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Inductive effects of dexamethasone on the mineralization and the osteoblastic gene expressions in mature osteoblast-like ROS17/2.8 cells

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Abstract

We examined the effects of dexamethasone (Dex), a synthetic glucocorticoid, on the formation of mineralized bone nodules and the gene expressions of the late osteoblastic markers, bone sialoprotein (BSP), osteocalcin (OC), and osteopontin (OPN) in mature osteoblast ROS17/2.8 cells. Treatment of ROS17/2.8 cells with Dex resulted in the induction of mineralization accompanied with increasing BSP and OC expressions. Previous reports have demonstrated that BSP and OC expressions are regulated by Runx2. Then, we hypothesized that Dex might promote osteoblastic differentiation and mineralization on ROS17/2.8 by Runx2. In this study, no effect was observed in mRNA and protein expression of Runx2. However, the transcriptional activity of Runx2 was enhanced by Dex treatment. Furthermore, the Dex-induced BSP and OC expressions decreased after the transfection of Runx2 small-interfering RNAs (siRNAs). These results suggested that the enhancement of Runx2 transcriptional activity by Dex treatment may be followed by the activation of osteoblast marker genes, such as BSP and OC to thereby produce a bone-specific matrix that subsequently becomes mineralized.

Keywords: Dexamethasone; Mineralization; ROS17/2.8; Runx2

Dexamethasone (Dex) is a synthetic glucocorticoid (GC) used frequently for the treatment of severe inflammatory diseases such as rheumatoid arthritis and lupus erythematosus [1]. However, the long-term use of Dex for patients causes severe osteoporosis as a secondary effect [2,3]. One of the mechanisms of Dex-induced osteoporosis is thought to be the result of a direct inhibitory effect on the differentiation of osteoblasts [4]. However, in osteoblast cultures, Dex exhibits both negative and positive effects on osteoblast differentiation and mineralization, depending on the level of cell maturity or cell density [5,6]. Dex promotes phenotypic markers of osteoblast differentiation, such as alkaline phosphatase (ALP), osteopontin (OPN), osteocalcin (OC), and bone sialoprotein (BSP) [7–9]. Therefore, Dex is a potent stimulator of osteoblast differentiation *in vitro*. However, the mechanism(s) by which Dex promotes osteogenesis remains poorly understood.

The Runx2 gene represents transcription factors that master-regulate osteogenesis [10]. The overexpression of exogenous Runx2 upregulates ALP activity, and mRNA expression of BSP and OC [11]. This regulation is thought to occur when Runx2 binds to an osteoblast-specific cis-acting element, termed OSE2, in the promoter region of skeletal target genes, and regulates their expression [11].

In the present study, we investigated the effects of Dex on terminal osteoblast differentiation with mineralization in the mature osteoblast-like ROS17/2.8 cells. The treatment of ROS17/2.8 with Dex resulted in the induction of mineralization. The results of an expression analysis and luciferase assay using the (OSE2)x6-Luc construct showed Dex to enhance the Runx2 transcriptional activity.

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Furthermore, Dex-induced BSP and OC expressions were reduced by the transfection of Runx2 small-interfering RNAs (siRNAs). These observations indicate that the facilitation of Runx2 transcriptional activity by Dex may therefore be followed by the induction of osteoblast marker genes such as BSP and OC, thus promoting a bone-specific matrix that subsequently becomes mineralized.

Materials and methods

ROS17/2.8 cell cultures. ROS17/2.8 cells were precultured until they reached confluence in α-modified essential medium (α-MEM, Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of CO_2 in air. Subsequently, the cells were cultured for the indicated period with or without treatment of the indicated concentrations of Dex (Sigma Chemical Co., MO) in the medium, supplemented with 50 μg/ml L-ascorbate phosphate and 10 mM β-glycerophosphate (Sigma–Aldrich, Gillingham, UK). The culture medium was changed at an interval of 2 days. The confluent ROS17/2.8 cells were pretreated in a medium containing 10 μg/ml cycloheximide (CHX) (Wako, Japan), a protein synthesis inhibitor, for 30 min and then cultured in the presence or absence of Dex for 24 h additionally. Mifepristone, a specific antagonist of classic cytosolic steroid hormone receptors (RU486, Sigma–Aldrich) was dissolved in 100% ethanol to make a stock solution of 10^{-3} M, which was then diluted in a culture medium to obtain the desired concentration.

Mineralization/bone nodule/Ca^{2+} releasing assay. After confluent ROS17/2.8 cells were cultured for 6 days in 6-well plates, then washed with 0.1 M cacodylate buffer (pH 7.4) and fixed with 4% (w/v) formalin in 0.1 M cacodylate buffer (pH 7.4) for 30 min. Subsequently, Alizarin red S solution was added and incubated for 10 min. Plates were washed with distilled water and the mineralization level was compared between Dex-treated and Dex-untreated cells. The amount of Ca^{2+} released from mineralization nodules was also determined between the Dex-treated and Dex-untreated cells. After removing supernatants, cells were washed with 10 mM Tris–HCl (pH 7.2) solution and 1 N HCl solution was added into each well, and then incubated until drying. Twenty microliters of distilled water were added to each well, and the amount of Ca^{2+} was determined using the Calcium E-test (Wako Co., Osaka, Japan) according to manufacturer's instructions.

Alkaline phosphatase activity. Alkaline phosphatase activity was measured using *p*-nitrophenyl phosphate as a substrate as described by Partridge et al. (1983) [12]. Protein concentration was also determined by a Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA), using bovine serum albumin as the standard.

Real time reverse transcription (RT)-polymerase chain reaction (PCR). First-strand cDNA was synthesized from 1 μg of DNase1-treated total RNA in 20 μl of solution. Two microliters of cDNA solution were mixed in 20 μl of PCR buffer containing 1× SYBER Green1 (Bio Wittaker Molecular Applications, Rockland, ME, USA), 1.5 mM dNTP Mix, 15 mM Mg^{2+}solution, 0.25 U Ex *Taq* DNA polymerase (Takara, Kyoto, Japan), and 20 μM primers (sense and antisense). Real time RT-PCR was performed using a Smart Cycler (Cepheid, Sunnyvale, CA, USA) under the following conditions (40 cycles): denaturation for 3 s at 95 °C, annealing and extension for 20 s at 68 °C. Sense and antisense primers used were as follows: BSP (forward, 5′-GAT AGT TCG GAG GAG GAG GG-3′; reverse, 5′-ACT CCA ACT TTC CAG CGT-3′), OC (forward, 5′-GAA CAG ACA AGT CCC ACA C-3′; reverse, 5′-GAG CTC ACA CAC CTC CCT G-3′), OPN (forward, 5′-AGA CCA TGC AGA GAG CGAG-3′, reverse, 5′-ACG TCT GCT TGT GTG CTG G-3′), Runx2 (forward, 5′-ACA ACC ACA GAA CCA CAA G-3′; reverse, 5′-TCT CGG TGG CTG GTA GTG A-3′), β-actin (forward, 5′-CTT TCT ACA ATG AGC TGC GTG-3′; reverse, 3′,5′-ATG GCT GGG GTG TTG AAG G-3′). mRNA expression levels were normalized by values obtained by that of β-actin mRNA.

Western blot analysis. The cell lysates were prepared in a buffer consisting of 50 mM Tris–HCl (pH 7.4), 0.1% Triton X-100, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, and sonicated three times (10 s each time) on ice, and then centrifuged at 10,000*g* for 10 min to remove the debris. Ten micrograms of proteins from cell lysates were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidine difluoride membrane (ATTO Co., Tokyo, Japan). Membranes were incubated in 1:500 diluted primary antibodies. The antibody against Runx2 (sc-10758), OC (sc-18319), actin (sc-1615) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), that against BSP (A2-20128) from American Research Products (Belmont, MA). After washing, the membranes were incubated with second antibodies for 1 h. After washing, bands were visualized by chemiluminescence using an ECL kit (Amersham-Pharmacia Biotech, NJ) according to the manufacturer's instructions.

Luciferase assay of (OSE2)x6-Luc construct. A promoter construct, (OSE2)x6-Luc, fused to firefly luciferase was kindly provided by Dr. Gerard Karsenty (Research Associate, Baylor College of Medicine, USA). A plasmid, pGL3-Basic (Promega, Madison, WI, USA), was also used as a negative control. ROS17/2.8 cells were seeded on a 6-well plates at 90% confluence and then were incubated for 18 h. Two micrograms of (OSE2)x6-Luc or pGL3-Basic were transfected in the cells using a lipofectamine™2000 (Invitrogen). Forty-eight hours after transfection, Luciferase assay was performed using a Dual Luciferase Assay Kit (Promega) according to the manufacturer's instructions. The values of luciferase activity of the (OSE2)x6-Luc and pGL3-Basic were normalized by values obtained by *Renilla* luciferase activity as an internal control.

Small-interfering RNA (siRNA) duplex preparation and transfection. The rat Runx2-siRNA was chosen using The BLOCK-iT™ RNAi Designer (Invitrogen). The sequences of the Runx2 siRNA are as follows: 5′-UAA CAG CAG AGG CAU UUC GUA GCU C-3′,5′-GAG CUA CGA AAU GCC UCU GCU GUU A-3′. A nonsense random sequence of siRNA was also synthesized as a negative control. ROS17/2.8 cells were plated on 6-well plates (1.5 ml medium/plate). The siRNAs were transfected into 50% confluent ROS17/2.8 cells with an oligofectamine (Invitrogen), and then cells cultured for 24 h. After 24 h, cells were additionally cultured for 24 h in the presence or absence of Dex (10^{-8} M).

Statistical analysis. Results were presented as means ± SD of triplicate cultures, and the difference in each time point was assessed by a Student's *t*-test. Asterisks (∗) indicate a significant difference, $^{*}p < 0.05$. Sharps (#) indicate no statistical significance, $^{\#}p > 0.05$.

Results

Alizarin red S staininglassessment of mineralization

To examine the effect of Dex on forming mineralization in ROS17/2.8, Alizarin red S staining was performed. A time course experiment revealed that the mineralization in Dex-treated cells was accelerated as compared with that of control cultures (Fig. 1A). Alizarin red S-positive mineralization nodules were visible at day 4 in the Dex-treated cells, whereas the same nodules of the Dex-untreated cells were not detectable at day 4. Subsequently, the mineralization nodules increased markedly up to 6 days in the presence of Dex.

ROS17/2.8 cells were cultured in the presence, or absence, of the various concentrations of Dex for 6 days (Fig. 1B). This dose-dependent experiment revealed that all effective concentrations (10^{-5}–10^{-11} M) were stimulatory on the mineralization of ROS17/2.8 cells. Dex increased mineralization nodules similarly in concentrations from 10^{-6} to