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Glucocorticoid enhances the expression of dickkopf-1 in human osteoblasts: novel mechanism of glucocorticoid-induced osteoporosis

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Abstract

To clarify the underlying mechanism of glucocorticoid-induced osteoporosis, we investigated the effect of glucocorticoid on the expression of dickkopf-1 (Dkk-1), an antagonist of Wnt signaling, in primary cultured human osteoblasts. Dexamethasone markedly induced the expression of mRNA for Dkk-1 in a dose- and time-dependent manner. The expression of Kremen1, a receptor for Dkk, did not change by the treatment with dexamethasone, while that of low-density lipoprotein receptor-related protein 5 (LRP5), a Wnt coreceptor, slightly decreased by the treatment with dexamethasone. Dexamethasone increased the transcriptional activity of the Dkk-1 gene promoter in human osteoblasts. Serial deletion and mutation analyses of the Dkk-1 promoter showed that one putative glucocorticoid responsive element-like sequence located from −788 to −774 bp is essential for the enhancement of the Dkk-1 promoter activity by dexamethasone in human osteoblasts. Since the Wnt signal is now recognized as a crucial regulator for bone formation, the Dkk-1 enhanced by glucocorticoid may inhibit the Wnt signal in osteoblasts, which may be involved in the pathogenesis of glucocorticoid-induced osteoporosis.

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

The Wnt family is a secreted glycoprotein that participates in morphogenesis, determination of cell polarity, and regulation of cell proliferation and differentiation during embryogenesis [1,2]. The Wnt proteins bind to frizzled family of seven transmembrane domain receptor and its coreceptor low-density lipoprotein receptor-related protein 5 (LRP5) and LRP6 [3]. Canonical Wnts inactivate glycogen synthase kinase-3β (GSK-3β) and inhibit phosphorylation and consequential degradation of intracellular β-catenin [1–3]. Accumulated β-catenin translocates into the nucleus and activates target genes by complex with transcription factors of the T-cell factor (Tcf)/lymphoid enhancer factor (Lef) family [1–3]. The Wnt signal is regulated by two classes of extracellular antagonists [4]. Secreted frizzled-related protein (sFRP), Cerberus, and Wnt inhibitory factor-1 (WIF-1) are inhibitors that bind Wnts and restrict the Wnt function. Dickkopf (Dkk) family is another class of secreted Wnt antagonist. Dkk interacts with the Wnt coreceptor LRP5 and LRP6, and inhibits Wnt signaling by disturbing the binding of LRP5/6 to the Wnt/frizzled ligand–receptor complex [5].

Recent analyses of patients with the LRP5 gene mutation and LRP5 knockout mice revealed that LRP5 plays pivotal roles in bone metabolism [6–8]. It is reported that the missense mutations of LRP5 gene cause osteoporosis-pseudoglioma syndrome in which bone and eyes are abnormally developed [6]. LRP5 knockout mice also showed similar phenotype in which low bone density, decreased osteoblast proliferation that is independent of *Runx2*/*Cbfa1*, and abnormal eye development were observed [7]. On the other hand, it is demonstrated that patients with other mutation of LRP5 gene have high bone mass [8]. In this mutation,

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the mutated LRP5 receptor has a low binding affinity to Dkk-1 and causes decrease in inhibitory function of Dkk-1 against Wnt signaling. Thus, the Wnt signal is now recognized as a novel regulator of bone formation and an important molecular target for the treatment of osteoporosis [9,10].

Glucocorticoid-induced osteoporosis is one of the serious problems during glucocorticoid therapy [11,12]. The major cause of glucocorticoid-induced osteoporosis is considered to be impairment of bone formation [11,12]. Glucocorticoid in an excess dose has inhibitory actions on osteoblastic replication, maturation, and differentiation. Glucocorticoid suppresses the expression of *Runx2*/*Cbfa1*, a critical factor for osteoblastogenesis, and reduces the synthesis of osteocalcin, type I collagen, and insulin-like growth factor-I (IGF-I) in osteoblasts [11,12]. It also promotes the apoptosis of osteoblasts and osteocytes [13]. However, detailed mechanism underlying glucocorticoid-induced osteoporosis remains to be fully elucidated.

In the present study, we hypothesized that glucocorticoid would affect the Wnt signal of bone formation in osteoblasts, and examined the effect of glucocorticoid on the expression of Wnt signal-related molecules in primary cultured human osteoblasts. We found that dexamethasone induces the expression of Dkk-1, an antagonist of Wnt, through the activation of trascription via glucocorticoid responsive element (GRE) of the Dkk-1 gene promoter.

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, and 1,25-dihydroxyvitamin D3 were purchased from Sigma (St. Louis, MI). All other reagents were of analytical grade.

Cell culture. Human osteoblasts were prepared from the bone fragments of femur neck as described previously [14]. The cells were grown in Eagle's α MEM with 10% FCS, 100 mU/ml penicillin, and 100 mU/ml streptomycin. Cells at ~80% confluence in 100-mm culture dishes (Falcon, Lincoln Park, NJ) were made quiescent through incubation with serum-free medium for 1 day before experiments.

Quantification of RNA. Total RNA was isolated from cultured cells using a RNeasy RNA Extraction kit (Qiagen, Germany). Reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blot analysis were performed as described previously [14,15]. The primer sets for DKK-1 (396 bp) were 5′-TGATGAGTACTGCGCTAGTC-3′ (sense) and 5′-CTCCTATGCTTGGTACACAC-3′ (antisense), the primer sets for LRP5 (377 bp) were 5′-CCGTCATTGGCATCA TCCTC-3′ (sense) and 5′-GTCCATGTTGTACAGGGAGG-3′ (antisense), the primer sets for Kremen1 (354 bp) were 5′-GTTTGCTGGG ATGGAGTCAG-3′ (sense) and 5′-GTGTAGCCATCCAGAAGC TC-3′ (antisense), and the primer sets for GAPDH (321 bp) were 5′-GGGCTCTCCAGAACATCATC-3′ (sense) and 5′-CAAAGTGGTC GTTGAGGGCA-3′ (antisense). The PCR products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI), sequenced, and then used as cDNA probes for Northern blot analysis.

Determination of the initiation site of transcription for the Dkk-1 gene was performed by a RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) kit (Ambion, Austin, TX) according to the manufacturer's instruction. The outer and inner antisense primers used for 5′ RLM-RACE for Dkk-1 were 5′-CTGCAGGCGAGACAGA TTTG-3′ and 5′-GGCTGGTAGTTGTCAATGGT-3′, respectively.

Construction of human Dkk-1 promoter/luciferase chimeric plasmids. Human gemonic DNA was purified from a Japanese man with a QIAamp DNA Blood Kit (Qiagen). The approximately 0.8 kb upstream region of the human Dkk-1 promoter was amplified by PCR using KOD-plus DNA polymerase (Toyobo, Tokyo, Japan), subcloned into the pCR-Blunt II-TOPO vector (Invitrogen), and sequenced to confirm the validity of the PCR product. The primer sets were 5′-CTCACGCGTCTGCCTAATCA-3′ (sense) and 5′- AAGCTTTCAG AAGGACTCAAGAGGGA-3′ (antisense, *Hin*dIII-linker added). After digestion with *Mlu*I and *Hin*dIII, the fragment was subcloned into the *Mlu*I/*Hin*dIII site of a promoterless luciferase expression vector, pGL3-Basic vector (Promega) and designated as pGL3-Dkk-1(−837/+151). Serial 5′-deletion constructs of the Dkk-1 promoter were generated by PCR, using pGL3-Dkk-1 (−837/+151) as template. To generate the mutant, PCR-mediated site-directed mutagenesis was performed by a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All the deletion and mutation constructs were confirmed by DNA sequencing.

Transient transfection and reporter assay. Human osteoblasts were transiently transfected by means of calcium phosphate precipitation [16]. Briefly, cells (5×10^4 cells/well) were seeded in a 12-well plate (Falcon) prior to transfection: each well received 5 μg of the Dkk-1 promoter-reporter firefly luciferase plasmid (pGL3) and 100 ng pRL-CMV (a *Renilla* luciferase vector, Promega) as an internal control. Two hours after transfection, the cells were incubated in Eagle's α MEM with 10% dextran/charcoal-treated FCS in the presence or absence of 10^{-7} M dexamethasone. Firefly and *Renilla* luciferase activities were measured at 36 h after transfection with a dual luciferase assay kit (Promega), and the values were adjusted for the activity of the internal control (*Renilla* luciferase activity).

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

We first screened the mRNA expression for the Dkk family (Dkk-1, -2, -3, and -4) by RT-PCR, and found that only Dkk-1 mRNA is expressed in primary cultured human osteoblasts (data not shown). We then examined the expression level of Dkk-1 mRNA by Northern blot analysis (Fig. 1). Although quiescent human osteoblasts expressed a low level of Dkk-1 mRNA transcript (1.8 kb), dexamethasone (10^{-7} M) significantly induced the expression of Dkk-1 mRNA (more than 10-fold) compared to that in unstimulated condition (Fig. 1A). This effect was observed at 6 h and reached maximum at 24 h (Fig. 1B). This increase was in a dose-dependent manner (10^{-9}–10^{-7} M of dexamethasone) (Fig. 1A). We also examined the effect of other steroid hormones on the expression of Dkk-1 mRNA. Addition of 10^{-7} M of 17β-estradiol (E2), dihydrotestosterone (DHT) or 1,25-dihydroxyvitamin D3 (VD3) did not affect the expression level of Dkk-1 mRNA (Fig. 2). These results

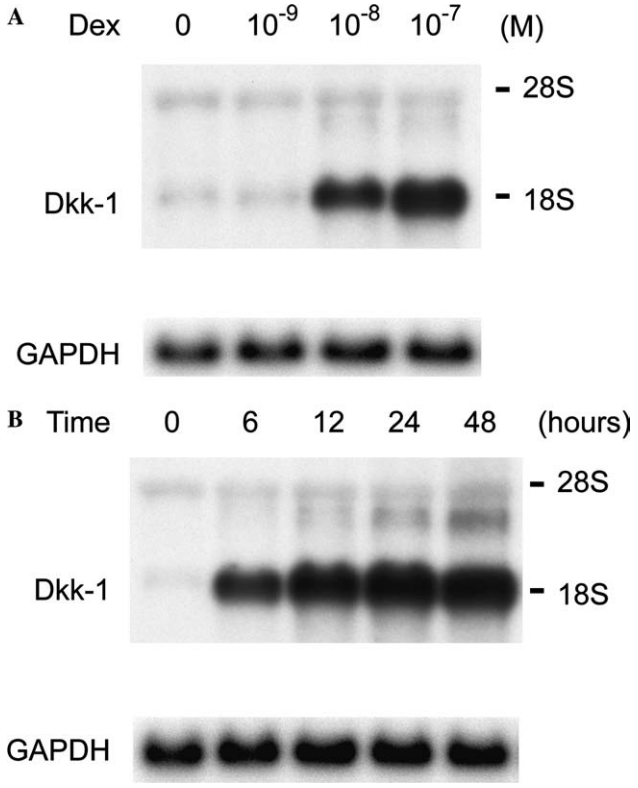

Fig. 1. Effect of dexamethasone on the expression level of Dkk-1 mRNA in primary cultured human osteoblasts. (A) Human osteoblasts were incubated for 24 h with vehicle (ethanol) or dexamethasone (Dex, 10^{-9}–10^{-7} M). (B) Human osteoblasts were incubated for 6–48 h with dexamethasone (10^{-7} M). Extracted total RNA (2 μg in each lane) was subjected to Northern blot analysis. A representative autoradiograph of three independent experiments are shown.

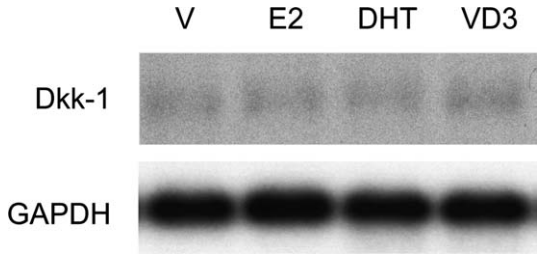

Fig. 2. Effect of various steroid hormones on the expression level of Dkk-1 mRNA in primary cultured human osteoblasts. Human osteoblasts were incubated for 24 h with vehicle (V, ethanol), 17β-estradiol (E2, 10^{-7} M), dihydrotestosterone (DHT, 10^{-7} M), or 1,25-dihydroxyvitamin D3 (VD3, 10^{-7} M). Extracted total RNA (2 μg in each lane) was subjected to Northern blot analysis. A representative autoradiograph of three independent experiments is shown.

suggest that glucocorticoid specifically induces the expression of Dkk-1 mRNA in primary cultured human osteoblasts.

We then examined whether dexamethasone affects the expression of other Wnt signal-related molecules in cultured human osteoblasts. Cultured human osteoblasts expressed the mRNA transcript for Kremen1, a receptor for Dkk [17], and that for LRP5. As shown in Fig. 3, dexamethasone (10^{-7} M) did not alter the expression level of the mRNA for Kremen1. On the other hand, the expression of LRP5 mRNA slightly decreased by the treatment with dexamethasone (10^{-7} M).

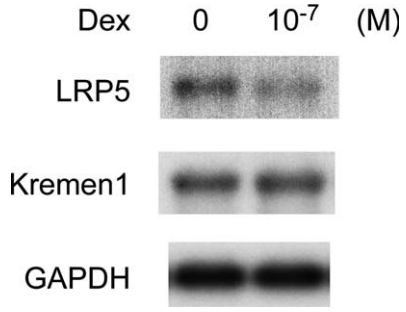

Fig. 3. Effect of dexamethasone on the expression levels of LRP5 and Kremen1 mRNA in primary cultured human osteoblasts. Human osteoblasts were incubated for 24 h with vehicle (ethanol) or dexamethasone (Dex, 10^{-7} M). Extracted total RNA (2 μg in each lane) was subjected to Northern blot analysis. A representative autoradiograph of three independent experiments is shown.

To clarify the mechanism by which dexamethasone up-regulates the expression of the Dkk-1 mRNA, we investigated the effect of dexamethasone on the promoter activity of the human Dkk-1 gene in cultured human osteoblasts. At first, we determined the transcription initiation site for the Dkk-1 gene in human osteoblasts by RLM-RACE. One major product was observed by PCR, and the sequence analysis of this product revealed that the transcription initiation site is 'A' at −152 bp relative to the translation start site. We then examined the 5′-promoter region on the transcriptional activity. When the reporter plasmid containing of the 0.8 kb upstream region was transfected into human osteoblasts, the basic promoter activity was 8–10 times higher than that of promoterless control plasmid. The addition of dexamethasone (10^{-7} M) increased 6–8 times the luciferase activity of the 0.8 kb construct (Fig. 4). The magnitude of the enhancement of the dexamethasone-induced transcription was comparable to that of the Dkk-1 mRNA induction by dexamethasone (Fig. 1A). Deletion of the 5′-promoter region from −837 to −540 bp, which contains one of three putative GRE-like sequences located within the 0.8 kb upstream region, abolished the effect of dexamethasone. No significant change of the dexamethasone effect on the promoter activity was observed by the deletion from −540 to −409 bp and that from −409 to −314 bp, each of which contains one putative GRE (Fig. 4). Mutation of the GRE-like sequence (−788 to −774 bp) in the promoter region from −837 to −540 bp also abrogated the induction effect of dexamethasone. These results suggest that the GRE located from −788 to −774 bp functions in the induction of Dkk-1 in response to glucocorticoid in human osteoblasts.

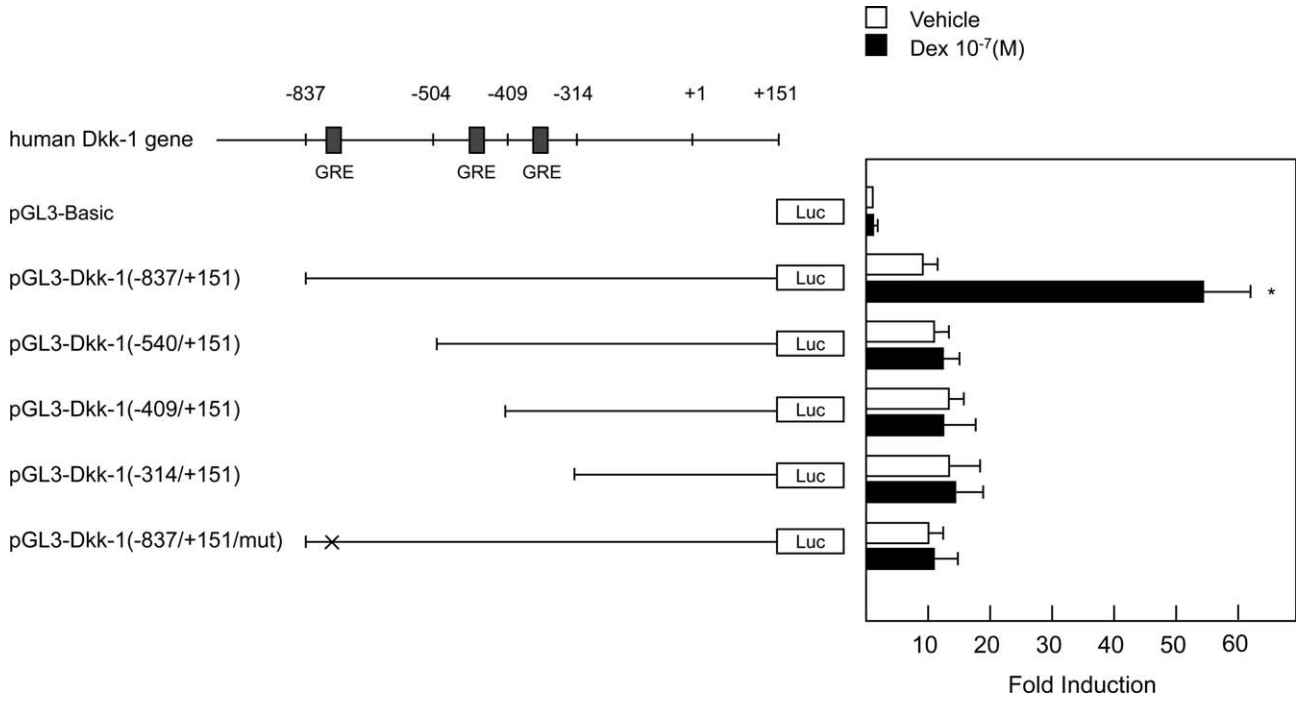

Fig. 4. Effect of dexamethasone on the promoter activity of the human Dkk-1 gene in primary cultured osteoblasts. Human osteoblasts were transiently transfected with various lengths of Dkk-1 promoter region/luciferase (Luc) chimeric plasmids or GRE-mutated Dkk-1 promoter region/luciferase chimeric plasmid as described in Materials and methods. Three putative GRE-like sequences are located in the human Dkk-1 gene promoter regions from −788 to −774 bp, from −448 to −434 bp, and from −380 to −366 bp. The sequence of GRE from −788 to −774 bp (5′-AGAACAACATTAAAT-3′) of pGL3-Dkk-1(−837/+151) was mutated to 5′-AGAGTTACATTAAAT-3′, and designated as pGL3-Dkk-1(−837/+151/mut). The reporter luciferase activity was expressed as fold over the activity of pGL3-Basic in the absence of dexamethasone. Data are shown as means ± SD ($n = 4$). *$P < 0.01$ vs. vehicle. One representative data of three independent experiments is shown. Vehicle, ethanol (open column) and Dex, dexamethasone (closed column).

Discussion

In the present study, we clearly demonstrated that dexamethasone markedly induces the expression of Dkk-1 mRNA in primary cultured human osteoblasts. We also showed that this induction is mainly mediated through the activation of transcription via GRE of the Dkk-1 gene promoter.

In this study, we found that only the mRNA for Dkk-1 was expressed in cultured human osteoblasts by RT-PCR and Northern blot analysis. Dkk-1 was originally identified as a head inducer in *Xenopus* [18] and was shown to be involved in skeletal development [19]. The Dkk family consists of Dkk-1, Dkk-2, Dkk-3, Dkk-4, and Dkk-3-related protein Soggy, and each Dkk possesses different properties on Wnt signaling [20,21]. It is important to address which Dkk plays a role in bone physiologically. Developmental analysis of mouse embryo showed that Dkk-1, Dkk-2, and Dkk-3 are expressed in undifferentiated mesenchymal cells on day E12.5 post coitum (p.c.), but only Dkk-1 is expressed in mesenchymal cells of mineralizing bone on day E15.5 p.c. [22]. These results may suggest a possible regulatory role of Dkk-1 in osteoblasts.

The expression of Dkk-1 is regulated both developmentally and tissue-specifically [18,22,23], and also induced by several factors such as bone morphogenetic protein-4 (BMP-4), *c-Jun*, p53 tumor suppressor, UV ray, and DNA damaging agents [24–26]. However, to our knowledge, this is the first report about the regulation of Dkk-1 expression by glucocorticoid in human osteoblasts.

Since Dkk-1 is a potent inhibitor of Wnt signaling, it is considered that the Dkk-1 induced markedly by dexamethasone suppresses the Wnt signal of bone formation in osteoblasts, which may be involved in the impairment of bone formation by glucocorticoid. Indeed, over-expression of Dkk-1 repressed the Wnt3a-induced alkaline phosphatase activity and reduced the extracellular matrix mineralization in mouse preosteoblastic MC3T3-E1 cells [27]. The recent study also demonstrated that Dkk-1 produced by multiple myeloma cells is associated with development of osteolytic lesions in bone [28]. The results that dexamethasone did not change the expression level of Kremen1, which participates in endocytosis of the LRP/Dkk complex from the cell surface [17], and slightly suppressed the expression of LRP5 may support a possibility that glucocorticoid suppresses bone formation by the induction

of Dkk-1, although we did not examine whether dexamethasone affects the expression of the Wnt receptor, frizzled. It remains to be fully elucidated which frizzled receptor functions for the Wnt signal in osteoblasts and further studies will be required.

Analysis of the Dkk-1 promoter region revealed that the induction of Dkk-1 by dexamethasone mainly resulted from activation of transcription through GRE in the Dkk-1 gene promoter. There are several putative GRE-like sequences within approximately 1 kb upstream from the transcription initiation site in the human Dkk-1 gene. Deletion and mutation analyses showed that the GRE located from −788 to −774 bp is responsible for the activation of the promoter activity in response to dexamethasone in human osteoblasts.

The recent report about LPR5 null mice demonstrated that loss of function in LRP5 causes low bone mass by decrease in osteoblastic proliferation and function [7]. It is noteworthy that the Wnt/LRP5 signal is independent of *Runx2*/*Cbfa1*, a master transcriptional factor for osteoblastic differentiation [7]. On the other hand, patients with the mutation of LRP5 (G171V), a gain-of-function mutation of LRP5, show high bone mass with no fragility, probably because of normal osteoclastic function [8]. Therefore, the enhancement of the Wnt signal in osteoblast by a specific inhibitor for Dkk-1 interacting with LRP5 is expected to promote bone formation by stimulating osteoblastic proliferation and function in the *Runx2*/*Cbfa1*-independent pathway. Our present findings and further study about the regulation of LRP5 function by Dkk-1 in osteoblasts may lead to the development of new drugs to promote bone formation for the treatment of glucocorticoid-induced osteoporosis.

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