Dexamethasone suppresses Smad3 pathway in osteoblastic cells

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Abstract

Central in the pathogenesis of glucocorticoid (GC)-induced osteoporosis is the effects of GC on bone formation. However, the mechanism of GC-inhibited bone formation is not well known. Transforming growth factor (TGF)-β is most abundant in bone matrix compared with other tissues, and we have recently proposed that Smad3, a TGF-β signaling molecule, is important for promoting bone formation. However, no reports have been available about the effects of GC on Smad3 in osteoblasts. In the present study, we investigated whether dexamethasone (Dex), an active GC analog, would affect the expression and activity of Smad3 in mouse osteoblastic MC3T3-E1 and rat osteoblastic UMR-106 cells. Dex significantly suppressed Smad3-stimulated alkaline phosphatase (ALP) activity, although it did not affect TGF-β-inhibited ALP activity in MC3T3-E1 cells. Moreover, pretreatment with Dex suppressed TGF-β-enhanced expression of type I collagen in MC3T3-E1 and UMR-106 cells. In the luciferase assay using p3TP-Lux with a Smad3-specific response element, Dex significantly suppressed the transcriptional activity induced by TGF-β as well as Smad3. However, Dex did not affect the expression of Smad3 in these cells at both mRNA and protein levels. In conclusion, the present study indicates that Dex inhibits ALP activity and type I collagen expression, presumably by suppressing Smad3-induced transcriptional activity but not by modulating Smad3 expression in osteoblastic cells.

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Introduction

Glucocorticoid (GC) causes bone loss and an increase in bone fragility, resulting in a great increase in fracture risk (Dempster *et al.* 1983, Dykman *et al.* 1985, Manolagas & Weinstein 1999). About 50% of patients with Cushing's syndrome and 30–50% of patients taking long-term GC have atraumatic fracture due to osteopenia (Ross & Linch 1982, Adinoff & Hollister 1983). Although GC-induced osteoporosis (GIO) is frequently seen in patients with GC excess, numerous questions remain about its mechanism. Histomorphometric studies of GIO revealed an increase in the number of osteoclasts and bone-resorbing sites as well as a reduction in bone formation (Bressot *et al.* 1979). However, these changes in bone resorption may be the secondary events following the decreased number and activity of osteoblasts. Actually, the effect of GC on bone formation seems to be more crucial in the pathogenesis of GIO (Canalis 1996). Nevertheless, the mechanism of GC-inhibited bone formation is still not fully elucidated.

GC decreases the number of osteoblasts and osteocytes by enhanced apoptosis as well as suppressed turnover of cell cycle (Weinstein *et al.* 1998, Smith *et al.* 2000, 2002). Moreover, GC inhibits the synthesis of bone matrix proteins, such as type I collagen (COLI) and osteocalcin, although GC induces the differentiation of the osteoblast lineage into mature cells (Canalis 1996), suggesting that the effects of GC on osteoblast differentiation are inconsistent, probably varying with the stage of cell growth and differentiation. The proliferation and differentiation of osteoblasts are controlled by various local growth factors and cytokines produced in bone, as well as by systemic hormones. GC inhibits the synthesis or action of bone-derived local growth factors. Among them, insulin-like growth factor (IGF) and transforming growth factor-β (TGF-β) are the important targets of GC in bone formation. GC represses IGF-I production and IGF-II receptor transcription in osteoblasts (Rydziel & Canalis 1995, Delany *et al.* 2001).

TGF-β is produced by osteoblasts and appears to regulate bone metabolism in various ways, including skeletal development and bone remodeling (Sowa *et al.* 2002*a*). TGF-β modulates the proliferation, differentiation and production of bone matrix proteins of osteoblasts (Centrella *et al.* 1994); TGF-β also induces bone formation when locally administered into bone tissues in rats (Noda & Camilliere 1989, Joyce *et al.* 1990, Beck *et al.* 1993, Rosen *et al.* 1994, Massague & Chen 2000). We recently reported that Smad3, a TGF-β signaling molecule, is an important molecule for bone formation in mouse

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osteoblastic MC3T3-E1 cells (Sowa *et al.* 2002*a*,*b*). Moreover, the mice with the target disruption of Smad3 exhibited osteopenia caused by the decreased bone formation (Borton *et al.* 2001). However, no reports are available about the effects of GCs on Smad3 in osteoblasts. In the present study, therefore, we investigated whether dexamethasone (Dex), affects the expression and activity of Smad3 in mouse osteoblastic MC3T3-E1 and rat osteoblastic UMR-106 cells.

Materials and Methods

Materials

Dex and human recombinant TGF-β1 were purchased from Sigma. Anti-Smad3 and anti-COLI antibodies were from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA) and Calbiochem-Novabiochem Corp. (San Diego, CA, USA) respectively. All other chemicals used were of analytical grade.

Cell culture

MC3T3-E1 cells were kindly provided by Dr H. Kodama (Ohu Dental College, Japan). MC3T3-E1 and UMR-106 cells were cultured in α-mimimal essential medium (α-MEM; containing 50 μg/ml ascorbic acid) and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Gibco) respectively. The medium was changed twice a week. Confluent MC3T3-E1 cells employed in the present study were considered to be well-differentiated osteoblastic cells, because osteocalcin – a late-differentiation marker of osteoblasts – was well expressed in these cells under experimental conditions similar to those of the present study, as previously described (Sowa *et al.* 2002*a*).

Transfection

Myc-tagged Smad3 was prepared as previously described (Kaji *et al.* 2001). The constructs and empty vector (each 3 μg) were transfected to MC3T3-E1 cells with lipofectamine (Gibco). After 6 h, the cells were fed with fresh medium containing 10% FBS; 48 h later, the transfected cells were used for the experiments. To rule out the possibility of clonal variation, we characterized at least three independent clones for each transfection. V-transfected cells were used as the control. For stable transfection, myc-Smad3 and empty vector (each 3 μg) were transfected to MC3T3-E1 cells with lipofectamine, as previously described (Sowa *et al.* 2002*a*). Six hours after transfection, the cells were fed with fresh α-MEM containing 10% FBS. After 48 h, cells were passaged and clones were selected in α-MEM supplemented with G418 (0·3 mg/ml; Gibco) and 10% FBS.

Luciferase assay

Cells were seeded at a density of 2×10^5 per six-well plate; after 24 h, cells were transfected with 3 μg/well of the reporter plasmid (3TP-Lux) and with 1 μg/well of the pCH110 plasmid expressing β-galactosidase, using lipofectamine (Gibco), as previously described (Kaji *et al.* 2001). After a further 15 h, the medium was changed to the fresh one containing 4% FBS, and the cells were incubated for an additional 9 h. Thereafter, cells were cultured for 24 h in the presence or absence of 10^{-7} M Dex or 5 ng/ml TGF-β in the medium containing 0·2% FBS. Cells were lysed, and the luciferase activity was measured and normalized to the relative β-galactosidase activity as previously described (Kaji *et al.* 2001).

Protein extraction and Western blot analysis

Cells were lysed with radioimmunoprecipitation buffer containing 0·5 mM phenyl methyl sulfonyl fluoride (PMSF), complete protease inhibitor mixture, 1% Triton X-100 and 1 mM sodium orthovanadate. Cell lysates were centrifuged at 12 000 ***g*** for 20 min at 4 °C, and the supernatants were stored at − 80 °C. Protein quantitation was performed with BCA protein assay reagent (Pierce, Rockford, IL, USA). Proteins (20 μg) were denatured in SDS sample buffer and separated on 10% polyacrylamide-SDS gels. Proteins were transferred in 25 mM Tris, 192 mM glycine and 20% methanol to polyvinylidene difluoride. Blots were blocked with Tris-buffered saline (TBS; 20 mM Tris–HCl (pH 7·5) and 137 mM NaCl plus 0·1% Tween 20) containing 3% dried milk powder. The antigen–antibody complexes were visualized using the appropriate secondary antibodies (Sigma), and the enhanced chemiluminescence detection system, as recommended by the manufacturer (Amersham).

RNA extraction and semi-quantitative RT-PCR

Total RNA was prepared from cells using the acid guanidinium-thiocyanate-phenol-chloroform extraction method. Reverse transcription of 5 μg of cultured cell total RNA was carried out for 50 min at 42 °C and then 15 min at 70 °C, using the Super Script First-Strand Synthesis system for RT-PCR (Gibco), which contained RT buffer, oligo (dT)12–18, 5 × first-strand solution, 10 mM dNTP, 0·1 M dithiothreitol (DTT), Super Script II (RT enzyme) and RNaseH (RNase inhibitor). PCR using primers to unique sequences in each cDNA was carried out in a volume of 10 μl reaction mixture for PCR (as supplied by TaKaRa, Otsu, Japan), supplemented with 2·5 units of TaKaRa TaqTM, 1·5 mM of each dNTP (Takara)

and PCR buffer (10×) (100 mM Tris–HCl (pH 8·3), 500 mM KCl and 15 mM $MgCl_2$); 25 ng of each primer and 1 μl of template (from a 50 μl RT reaction) were used. Thermal cycling conditions and primer sequences are described below. (1) Initial denaturation at 96 °C for 2 min. (2) Cycling for cDNA-specific number of cycles: 96 °C for 1 min, cDNA-specific annealing temperature for 2 min, and 72 °C for 2 min. (3) Final extension at 72 °C for 5 min. Primer sequences, annealing temperature and cycle numbers were as follows: Smad3; 5′-GA GTAGAGACGCCAGTTCTACC-3′ and 5′-GGTTT GGAGAACCTGCGTCCAT-3′ (62 °C; 25 cycles) (Yanagisawa *et al.* 1998), and GAPDH; 5′-ATCCCA TCACCATCTTCCAGGAG-3′ and 5′-CCTGCTTCA CCACCTTCTTGATG-3′ (47 °C; 24 cycles). For semi-quantitative RT-PCR, the number of cycles was chosen so that amplification remained well within the linear range, as assessed by densitometry (NIH Image J, version 1·08i, public domain program). An equal volume from each PCR was analyzed by 6% non-denaturing PAGE and ethidium bromide stained PCR products were evaluated. Marker gene expression was normalized to GAPDH expression in each sample.

Alkaline phosphatase (ALP) assay

ALP activity was assayed with the standard method, as described previously (Sowa *et al.* 2002*a*). In brief, the assay mixtures contained 0·1 M 2-amino-2-methyl-1-propanol (Sigma), 1 mM $MgCl_2$ and 8 mM *p*-nitrophenyl phosphate disodium and cell homogenates. After 3-min incubation at 37 °C, the reaction was stopped with 0·1 N NaOH and the absorbance was read at 405 nm. A standard curve was prepared with *p*-nitrophenol (Sigma). Each value was normalized to the protein concentration.

[^{3}H]Thymidine incorporation (TdR) assay

MC3T3-E1 cells were seeded at 2×10^4 cells/well in 24-well plates. These cells were maintained in α-MEM with 10% FBS. After 48 h of culture, cells were labeled with 0·5 μCi/ml [^{3}H]thymidine (Amersham) for 4 h. The incubation was terminated by removal of the medium, washed with PBS twice, followed by the addition of 5% trichloroacetic acid on ice for 10 min. After removal of the trichloroacetic acid, the residue was dissolved in 20 mM NaOH at 37 °C and scintillation mixture was added. Each sample was counted in a liquid scintillation counter.

Statistical analyses

Data were expressed as mean ± S.E.M. Statistical analysis was performed using an unpaired *t*-test or ANOVA.

Results

Effects of Dex on Smad3-induced ALP activity and COLI expression

We first investigated whether Dex affects Smad3-induced ALP activity by using Smad3-transfected MC3T3-E1 cells. As shown in Fig. 1, ALP activity was significantly higher in Smad3-transfected cells, compared with empty vector-transfected cells; this confirmed our previous data (Sowa *et al.* 2002*a*,*b*). Dex (10^{-7} M) significantly suppressed Smad3-stimulated ALP activity. The effects of Dex were significant from 10^{-8} M. Our previous study revealed that TGFβ as well as Smad3 enhanced the expression of COLI, a most abundant bone matrix protein, in MC3T3-E1 cells. We, therefore, investigated whether Dex affects the expression of COLI induced by TGF-β in MC3T3-E1 cells using Western blots. Dex (10^{-7} M) inhibits COLI expression in MC3T3-E1 cells. In the experiments for COLI expression, therefore, we employed Dex only as a pretreatment before the stimulation with TGF-β. In the experiments for the expression of COLI, even 10^{-7} M Dex pretreatment inhibited the expression of COLI (data not shown). We, therefore, employed the lower dose (10^{-9} M) of pretreatment to observe the effects of Dex on COLI expression induced by TGF-β. As shown in Fig. 2, pretreatment with 10^{-9} M Dex suppressed TGF-β-enhanced expression of COLI in MC3T3-E1 cells, suggesting that Dex inhibits COLI expression induced by TGF-β as well as Smad3 in osteoblasts. Our previous study and others showed that TGF-β suppressed ALP activity in MC3T3-E1 cells. Therefore, we examined the effects of Dex on TGF-β-inhibited ALP activity in MC3T3-E1 cells. As shown in Fig. 3A, 10^{-7}M Dex did not affect TGF-β-inhibited ALP activity in wild-type MC3T3-E1 cells. Our previous study revealed that TGF-β inhibits ALP activity in a manner independent upon Smad3 in MC3T3-E1 cells (Sowa *et al.* 2002*b*). Taken together, the present findings indicated that Dex suppresses Smad-3-induced ALP activity, although Dex does not affect TGFβ-inhibited ALP activity in osteoblasts. We examined the effects of Dex on TGF-β-inhibited osteoblast proliferation by using a [^{3}H]thymidine incorporation assay. As shown in Fig. 3B, Dex did not affect osteoblast proliferation suppressed by TGF-β in MC3T3-E1 cells. These findings suggest that Dex primarily inhibits the bone anabolic actions of TGF-β, but not the bone catabolic ones. Alternatively, the effects of Dex itself on osteoblast proliferation might antagonize the effects of Dex on the TGF-β pathways.

Effects of Dex on the transcriptional activity induced by Smad3

In order to examine the mechanism by which Dex suppresses TGF-β–Smad3 pathways, we investigated

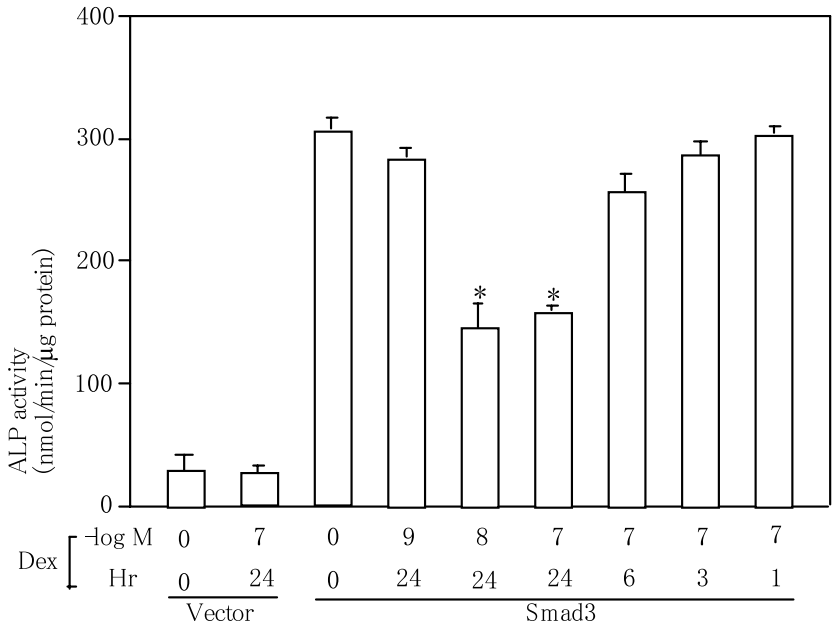

Figure 1 Dex inhibits Smad3-stimulated ALP activity in MC3T3-E1 cells. Stably Smad3- or empty vector (Vector)-transfected confluent MC3T3E1 cells were treated with the indicated concentrations (− log M) of Dex for the indicated times (Hr). ALP activity was measured as described in the Materials and Methods. Each bar represents the mean+S.E.M. of six determinations. *P<0·01, compared with Dex-untreated, Smad3-transfected group.

whether Dex affects the transcriptional activity induced by TGF-β as well as Smad3 in MC3T3-E1 cells. We employed a luciferase assay using 3TP-Lux containing the promoter of plasminogen inhibitor I with a Smad3-specific responsive element. As shown in Fig. 4A, TGF-β increased the transcriptional activity in the absence of Dex in MC3T3-E1 cells; 10^{-7} M Dex significantly suppressed TGF-β-induced transcriptional activity. Similar data were obtained in UMR-106 cells (Fig. 4B). Moreover, 10^{-7} M Dex significantly antagonized Smad3-induced transcriptional activity by transient transfection in MC3T3-E1 cells (Fig. 5). These results indicate that Dex negatively regulated the transcriptional activity of the TGF-β–Smad3 signaling pathway in osteoblasts.

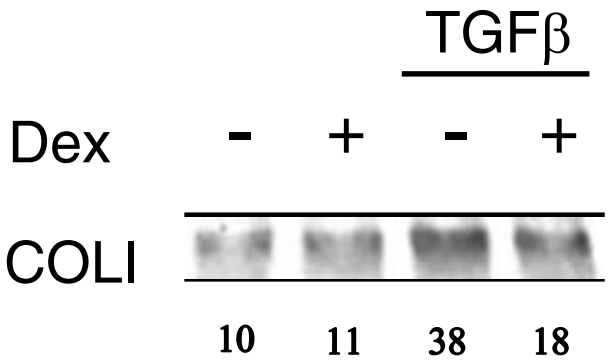

Figure 2 Dex inhibits the expression of COLI induced by TGF-β in MC3T3-E1 cells. After a 12-h preincubation with 10^{-9} M Dex, confluent cells were treated with 2·5 ng/ml TGF-β for 24 h. Protein extraction and Western blot analysis were then performed as described in the Materials and Methods. The results of densitometric analyses are indicated below the Western blot.

Effects of Dex on the expression of Smad3

Our previous study indicated that parathyroid hormone (PTH) enhanced Smad3 expression, resulting in the inhibition of apoptosis in MC3T3-E1 cells, suggesting that the osteotropic hormones modulate Smad3 expression in osteoblasts. We therefore examined the effects of Dex on the endogenous expression of Smad3 in MC3T3-E1 cells. As shown in Fig. 6A, Dex did not affect the expression of Smad3 mRNA in semi-quantitative RT-PCR in MC3T3-E1 cells. Moreover, Dex did not affect the protein level of Smad3 in Western blot analyses (Fig. 6B). Dex did not affect Smad3 expression from 1 to 48 h (data not shown). These results indicate that Dex did not affect the expression of Smad3 in osteoblasts.

Discussion

The TGF-β and GC signaling pathways interact both positively and negatively in regulating a variety of physiological and pathological processes. GC modifies the production of several bone growth factors. Selective inhibitory

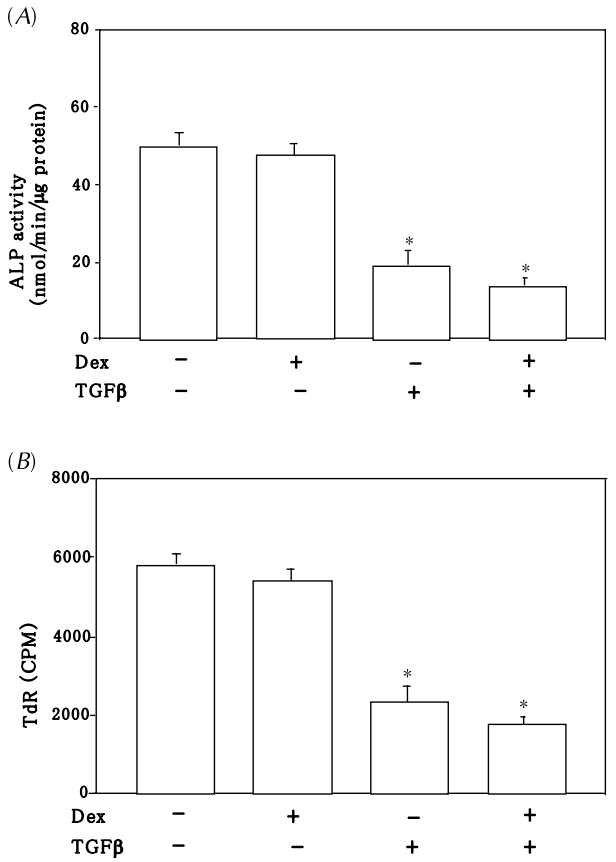

Figure 3 Effects of Dex on TGF-β-reduced ALP activity and proliferation in MC3T3-E1 cells. (A) After 24-h preincubation with 10^{-7} M Dex, confluent cells were treated with 2·5 ng/ml TGF-β for 24 h. ALP activity was measured as described in the Materials and Methods. Each bar represents the mean+S.E.M. of four determinations. *P<0·01, compared with TGF-β-untreated group. (B) After 24-h preincubation with 10^{-7} M Dex, confluent cells were treated with 2·5 ng/ml TGF-β for 24 h. TdR was measured as described in the Materials and Methods. Each bar represents the mean+S.E.M. of four determinations.

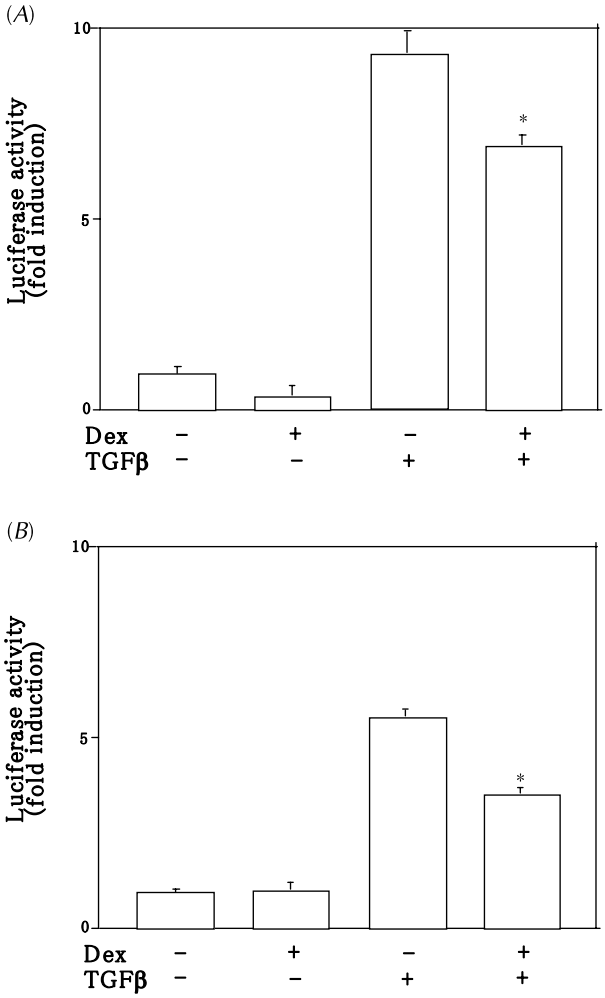

Figure 4 Dex inhibits TGF-β-induced transcriptional activity of Smad3. MC3T3-E1 (A) and UMR-106 cells (B) were transfected with 3 μg/well of the reporter plasmid (3TP-Lux), and with the pCH110 plasmid expressing β-galactosidase (1 μg/well) in six-well plates. After 48 h, cells were treated with 10^{-7} M Dex for 12 h and fed with fresh α-MEM with or without 5 ng/ml TGF-β. Then, 24 h later, cells were harvested and relative luciferase activity was measured. Values of relative luciferase activity represent the mean+S.E.M. of four determinations. *P<0·01, compared with the group treated with TGF-β alone.

actions of GC on bone formation are partly secondary to the regulation of the IGF axis (Canalis 1996). IGF-I has stimulatory effects on bone formation and GC inhibits IGF-I production and transcription in osteoblasts (Rydziel & Canalis 1995). On the other hand, GC does not modify the expression of TGF-β1 in osteoblasts, but induces the activation of a latent form by increasing the levels of proteases in bone (Oursler *et al.* 1993). Moreover, Chang *et al.* (1998) reported that GC suppresses Runx2, with a resultant decrease in the expression and activity of the TGF-β type-I receptor on matrix-producing bone cells. Thus, the effects of GC on the TGF-β pathway seem a little complex, and the relationship between a TGF-β signaling molecule, Smad3, and GC has not been elucidated in bone.

The present study revealed that Dex inhibited the transcriptional activity induced by TGF-β as well as Smad3 in mouse osteoblastic cells. Moreover, Dex suppressed ALP activity as well as COLI expression induced by Smad3 in these cells. Numerous transcriptional regulators – such as FAST1, AP-1, TFE3 and CBP/p300 – affect Smad3-induced transcriptional activity (Massague & Wotton 2000). Indeed, Song *et al.* (1999) demonstrated that the GC receptor inhibits TGF-β signaling by directly targeting the transcriptional activity of Smad3 in human hepatoma Hep3B cells. They also showed that the GC

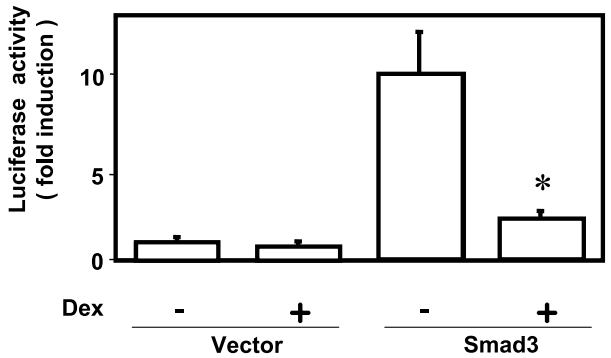

Figure 5 Dex inhibits the transcriptional activity of Smad3 in MC3T3-E1 cells. MC3T3-E1 cells were transiently transfected with 3 μg/well of the reporter plasmid (3TP-Lux), and with the pCH110 plasmid expressing β-galactosidase (1 μg/well) with or without Smad3 cotransfection (3 μg/well) in six-well plates. After 48 h, cells were treated with 10^{-7} M Dex for 12 h and fed with fresh α-MEM. Cells were then harvested and relative luciferase activity was measured. Values of relative luciferase activity represent the mean+S.E.M. of four determinations. *$P<0{\cdot}05$, compared with Dex-untreated and Smad3-transfected group.

receptor interacts with Smad3 physically. These findings support our present data in osteoblasts.

Our recent study revealed that PTH increases Smad3 expression within 1 h, through cAMP and protein kinase C (PKC) pathways (Sowa *et al.* 2003). Therefore, the regulation of Smad3 expression might mediate the effects of Dex on Smad3 pathways in osteoblasts. However, the present study showed that Dex did not affect Smad3 expression in both protein and mRNA levels in MC3T3-E1 cells. Dex did not affect Smad3 expression even after 6 h of treatment; this was compatible with the previous findings that GC did not affect the production of TGF-β in osteoblasts (Oursler *et al.* 1993). Taken together, the effects of GC on Smad3-induced transcriptional activity seemed to be important in the interaction between GC and TGF-β pathways in osteoblasts. These findings also suggest that the Smad3 pathway might be involved as a direct target of bone catabolic factors in osteoblasts. As for bone resorption, a recent study revealed that Dex enhanced osteoclast formation synergistically with TGF-β by stimulating the priming of osteoclast progenitors for differentiation into osteoclasts (Takuma *et al.* 2003). Therefore, the interactions of GC and Smad3 might be different between bone-forming cells and bone-resorbing cells.

Previous studies have suggested that nuclear receptor families interact with Smad3 (Yanagisawa *et al.* 1999, Matsuda *et al.* 2001, Chipuk *et al.* 2002, Pendaries *et al.* 2003), although the biological significance of this remains unknown. The present study indicated that GC repressed Smad3-induced transcriptional activity in osteoblasts. Since other factors are also important in bone metabolism, physiologically and pathologically, the interaction between steroid hormone and Smad3 may be important in the actions of estrogens, androgens, vitamin D and retinoic acid in bone. Further study is necessary to clarify these issues.

The present study indicated that Dex inhibited Smad3-stimulated ALP activity in MC3T3-E1 cells, although it did not affect TGF-β-inhibited ALP activity. In our previous study, stimulation of increased COLI synthesis was the common effect of TGFβ and Smad3 on osteoblasts. However, Smad3 greatly increased ALP activity and mineralization, whereas TGF-β inhibited them in these cells. The reason for the differing effects of TGF-β and Smad3 on ALP activity and mineralization remains unknown. Our previous study suggested that TGF-β-induced and Smad-independent extracellular regulated kinase (ERK)1/2 and Jun N-terminal kinase (JNK) signaling pathways negatively regulate the Smad3 signaling pathway, resulting in the suppression by TGF-β of Smad3-induced ALP activity and mineralization in osteoblasts (Sowa *et al.* 2002*b*). The negative signal of TGF-β-responsive ERK1/2 and JNK for the Smad3 signaling pathway might explain the differing effects of TGF-β and Smad3 on ALP activity and mineralization in MC3T3-E1 cells. TGF-β possesses Smad-dependent and -independent

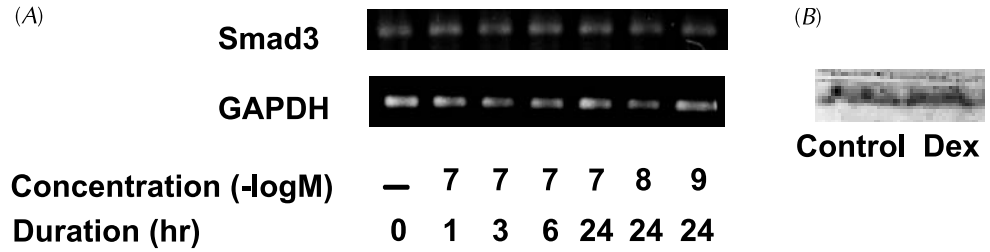

Figure 6 Effects of Dex on the level of Smad3 in MC3T3-E1 cells. (A) After confluent MC3T3-E1 cells were cultured in serum-free α-MEM for 12 h, cells were treated with the indicated concentrations of Dex for 24 h or 10^{-7} M Dex for the indicated times. RNA extraction and semi-quantitative RT-PCR assay were then performed as described in the Materials and Methods. (B) After confluent MC3T3-E1 cells were cultured in serum-free α-MEM for 12 h, cells were treated with 10^{-7} M Dex for 24 h. Protein extraction and Western blot analysis were then performed as described in the Materials and Methods.

pathways in cultured cells, as recently described (Derynck & Zhang 2003). Therefore we believe that the differing effects do not necessarily mean that it is paradoxical to use the MC3T3-E1 cell-line to examine the effects of TGF-β on osteoblasts. Moreover, the transformed state or differentiation stage of cells might affect the response to TGF-β and Smad3 on ALP activity in the present study. Further studies are in progress in our laboratory to examine the influence of osteoblast differentiation on the differing effects of TGF-β and Smad3 on osteoblast phenotype, such as ALP activity. We confirmed the effects of Dex on the TGF-β pathway by using UMR-106 cells. As shown in Fig. 4B, Dex significantly suppressed the transcriptional activity induced by TGF-β in UMR-106 cells. Moreover, Dex inhibited COLI expression induced by TGF-β, and it did not affect the expression of Smad3 in these cells (data not shown). In addition, a recent preliminary abstract revealed that GC antagonizes TGF-β actions in 2T3 preosteoblast cells (Shi *et al.* 2004), when our present manuscript was in preparation. These findings suggest that the effects of Dex on the TGF-β–Smad pathway are common in several osteoblastic cell-lines.

Recent reports have indicated, therefore, that the mechanism of GIO is mainly through direct effects of GC on bone formation, possibly through osteoblasts. The present study suggested that TGF-β–Smad pathways are involved in the suppression of GC on bone formation. Further investigation of the detail of the molecular mechanisms involved is necessary.

In conclusion, the present study indicated that Dex inhibits ALP activity and COLI expression presumably by suppressing Smad3-induced transcriptional activity, but not by modulating Smad3 expression in osteoblastic cells.

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