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**Statin Suppresses Apoptosis in Osteoblastic Cells: Role of Transforming Growth Factor-β-Smad3 Pathway**

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**Running title**: Statin and osteoblast

**Abstract**

Statins possess pleiotropic effects in several tissues. Among them, their bone anabolic actions have been recently noted. Moreover, we proposed that Smad3, a TGF-β-signaling molecule, is a promoter of bone formation. However, whether statins would affect TGF-β-Smad3 pathway in osteoblasts is still unknown. The present study was therefore performed to examine the effects of statin on Smad3 expression and cell apoptosis by employing mouse osteoblastic MC3T3-E1 and rat osteoblastic UMR-106 cells. Statins (pitavastatin, mevastatin, and simvastatin) as well as alendronate increased the levels of Smad3 in MC3T3-E1 cells. The effects of pitavastatin on Smad3 levels were observed from 3 hr and later. Pitavastatin induced the expression of TGF-β, and cyclohexamide, a protein synthesis inhibitor, antagonized the increased levels of pitavastatin on Smad3. On the other hand, pitavastatin antagonized dexamethasone- or etoposide-induced apoptosis in a dose dependent manner, and Smad3 inactivation by dominant negative Smad3 or an inhibition of endogenous TGF-β action by SB431542 antagonized anti-apoptotic effects of pitavastatin, indicating that pitavastatin suppressed osteoblast apoptosis partly through TGF-β-Smad3 pathway.

In conclusion, the present study first demonstrated that statin suppressed cell apoptosis partly through TGF-β-Smad3 pathway in osteoblastic cells.

**Key words;** Statin, Smad3, Osteoblast, TGF-β, Apoptosis

## Introduction

Statins are highly effective cholesterol-lowering drugs that are widely appreciated to reduce incidence from coronary artery disease, and they possess pleiotropic effects other than their effects on the lipid metabolism [1]. Among them, the osteotropic effects of statins are expected. Several studies [2-4] indicated the positive effects of statins on bone mineral density and fractures. In rodents, Mundy et al [5] reported that lovastatin and simvastatin increased bone formation when injected subcutaneously over the calvaria of mice and increased trabecular bone volume when orally administered to rats by inducing bone morphogenetic protein (BMP)-2 expression. Other statins also induced BMP-2 in osteoblastic cells, although pravastatin did not affect it [6, 7]. Woo et al [8] reported that mevastatin suppressed bone resorption by inhibiting the fusion of prefusion osteoclasts and disrupting the actin ring in osteoclasts. Moreover, we recently reported that statins induce and reduce the levels of osteoprotegerin and receptor activator of NFkB ligand mRNA in mouse bone cell cultures, respectively [9].

As for the effects of statins on osteoblast, other groups confirmed by using osteoblastic cell-lines that statins stimulate the expression of BMP-2 and osteocalcin in vitro [7, 10-12]. Moreover, statins induced mineralization in mouse osteoblastic MC3T3-E1 cells [10]. In addition, the recent study indicated that statins stimulate vascular endothelial growth factor expression in osteoblasts via reduced protein prenylation and the phosphatidylinositide-3 kinase pathway [13]. These previous findings suggested that statins stimulate bone formation partly by inducing osteoblast differentiation. Although a study by Mendoza et al suggested that pravastatin possesses anti-apoptotic effect in osteoblasts in vivo

[14], there have been no reports available about the effects of statins on osteoblast apoptosis in vitro.

We recently reported that Smad3, a crucial TGF-β-signaling molecule, promotes the production of type I collagen (Col1), alkaline phosphatase (ALP) activity and mineralization in mouse osteoblastic cells [15-17]. Moreover, mice with targeted disruption of Smad3 exhibit osteopenia caused by decreased bone formation [18]. These findings suggest that the Smad3 molecule is a promoter of bone formation. In addition, we recently reported that hormones, such as parathyroid hormone (PTH) and glucocorticoid modulate Smad3 pathway in osteoblastic cells [19-21]. However, whether statins would affect Smad3 expression is unknown.

The present study was therefore performed to examine the effects of statin on Smad3 expression and cell apoptosis by employing mouse osteoblastic MC3T3-E1 and rat osteoblastic UMR-106 cells.

## Materials and Methods

### Materials

Pitavastatin was kindly provided from Kowa Company Ltd. (Tokyo, Japan). Mevastatin, simvastatin, cyclohexamide and anti-β-actin antibody were purchased from Sigma. Anti-TGF-β and anti-Smad3 antibodies were purchased from Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. SB431542 was obtained from Clbiochem-Nova-biochem Corp. (San Diego, CA). All other chemicals used were of analytical grade.

**Cell Culture**

MC3T3-E1 and UMR-106/NIH-3T3 cells were cultured in α-minimal essential medium (α-MEM; containing 50 mg/ml of ascorbic acid) and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin (Gibco BRL, Rockville, MD, USA), respectively, as previously described [19, 22]. The medium was changed twice a week. Statins were added to overnight serum-starved cells at confluent stage for the experiments of Western blot analysis and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR).

**Transient transfection**

The vectors expressing Myc-tagged Smad3 and a mutant form of Myc-tagged Smad3 (Smad3 Δ C), in which the MAD homology 2 (MH2) domain corresponding to amino acid residues 278-425 had been removed, were described previously [19, 21]. Myc-Smad3ΔC, and empty vector (pcDNA3.1+; each 3 μg) were transfected using Lipofectamine (Invitrogen). Fifteen hr later, the medium was changed to medium containing 4% FBS, and the cells were incubated for an additional 9 hr. Thereafter, the cells were cultured for the experiments for apoptosis.

**Protein extraction and Western blot analysis**

Cells were lysed with radioimmunoprecipitation buffer containing 0.5 mM phenylmethylsulfonylfluoride, complete protease inhibitor mixture (Roche Applied Science, Tokyo, Japan), 1% Triton X-100, and 1 mM sodium

orthovanadate. Cell lysates were centrifuged at 12,000 x *g* for 20 min at 4 C, and the supernatants were stored at -80 C. Protein quantitation was performed with bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, IL). Twenty-microgram protein aliquots were denatured in sodium dodecyl sulfate sample buffer and separated on 10% polyacrylamide-sodium dodecyl sulfate gels. Proteins were transferred in 25 mM Tris, 192 mM glycine, and 20% methanol to polyvinylidene difluoride. Blots were blocked with 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-Aldrich Corp.) and the enhanced chemiluminescence detection system, as recommended by the manufacturer (Amersham Biosciences, Arlington Heights, IL). The results depicted in each figure are representative of at least three separate cell preparations. Each experiment was repeated three times.

**Semiquantitative RT-PCR**

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 SuperScriptTM first strand synthesis system for RT-PCR (Invitrogen), which contained RT buffer, oligo(dT)12-18, 5x first strand solution, 10 mM dNTP, 0.1 M dithiothreitol, SuperScript II (RT-enzyme), and RNase H (RNase inhibitor). PCR using primers to unique sequences in each cDNA was carried out in a volume of 10 μl of reaction mixture for PCR (as supplied by TaKaRa, Otsu, Japan), supplemented with 2.5 units of TaKaRa TaqTM, 1.5 mM each dNTP (TaKaRa), and PCR buffer (10x), which contained 100 mM Tris-HCl (pH 8.3), 500 mM KCl, and 15 mM

$MgCl_2$. 25 ng of each primer and 1 $\mu$l of template (from a 50-$\mu$l 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); and 3) final extension at 72 ° C for 5 min. Primer sequences, annealing temperature, and cycle numbers were as follows: Smad3, 5'-GAGTAGAGACGCCAGTTCTACC-3' and 5'-GGTTTGGAGAACCTGCGTCCAT-3' (62 ° C; 25 cycles); TGF-$\beta$; 5'-AAGTGGATCCACGAGCCCAA-3' and 5'-GCTGCACTTGCAGGAGCGCA-3' (58 ° C; 27 cycles); glyceraldehyde-3-phosphate dehydrogenase (GA-PDH), 5'-ATCCCATCACCATCTTCCAGGAG-3' and 5'-CCTGCTTCACCACCTTCTTGATG-3' (47 ° C; 24 cycles). For semiquantitative 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% nondenaturing polyacrylamide gel electrophoresis, and ethidium bromide-stained PCR products were evaluated. Marker gene expression was normalized to GAPDH expression in each sample.

**Determination of osteoblast apoptosis**

Triransferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining was used to detect apoptosis. In brief, cells were cultured on round coverglasses (Fisher Scientific, Pittsburgh, PA) in six-well plates, and the coverglasses with cells attached were rinsed twice with ice-cold PBS, followed

by fixation with 4% neutral formaldehyde. Then the fixed cell layers on the glasses were stained. The TUNEL reaction was performed using an Apoptosis In Situ Detection kit (Wako Biochemicals, Osaka, Japan) following the standard protocol, as previously described [19]. In each experiment, apoptotic cells were counted in randomly selected fields using a hemocytometer and were expressed as percentages compared with total cells. Each experiment was performed at least three times.

**Statistics**

Data were expressed as the mean ± SEM. Statistical analysis was performed using ANOVA.

**Results**

We first examined the effects of statins as well as alendronate, a bisphosphonate, on the level of Smad3 expression in osteoblastic cells. As shown in Fig. **1A**, pitavastatin ($10^{-5}$ M) as well as alendronate ($10^{-5}$ M) increased the level of Smad3 in MC3T3-E1 cells. The effects of pitavastatin on Smad3 levels were observed from 3 hr and later (Fig. **1B**). The other statins, such as mevastatin and simvastatin, also induced the levels of Smad3 in these cells (Fig. **1C**). These effects were also observed in UMR-106 cells (data not shown). As shown in Fig. **1D**, pitavastatin induced the level of Smad3 mRNA from 3 hr and at $10^{-5}$M in MC3T3-E1 cells in semi-quantitative RT-PCR. As shown in Fig. **1E**, pitavastatin ($10^{-5}$M) induced the levels of phosphorylated Smad3 in MC3T3-E1 cells, although SB431542 antagonized Smad3 phosphorylation by pitavastatin. Pitavastatin did not affect Smad3 expression in mouse fibroblastic NIH3T3 cells

(Fig. **1F**), suggesting that the effects of statin on Smad3 expression are not common in all tissues.

Smad3 is a crucial signaling molecule of TGF-β pathway, and statins might induce Smad3 through the induction of endogenous TGF-β in osteoblasts. We therefore investigated whether pitavastatin would affect the levels of TGF-β in osteoblastic cells. As shown in Fig. **2A**, pitavastatin induced the expression of TGF-β within 3 hr in MC3T3-E1 cells. Moreover, cyclohexamide, a protein synthesis inhibitor, antagonized the increased effects of pitavastatin on Smad3 level, indicating that pitavastatin induces Smad3 levels through a protein synthesis (Fig. **2B**). As shown in Fig. **2C**, cyclohexamide did not affect the induction of TGF-β mRNA by pitavastatin, although it antagonized Smad3 mRNA increased by pitavastatin. Moreover, SB432542 decreased the levels of Smad3 induced by TGF-β(Fig. **2D**).

Our previous studies revealed that Smad3 overexpression significantly antagonized dexamethasone- or etoposide-induced apoptotic effects in osteoblastic cells [19, 21]. Therefore, we examined the effects of statins on osteoblast apoptosis in MC3T3-E1 and UMR-106 cells. We examined the effects of statins on osteoblast apoptosis by employing Tunel assay. As shown in Fig. **3**, pitavastatin antagonized dexamethasone-induced apoptosis in a dose dependent manner in MC3T3-E1 cells, and these effects were significant from $10^{-6}$M. Mevastatin and simvastatin exerted similar effects with pitavastatin in these cells. Moreover, Smad3 inactivation by dominant negative Smad3 (Smad3 Δ C) antagonized anti-apoptotic effects of pitavastatin, indicating that pitavastatin suppressed osteoblast apoptosis induced by dexamethasone through Smad3. Moreover, pitavastatin as well as Smad3 over expression

significantly inhibited cell apoptosis induced by etoposide and dexamethasone in UMR-106 cells (Fig. **4A** and **4B**). An activation of type II receptors by TGF-β led to activation of the type I receptor. TGF-β type I receptor-like kinase [activin receptor-like kinase 5 (ALK5)] phosphorylated and activated TGF-β receptor-regulated Smad2/3. We used SB431542, which specifically inhibits ALK5 and results in an inhibition of endogenous TGF-β action and subsequent phosphorylation of Smad2/3. As shown in Fig. **4B**, SB431542 significantly reversed an inhibition of pitavastatin on dexamethasone-induced apoptosis.

## Discussion

The Smad family proteins are critical components of the TGF-β signaling pathways, and TGF-β regulates the transcriptional response of the target genes through the two receptor-regulated Smads, Smad2 and Smad3 [22]. We have recently proposed that Smad3 is a molecule of promoting bone formation [15, 16]. Our study indicated that PTH stimulates the expression of Smad3 in osteoblasts [19]. In that study, the pathway, the "PTH-Smad3 axis," was essential in PTH-induced anti-apoptotic effects and reinforced the anabolic effects of Smad3. Moreover, our recent studies revealed that PTH stimulates β -catenin levels via Smad3 in osteoblasts [21]. The present study first demonstrated that pitavastatin induces the level of Smad3 in osteoblastic cells. Moreover, both mevastatin and simvastatin also induced the levels of Smad3. These findings indicate that statins increase the expression of Smad3 in osteoblasts. Taken into account with the bone anabolic action of Smad3, these effects of statins on Smad3 might be some clue for bone anabolic actions.

Statins suppress mevalonate pathway by inhibiting

3-hydroxy-3-glutaryl-coenzyme A (HMG-CoA) reductase activity, which is required for the conversion of HMG-CoA to mevalonate, and previous study indicated that statins affect osteoblastic cells partly by affecting mevalonate pathway [11]. Nitrogen-containing bisphosphonates, such as alendronate and risedronate, induce osteoclast apoptosis by suppressing the mevalonate pathway [24]. On the other hand, bisphosphonate administration prevented glucocorticoid-induced osteoblast apoptosis in mice [25]. Moreover, our studies indicated that Smad3 overexpression suppresses dexaemethasone- or etoposide-induced apoptosis in osteoblastic cells, and that PTH suppresses osteoblast apoptosis through Smad3, followed by induction of β-catenin [21]. In addition, the previous study suggested anti-apoptotic effects of statins in other tissue [26]. Taken together, the possibility was raised that statins possess anti-apototic effects on osteoblasts, as bisphosphonate does. In the present study, pitavastatin significantly suppressed apoptosis induced by dexamethasone or etoposide. Moreover, an inactivation of Smad3 by dominant negative Smad3 significantly antagonized the inhibitory effects induced by pitavastatin on apoptosis. Moreover, our previous study revealed that Smad3 overexpression suppressed apoptosis in MC3T3-E1 and UMR-106 cells [19, 21]. These findings indicated that pitavastatin suppresses apoptosis through Smad3 induction in osteoblastic cells. In addition, an inhibition of endogenous TGF-β action by SB431542 antagonized the suppressive effects of pitavastatin on osteoblast apoptosis, indicating that pitavastatin suppresses apoptosis through an induction of TGF-β in osteoblasts. The anti-apoptotic effects of pitavastatin seemed to be potent, compared with its effects on Smad3 levels in osteoblastic cells. Since the antagonism of dominant negative Smad3 or SB431542 on

anti-apoptotic effects of statin is only partial, the mechanism other than TGF-β or Smad3 pathways might be responsible for these discrepancies. Taken together, statins might stimulate osteoblastic bone formation by increasing the number of cells through TGF-β-Smad3 pathways as well as by inducing the differentiation through increased BMP-2 and osteocalcin expression. The previous study suggested that statins stimulate ALP activity and mineralization in osteoblastic cells [9]. Taken into account with our previous evidence that Smad3 induced ALP activity and mineralization in MC3T3-E1 cells, it is possible that statins induces ALP activity and mineralization through Smad3 in osteoblasts. Further study is necessary to clarify this issue.

Our recent study suggests that PTH induces the level of β-catenin through Smad3 in osteoblastic cells [21]. Moreover, PTH stimulated osteoblast β-catenin levels via Smad3 independently of and dependently of TGF-β in the early and later induction phases, respectively. In the present study, the induction of Smad3 by pitavastatin seemed to start from 3 hour and later. These effects were different from the early induction of Smad3 and β-catenin by PTH within 1 hr. Moreover, a protein synthesis inhibitor suppressed the Smad3 level induced by pitavastatin, and pitavastatin induced the level of TGF-β expression in osteoblasts. These findings indicated that pitavastatin elevated the level of Smad3 partly through protein synthesis and TGF-β induction in osteoblastic cells. These findings were not identical to the effects of PTH on Smad3. Therefore, statins might suppress osteoblast apoptosis by the mechanism different from PTH.

Maeda et al [12] showed that $10^{-7}$ M simvastatin did not affect the level of TGF-β mRNA in long-term cultured MC3T3-E1 cells. However, we showed that

$10^{-5}$ M pitavastatin induced the levels of TGF-β mRNA and protein in confluent MC3T3-E1 cells. $10^{-7}$ M pitavastatin did not affect the levels of TGF-β in these cells (data not shown). These discrepancies might be due to the dose of statins, differentiation stage of cells or culture conditions.

Statins, such as lovastatin and simvastatin induced bone formation in rodent in vivo study [5]. However, the controversy existed about the effects of statins on bone mineral density and fracture risk in clinical studies [2-4]. A potential explanation of these discrepancies is that most statins were metabolized in liver after oral administration; subsequently the local concentration of statins in bone is appreciated to be not enough for the induction of bone formation. On the other hand, pitavastatin is relatively less metabolized in liver, compared with other statins [27]. Therefore, higher concentration ($10^{-6}$ -$10^{-5}$ M) of pitavastain to induce TGF-β-Smad3 pathway may be possible in bone microenvironment. Although there are no reports available about the effects of pitavastain in vivo and in human, the further clinical and in vivo studies will be necessary.

In conclusion, the present study first demonstated that statin increased the level of Smad3 possibly through TGF-β induction in osteoblastic cells. Moreover, it suppressed osteoblast apoptosis through TGF-β-Smad3 pathway.

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**Figure Legends**

Fig.**1** Effects of statins on Smad3 levels in mouse osteoblastic cells.

Confluent MC3T3-E1 cells were cultured in serum-free α-MEM with (**A**) alendronate or pitavastatin at the indicated concentrations for the 6 Hr or (**B**) pitavastatin at $10^{-5}$ M for the indicated times (Hrs), or (**C**) pitavastatin (Pitava), mevastatin (Meva), or simvastatin (Simva) at $10^{-5}$ M for 6 Hr. Protein extraction of the cells and Western blot analysis were performed as described in Materials and Methods. (**D**) Confluent MC3T3-E1 cells were cultured with pitavastatin at the indicated concentrations for 6 Hr (upper panel) or with pitavastatin at $10^{-5}$ M for the indicated times (Hrs) (lower panel). RNA extraction and semiquantitative RT-PCR analyses were performed as described in Materials and Methods. (**E**) Confluent MC3T3-E1 cells were cultured in the presence or absence of $10^{-5}$ M pitavastatin (Pitava) with or without pretreatment with 5 μM SB431542 for 30 min. Protein extraction of the cells and Western blot analysis were performed as described in Materials and Methods. (**F**) Confluent NIH-3T3 cells were cultured in DMEM with $10^{-5}$ M pitavastatin for 6 Hr. Protein extraction of the cells and Western blot analysis were performed as described in Materials and Methods.

Fig.**2** Role of endogenous TGF-β in an induction of Smad3 levels by pitavastatin

Confluent MC3T3-E1 cells were cultured in serum-free α-MEM with (**A**) pitavastatin at $10^{-5}$ M for the indicated times (Hrs), or (**B**) $10^{-5}$ M pitavastatin (Pitava) with or without 10 μM cycloheximide (CHX) pretreatment for 6 Hr. Protein extraction of the cells and Western blot analysis were performed as described in Materials and Methods. (**C**) Confluent MC3T3-E1 cells were cultured in the presence or absence of $10^{-5}$ M pitavastatin (Pitava) with or

without pretreatment with 10 μM cycloheximide (CHX) for 6 Hr. RNA extraction and semiquantitative RT-PCR analyses were performed as described in Materials and Methods. (**D**) Confluent MC3T3-E1 cells were cultured in the presence or absence of 2.5 ng/ml TGF-β with or without pretreatment with 5 μM SB431542 for 30 min. Protein extraction of the cells and Western blot analysis were performed as described in Materials and Methods.

Fig.**3** Pitavastatin reverses dexamethasone-mediated cell apoptosis through Smad3 in MC3T3-E1 cells.

MC3T3-E1 cells with or without transient Smad3ΔC transfection were treated without or with $10^{-7}$M dexamethasone (Dex) for 6 Hr without or with pretreatment with $10^{-5}$M mevastatin (Meva), $10^{-5}$M simvastatin (Simva) the indicated concentrations of pitavastatin (Pitava) for 6 Hr in serum-free α-MEM. The relative number of apoptotic cells was determined by TUNEL assay as described in Materials and Methods. Values are the mean ± SEM ratio of TUNEL positive/negative cell numbers compared with Dex-untreated cells. *, $P < 0.01$ compared with Dex-only-treated cells. **, $P<0.01$ compared with Dex and $10^{-5}$ M pitavastatin-treated cells.

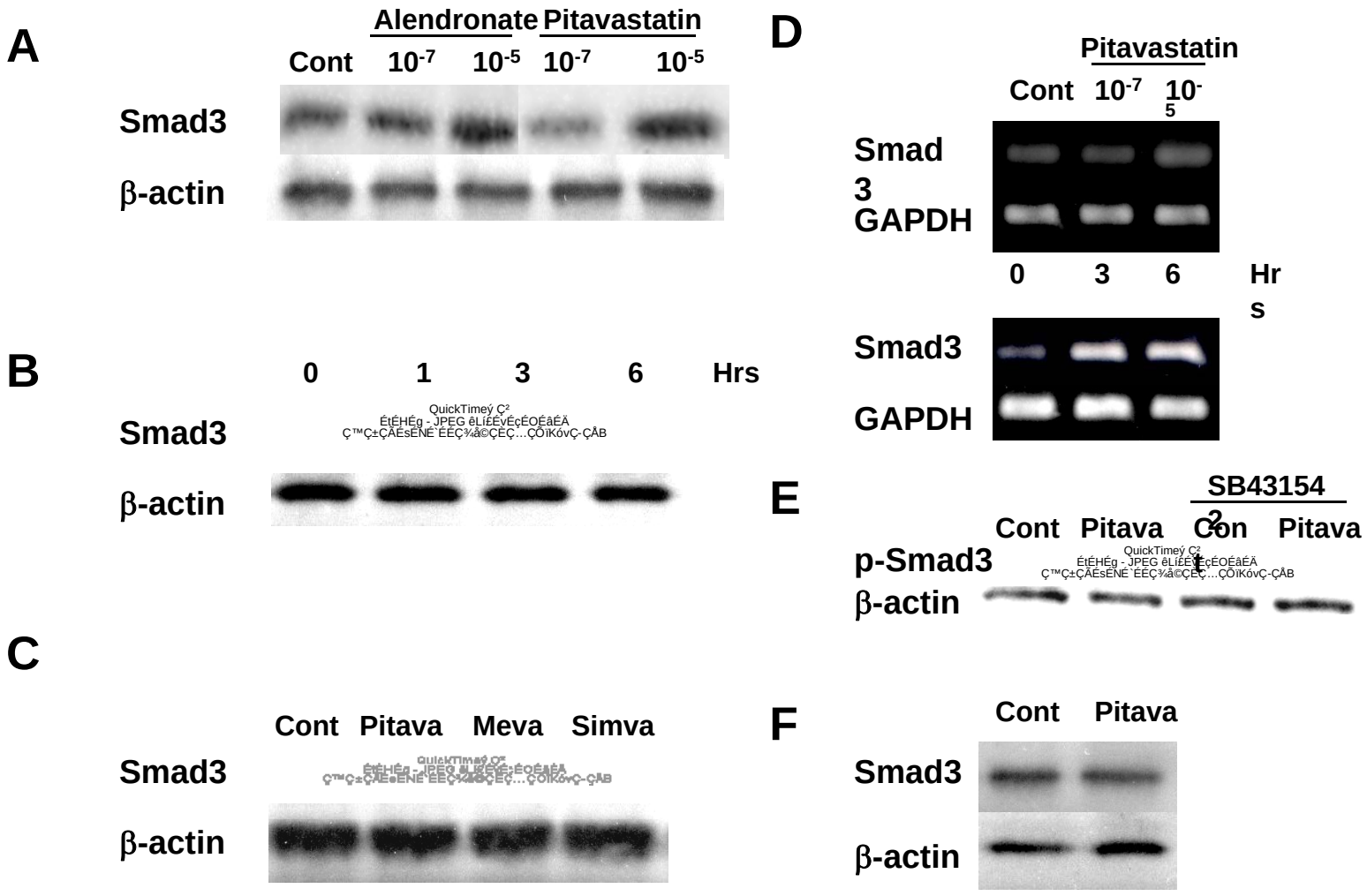
Fig.**4** Pitavastatin reverses etoposide or dexamethasone-mediated cell apoptosis through endogenous TGF-β inUMR-106 cells.

(**A**) UMR-106 cells with or without transient Smad3 transfection were treated without or with 5 x $10^{-5}$ M etoposide for 6 Hr with or without pretreatment with $10^{-5}$ M pitavastatin for 6 Hr in serum-free DMEM. The relative number of apoptotic cells was determined by TUNEL assay as described in Materials and

Methods. Values are the mean±SEM ratio of TUNEL positive/negative cell numbers compared with etoposide-untreated cells. *, P < 0.01 compared with etoposide-only-treated cells.

(**B**) UMR-106 cells were treated without or with $10^{-7}$ M Dex or 5 x $10^{-5}$ M etoposide for 6 Hr without or with pretreatment with the indicated concentrations of pitavastatin for 6 Hr in the presence or absence of 5 μM SB431542 in serum-free DMEM. The relative number of apoptotic cells was determined by TUNEL assay as described in Materials and Methods. Values are the mean ± SEM ratio of TUNEL positive/negative cell numbers compared with Dex- or etoposide-untreated cells (control group). *, *P* < 0.01 compared with Dex or etoposide-only-treated cells. **, P<0.01 compared with Dex and $10^{-5}$ M pitavastatin-treated cells.

# Fig. 1


A
Alendronate
Pitavastatin
Cont
10-7
10-5
10-7
10-5
Smad3
β-actin
B
0
1
3
6
Hrs
Smad3
β-actin
C
Cont
Pitava
Meva
Simva
Smad3
β-actin
D
Pitavastatin
Cont
10-7
10-5
Smad3
GAPDH
0
3
6
Hrs
Smad3
GAPDH
E
SB431542
Cont
Pitava
Con
Pitava
p-Smad3
β-actin
F
Cont
Pitava
Smad3
β-actin

# Fig. 2

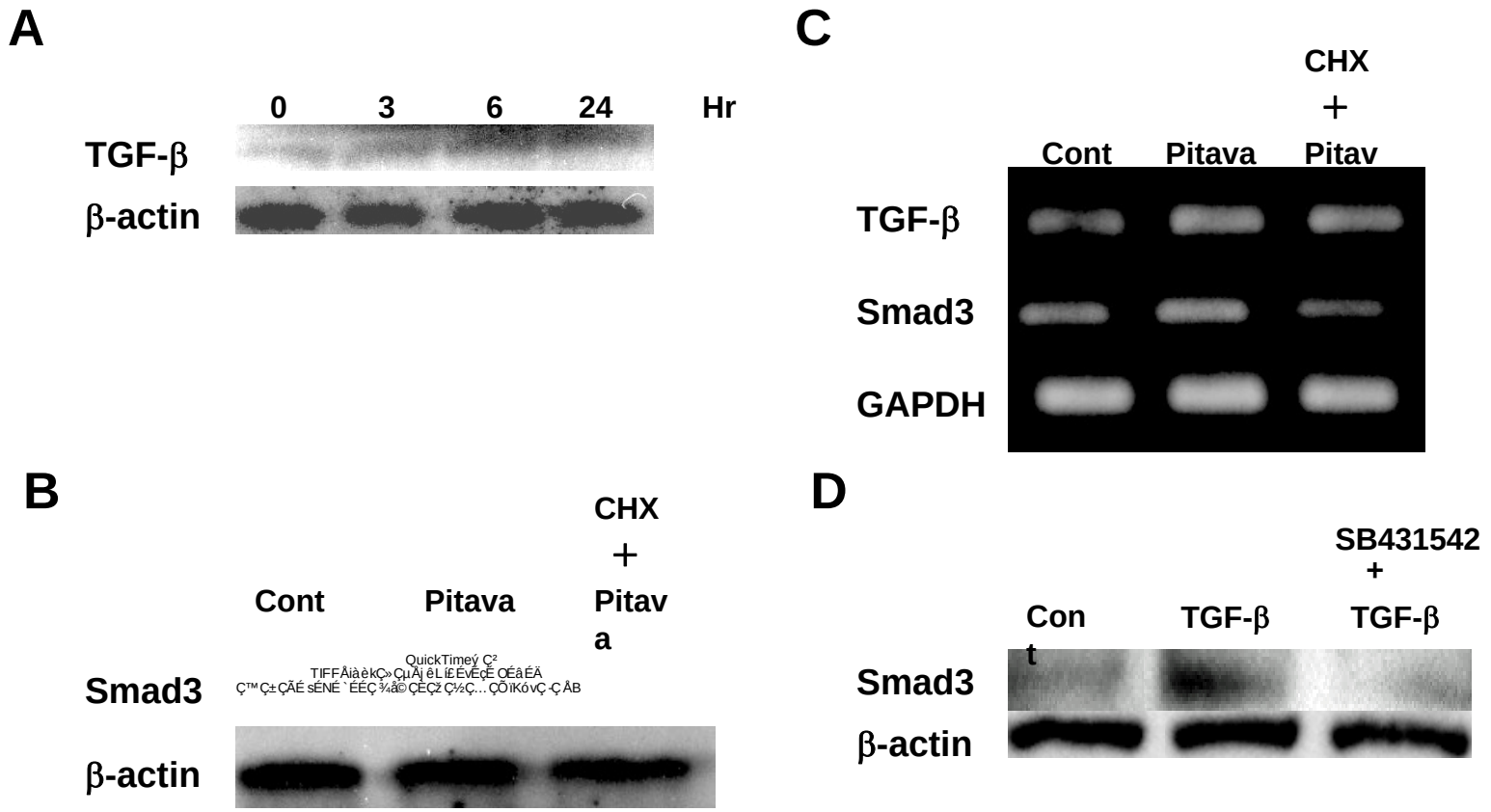

A
0
3
6
24
Hr
TGF-β
β-actin
C
CHX
+
Cont
Pitava
Pitav
TGF-β
Smad3
GAPDH
B
CHX
+
Cont
Pitava
Pitav
a
Smad3
β-actin
D
SB431542
+
Con
t
TGF-β
TGF-β
Smad3
β-actin

**Fig.3**

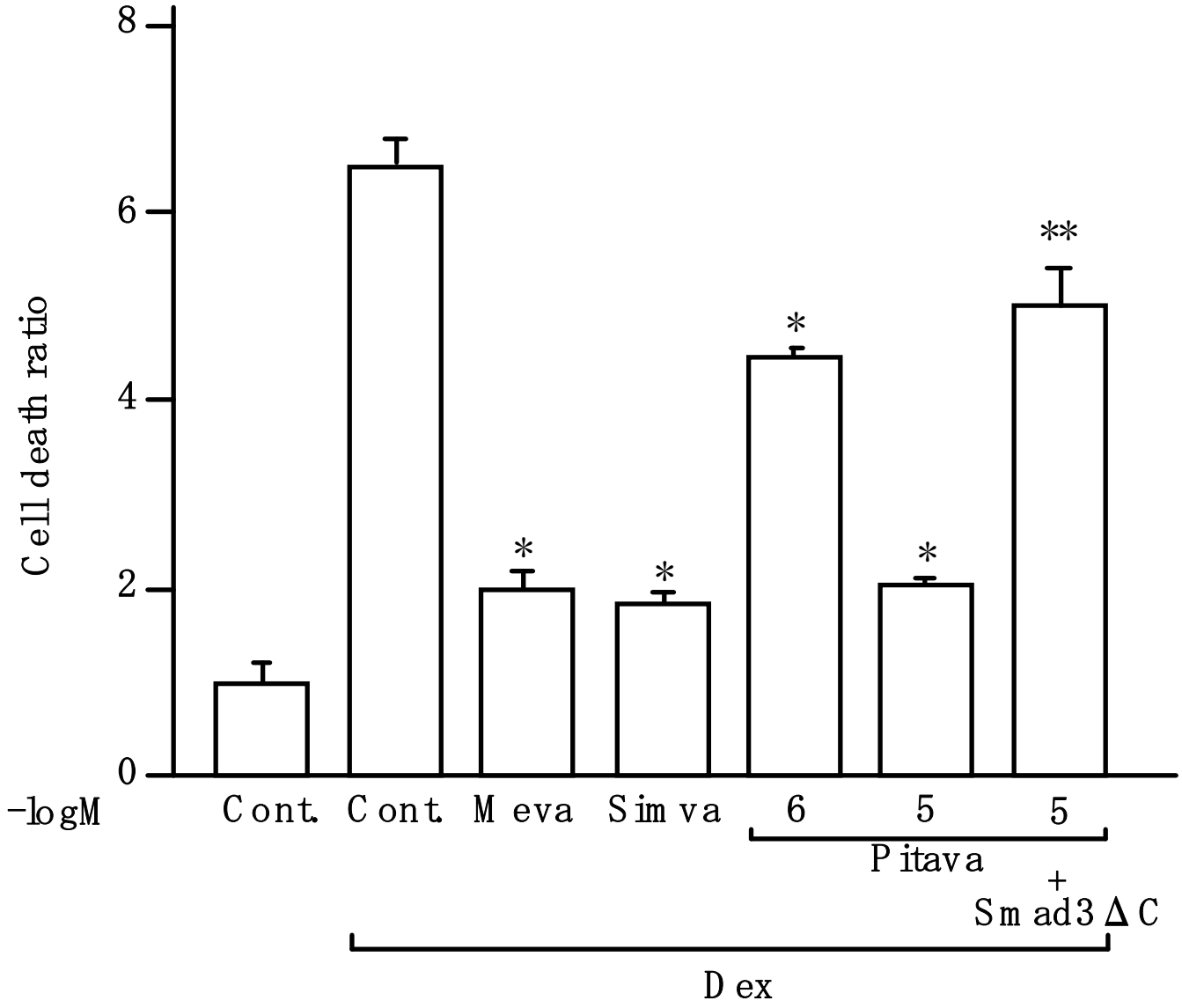
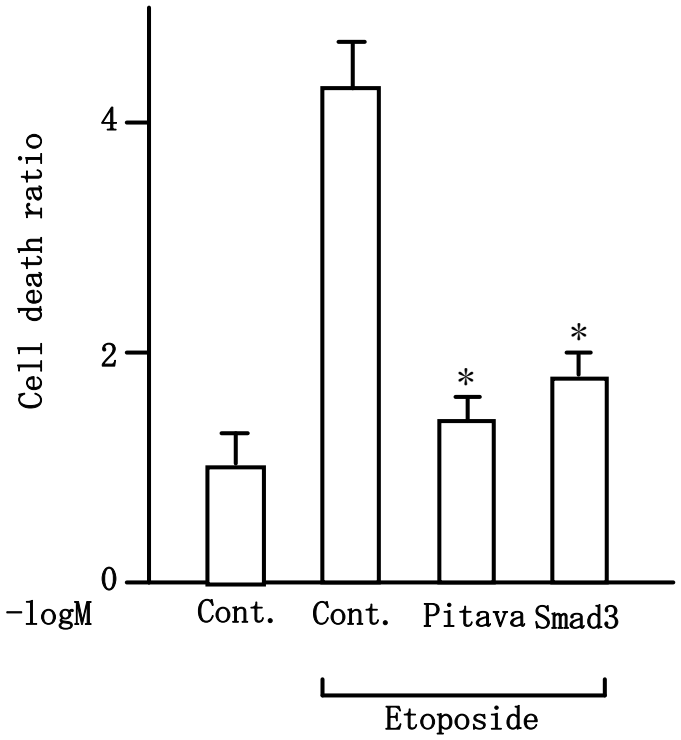
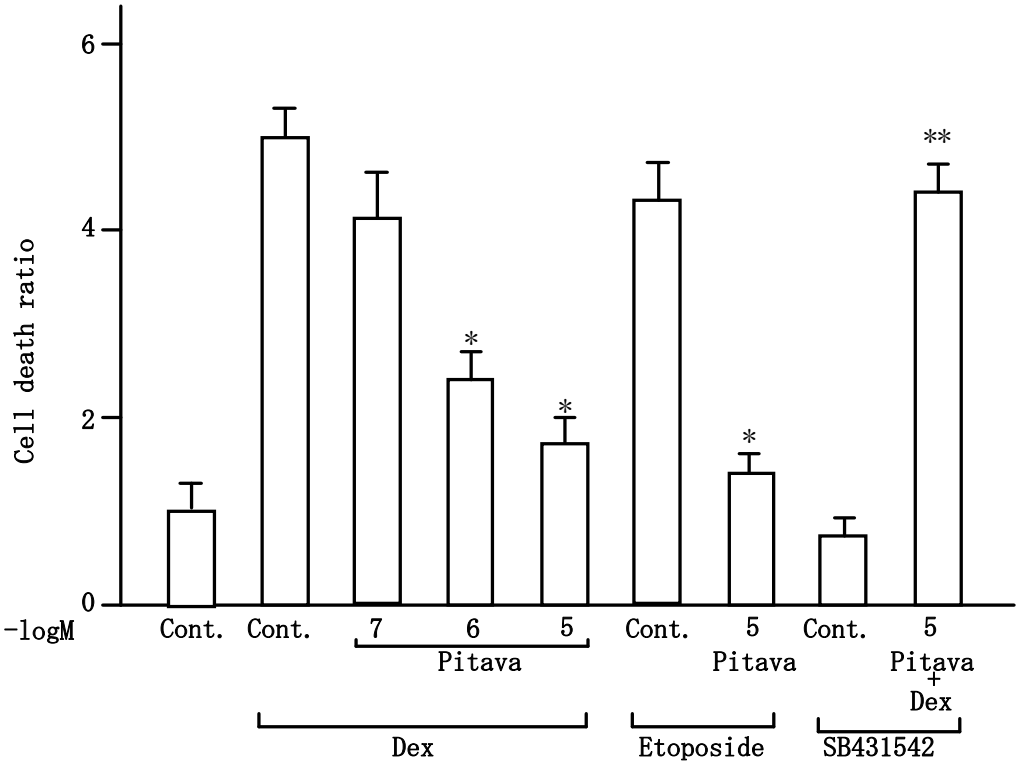

8
6
4
2
0
Cell death ratio
*
*
*
*
**
-logM
Cont.
Cont.
Meva
Simva
6
5
5
Pitava
+
Smad3ΔC
Dex

Fig. 4

A


Cell death ratio
0
2
4
-logM
Cont.
Cont.
Pitava
Smad3
*
*
Etoposide

B


Cell death ratio
0
2
4
6
-logM
Cont.
Cont.
7
6
5
Pitava
Cont.
5
Pitava
Cont.
5
Pitava
+
Dex
*
*
*
**
Dex
Etoposide
SB431542