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Glucocorticoid suppresses the canonical Wnt signal in cultured human osteoblasts

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Abstract

To explore the mechanism of glucocorticoid-induced osteoporosis, we investigated the effect of glucocorticoid on canonical Wnt signaling that emerged as a novel key pathway for promoting bone formation. Wnt3a increased the T-cell factor (Tcf)/lymphoid enhancer factor (Lef)-dependent transcriptional activity in primary cultured human osteoblasts. Dexamethasone suppressed this transcriptional activity in a dose-dependent manner, while 1,25-dihydroxyvitamin D3 increased this transcriptional activity. LiCl, an inhibitor of glycogen synthase kinase-3β, also enhanced the Tcf/Lef-dependent transcriptional activity, which was, however, not inhibited by dexamethasone. The addition of anti-dickkopf-1 antibody partially restored the transcriptional activity suppressed by dexamethasone. Dexamethasone decreased the cytosolic amount of β-catenin accumulated by Wnt3a and also inhibited the nuclear translocation of β-catenin induced by Wnt3a. These data suggest that glucocorticoid suppresses the canonical Wnt signal in cultured human osteoblasts, partially through the enhancement of the dickkopf-1 production.

Keywords: Glucocorticoid; Wnt; Dickkopf-1; Osteoblast; Osteoporosis

Osteoporosis is one of the most frequent and serious side effects of long-term glucocorticoid therapy [1]. Glucocorticoids have profound effects on bone metabolism [2]. Glucocorticoid excess increases bone resorption and decreases bone formation; consequently rapid bone loss occurs. Nowadays, a direct inhibition of osteoblast activity by glucocorticoids is the most favored principal mechanism of glucocorticoid-induced osteoporosis [1–3]. However, detailed mechanism by which they inhibit osteoblast function remains to be fully elucidated.

Recent progress uncovers the importance of Wnt signaling in skeletal biology [4,5]. The loss-of-function mutations in human LDL receptor-related protein 5 (LRP5) gene cause osteoporosis-pseudoglioma syndrome (OPPG) characterized by low bone mass and abnormal eye development, while the gain-of-function mutations in this gene give rise to high bone mass syndrome [6–8]. Furthermore, the LRP5 gene knockout mice show the phenotype of low bone mass resembling that of human OPPG [9]. These findings highlighted Wnt signaling as another key pathway involved in the regulation of postnatal bone mass.

The Wnt signal transduction comprises three intracellular pathways: the canonical pathway, the Wnt/planar-cell-polarity (PCP) pathway, and the Wnt/Ca^{2+} pathway [10,11]. Although which pathway is involved in bone formation remains to be fully elucidated, recent studies strongly suggest that the canonical pathway plays a central role in promoting bone formation [4,5,12]. Canonical Wnts bind to frizzled/LRP5 receptor complex, inactivate glycogen synthase kinase-3β

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(GSK-3β), and inhibit phosphorylation and consequential degradation of intracellular β-catenin [10,11]. Accumulated β-catenin translocates into the nucleus and activates target genes by a complex formed with transcription factors of the T-cell factor (Tcf)/lymphoid enhancer factor (Lef) family [10,11].

Wnt signals are extracellularly regulated by several secreted antagonists including secreted frizzled-related protein (sFRP), Cerberus, Wnt inhibitory factor-1 (WIF-1), and dickkopf (Dkk) [13]. We have reported that glucocorticoid enhances the expression of dickkopf-1 (Dkk-1) in cultured human osteoblasts [14]. We extended our exploration of the effect of glucocorticoid on Wnt signaling, and found that glucocorticoid suppresses the canonical Wnt signal, in part mediated by the enhancement of the Dkk-1 production, in cultured human osteoblasts.

Materials and methods

Materials. Eagle's α-MEM, penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal calf serum (FCS) was purchased from Sanko Junyaku (Tokyo, Japan). Dexamethasone, 17β-estradiol, dihydrotestosterone, 1,25-dihydroxyvitamin D3, LiCl, and goat immunoglobulin (IgG) were purchased from Sigma (St. Louis, MI). Anti-β-catenin monoclonal antibody and anti-Dkk-1 goat polyclonal antibody were purchased from Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. All other reagents were of analytical grade.

Cell culture. Human osteoblasts were prepared from the bone fragments of femur neck as described previously [15]. The cells were grown in Eagle's α-MEM with 10% FCS, 100 mU/ml penicillin, and 100 mU/ml streptomycin. The Wnt3a-expressing cell line (L Wnt-3A cell) and the control cell line (L cell) were obtained from American Type Culture Collection (Manassas, VA). Wnt3a-conditioned medium (Wnt3a-CM) and the control-conditioned medium (C-CM) were harvested according to the manufacturer's instructions. Wnt3a-CM was used in experiments at 10% final concentration, which gave the maximal effect on the Tcf/Lef-dependent transcriptional activity in preliminary studies (data not shown).

Plasmid constructs. The entire coding region of human β-catenin was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using KOD-plus DNA polymerase (Toyobo, Tokyo, Japan), confirmed by DNA sequencing, and subcloned into *Sca*I/*Bam*HI sites of pEGFP-C3 (Clontech, Palo Alto, CA) expression vector (designated as pEGFP-β-catenin). TOPflash, a Tcf-binding site reporter plasmid, was purchased from Upstate Biotechnology (Lake Placid, NY).

Transient transfection and reporter assay. Human osteoblasts were transiently transfected by means of calcium phosphate precipitation as described previously [14]. Reporter assay was performed by a dual luciferase assay kit (Promega, MI) according to the manufacturer's instructions.

Subcellular fractionation and immunoblot analysis. Subcellular fractionation and immunoblot analysis were performed essentially as described previously [15]. Soluble (cytosolic) proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and proteins in the gel were transferred to a Hybond ECL nitrocellulose membrane (Amersham Biosciences Corp., Piscataway, NJ) through electroblotting. For detection of β-catenin, blots were probed with an anti-β-catenin monoclonal antibody at a dilution of 1:1000. The protein concentration was determined by a BCA protein assay kit (Pierce, Rockford, IL).

Confocal laser microscopic imaging. Human osteoblasts were cultured in 35-mm glass-bottomed dishes (Asahi Techno Glass, Tokyo, Japan) and transfected with pEGFP-β-catenin plasmid vector. The cells were maintained in α-MEM supplemented with 10% charcoal-treated FCS for 24 h and observed with a confocal laser scanning microscope (LSM 510 META, Carl Zeiss, Jena, Germany) as described previously [16].

Statistical analysis. Data are expressed as means ± SD. Statistical analyses were performed with ANOVA followed by Fisher's protected least significant difference test. Significance was accepted at $P < 0.05$.

Results

To investigate the effect of glucocorticoid on canonical Wnt signaling, we first examined whether glucocorticoid would affect the Tcf/Lef-dependent transcriptional activity by a Tcf-reporter gene (luciferase) assay in cultured osteoblasts (Fig. 1A). In primary cultured human osteoblasts, the addition of Wnt3a-conditioned medium (Wnt3a-CM) enhanced the Tcf/Lef-dependent transcriptional activity (approximately 3.5-fold). Dexamethasone suppressed the Wnt3a-induced Tcf/Lef-dependent transcriptional activity in a dose-dependent manner, and dexamethasone at 10^{-7} M suppressed the Wnt3a-stimulated transcriptional activity to the unstimulated basal level.

We also examined the effect of other steroid hormones on the Tcf/Lef-dependent transcriptional activity (Fig. 1B). The addition of 17β-estradiol or dihydrotestosterone did not affect the transcriptional activity stimulated by Wnt3a. The addition of 1,25-dihydroxyvitamin D3 (10^{-7} M) enhanced the Wnt3a-induced reporter activity in cultured human osteoblasts. Furthermore, 1,25-dihydroxyvitamin D3 partially restored the suppressed Wnt3a-induced transcriptional activity by dexamethasone.

Wnt proteins enhance Tcf/Lef-dependent transcription by the canonical signal cascade, namely, the inhibition of GSK-3β, its consequential accumulation of cytosolic β-catenin, translocation of the accumulated β-catenin into the nucleus, and its activation of Tcf/Lef. Therefore, we examined whether or not dexamethasone would affect the intracellular β-catenin level in cultured human osteoblasts. As shown in Fig. 2, the addition of Wnt3a-CM increased the amount of cytosolic β-catenin protein in human osteoblasts. Co-treatment with dexamethasone (10^{-9}–10^{-7} M) dose-dependently decreased the cytosolic level of β-catenin and dexamethasone at 10^{-7} M almost completely reduced to the unstimulated basal level, which is parallel to the suppressive effect of dexamethasone on the reporter luciferase activity. When pEGFP-β-catenin was transfected into human osteoblasts, the GFP-β-catenin was localized most abundantly in the cytosol (Fig. 3). The addition of Wnt3a-CM translocated the GFP-β-catenin into the nucleus, however, co-treatment with

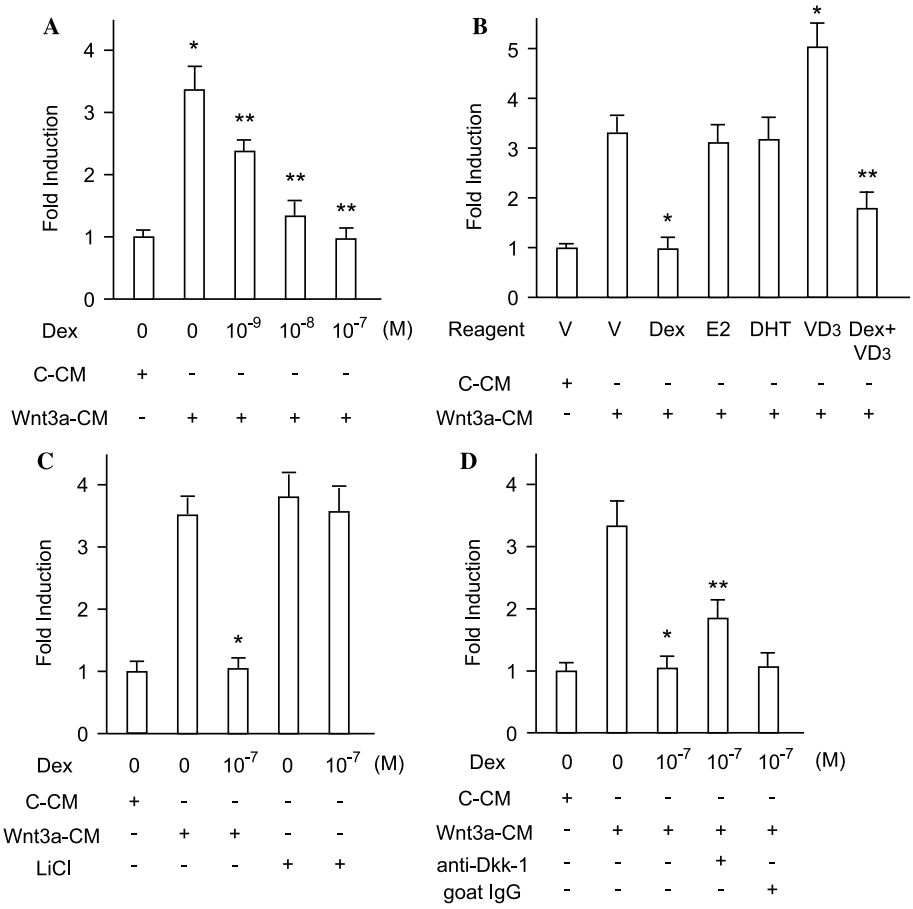

Fig. 1. Effects of Wnt3a, steroid hormones, LiCl, and anti-Dkk-1 antibody on the Tcf/Lef-dependent transcriptional activity in primary cultured human osteoblasts. Human osteoblasts were transfected with TOPflash plasmid vector, and incubated for 36 h with the control-conditioned medium (C-CM) or Wnt3a-conditioned medium (Wnt3a-CM) in the presence of vehicle (ethanol) or various reagents indicated. The reporter luciferase activity was expressed as fold over the activity of TOPflash with C-CM and vehicle. Data are expressed as means ± SD ($n = 4$). One representative data of three independent experiments are shown. (A) 10^{-9}–10^{-7} M dexamethasone (Dex). *$P < 0.01$ vs. C-CM with vehicle. **$P < 0.01$ vs. Wnt3a-CM with vehicle. (B) Vehicle (V, ethanol), 10^{-7} M dexamethasone (Dex), 10^{-7} M 17β-estradiol (E2), 10^{-7} M dihydrotestosterone (DHT), or 10^{-7} M 1,25-dihydroxyvitamin D3 (VD3). *$P < 0.01$ vs. Wnt3a-CM with vehicle. **$P < 0.05$ vs. Wnt3a-CM with Dex. (C) Twenty-five millimolar of LiCl in the presence of vehicle (ethanol) or 10^{-7} M dexamethasone (Dex). *$P < 0.01$ vs. Wnt3a-CM with vehicle. (D) 10^{-7} M dexamethasone (Dex) in combination with anti-Dkk-1 goat polyclonal antibody (anti-Dkk-1) or non-immune goat IgG (goat IgG). *$P < 0.01$ vs. Wnt3a-CM with vehicle. **$P < 0.05$ vs. Wnt3a-CM with Dex.

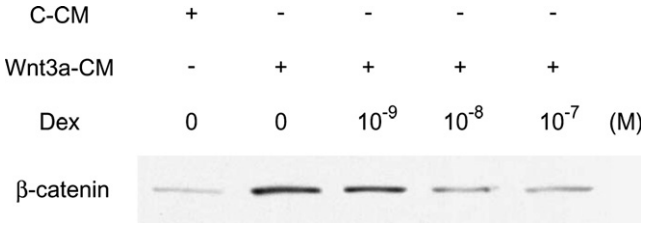

Fig. 2. Effect of dexamethasone on the level of the cytosolic β-catenin protein in primary cultured human osteoblasts. Human osteoblasts were incubated with the control-conditioned medium (control-CM) or Wnt3a-conditioned medium (Wnt3a-CM) in the presence of vehicle (ethanol) or 10^{-9}–10^{-7} M dexamethasone (Dex) for 24 h, and fractionated to soluble and particulate fractions. Soluble proteins (20 μg) were loaded in each lane and subjected to SDS–PAGE (7.5% separating gel). Immunoblot analyses were performed using a specific anti-β-catenin monoclonal antibody. Results shown are representative of three independent experiments.

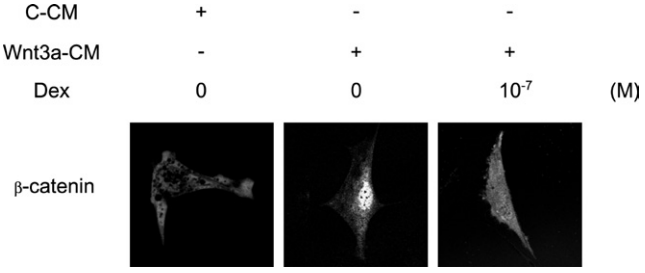

Fig. 3. Effect of dexamethasone on the localization of β-catenin in primary cultured human osteoblasts. Human osteoblasts were transfected with the pEGFP-β-catenin expression vector, and incubated for 24 h with the control-conditioned medium (control-CM) or Wnt3a-conditioned medium (Wnt3a-CM) in the presence of vehicle (ethanol) or 10^{-7} M dexamethasone (Dex). The cells were analyzed by laser confocal microscopy. A representative imaging of three independent experiments is shown (magnification, 100×).

dexamethasone at 10^{-7} M completely inhibited the nuclear translocation of cytosolic β-catenin (Fig. 3). These data suggest that dexamethasone suppressed the Tcf/Lef-dependent transcription through the canonical Wnt signaling cascade rather than by affecting via other signaling pathway(s) or inhibiting directly the Tcf/Lef expression in human osteoblasts.

To further assess the effect of dexamethasone on canonical Wnt signaling, we observed the effect of dexamethasone on the Tcf reporter activity in the presence of LiCl, an inhibitor of GSK-3β, in human osteoblasts (Fig. 1C). The addition of 25 mM LiCl increased the Tcf/Lef-dependent transcriptional activity comparable to that by Wnt3a-CM. However, this increase by LiCl was not suppressed by the treatment with dexamethasone at 10^{-7} M. Since glucocorticoid enhances the expression of Dkk-1, an antagonist of Wnt signaling in human osteoblast [14], we examined the involvement of Dkk-1 in suppressive effect of dexamethasone on canonical Wnt signaling. The addition of anti-Dkk-1-specific goat polyclonal antibody in part (35–45%) restored the suppression of Wnt3a-induced Tcf/Lef-dependent transcriptional activity by dexamethasone, while non-specific goat IgG had no effect on it (Fig. 1D).

Discussion

In the present study, we demonstrated that dexamethasone suppressed the Tcf/Lef-dependent canonical Wnt signaling pathway in primary cultured human osteoblasts. This effect was in part attributed to the increase of Dkk-1 expression by dexamethasone.

Glucocorticoid suppresses osteoblastic differentiation and proliferation by affecting multiple aspects of osteoblast function [1–3]. One well-known effect of glucocorticoid on osteoblast is the inhibition of the expression for Runx2/Cbfa1 [17], a crucial transcriptional factor for differentiation of osteoblast lineage [18]. Runx2/Cbfa1 promotes early osteoblast differentiation from undifferentiated mesenchymal cells, but rather inhibits late osteoblast maturation [18,19]. On the other hand, the Wnt signal plays an essential role in postnatal bone accrual in a Runx2/Cbfa1-independent manner [9]. Our data suggest that glucocorticoid at a therapeutic pharmacological dose may almost completely suppress the canonical Wnt signaling pathway promoting postnatal bone formation in human osteoblasts.

Dexamethasone did not affect the enhancement of Tcf/Lef-dependent transcriptional activity by LiCl, a GSK-3β inhibitor. Therefore, it is presumed that the dexamethasone affects canonical Wnt signaling through GSK-3β itself or upstream of GSK-3β. Indeed, it was reported that glucocorticoid activates GSK-3β and inhibits cell cycle progression in murine preosteoblastic MC3T3-E1 cells [20]. We have previously shown that glucocorticoid enhances the expression of Dkk-1, a secreted antagonist of Wnt signaling, in cultured human osteoblasts [14]. Treatment with anti-Dkk-1-specific antibody partially (approximately 40%) restored the suppression by dexamethasone of Wnt3a-induced Tcf/Lef-dependent transcriptional activity. Although there are several secreted antagonists of Wnt signaling [13] and we did not examine the involvement of other antagonists than Dkk-1 proteins, our data suggest that the inhibition of the canonical Wnt signal by glucocorticoid may be in part through the antagonistic effect of the enhanced Dkk-1 production in cultured human osteoblasts.

Interestingly, 1,25-dihydroxyvitamin D3 enhanced the Tcf/Lef-dependent transcriptional activity induced by Wnt3a in cultured human osteoblasts. Furthermore, 1,25-dihydroxyvitamin D3 restored the suppressed Wnt3a-induced transcriptional activity by dexamethasone. Previous study reported that the vitamin D receptor with its ligand inhibits β-catenin-Tcf/Lef-dependent gene transcription in colon carcinoma cells [21]. The vitamin D effect on Wnt signaling may be different by the types of cell and target genes, which will require further investigations. Active vitamin D metabolites are used for prevention and treatment of glucocorticoid-induced osteoporosis [22,23]. Besides the known effects of vitamin D3 on bone and mineral metabolism [23], the effect on Wnt signaling may contribute to the clinical effect of vitamin D3 for the treatment of osteoporosis.

LRP5 knockout mice show low bone mass due to decreased osteoblast proliferation [9], but the precise mechanism whereby the Wnt/LRP5 signal promotes bone formation remains to be fully clarified. However, given the significance of the canonical Wnt signaling pathway in postnatal control of bone formation, strong inhibition of this pathway by glucocorticoid may in part explain the impairment of osteoblastic function and bone formation induced by glucocorticoid excess. Our findings in this study and further investigations will provide clues to new strategies for the treatment of glucocorticoid-induced osteoporosis.

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