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Original article

Secreted frizzled related protein 2 protects cells from apoptosis by blocking the effect of canonical Wnt3a

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ABSTRACT

We have demonstrated that mesenchymal stem cells overexpressing the survival gene Akt can confer paracrine protection to ischemic myocytes both in vivo and in vitro through the release of secreted frizzled related protein 2 (Sfrp2). However, the mechanisms mediating these effects of Sfrp2 have not been fully elucidated. In this study, we studied rat cardiomyoblasts subjected to hypoxia reoxygenation (HR) injury to test the hypothesis that Sfrp2 exerts anti-apoptotic effect by antagonizing pro-apoptotic properties of specific Wnt ligands. We examined the effect of Wnt3a and Sfrp2 on HR-induced apoptosis. Wnt3a significantly increased cellular caspase activities and TUNEL staining in response to HR. Sfrp2 attenuated significantly Wnt3a-induced caspase activities in a concentration dependent fashion. Using a solid phase binding assay, our data demonstrates that Sfrp2 physically binds to Wnt3a. In addition, we observed that Sfrp2 dramatically inhibits the beta-catenin/TCF transcriptional activities induced by Wnt3a. Impressively, Dickkopf-1, a protein that binds to the Wnt coreceptor LRP, significantly inhibited the Wnt3a-activated caspase and transcriptional activities. Similarly, siRNA against beta-catenin markedly inhibited the Wnt3a-activated caspase activities. Consistent with this, significantly fewer TUNEL positive cells were observed in siRNA transfected cells than in control cells. Together, our data provide strong evidence to support the notion that Wnt3a is a canonical Wnt with pro-apoptotic action whose cellular activity is prevented by Sfrp2 through, at least in part, the direct binding of these molecules. These results can explain the in vivo protective effect of Sfrp2 and highlight its therapeutic potential for the ischemic heart.

1. Introduction

It has been shown that intramyocardial transplantation of mesenchymal stem cells (MSCs) over-expressing the survival gene Akt result in significant increase in cell viability and engraftment, reduction of infarct size and improvement of cardiac function [1,2]. Recently, our laboratory has demonstrated that Akt MSCs release Sfrp2 that mediate the pro-survival effects of these cells on ischemic cardiomyocytes. Indeed the cytoprotective effects of these cells were markedly attenuated upon knockdown of Sfrp2 with siRNA [3]. Taken together, these data support a key role played by Sfrp2 in mediating the paracrine effects of Akt-MSC on myocardial protection.

The mechanisms mediating Sfrp2's cellular survival effect has not been precisely elucidated. It is currently thought that Sfrps compete with the Frizzled receptor for Wnt ligands thereby preventing the activation of Wnt signaling [4]. Through this interaction, Sfrps could potentially influence cell fate and survival. Accordingly, we hypothesize that Sfrp2 exerts anti-apoptotic effect by antagonizing Wnts with pro-apoptotic properties. To further explore this possibility, we studied rat cardiomyoblasts subjected to hypoxia reoxygenation (HR). We examined the effect of Wnt3a on HR-induced apoptosis and then studied the nature of the interaction of Sfrp2 with Wnt3a. Wnt3a was selected since its expression is up-regulated in response to hypoxia [3] and it has been shown to induce apoptosis.

We examined the effect of Sfrp2 on canonical pathway activation induced by Wnt3a. Wnt ligands bind to the frizzled receptors and co-receptors LRP5/6, leading to phosphorylation of the disheveled protein, which, through its association with Axin and the adenomatous polyosis coli (APC) tumor suppressor, prevents glycogen synthase kinase 3beta from phosphorylating beta-catenin. Unphosphorylated beta-catenin is stabilized by escaping recognition by beta-TrCP, a component of an E3

Abbreviations: Sfrp2, secreted frizzled related protein 2; HR, hypoxia reoxygenation; MSCs, mesenchymal stem cells; APC, adenomatous polyosis coli; TCF/LEF, T cell factor/lymphoid enhancer factor; DKK1, Dickkopf-1; TdT, terminal deoxynucleotidyl transferase; AMC, 7-amino-4-methyl coumarin; BSA, bovine serum albumin; RT, room temperature; CRD, cysteine rich domain.

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ubiquitin ligase. Free beta-catenin translocates to the nucleus, where it engages transcription factors T cell factor/lymphoid enhancer factor (TCF/LEF) to activate downstream genes [5,6]. This signaling pathway can be modulated by Sfrp [4,7–11]. In this article, we demonstrated that Wnt3a induces apoptosis via the canonical pathway and that Sfrp2 protects hypoxic and reoxygenated cells by inhibiting the pro-apoptotic effect of canonical Wnt3a. Furthermore, our data showed that Sfrp2 physically binds to Wnt3a. This may explain, at least in part, how Sfrp2 antagonizes the Wnt3a cellular effect. In addition, we observed that Sfrp2 inhibits the beta-catenin/TCF transcriptional activities induced by Wnt3a. Impressively, Dickkopf-1 (DKK1) and siRNA against beta-catenin significantly inhibited the Wnt3a-activated apoptosis and transcriptional activities. Together, our data provide strong evidence to support the notion that Wnt3a is a canonical Wnt with pro-apoptotic action whose cellular activity is prevented by Sfrp2 through, at least in part, direct binding of these molecules.

2. Materials and methods

2.1. Cell cultures and hypoxia/reoxygenation treatment

H9C2, a clonal line of rat embryonic heart-derived myoblasts, was obtained from American Type Culture Collection (CRL-1446, Manassas, VA USA). The cells were maintained in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, N.Y. USA), 10% fetal bovine serum (Hyclone, Logan, UT USA), penicillin (100 U/ml), streptomycin (100 U/ml), and glutamine (4 mM). To prevent loss of differentiation potential, cells were not allowed to become confluent. Passages 12–25 were used for all experiments described in this article. Cell density and viability were determined by Trypan blue dye exclusion test. After changing to hypoxic serum-free medium, cells were incubated under hypoxia and then put into normoxia for indicated hours.

2.2. Western blotting

Nuclear fractions of protein were isolated according to the protocol of FractionPREP Cell Fractionation System (Biovision, Mountain View, CA USA). Proteins from H9C2 cell lysates were separated by SDS page gel (Invitrogen, Carlsbad, California USA) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA USA). The blots were incubated with beta-catenin primary antibody (Cell Signaling, Danvers, MA USA) and then with appropriate second antibody conjugated with horseradish peroxidase (Amersham Biosciences, Piscataway, NJ USA). Complexes were detected by chemiluminescence (LumiGLO, Cell Signaling, Danvers, MA USA).

2.3. In situ cell death (TUNEL) analysis

The TUNEL assay (terminal deoxynucleotidyl transferase [TdT]-mediated 3′ ends of cleaved DNA labeling) was carried out according to the protocols of DeadEnd Colorimetric TUNEL System (Promega, Madison, WI USA). H9C2 cells, transfected with siRNAs against rat beta-catenin or scrambled control siRNA, were seeded on glass slide chambers and then treated with hypoxia/reoxygenation. Cells were fixed with 4% formaldehyde, permeabilized with proteinase K 20 μg/ml following by the incubation with rTdT enzyme and biotinylated nucleotides. Then strepavidin-horseradish peroxidase was developed. TUNEL staining is quantified by calculating the percentage of TUNEL positive nuclei in total nuclei in each low magnification field.

2.4. Quantification of caspases

Apoptosis was determined by measuring the activity of cleaved-caspase 3/7 using a caspase-specific fluorogenic substrate according to the protocol for Caspas 3/7 assay kit (Sigma, St. Louis, MO USA). H9C2 cells were lysed after treatment with Wnt3a and/or *Sfrp2* for 24 h under hypoxia/reoxygenation. 5 μl of cell extract was incubated in reaction buffer at room temperature for 1 h. The enzyme-catalyzed release of 7-amino-4-methyl coumarin (AMC) was measured by a fluorescence microplate reader. Fluorescent units were converted to pmole AMC/h/μg protein, using a standard curve of AMC.

2.5. Luciferase reporter assay

Cells were plated to for 60–80% confluent cultures in 12-well dishes. All transfection experiments were performed *in triplicate with Lipofectamine kit* (Invitrogen, Carlsbad, California USA), in accordance with the manufacture's instructions. TopFlash and the negative control counterpart FopFlash contain TCF binding sites (Top) and inactive TCF binding sites (Fop) (catalog #17-285, Upstate, Billerica, MA USA), *were* used to report TCF/beta-catenin signaling in *the* cells. The pRL-CMV renilla luciferase (Promega, Madison, WI USA) was co-transfected to normalize for transfection efficiency and total amount of transfected plasmid was made equal by addition of pUC18 vector (Stratagene, La Jolla, CA USA). Luciferase activity was assayed 48 h after transfection, using a dual-luciferase reporter assay system (Promega, Madison, WI USA).

2.6. Sfrp2/Wnt3a ELISA binding assays

Different doses of Sfrp2 (R&D System, Minneapolis, MN USA) diluted in 0.1% bovine serum albumin(BSA)/PBS was incubated in 96-well Falcon ELISA (100 μl/well) for over night at room temperature (RT). After decanting, all wells were filled with 5%BSA/PBS (300 μl/well) and incubated for an additional 3 h at 37 °C to block the plate. Following three washes with wash buffer (0.05% Tween 20 in PBS, pH 7.4), 100 μl aliquots of 3 or 15 nM of wnt3a (R&D Systems, Minneapolis, MN USA) diluted in 0.1% BSA/PBS were added and incubated *for* 2 h at RT. After three washes, 100 μl/well of Wnt3a antibody (R&D Systems, Minneapolis, MN USA) diluted in 01% BSA/PBS to a final concentration of 1 μg/ml *was* incubated *for* 1 h at RT. Another three washes were followed by a 1-h treatment at RT with 1: 10000 dilution of conjugated HRP goat anti-rat IgG (abcam, Cambridge, MA USA). After a final set of three washes, 100 μl/well of Color reagent (R&D Systems, Minneapolis, MN USA) was added and incubated for 20 min at RT following by 50 μl/well of Stop Solution (R&D Systems, Minneapolis, MN USA). Absorbance at 450 nm was measured with an ELISA plate reader (BMG LABTECH, Durham, NC USA).

2.7. Sfrp2/Wnt3a immuno-precipitation

15 nM of Sfrp2 and/or Wnt3a were incubated in binding buffer (0.1% BSA/PBS) at RT for overnight following by pre-cleaning with 50 μl of Protein G beads (Pierce, Rockford, IL). At 4 °C, the samples were reacted for overnight with Wnt3a antibody (R&D Systems, Minneapolis, MN USA) diluted to a final concentration of 1 μg/ml. 50 μl of Protein G beads was added in the samples and incubated with mixing at RT for 1 h. Collected beads were denatured for SDS page and blotted by Sfrp2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Recombinant Sfrp2 (R&D System, Minneapolis, MN USA) was loaded as a size control.

2.8. Electroporation

Pelleted cells were resuspended in fresh medium and counted, *and* sufficient cells for all transfections (1×10^6 cells per transfection) were transferred to a fresh tube and centrifuged at 80 g for 10 min. After carefully removing the supernatant, the cell pellet was resuspended in 100 μl of cell line nucleofector solution L (Amaxa biosystems, Gaithersburg, MD USA) per transfection, then each transfection was