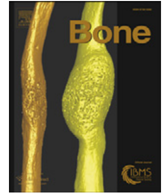
Bone 44 (2009) 1069–1077

Contents lists available at ScienceDirect

Bone

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



Wnt inhibitory factor (WIF)-1 inhibits osteoblastic differentiation in mouse embryonic mesenchymal cells

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ARTICLE INFO

Article history:
Received 31 July 2008
Revised 31 January 2009
Accepted 11 February 2009
Available online 27 February 2009

Edited by: R. Baron

Keywords:
Wnt inhibitory factor-1
Wnt
Osteoblastic differentiation
Mesenchymal cell
Adipogenesis

ABSTRACT

Wnt inhibitory factor (WIF)-1 belongs to the members of secreted modulators of Wnt proteins. Secreted frizzled-related proteins (sFRPs), another member of Wnt modulators, have been shown to play differential roles in Wnt signaling depending on the subtypes and cell models. This study was undertaken to investigate the functional role of WIF-1 in osteoblastic differentiation of mouse mesenchymal C3H10T1/2 cells. C3H10T1/2 cells express endogenous WIF-1 and its expression level decreases during osteoblastogenesis. Treatment of C3H10T1/2 cells with WIF-1 significantly reduced alkaline phosphatase (ALP) activities induced by either osteogenic medium (OM, ascorbic acid and β-glycerophosphate) or Wnt-3a conditioned medium (CM) in a dose-dependent manner. In contrast, the expression level of endogenous WIF-1 increased during adipogenesis and WIF-1 treatment resulted in increased adipogenesis. C3H10T1/2 cells transduced with WIF-1 retrovirus also exhibited reduced ALP activity and decreased mRNA expression of Runx2, collagen type 1, ALP and osteocalcin during osteoblastic differentiation compared to empty virus-transduced cells. Moreover, treatment with WIF-1 dose-dependently attenuates β-catenin/T-cell factor (TCF) transcriptional activity in this cell line. Finally, knockdown of WIF-1 in C3H10T1/2 cells by RNA interference leads to increase in ALP activities. Collectively, these results indicate that WIF-1 plays as a negative regulator of osteoblastic differentiation in mouse mesenchymal C3H10T1/2 cells in vitro.

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Introduction

Wnt proteins constitute a large family of 19 secreted glycoproteins that regulate many aspects of cell physiology, including proliferation, migration and differentiation in embryogenesis and adult tissue homeostasis (for review see Ref. [1]). The best characterized signaling of Wnts is canonical Wnt/β-catenin pathway which is initiated when Wnts bind to frizzed receptor and lipoprotein receptor-related protein 5/6 (LRP5/6), a coreceptor of frizzled [2]. Upon binding of Wnt ligand to its receptors, activated intracellular Dishevelled inhibits the kinase activity of a multi-protein complex containing glycogen synthetase kinase-3 (GSK3), Axin and β-catenin. In the presence of Wnt protein, stabilized β-catenin is translocated into the nucleus and binds to TCF family of transcription factors with subsequent regulation of Wnt target genes [1].

It has been well established that a repertoire of Wnts and its membrane receptor complex are expressed in bone and have crucial roles in regulation of bone accrual and bone remodeling (for review see ref. [3]). A gain-of-function mutation at the beta-propeller module of LRP5 (G171V) results in high bone mass phenotype [4], whereas non-sense or frame shift mutation of LRP5 leads to autosomal recessive osteoporosis–pseudoglioma syndrome with increased risk of fracture [5]. Same pattern of high or low bone mass phenotype has also been reproduced in mice model, using transgenic mice expressing LRP5 (G171V) [6] or knock-out model that lacks LRP5 gene [7], respectively.

Regulation of Wnt signaling pathway may occur at different levels, including extracellular components of the signaling cascade [8–10]. Secreted frizzled-related proteins (sFRP) are most thoroughly studied regulators of Wnt signaling, working at the extracellular environment. The binding of Wnt to frizzled receptor is prevented by competitive binding of sFRPs to Wnt ligand, thereby preventing signaling through frizzled receptors. In contrast, Dkk family proteins bind to LRP5 coreceptor, which triggers rapid internalization and depletion of cell-surface LRP5, leading to inhibition of the canonical Wnt signaling [11].

Wnt inhibitory factor-1 (WIF-1) is an evolutionary conserved protein of 379 amino acid residues and constitutes another member of the secretary Wnt modulators that directly bind to Wnt proteins similar to sFRPs [12]. However, unlike sFRPs, it dose not share sequence similarities with cysteine-rich domain of the frizzled

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doi:10.1016/j.bone.2009.02.012

receptors. WIF-1 has five epidermal growth factor-like repeats and one WIF domain, which mediates Wnt binding of WIF-1 [12]. Overexpression of hWIF-1 in *Xenopus* embryos inhibits the activity of XWnt8 and Xwnt3a in a dose-dependent manner [12] and results in phenotype closely resembling that of Frzb (sFRP-3) overexpression [13]. Although only limited data are available on the role of WIF-1 in osteoblasts, a recent microarray study showed that treatment with BMP-2 in C2C12 cells resulted in increase in the expression level of WIF-1 more than 1,000 times [14]. Same results were confirmed in MC3T3-E1 cells [14] as well as KS483 cells [15], suggesting that WIF-1 may be regulated in mesenchymal cells, including osteoblasts. This study was carried out to determine the role of WIF-1 on osteoblastic differentiation in mouse mesenchymal progenitor cells.

Materials and methods

Materials

Recombinant human WIF-1, human Dkk-1 and anti-mouse WIF-1 antibody were purchased from R and D system (Minneapolis, MN). Monoclonal anti-HA antibody (MMS 101-P) was obtained from Covance Inc. (Princeton, NJ). TOPflash plasmid which contains 8 copies of the optimal Tcf motif (CCTTTGATC) upstream of a minimal thimidine kinase promoter driving luciferase expression and FOPflash plasmid that contains a mutated motif (CCTTTGGCC) of the Tcf-Lef-sensitive elements were obtained from Dr. Roberto Civitelli (Washington University, St. Louis). Western blotting detection reagents was obtained from Amersham Int. (Buckinghamshire, UK). Random priming kits and reagents for the luciferase assay were purchased from Promega Corp. (Madison, WI) and Lipofectamine Plus from Invitrogen Corp. (Carlsbad, CA). Oligonucleotides were synthesized by Bioneer Corp. (Chungwon, Korea), and unless otherwise indicated, all other chemicals, including tissue culture medium, were from Sigma-Aldrich Chemical company (St. Louis, MO).

Cell culture and induction of osteoblastogenic and adipogenic differentiation

Murine mesenchymal cells C3H10T1/2 (American Type Culture Collection, Manassas, VA) are pluripotent cells and were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). The mouse MC3T3-E1 osteoblastic cells (RIKEN cell bank, Tsukuba, Japan) were derived from spontaneously immortalized calvaria cells and maintained in DMEM medium with 10% FBS. Mouse myoblast cell line, C2C12, is a more committed cell that differentiates rapidly into muscle cells. The 3T3-L1 preadipocytic cell line was a kind gift from Dr. Jae Bum Kim (Seoul National University, Seoul, Korea) and maintained in an immature state by culturing in DMEM supplemented with 20% FBS and 2.0 mM glutamine. Rat and human osteosarcoma cell line, ROS 17/2.8 and MG 63, respectively, were cultured in DMEM/F12 with 10% FBS. Normal mouse osteoblasts were isolated from 21-day-old fetal mouse calvariae using a well characterized technique essentially as described previously [16].

For osteoblastic differentiation studies, C3H10T1/2 cells were cultured in DMEM with 10% FBS supplemented with osteogenic medium (OM, 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate) or 0.5× Wnt-3a conditioned medium (CM). In some experiment, the cells were treated with 150 ng/ml of BMP-2 for osteogenic differentiation. Adipogenic differentiation was initiated 24–48 h after confluence by treatment with 5 μg/ml insulin, 0.1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 7–9 days.

Preparation of Wnt-3a conditioned media (CM)

L Wnt-3a cells [17], that stably secrete biologically active mouse Wnt-3a were grown in DMEM supplemented with 10% FBS, 4 mM L-glutamine supplemented with 0.4 mg/ml G418. Upon reaching 80% confluence, cells were washed once with PBS and transferred into DMEM containing 2% FBS, 1× penicillin/streptomycin and 4 mM L-glutamine. Conditioned media (CM) was collected one day past confluence. We refer to the concentration of this media to be 1×. The concentration of active Wnt-3a in the 1× Wnt-3a CM is estimated to be 5 nM [18].

Retroviral vector construction

The mouse WIF-1 cDNAs were obtained from Dr. Jen-Chih Hsieh (State University of New York, Stony Brook, NY), and mouse Dkk-1 cDNAs was a kind gift from Dr. Christof Niehrs (Deutsches Krebsforschungszentrum, Heidelberg, Germany). A hemagglutinin (HA) epitope was incorporated at the C-terminal of WIF-1 and Dkk-1 using PCR, then ligated into the BglII/EcoRI site of the pMSCV-GFP plasmid (a kind gift from Dr. Neil A. Clipstone at the Northwestern University, Chicago, IL), upstream of internal ribosomal entry site (IRES), giving pMSCV-WIF-1-HA-GFP and pMSCV-Dkk-1-HA-GFP, respectively. The nucleotide sequences of the amplified WIF-1 or Dkk-1 coding region were confirmed by sequencing.

Since the same promoter was used for the transcription of both genes, higher expressing level of GFP in cells could be proportionate to the expressing level of WIF-1 thereby these expressions of GFP allowed rapid sorting of successfully transduced cells using FACS (FACSVantage SE, Beckton-Dickinson).

Retrovirus production and transduction of cell lines

To generate retroviral particle, 293T cells were transfected with DNA (4 μg pMD-gag-pol, 4 μg pMD-VSVG, and 4 μg retroviral vector pMSCV-WIF-1-HA-GFP, pMSCV-Dkk-1-HA-GFP, or pMSCV-GFP alone), Lipofectamine PLUS, and serum-free DMEM. Viral supernatant fractions were collected at 48 h and transduced into C3H10T1/2 cells as described previously [19].

Alkaline phosphatase (ALP) activity assay

To assess ALP activities, cells were washed three times with ice-cold Tris-buffered saline (TBS), pH 7.4 and scraped immediately after adding 0.5 ml of ice-cold 50 mM TBS; the collected lysates were then sonicated for 20 s at 4 °C. Enzyme activity assay was performed in assay buffer (10 mM $MgCl_2$ and 0.1 M alkaline buffer, pH 10.3) containing 10 mM p-nitrophenylphosphate as substrate. The reaction was stopped by adding 0.3 N NaOH and absorbance was read at OD405. Relative ALP activity is defined as mmol of p-nitrophenol phosphate hydrolyzed per min per mg of total protein.

ALP stain

ALP staining was carried out using an alkaline phosphatase kit according to the manufacturer's instructions (Promega, Southampton, U.K.). Briefly, after a 5 min fixation with 10% buffered neutral formalin the osteogenic culture was incubated in a mixture of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate within 1 h. The resulting blue, insoluble, granular dye deposit indicated sites of alkaline phosphatase activity.

Oil Red O stain

C3H10T1/2 cells were fixed with 10% buffered neutral formalin for 5 min room temperature and rinsed in distilled water. Working stock solution of Oil Red O was prepared from 1% (wt/vol) Oil Red O in 99% isopropanol and diluted to 0.3% (vol/vol) with distilled water. Cultures were stained with Oil Red O working solution for 60 min with gentle rocking and rinsed with distilled water. For quantitative

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