Differential effects of secreted frizzled-related proteins (sFRPs) on osteoblastic differentiation of mouse mesenchymal cells and apoptosis of osteoblasts

Sun Wook Cho, Sun Ju Her, Hyun Jin Sun, Ok Kyong Choi, Jae-Yeon Yang, Sang Wan Kim, Seong Yeon Kim, Chan Soo Shin *

Department of Internal Medicine, Seoul National University College of Medicine, 28 Yungun-Dong, Chongno-Gu, Seoul 110-744, Republic of Korea

Received 19 December 2007
Available online 31 December 2007

Abstract

Secreted frizzled-related proteins (sFRPs) are modulators of Wnt signaling. This study was undertaken for definitive assessment of contribution of different sFRPs in osteoblastic differentiation of mesenchymal progenitor cells and apoptosis of osteoblasts. Treatment of C3H10T1/2 cells with sFRP-2 at concentrations of 10, 50, and 100 nM and sFRP-4 at low concentrations (5 nM) significantly increased Wnt-3A-induced alkaline phosphatase (ALP) activities, whereas sFRP-1 or 3 did not. Retroviral transduction of the sFRP-2 but not other sFRPs also significantly enhanced ALP activity induced by β-glycerophosphate and ascorbic acid. Furthermore, transfection of all the sFRP expression vectors significantly increased β-catenin/TCF reporter activity and the effects were most prominent with sFRP-2 and -4. In osteoblast apoptosis assay, only sFRP-3 increased etoposide-induced apoptosis in MC3T3-E1 mouse osteoblasts. In conclusion, we found that different repertoires of sFRPs exert differential effects on osteoblastic differentiation of mouse mesenchymal cells and cellular apoptosis of mouse osteoblasts in vitro.

© 2007 Elsevier Inc. All rights reserved.

Keywords: Secreted frizzled-related proteins; Wnt; Osteoblastic differentiation; Apoptosis

Wnts are a family of secreted glycoproteins that regulate various normal and pathological developmental process of many organs including bone (for review see Ref. [1]). In the canonical Wnt/β-catenin pathway, binding of Wnt proteins to the frizzled receptors activates the intracellular Dsh-elled, which inhibits the kinase activity of a complex containing glycogen synthetase kinase-3, Axin, β-catenin, and other proteins [2]. Since these complex targets β-catenin for rapid degradation through phosphorylation, Wnt signaling allows the stabilization, and nuclear translocation of β-catenin. β-Catenin then binds to TCF/LEF family of transcription factors to regulate the expression of Wnt target genes [1].

Growing number of evidences suggest that Wnt signaling plays a critical role in the regulation of bone accrual during growth and the establishment of peak bone mass as well as bone remodeling. A point mutation (G171V) at the β-propeller module of LRP5, a Wnt co-receptor, results in high bone mass phenotype, whereas targeted disruption of LRP5 in mice or inactivating mutation in human leads to decreased bone formation [3]. Wnt5a is required for longitudinal skeletal outgrowth and both Wnt5a and Wnt5b regulate the transition between different chondrocyte zones [4].

Activity of Wnt signaling pathway is regulated by secreted inhibitors, including secreted frizzled-related protein (sFRP) [5], Wnt inhibitory factor-1 (WIF-1) [6], and Dickkopf (Dkk) [7]. The sFRP family has an amino-terminal cysteine-rich domain (CRD) that has high homology with the ligand-binding domain of frizzled Wnt receptor
Materials. Recombinant human sFRP-1, 3, 4, Dkk-1, and mouse sFRP-2 were purchased from R&D system (Minneapolis, MN). Monoclonal anti-HA antibody (MMS 101-P) was obtained from Covance Inc. (Princeton, NJ). TOPlash plasmid which contains eight copies of the optimal Tef motif CTTTTGGATC upstream of a minimal thymidine kinase promoter driving luciferase expression and FOPflash plasmid that contains a mutated motif CTTTTGCGCC of the Tcf-Lef-sensitive elements was obtained from Dr. Roberto Civitelli (Washington University, St. Louis). Western blotting detection reagents and [α-32P]dCTP 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. 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% FBS. During osteoblast differentiation studies, C3H10T1/2 cells were cultured in DMEM with 10% FBS supplemented with either Wnt-3A conditioned medium or 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate. The mouse MC3T3-E1 osteoblastic cells (RIKEN cell bank, Tsukuba, Japan) were derived from spontaneously immortalized calvarial cells [12] and maintained in DMEM medium with 10% FBS. The 3T3-L1 pre-adipocytic 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. Normal mouse osteoblasts were isolated from 21-day-old fetal mouse calvariae using a well-characterized technique essentially as described previously [13].

Preparation of Wnt-3A conditioned media (CM). 1 L Wnt-3A [14] cells, 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 G-418. 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 1-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 [15].

Retroviral vector construction. The mouse sFRP-1, 2, 3, and 4 cDNAs were obtained from Dr. Jeremy Nathans (Johns Hopkins University, Baltimore, MD). The 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 sFRPs using PCR, then ligated into the BglII/EcoRI site of the pMSCV-GFP plasmid (a kind gift from Dr. Neil A. Clipastone at the Northwestern University, Chicago, IL), upstream of internal ribosomal entry site (IRES), giving pMSCV-sFRP-1–4-HA-GFP, and pMSCV-Dkk-1-HA-GFP. The nucleotide sequences of the amplified sFRP coding region were confirmed by sequencing.

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-sFRP-1–4-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 [16]. Transduced cells expressing higher level of GFP were sorted using FACS (FACSVantage SE, Beckton-Dickinson) and used for studies.

Alkaline phosphatase (ALP) activity. 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 MgCl2 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 absorbance was read at OD405. Relative ALP activity is defined as nmol of p-nitrophenol phosphate hydrolyzed per min per mg of total protein.

Quantification of apoptosis. MC3T3-E1 cells were cultured in serum-starved condition for 24 h, and sequentially treated with 20 nM of sFRP-1 or Dkk-1 in the presence or absence of Wnt-3A CM. Forty-eight hours later, cell lysates were prepared using the Promega Luciferase assay kit, and enzyme activity was measured using a luminometer (Lumat LB9907, Berthold, Germany). The proliferation rates were normalized to the control and calculated using the Student–Newman–Keuls multiple comparison test.
Results

Expression of sFRPs in murine cells of mesenchymal origin

We first investigated the endogenous expression of sFRPs in a number of murine cells of mesenchymal origin by RT-PCR analysis: pluripotent mesenchymal C3H10T1/2, osteoblastic MC3T3-E1, myoblastic C2C12, pre-adipocytic 3T3-L1, and primary cultures of mouse calvaria. Expected cDNA fragments corresponding to mouse sFRP-1, 2, 3, 4 were clearly amplified from RNA of these cells (Fig. 1A). The expression level of sFRP-1 was the highest in MC3T3-E1 cells whereas both sFRP-2 and -4 were most highly expressed in C2C12 cell compared to other cell types. The expression of sFRP-3 was comparable in C2C12, MC3T3-E1, and C3H10T1/2 cells.

Effects of sFRP treatment on osteoblastic differentiation of C3H10T1/2 cells

When C3H10T1/2 cells were treated with 0.5× Wnt-3A conditioned media (CM) for 72 h, the ALP activity was increased by 10- to 25-folds (Fig. 1B, Wnt-3A) as expected. Co-treatment with 5–100 nM of sFRP-1, 2, 3, or 4 in this setting, however, did not significantly inhibit the ALP activity (Fig. 1B). Actually, sFRP-2 at concentrations of 10, 50, and 100 nM (Fig. 1B-b) and sFRP-4 at low concentrations (5 nM, Fig. 1B-d) significantly increased ALP activities. The effects of sFRPs on ALP activity did not seem to have any dose-responsive pattern. In contrast, in a parallel experiment, the treatment with Dkk-1, a robust inhibitor of Wnt signaling, has consistently inhibited the ALP activity in a dose-responsive manner (Fig. 1B-e).

Fig. 1. Expression of sFRPs in mesenchymal cells and effect of sFRPs on the ALP activities induced by Wnt-3A in C3H10T1/2 cells. (A) RT-PCR analysis for sFRP-1, 2, 3, 4, and β-actin were performed from reverse-transcribed total RNA prepared from primary culture of calvarial osteoblasts, pre-adipocytic 3T3-L1 cells, myoblastic C2C12 cells, osteoblastic MC3T3-E1 cells, and mouse mesenchymal C3H10T1/2 cells. (B) C3H10T1/2 cells were seeded in 24-well plate and grown for 72 h in DMEM containing 10% FBS and treated with vehicle or varying concentrations of sFRP-1–4 or Dkk-1 in the presence or absence of Wnt-3A. ALP activity was determined and normalized to protein content. The results are representative of three independent experiments performed in triplicate. Each bar represents means ± SD. *p < 0.05 vs. vehicle.
Effects of retrovirally transduced sFRPs on osteoblastic differentiation of C3H10T1/2 Cells

To further verify the role of sFRPs in the osteoblastic differentiation of mesenchymal cells, we studied the effects of the sFRPs treatment during cultures in osteogenic medium, i.e., β-glycerophosphate and ascorbic acid, a more physiologic stimulator of osteoblastic differentiation. We used stable cell lines that endogenously overexpressed sFRPs in C3H10T1/2 cells using retrovirus. In this model, the overexpressed sFRPs are expected to work by autocrine and/or paracrine mechanism. The expression of sFRPs were examined by Western blot analysis using an antibody directed against the HA epitope (Fig. 2A).

As expected, culture of C3H10T1/2 cells transduced with empty virus (MSCV) in osteogenic medium for 14 days exhibited typical induction of ALP activity (Fig. 2B). Overexpression of sFRP-2 in this condition resulted in significant increase in the ALP activity on day 14 of culture, whereas other sFRPs did not. These results are analogous with the data we obtained from the exogenous treatment of the cells with sFRPs in the presence of Wnt-3A CM and suggest that the effects of sFRPs are not necessarily negative in the regulation of osteoblastic differentiation of mesenchymal cell line.

Effect of sFRPs on Wnt-β-catenin transcriptional activity

We next examined the effects of sFRPs on the β-catenin/Tcf transcriptional activity, a downstream target of the Wnt signaling. Transient transfection of TOPflash in C3H10T1/2 cells showed only a basal level of transactivation, and this activity was increased by 8-fold with the treatment with Wnt-3A CM (Fig. 3). Co-transfection with sFRP-1, -2, -3, or sFRP-4 expression plasmid in this setting resulted in significant increase in the transcriptional activity. Among the sFRPs, sFRP-2 and -4 exhibited most strong responses. As a control, Dkk-1 significantly inhibited the reporter activity as expected. Same experiments performed using the FOPflash reporter did not reveal any changes regardless of treatment with Wnt-3A.

Effect of sFRPs on apoptosis of osteoblastic MC3T3-E1 cells

Since none of the sFRPs has negatively regulated osteoblastic differentiation of mesenchymal C3H10T1/2 cells, we next evaluated whether sFRPs increases apoptosis of osteoblasts thereby contributing to net loss of bone as has been reported in sFRP-1 transgenic model [17]. Towards this end, we treated topoisomerase inhibitor etoposide to MC3T3-E1 cells after 1 h pre-conditioning with sFRPs or
Dkk-1. Quantification of apoptotic cells with DAPI staining showed that only sFRP-3 significantly increased etoposide-induced apoptosis in mouse osteoblastic cells (Fig. 4), whereas other subtypes did not affect cell death.

Discussion

In this study we have shown that the major secretory Wnt modulators, sFRPs, including sFRP-1, 2, 3, and 4, did not inhibit the Wnt-3A-mediated increase in ALP activities in C3H10T1/2 mesenchymal cells in vitro. On the contrary, both sFRP-2 and sFRP-4 have actually increased ALP activities.

We have found that all the mesenchymal cell lines express varying degree of sFRP expression. These results initially suggested that these molecules may serve physiologic functions in these cells as an auto- or paracrine modulators of Wnt signaling. We hypothesized that sFRPs, by binding directly to the Wnts proteins and disrupting of Wnt/β-catenin signaling, would inhibit the osteoblastic differentiation. However, in our study we were unable to identify the negative roles of sFRPs in the ALP activity induced by Wnt-3A in C3H10T1/2 cell line. Indeed, both sFRP-2 and -4 have significantly enhanced ALP activity at low concentrations, and at higher concentrations (>=50 nM), sFRP-2 has consistently increased ALP activities. This lack of inhibitory effects by all the sFRPs was also reproduced when we transduced the cells with retrovirus rendering the cells to produce sFRPs endogenously. This approach was employed because sFRPs tend to remain predominantly associated with the cell membrane or extracellular matrix [18]. In addition, transduction of sFRP-2 retrovirus has also resulted in increase in ALP activities compared to control virus. Therefore, the results from the retroviral transduction study further confirm that even endogenously secreted sFRPs that should have autocrine or paracrine effects, do not inhibit the osteoblastic differentiation capabilities in these C3H10T1/2 cells.

The lack of inhibition and even enhancement of ALP activity by sFRPs (sFRP-2 and -4) in our experiments is an unexpected result. The reason for this discrepancy with previous reports is currently unknown. One explanation might be that the sFRPs may have differential effects depending on the concentration. Indeed, Uren and his co-worker showed that sFRP-1 reduces Wingless (Wg, Drosophila homolog of Wnt) activity only at high concentrations around 300–600 nM [19] whereas at low concentrations it potentiates Wg activity in Drosophila S2 cells [19]. Moreover, Chung et al. also showed that sFRP-3 at a concentration of 10 nM increased Wnt-3A-mediated increase in ALP activity [11]. Alternatively, the sFRPs
may have stage-specific effects on the osteoblast differentiation with little or no effects on undifferentiated mesenchymal stage but significant effects on pre-osteoblasts or mature osteoblasts in vitro [17]. Lastly, there may be some specificity in interactions between particular sFRPs and Wnt molecules so that the sFRPs are not capable to specifically inhibit the action of Wnt-3A. In support of this notion, Wang et al. has demonstrated that sFRP-3 (Frzb-1) is not able to inhibit the action of Wnt-3A or -5A, whereas it inhibits Wnt-1 or -8 [20], suggesting that sFRPs modulates Wnt activity depending on the subtypes of both Wnt and sFRPs.

We have also shown that all the sFRPs increased TOP-flash activity induced by Wnt-3A. Moreover, the prominent effects by sFRP-2 and -4 suggest that the increase in ALP activities by both of sFRPs may result from the increased β-catenin/Tcf transcriptional activities. However, although ALP is one of the downstream targets of Wnt signaling [21], we have not further dissected molecular mechanisms whereby sFRPs exerted ALP activities and osteoblastic differentiation. Additional studies on the ligand and receptor-binding activities and the events downstream of frizzled receptors could help answer this question.

Since sFRPs did not inhibit osteoblastic differentiation of mesenchymal progenitors, we next reasoned that these secretory modulators may promote apoptosis of mature osteoblasts. This is based on the previous in vivo demonstration of increased apoptosis of osteoblasts in sFRP-1 overexpressing mice [17]. However, unlike the animal model, only sFRP-3 promoted apoptosis induced by etoposide in our experiment. Although differences in the experimental model preclude direct comparison, these results also indicate that there are a lot of disparities in the action of sFRPs depending on the study model and subtypes.

In conclusion, we have shown that different repertoires of sFRPs have differential effects on osteoblastic differentiation and TCF/LEF transcriptional activities in murine mesenchymal cells and also on the apoptosis of osteoblasts. We speculate that the contradictory effects of sFRPs result from difference in interactions between different sFRPs and Wnts, diverse repertoires of Wnt molecules, and tissue-specific or stage-specific responses, as well as biphasic response depending on the concentrations of sFRPs.

Acknowledgement

This work was supported by a grant from Ministry of Health and Welfare of Korea (Grant No. A060371).

Appendix A. Supplementary data


References


S.W. Cho et al. / Biochemical and Biophysical Research Communications 367 (2008) 399–405