). BP, the main metabolic of ligustilide, is the major component of Angelica sinensis [14]. BP has been reported to have a variety of pharmacological activities, including vasorelaxant, anti-anginal, anti-platelet, and antioxidant effects [15,16]. Previous studies have shown neuroprotective effects of Angelica sinensis extract via the inhibition of GSK-3β activity in cultured cortical neurons [17]. However, whether BP plays a role in inhibiting GSK-3β activity remained unknown. Furthermore, whether the effect of preconditioned ADSCs by BP on arrhythmias after infarction remained unclear. In this study, we assessed 1) whether in vivo transplantation of human ADSCs (hADSCs) can attenuate post-infarction arrhythmias, and 2) whether the anti-arrhythmic effect of BP-pretreated ADSCs is superior to ADSCs through inhibition of GSK-3β-mediated NGF expression in a rat MI model. Our results show that BP is a powerful new preconditioning strategy to enhance their therapeutic potential as shown by reduced sympathetic innervation and arrhythmias via a PI3K/Akt/GSK-3β dependent pathway in rats following MI.

2. Methods

The cell culture and animal experiment were approved and conducted in accordance with local institutional guidelines for the care and use of laboratory animals at the China Medical University (CMUH105-REC2-066 and 2016-070) and conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Culture of hADSCs

hADSCs were generously provided by Gwo Xi Stem Cell Applied Technology (Hsinchu, Taiwan) according to our previously described method [18]. In brief, human adipose tissue was removed from the transport medium, placed in a Petri dish, and cut into small pieces (1–2 mm^3) in the presence of Ca^{2+}/Mg^{2+}-free PBS. The tissues were dissociated with 0.1% collagenase I (Invitrogen-Gibco, Carlsbad, CA) and incubated for 60 min at 37 °C. After enzymatic digestion, the resultant cells were collected and cultured in keratinocyte serum-free media (Invitrogen-Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), *N*-acetyl-L-cysteine (Sigma-Aldrich, St. Louis, MO), L2 ascorbic acid, and phosphate (Sigma-Aldrich, St. Louis, MO). The supernatant and debris were removed from the culture dish after being cultured for 2 days. ADSCs at passages 3–5 were used in this study.

2.2. Characterization of ADSC surface phenotype

Trypsinized ADSCs were suspended in 100 μL PBS. Cells (1×10^5 per sample) were treated at room temperature for 20 min with the following specific anti-human antibodies: anti-Isotype IgG1-PE, -CD19-PE, -CD34-PE, -CD73-PE, -CD105-PE, -Isotype IgG1-FITC, -CD45-FITC, -CD90-FITC, -Isotype IgG2a-PE, -CD14-PE, -HLA-DR-PE (BD Biosciences, San Jose, CA, USA). Mouse IgG was used as a negative control condition. Fluorescent labeling was analyzed with a flow cytometer (Accuri C6; BD Biosciences, San Jose, CA, USA).

2.3. Induction of MI and cell transplantation

Male Wistar rats (250–300 g) were subjected to ligation of the anterior descending artery, resulting in infarction of the LV free wall as we previously described [19]. For surgery, hemodynamics measurements, electrophysiological studies and sacrifice, rats were intraperitoneally anesthetized with ketamine (90 mg/kg body weight) and xylazine (9 mg/kg). Anesthesia monitor was tested by rear foot reflexes before and during procedures, observation of respiratory pattern, and responsiveness to manipulations throughout the procedures. Animals were ventilated with 95% O_2 and 5% CO_2 using a ventilator (Harvard Apparatus 486).

One hour after ligation, rats were randomly assigned into groups of either vehicle or cell transplantation. For cell transplantation, ADSCs were detached from the plate, suspended in 30 μl of PBS (1×10^6 cells), and injected with a 30-G needle as three equal aliquots in the border zone. ADSC were primed with either 40 μg/ml BP (Alfa Aesar), BP + 3 mM lithium (a GSK-3β inhibitor), BP + 10 μM SB216763 (3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1Hpyrrole-2,5-dione, a selective ATP competitive GSK-3α/β antagonist), or BP + 20 μM LY294002 (a PI3K inhibitor) for 16 h before transplantation. Lithium was used as a positive control as it is known that lithium pretreatment attenuated infarct volumes in an experimentally-induced stroke, by inhibiting GSK-3 signaling [10]. The doses of BP [20], LiCl [21], SB216763 [22], and LY294002 [23] were used as previously described. Before cell transplantation, cells were washed for 3 times to eliminate the direct drug or media effects. The heart was excised at days 3 or 28 after MI as early and late stages of MI. The study duration was 4 weeks so as to extend beyond the majority of the myocardial remodeling process in the rat (70–80%) which is complete within 3 weeks [24]. Sham operation served as controls. Thus, together, the experimental groups studied were: sham group and infarction groups (vehicle, ADSC, BP-ADSC, (BP/lithium)-ADSC, (BP/SB216763)-ADSC, and (BP/LY294002)-ADSC).

2.4. Echocardiogram

At 28 days after ligation, rats were lightly anesthetized with intraperitoneal injection of ketamine–HCl (25 mg/kg). Echocardiographic measurements were done with a Vivid 7 scanner and a 14-MHz Linear Array Probe (GE Vingmed Ultrasound, Milwaukee, WI) as described previously [19]. M-mode tracing of the LV was obtained from the