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Wnt signaling enhances the activation and survival of human hepatic stellate cells

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Abstract Wnt signaling was implicated in pulmonary and renal fibrosis. Since Wnt activity is enhanced in liver cirrhosis, Wnt signaling may also participate in hepatic fibrogenesis. Thus, we determined if Wnt signaling modulates hepatic stellate cell (HSC) activation and survival. Wnt3A treatment significantly activated human HSCs, while this was inhibited in secreted frizzled-related protein 1 (sFRP1) overexpressing cells. Wnt3A treatment significantly suppressed TRAIL-induced apoptosis in control HSCs versus sFRP1 over-expressing cells. Particularly, caspase 3 was more activated in sFRP1 over-expressing cells following TRAIL and Wnt3A treatment. These observations imply that Wnt signaling promotes hepatic fibrosis by enhancing HSC activation and survival.

Keywords: Hepatic stellate cell; Fibrosis; Wnt; Secreted frizzled-related protein 1; Apoptosis

1. Introduction

Hepatic fibrosis is a common wound-healing response to chronic liver injuries, including persistent viral infection, alcoholic or drug toxicity and hereditary metal overload [1]. Activated hepatic stellate cells (HSCs) are the most important source of extracellular matrix proteins during this fibrotic process [2]. Therefore, the majority of anti-fibrotic therapies are designed to inhibit the activation, proliferation, or synthetic products of HSCs. More recently, the selective induction of HSC apoptosis by TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) has been proposed as an anti-fibrotic treatment [3].

Vertebrate Wnt and *Drosophila* wingless are homologous genes, and their protein products have been shown to participate in the regulation of cellular differentiation, proliferation, and polarity [4]. More recently, Wnt signaling was implicated in human fibrosing diseases, such as pulmonary and renal fibrosis [5,6]. Given that Wnt activity is enhanced in liver cirrhosis [7], it is also likely that Wnt signaling participates in hepatic fibrogenesis.

Secreted frizzled-related proteins (sFRPs) are soluble proteins that are capable of binding to Wnt and its receptor, frizzled (Fz), and thereby, interfere with Wnt signaling [8]. Diverse sFRP family members exhibit distinctive expression patterns, and modulate various aspects of Wnt signaling. In particular, sFRP1 plays a prominent role in the regulation of cellular apoptosis, differentiation, and angiogenesis [4,8]. If Wnt activation promotes hepatic fibrosis, the inhibition of this signaling by sFRP is likely to attenuate the Wnt-dependent activation of hepatic fibrosis.

In the present study, we hypothesized that Wnt signaling promotes hepatic fibrosis, and that the inhibition of this signaling by sFRP attenuates the Wnt-dependent activation of hepatic fibrosis. To test this hypothesis, we formulated the following questions: (i) Are Wnt receptors expressed in human HSCs? (ii) Does Wnt signaling enhance the activation or anti-apoptotic signaling of HSCs? And if so, (iii) Does sFRP inhibit these Wnt-dependent processes in HSCs? Collectively, our results demonstrate that Wnt signaling does participate in hepatic fibrosis by enhancing HSC activation and survival, and that this process is effectively prevented by sFRP1 over-expression, thus suggesting that the selective interruption of this signaling pathway may provide an efficient anti-fibrotic strategy in hepatic fibrosis.

2. Materials and methods

2.1. Cell culture and reagents

LX-2 cells, an immortalized human HSC line, were used in this study, and were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100,000 U/L penicillin, 100 mg/L streptomycin, and 100 nM insulin. Cells were plated at 5×10^4 cells/well in 1 mL of media or 10^6 cells in 5 mL of media in 6-well plate or 100-mm culture dish, respectively. Wnt3A-conditioned or control medium was prepared from a stably transfected mouse L cell clone, which secreted soluble Wnt3A proteins into the medium, or control L cells, respectively, as previously described with minor modification [9], and was diluted to a final concentration of 30%. TRAIL was purchased from Alexis (San Diego, CA).

2.2. Isolation of HSCs from normal human liver tissues

HSCs were isolated from a normal adult liver specimen obtained during the surgical resection of a metastatic tumor by collagenase/pronase digestion, followed by density gradient centrifugation using Nycodenz, as described previously [10]. The purity of the isolated HSCs, as determined by vitamin A autofluorescence, was more than 97% at 24 h after plating. The human material used in this study was a normally discarded specimen, and the study protocol was approved by the Institutional Review Board of Seoul National University Hospital.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using Trizol Reagent (Invitrogen, Carlsbad, CA). cDNA templates were prepared using oligo-dT random primers and MoMLV (Moloney Murine Leukemia Virus)

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reverse transcriptase. PCR was performed using primers specific for the Fz genes (forward, 5′-cagcgtcttgcccgaccagatcca; reverse, 5′-ctagcgccgctcttcgtgtacctg) [11]. To exclude the possibility of the presence of genomic DNA in the reaction, we performed control reactions without the RT step. RT-PCR products were subcloned using TOPO TA cloning kits (Invitrogen). Positive clones were sequenced using an ABI PRISM® 377 Genetic Analyzer (Applied Biosystems, San Francisco), and genes were identified by BLAST searching.

2.4. Vectors

The sFRP1 cDNA clone was a gift from Dr. Jeremy Nathans (Johns Hopkins University, Baltimore, MD). A hemagglutinin epitope was incorporated at the C-terminal of sFRP1 by PCR, and then ligated into the BglII/EcoRI site of pMSCV-IRES-GFP retroviral vector (a gift from Neil A. Clipstone, Northwestern University, Chicago, IL) upstream of IRES to give pMSCV-sFRP1-IRES-GFP.

2.5. Retrovirus generation and transduction

For the transient generation of VSV-G pseudo-typed retrovirus, 293T cells were transfected with pMD-gag-pol, pMD-VSVG (both gifts from Dr. Richard C. Mulligan (Harvard Medical School, Boston, MA)) and the retroviral vectors pMSCV-sFRP1-IRES-GFP or pMSCV-IRES-GFP using LipofectAMINE Plus reagents (Invitrogen, Carlsbad, CA). LX-2 cells were transduced with virus-containing supernatants in the presence of 8 μg/mL of Polybrene for 6 h and cells were collected 48 h later. GFP-positive fractions were FACS-sorted using a BD FACS Vantage Cell Sorter (Franklin Lakes, NJ).

2.6. Reporter gene assay

Cells were cotransfected over 24 h using 20 ng TK Renilla-CMV and 0.2 μg TCF reporter plasmid (Upstate Biotechnology Inc., Lake Placid, NY). Firefly and Renilla luciferase activities were quantitated using a dual luciferase reporter assay system (Promega, Madison, WI). Data are expressed as ratios of firefly to Renilla luciferase activity.

2.7. Real-time PCR

Total RNA was extracted and cDNA templates were prepared as described above. Collagen α1 mRNA was quantitated using real-time PCR technology and the following primers: forward, 5′-aacatgaccaaaaaccaaaagtg, reverse, 5′-cattgtttcctgtgtcttctgg. Universal 18S primers (Ambion Inc., Austin, TX) were used as a control for RNA integrity and as a "housekeeping gene". For quantitation, we used real-time PCR (LightCycler, Roche Molecular Biochemicals, Mannheim, Germany) and SYBR green as the fluorophore (Molecular Probes, Eugene, OR).

2.8. Apoptosis

Apoptosis was induced in LX2 cells using TRAIL [3], and assessed by examining characteristic nuclear changes (i.e., chromatin condensation and nuclear fragmentation) using the nuclear binding dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and fluorescence microscopy (Zeiss, Germany).

2.9. Immunoblotting

Cell lysates were resolved by SDS–PAGE, and blotted using appropriate primary antibodies and peroxidase-conjugated secondary antibodies (Biosource International, Camarillo, CA). The primary antibodies used were; rabbit anti-caspase 9 from Cell Signaling Technology Inc. (Beverly, MA); rabbit anti-caspase 8, mouse anti-cytochrome *c* and rabbit anti-caspase 3 from Pharmingen (San Diego, CA); mouse anti-α-smooth muscle actin from BioGenex (San Ramon, CA); and rabbit anti-sFRP1 and goat anti-β-actin from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

2.10. Immunoprecipitation analysis

Cells were treated with Wnt3A-conditioned media for 24 h, and apoptosis was induced using TRAIL. The cell lysates obtained were mixed with anti-sera for XIAP (X chromosome-linked inhibitor of apoptosis protein) (Cell Signaling Technology Inc.), and then incubated overnight at 4 °C. Immune complexes were immunoprecipitated with protein A/G PLUS-Agarose (Santa Cruz) and then washed for 5×10 min with 1 mL of washing buffer. After washing, polypeptides were resolved by boiling with Laemmli sample buffer, and then immunoblotted for caspase 9.

2.11. Statistical analysis

All numerical data represent at least three independent experiments using cells from a minimum of three separate isolations and are expressed as means ± S.D. Groups were compared using two-tailed Student's *t*-tests.

3. Results

3.1. Identification of Wnt receptors in human HSCs

RT-PCR using primers that bind to the conserved sequences of all Fz genes were used to visualize the expressions of these genes in both LX-2 cells and primarily isolated human HSCs (Fig. 1a). PCR products from LX-2 cells were cloned using the TA cloning procedure, and random clones were analyzed by sequencing. Six out of sixteen clones sequenced were identified as containing Fz gene sequences, such as Fz-2, Fz-7, and Fz-10, by BLAST searching (Fig. 1b). These findings indicate that human HSCs are capable of responding to a Wnt stimulus.

3.2. Establishment of a human HSC line over-expressing sFRP1

To inhibit Wnt signaling in HSCs, we established a human HSC line over-expressing sFRP1 by infecting LX-2 cells with an sFRP1/GFP expression vector and by the flow cytometric cloning of GFP-expressing cells. sFRP1 over-expression in these cells was confirmed by immunoblot analysis (Fig. 2a).

3.3. Functional analysis of Wnt activity in human HSCs

We next evaluated whether canonical Wnt signaling is functionally active in human HSCs, and whether this is inhibited in sFRP1 over-expressing cells. For this purpose, cells were stimulated using Wnt3A, which is capable of activating the canonical Wnt pathway. Canonical Wnt signaling activity was evaluated by TOPflash TCF-luciferase reporter gene assay. Following Wnt3A-conditioned media treatment, a twofold increase in TOPflash reporter gene activity was observed in control cells, whereas this was significantly suppressed in sFRP1 over-expressing cells (Fig. 2b). These findings indicate that the canonical Wnt signaling pathway is functionally active in human HSCs.

3.4. Wnt regulation of HSC activation

The expressions of collagen α1 and α-smooth muscle actin (fibrosis-related markers during HSC activation) were compared in control and sFRP1 over-expressing HSCs. Treatment of control cells with Wnt3A-conditioned media for 24 h increased collagen α1 mRNA levels, whereas this was not observed in sFRP1 over-expressing cells (Fig. 3a). In a similar way, α-smooth muscle actin expression was increased in control cells following Wnt3A treatment, whereas this was not evident in sFRP1 over-expressing cells (Fig. 3b). These findings indicate canonical Wnt signaling participates in HSC activation.

3.5. Wnt signaling in HSC apoptosis

We next evaluated if Wnt signaling modulates HSC survival by regulating cellular apoptotic processes. When cells were