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The anti-apoptotic effect of IGF-1 on tissue resident stem cells is mediated via PI3-kinase dependent secreted frizzled related protein 2 (Sfrp2) release ☆

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ABSTRACT

Previous studies suggest that IGF-1 may be used as an adjuvant to stem cell transfer in order to improve cell engraftment in ischemic tissue. In the current study, we investigated the effect of IGF-1 on serum deprivation and hypoxia induced stem cell apoptosis and the possible mechanisms involved. Exposure of adipose tissue derived stem cells (ASCs) to serum deprivation and hypoxia resulted in significant apoptosis in ASC which is partially prevented by IGF-1. IGF-1's anti-apoptotic effect was abolished in ASCs transfected with Sfrp2 siRNA but not by the control siRNA. Using Western blot analysis, we demonstrated that serum deprivation and hypoxia reduced the expression of nuclear β-catenin, which is reversed by IGF-1. IGF-1's effect on β-catenin expression was abolished by the presence of PI3-kinase inhibitor LY294002 or in ASCs transfected with Sfrp2 siRNA. These results suggest that IGF-1, through the release of the Sfrp2, contributes to cell survival by stabilizing β-catenin.

Severe acute myocardial ischemia may result in heart failure due to irreparable loss of cardiomyocytes and heart function [1]. Clinical studies suggest that stem cells may contribute to the healing of myocardial infarction (MI) [2–6]. However, the reported functional improvements are generally modest partly due to the low cellular survival rate after transplantation [7,8]. In order to improve the efficacy of cell therapy, it is necessary to reinforce stem cells against the hostile microenvironment incurred from inflammatory response and proapoptotic milieu as a result of ischemia reperfusion injury. Insulin like growth factor-1 (IGF-1) has been shown to have a number of beneficial effects on the heart including protection against apoptosis [9–12]. It has been suggested that IGF-1 may be used as an adjuvant to cell transfer for myocardial restoration [13,14]. When added to a cell suspension prior to injection, IGF-1 promotes engraftment, differentiation, and functional improvement after transfer of embryonic stem cells for myocardial restoration [14]. However, the mechanisms underlying such an observed improvement are far from clear. It has recently been shown that intracardiac implantation of genetically engineered MSCs overexpressing the Akt gene (Akt-MSCs) yielded dramatic diminution of infarct size and restoration of cardiac function in rodent hearts after myocardial injury [15] and Sfrp2 was identified as a major paracrine mediator of Akt-MSCs myocardial survival and reparative effects [16]. It is known that IGF-1 is an up stream activator for Akt, we reasoned that IGF-1's anti-apoptotic effect might be mediated by Sfrp2.

Materials and methods

Isolation and culture of stem cells. Cells were isolated from either mouse adipose tissue (ASCs) or skeletal muscle as described previously [17]. Briefly, adipose tissue or muscle was minced and incubated for 90 min at 37 °C on a shaker with Liberase Blendzyme 3 (Roche) at a concentration of four units per gram of fat tissue in PBS. The digested tissue was sequentially filtered through 100 μm and 40 μm filters (Fisher Scientific) and centrifuged at 450*g* for 10 min. The supernatant containing adipocytes and debris was discarded and the pelleted cells were washed twice with Hanks Balanced Salt Solution (Cellgro) and finally resuspended in growth media. Growth media contained alpha-modification of Eagle's medium (αMEM, Cellgro), 20% FBS (Atlanta Biologicals), 2 mM glutamine (Cellgro), 100 U/ml penicillin with 100 μg/ml streptomycin (Cellgro).

Quantitative PCR. Total RNA of ASCs was isolated using RNAqueous kit (Ambion) according to the manufacturer's instructions and then reverse transcribed using iScript™ cDNA Synthesis kit (Bio-rad). To assess the mRNA expression level of Sfrp2, quantitative PCR was performed. GAPDH was used as a reference gene for relative quantification. Primers used for realtime PCR: Sfrp2: 5′AGATG AA CGACATCAACGCTCCGT3′ and 5′GAAACTAGCATTGCAGCTTGC

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GGA3′;GAPDH: 5′ATCGTGGAAGGGCTAATGACCACA3′ and 5′TTGAGCTCTGGGATGACTTTGCCT3′.

Suppression of Sfrp2 by siRNA. ASCs were transfected with Sfrp2 siRNA using the transfection protocol recommended by Santa Cruz Tenologies, Inc. Briefly, 50 pmols of the Sfrp2 siRNA duplex (sc-40001, Santa Cruz) or control siRNA (scrambled sequence, sc-37007) was diluted into 100 μl transfection medium (sc-36868) which are then mixed with transfection reagent (sc-29528) diluted in 100 μl transfection medium (sc-36868). The siRNA was incubated with the transfection reagent for 30 min at room temperature then added to 0.8 ml transfection medium. The mixture was then overlayed onto 2×10^5 cells in a 6-well plate and incubates for 7 h. After adding 1 ml of 2× normal growth medium (αMEM), the cells were cultured for an additional 24 h. The medium was then replaced with 1× normal growth medium and cultured for 48 h.

In order to verify the specificity of the siRNA approach, Sfrp2 expression was knocked down by transduction with a lentivirus containing short hairpin RNA (shRNA) construct targeting an independent Sfrp2 coding sequence (Sigma, Cat# TRCN0000034474). ASCs or muscle derived cells were transduced with a lentivirus for 24 h and transfected cells were selected by Puromycin (6 μg/ml) as described previously [18]. Cells transduced with non-target shRNA served as negative control in the experiment.

The efficiency of the siRNA-mediated reduction of Sfrps was assessed by realtime PCR using GAPDH as a control.

Apoptosis. Apoptosis was induced by subjecting ASCs to serum deprivation and hypoxia for 48 h. Cells (passage 3) were placed in a humidified modular hypoxia chamber (Billups-Rothenberg) which was purged for 4 min with a 95% N_2 and 5% CO_2 gas mixture. FITC Annexin V (BD Bioscience) was used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis per manufacture's instruction. Cells that stain positive for FITC Annexin V and negative for PI are considered undergoing apoptosis. Recombinant mouse IGF-1 and Sfrp2 were purchased from R&D Systems. LY294002 was purchased from Cell Signaling, Inc.

Western Blot. Passage 3 ASCs were lysed in Lysis Buffer including protease inhibitor cocktail (Cell Signaling). For Sfrp2 detection, the medium was concentrated up to 50× by using a Millipore Amicon Ultra-15 (Billerica, MA) system with membrane. Nuclear protein extract was prepared using the NE-PER nuclear extraction reagents (Pierce, Rockford, IL, USA) for the detection nuclear translocation of β-catenin. The proteins were separated on sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) gels and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). After blocking with 5% non-fat milk, the membranes were incubated for 1 h at room temperature with primary antibody. Following incubation with goat anti-rabbit horseradish peroxidase (HRP) secondary antibody (Amersham Biosciences), labeled proteins were detected using the SuperSignal chemiluminescence detection system (Pierce). Sfrp2 antibody (H-140) was purchased from Santa Cruz Biotechnology, Inc. Rabbit anti-β-catenin was from Cell Signaling, Inc.

Statistical analysis. All values are presented as means ± standard deviation (SD). Statistical analysis was performed using Student's *t*-tests. A level of $P < 0.05$ was considered as statistically significant.

Result

IGF-1 induces the secretion of Sfrp2 in ASC

ASCs are characteristically plastic adherent, spindle-shaped cells (Fig. 1A). They are positive for CD44, CD90, CD105 and negative for CD11b, CD14, CD34, CD45 as we have described previously [19].

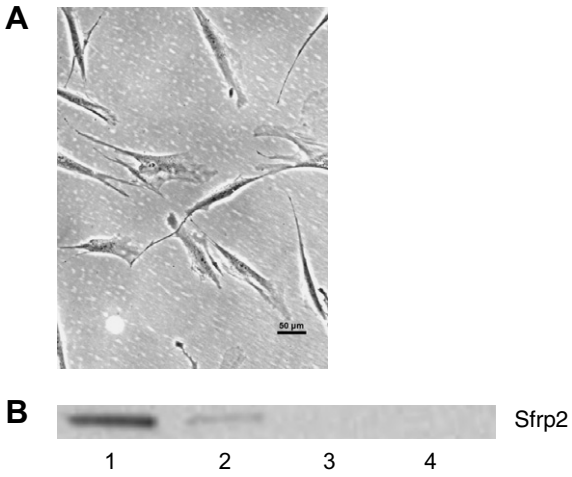

Fig. 1. (A) Phase contrast photomicrographs of mouse passage 3 ASCs cultured in αMEM containing 20% FBS. Mouse ASCs are spindle shaped and plastic adherent. (B) Western blot analysis of Sfrp2 expression. Lane 1: IGF-1 treated ASC; lane 2: untreated ASC; lane 3: ASC treated with IGF-1 and LY294002; lane 4: ASC treated with LY294002. IGF-1 concentration: 10 ng/ml, LY294002: 50 μM.

ASCs were cultured for 12 h with IGF-1 in the presence or absence of 50 μM phosphatidylinositol 3-kinase (PI3-kinase) inhibitor (LY294002). The conditioned medium was collected and concentrated for Western blot protein analysis. As shown in Fig. 1B, Sfrp2 was secreted into the conditioned medium from the IGF-1 treated cells. The levels of Sfrp2 were low in the conditioned medium of untreated ASCs. Furthermore, the release of Sfrp2 in the IGF-1 treated ASCs depended on the PI3-kinase pathway because inhibition of the PI3-kinase abolished Sfrp2 accumulation in the medium.

Sfrp2 knockdown in ASC reduces IGF-1's anti-apoptotic effect

IGF-1 is a known anti-apoptotic hormone. We investigated whether IGF-1 can promote ASC survival and whether the effect is mediated by Sfrp2. ASCs are treated with siRNA against Sfrp2 or control siRNA. As shown in Fig. 2A, the mRNA expression of Sfrp2 was significantly reduced in ASCs transfected with siRNA against Sfrp2 but not by control siRNA. Serum deprivation and hypoxia induced significant apoptosis in ASC (43.2 ± 3.39%) which is partially prevented by IGF-1 (25.05 ± 4.31%) (Fig. 2B). IGF-1's anti-apoptotic effect was abolished in ASCs transfected with Sfrp2 siRNA (41.2 ± 1.13%) but not by the control siRNA (26.05 ± 4.74%). These data indicate that IGF-1's anti-apoptotic effect is mediated by Sfrp2 (Fig. 2B). In order to verify the specificity of the siRNA approach, Sfrp2 expression was knocked down by transduction with a lentivirus containing short hairpin RNA (shRNA) construct targeting an independent Sfrp2 coding sequence. Efficiency of silencing was 86% as assessed by quantitative real-time PCR. Again, we showed that IGF-1's anti-apoptotic effect was abolished in ASCs transfected with Sfrp2 lentiviral shRNA but not by the control shRNA (Fig. 3A). In order to find out whether Sfrp2 release also mediate IGF-1's anti-apoptotic effect in other cell types, we performed a similar experiment using muscle derived cells. As shown in Fig. 3B, IGF-1's anti-apoptotic effect was also abolished in muscle derived cells transduced with Sfrp2 lentiviral shRNA but not by the control shRNA .

IGF-1 induces up-regulation of β-catenin in hypoxic ASCs via the release Sfrp2

It has been shown that Sfrp2 can enhance the survival response of cardiomyocytes against hypoxia-induced apoptosis by increas-