International Journal of Oral Biology, Vol. 31, No. 3, September 30 2006, p. 93~98

International Journal of Oral Biology

Regulation of ADAMTS-2 by 1,25-Dihydroxyvitamin D3 in Osteoblastic Cells

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(Received August 16, 2006 ; Accepted August 28, 2006)

Biosynthetic processing of fibrillar procollagens is essential for producing mature collagen monomers that polymerize into fibrils by a self-assembly process. The metalloproteinase ADAMTS-2 is the major enzyme that processes the N-propeptide of type I procollagen in the skin and also of type II and type III procollagens. Mutations in the ADAMTS-2 gene cause dermatospraxis in animals and Ehlers-Danlos syndrome VIIC in humans, both of which are characterized by the accumulation of type I pN-collagen and the formation of abnormal collagen fibrils in the skin. Despite its importance in procollagen processing, little is known about the regulation of ADAMTS-2 expression. Here, we demonstrate that ADAMTS-2 can be regulated by 1,25-dihydroxyvitamin D3, an inducer of type I procollagen synthesis. This steroid hormone induced ADAMTS-2 mRNA ~3-fold in MG-63 human osteosarcoma cells and MC3T3-E1 murine osteoblastic cells. This induction was dose- and time-dependent in MG-63 cells. In contrast, secreted ADAMTS-2 protein was increased only 1.4-fold with 1,25-dihydroxyvitamin D3. Finally, 1,25-dihydroxyvitamin D3 in the presence of ascorbate increased levels of secreted ADAMTS-2 1.9-fold over ascorbate treatment alone, which did not appreciably change ADAMTS-2 expression. These data indicate that the regulation of ADAMTS-2 is coupled with the synthesis of type I procollagen through 1,25-dihydroxyvitamin D3 signaling and may involve translational or posttranslational control.

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Introduction

Fibrillar collagens (types I, II, and III) are synthesized and secreted as procollagen precursors with N- and C-propeptides that are extracellularly cleaved by procollagen N-proteinases (PNPs) and procollagen C-proteinases (PCPs), respectively. Three metalloproteinases in the astacin family (BMP-1, mTLD, and mTLL-1) provide PCP activity for procollagens I-III (Kessler *et al.*, 1996; Li *et al.*, 1996; Scott *et al.*, 1999; Suzuki *et al.*, 1996). Other metalloproteinases in the ADAMTS (A Disintegrin And Metalloproteinase with ThromboSpondin motifs) family are responsible for PNP activity: ADAMTS-2 acts on procollagens I-III (Colige *et al.*, 1997; Wang *et al.*, 2003), and ADAMTS-3 and -14 process only type II and type I procollagens, respectively (Colige *et al.*, 2002; Fernandes *et al.*, 2001). Processing of fibrillar procollagens produces mature monomers which are self-assembled into collagen fibrils.

There are indications that the processing of fibrillar procollagens by ADAMTS-2 is critical for the structural integrity of tissues and organs. Mutations in the ADAMTS-2 gene lead to the recessively inherited human connective-tissue disorder Ehlers-Danlos syndrome (EDS) type VIIC and the related animal disease, dermatosparaxis (Colige *et al.*, 1999). The main clinical feature of these diseases is severe skin fragility due to the accumulation of type I pN-collagen, the processing intermediate of type I procollagen that retains the N- but not the C-propeptide (Nusgens *et al.*, 1992; Smith *et al.*, 1992). Participation of type I pN-collagen monomers in collagen fibrillogenesis gives rise to highly irregular type I collagen fibrils with decreased tensile strength (Holmes *et al.*, 1993; Hulmes *et al.*, 1989; Watson *et al.*, 1998). In the skin of *ADAMTS-2* null mice, a large fraction of type I collagen retains uncleaved N-propeptides

(Li *et al.*, 2001), suggesting that ADAMTS-2 is the major PNP for type I procollagen in this tissue.

Despite its importance in biosynthetic processing of fibrillar procollagens, little is known about how ADAMTS-2 expression is regulated. In a previous study, we showed that transforming growth factor-β1 (TGF-β1), a potent inducer of osteoblast differentiation and bone formation, increases expression of ADAMTS-2 mRNA in MG-63 human osteosarcoma cells (Wang *et al.*, 2003). As TGF-β1 also stimulates synthesis of procollagens I-III (Massague, 1990), as well as procollagen C-proteinases BMP-1 and mTLD (Lee *et al.*, 1997), induction of ADAMTS-2 by TGF-β1 is followed by net increases in the formation of the collagenous extracellular matrix (ECM). Synthesis of type I procollagen is also stimulated by 1,25-dihydroxyvitamin D3, another potent inducer of osteoblast differentiation (Beresford *et al.*, 1986; Franceschi *et al.*, 1988; Kojima *et al.*, 1997; Kurihara *et al.*, 1986). However, it has not been previously determined whether like TGF-β1, 1,25-dihydroxyvitamin D3 also induces procollagen processing enzymes for the efficient production of collagenous ECM. Therefore, the present study was undertaken to determine the effect of 1,25-dihydroxyvitamin D3 on the expression of ADAMTS-2.

Materials and Methods

Antibody

The polyclonal peptide antibody 1435, which was directed against sequence in the protease domain of ADAMTS-2 (Wang *et al.*, 2003), was kindly provided by Dr. Mitch Brenner (FibroGen, South San Francisco, CA, USA).

Cell culture

MG-63 human osteosarcoma and MC3T3-E1 murine osteoblastic cells were grown in Dulbecco's modified Eagle's medium (DMEM) and α-minimum essential media (α-MEM), respectively, supplemented with 10% heat-inactivated (30 min, 55°C) fetal bovine serum (FBS), 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin. For treatment with 1,25-dihydroxyvitamin D3 (Sigma, St.Louis, MO, USA), just-confluent cells were rinsed once with serum-free DMEM and then fed with DMEM containing 0.5% heat-inactivated FBS. Unless otherwise noted, 1,25-dihydroxyvitamin D3 treatment of cells was at 10^{-10} M for 24 h (RNA preparation) or 48 h (protein preparation). Ascorbate treatment of cells was at 75 μg /ml.

Reverse transcription (RT)-PCR

Total RNA was extracted from confluent cells in six-well plates using the TRIzol reagent (Invitrogen, Carlsbad, California, USA), treated with DNase I (Takara, Otsu, Shiga, Japan), and quantified photometrically. Before use in PCR, 1 μg of total RNA was reverse transcribed for 1 h at 42°C in a 20 μl reaction containing 100 pmol Poly(dT) and AccuPower RT premix (Bioneer, Daejeon, Korea). The resulting cDNA (1 μl) was amplified by PCR using Ex Taq polymerase (Takara, Otsu, Shiga, Japan) and the following primers: human ADAMTS-2 (sense, 5'-GCTGACACTCATGAACATTG-3'; antisense, 5'-GTCATAGGAGTGCAGGTAGC-3'), mouse ADAMTS-2 (sense, 5'-GACACCAGGATCTCACTG-3'; antisense, 5'-TAGCGAGACAAGACTTCC-3'), human osteocalcin (sense, 5'-AGAGCGACACCCTAGACC-3'; antisense, 5'-AGAGCCCTCACACTCCTC-3'), human COL1A1 (sense, 5'-CCAGAACATCACCTACCACT-3'; antisense, 5'-AGAGATGAATGCAAAGGAAA-3'), human COL3A1 (sense, 5'-TGGTCAAAACTCAGTCTCCT-3'; antisense, 5'-TAATCCTCAAAGGGCAATAA-3'), human β-actin (sense, 5'-GGCGGACTATGACTTAGTTG-3'; antisense, 5'-AGACCAAAAGCCTTCATACA-3'), and mouse β-actin (sense, 5'-AGAAGGACTCCTATGTGG-3'; antisense, 5'-GATCTTGATGGTGCTAGG-3'). PCR reactions were heated at 94°C for 5 min, and then run for 25°C cycles at 94°C for 30 sec, 56°C for 1 min, and 72°C for 1 min. PCR products were resolved on 1% agarose gels and then visualized with ethidium bromide. For relative quantification of gene expression, intensity of each specific-band was estimated using the Gel Documentation System (Vilber Lourmat, Marne-la-Vallee, France).

Western blot analysis

Post culture media were treated with protease inhibitors (2.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM *p*-aminobenzoic acid, 1 mM *N*-ethylmaleimide) and with 10% trichloroacetic acid to precipitate proteins. Pellets were washed with ice-cold acetone and then washed twice with 75% ethanol, 12.5 mM Tris (pH 7.5). The samples were air-dried, resuspended in hot SDS sample buffer with 5% β-mercaptoethanol, and subjected to 5% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). Blots were blocked for 1 h with 2% bovine serum albumin (BSA) in T-PBS (PBS, 0.05% Tween 20) and incubated overnight with anti-ADAMTS-2 1435 antibody diluted 1 : 1000 in T-PBS, 1% BSA. After washing with T-PBS, blots were incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1 : 5000 in T-PBS, 1% BSA. Blots were washed with T-PBS, incubated with SuperSignal West Pico substrate (Pierce, Rockford, IL, USA), and exposed to film.

Results

1,25-Dihroxyvitamin D3 increases steady state levels of ADAMTS-2 mRNA in osteoblastic cells

Previous studies have shown that 1,25-dihydroxyvitamin

D3 is a potent regulator of osteoblast differentiation and function (Franceschi *et al.*, 1985; Franceschi *et al.*, 1987; Franceschi *et al.*, 1988). In MG-63 osteosarcoma cells, this hormone induces expression of the osteoblast phenotype that includes elevated levels of type I procollagen (Franceschi *et al.*, 1988). To determine whether increases in synthesis of type I procollagen by 1,25-dihydroxyvitamin D3 is paralleled by induction of procollagen N-proteinase (PNP) activity, we attempted to examine levels of ADAMTS-2 mRNA in 1,25-dihydroxyvitamin D3-treated or untreated MG-63 cells. When just-confluent MG-63 cells were treated for 24 h with 100 pM 1,25-dihydroxyvitamin D3, levels of ADAMTS-2 mRNA increased 3.5-fold, while this induction was not observed for the β-actin RNA control (Fig. 1A,C). Consistent with previous reports (Bonewald *et al.*, 1992; Lajeunesse *et al.*, 1991), 1,25-dihydroxyvitamin D3 also increased steady state levels of osteocalcin, COL1A1 [the gene of pro-α1(I) chain], and COL3A1 [the gene of pro-α1(III) chain] RNA (Fig. 1A), suggesting differentiation of MG-63 cells into a more osteoblastic phenotype. 1,25-Dihydroxyvitamin D3 similarly induced ADAMTS-2 mRNA in MC3T3-E1 osteoblastic mouse cells (Fig. 1B,C)

Incubations of MG-63 cells with 1,25-dihydroxyvitamin D3 concentrations, ranging from 1 to 500 pM, showed induction of ADAMTS-2 RNA to be dose-dependent (Fig. 2), with maximum induction at 100 pM. We then determined kinetics of ADAMTS-2 mRNA induction by treating MG-63 cells 100 pM 1,25-dihydroxyvitamin D3 for varying times. As can be seen in Fig. 3, substantial increases were first noticed at 6~12 h, with maximal induction at ~24 h post-treatment.

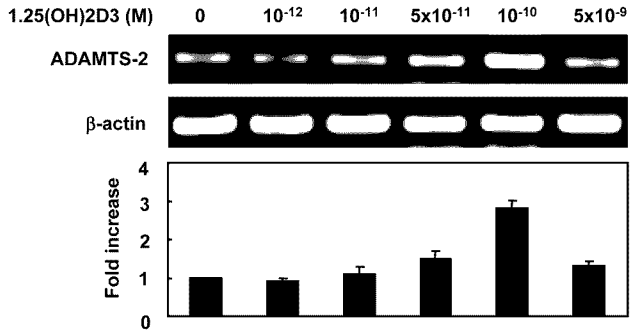

Fig. 2. 1,25-Dihydroxyvitamin D3 elevates steady state levels of ADAMTS-2 mRNA in a dose-dependent manner. MG63 cells were treated with increasing amounts of 1,25-dihydroxyvitamin D3 for 24 h. Total RNA was isolated from the cells and analyzed by RT-PCR for ADAMTS-2 (*top panel*) and β-actin (*middle panel*) mRNA expression. Fold quantitation of ADAMTS-2 mRNA induction is shown in the *bottom panel*. Values represent mean± S.D. of three independent experiments.

Levels of secreted ADAMTS-2 protein are increased by 1,25-dihroxyvitamin D3 in MG-63 cells

We next determined whether induction of ADAMTS-2 RNA in MG-63 cells is followed by increases in the production of its cognate protein. Therefore, levels of ADAMTS-2 protein secreted into culture media were determined by Western blot analysis using an anti-

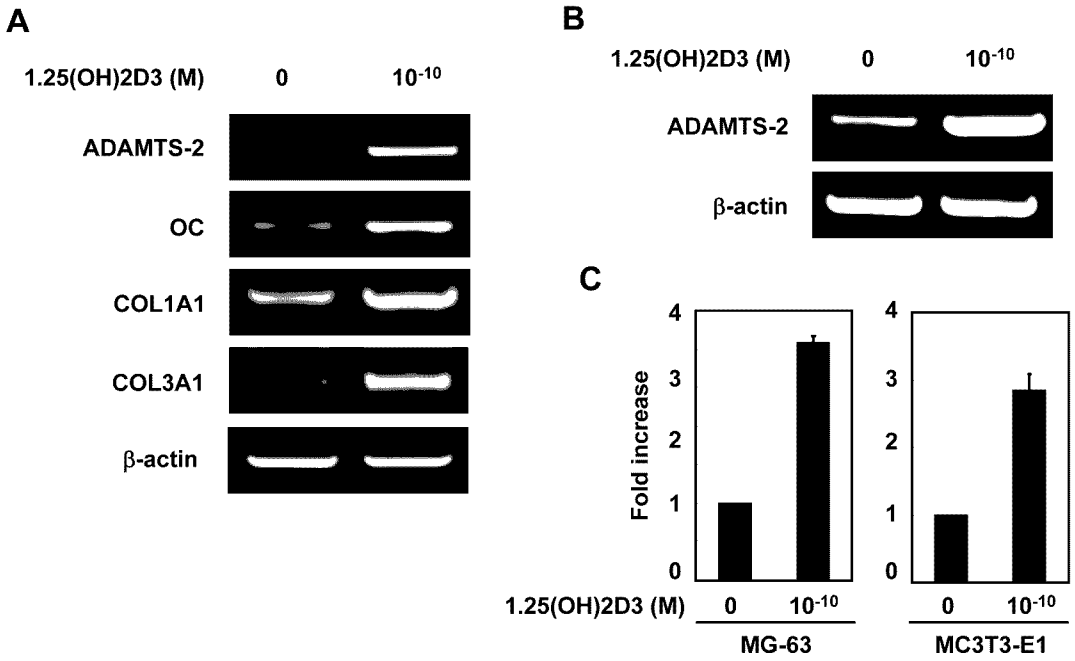

Fig. 1. 1,25-Dihydroxyvitamin D3 [1,25(OH)2D3] induces ADAMTS-2 mRNA in osteoblastic cells. Total RNA from MG-63 cells (A) and MC3T3-E1 cells (B), cultured in the absence and presence of 100 pM 1,25$(OH)_2$-vitamin D3 for 24 h, was analyzed by RT-PCR for the expression of ADAMTS-2, osteocalcin (OC), COL1A1, COL3A1, and β-actin mRNA. (C) Fold quantitation of ADAMTS-2 mRNA induction. Values represent mean± S.D. of three independent experiments.

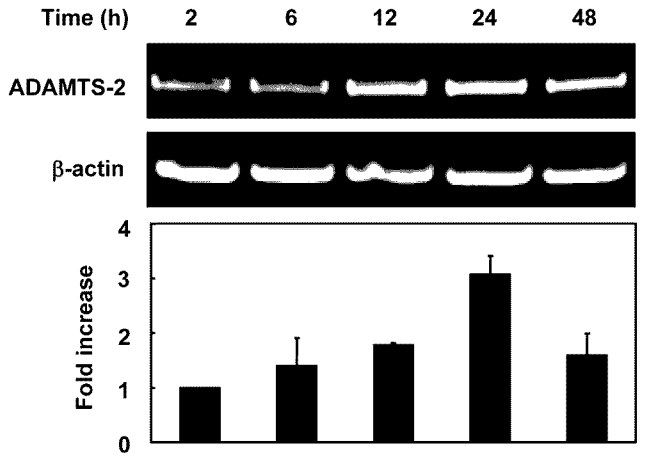

Fig. 3. 1,25-Dihydroxyvitamin D3 elevates steady state levels of ADAMTS-2 mRNA in a time-dependent manner. MG63 cells were treated with 100 pM 1,25-dihydroxyvitamin D3 for varying amounts of time. Total RNA was isolated from the cells and analyzed by RT-PCR for ADAMTS-2 (*top panel*) and β-actin (*middle panel*) mRNA expression. Fold quantitation of ADAMTS-2 mRNA induction is shown in the *bottom panel*. Values represent mean± S.D. of three independent experiments.

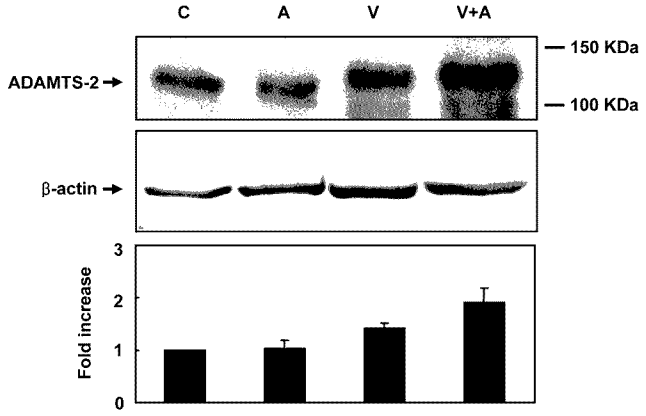

Fig. 4. Induction of secreted ADAMTS-2 protein by 1,25-dihydroxyvitamin D3 and ascorbate. Media from MG-63 cell culture were examined by Western blot analysis using peptide antibody 1435 which is directed against sequences in the ADAMTS-2 protease domain (Wang *et al.*, 2003). Lanes C, C+A, V, and V+A are from untreated control cultures or from cultures treated with ascorbate, 1,25-dihydroxyvitamin D3, or ascobate plus 1,25-dihydroxyvitamin D3, respectively. The anti-β-actin blot is shown as a loading control. Fold quantitation of ADAMTS-2 protein induction is shown in the *bottom panel*. Values represent mean± S.D. of three independent experiments.

ADAMTS-2 antibody 1435, which is able to detect the endogenous ~132 kDa ADAMTS-2 protein (Wang *et al.*, 2003). As shown in Fig. 4, ADAMTS-2 protein was up-regulated by 1,25-dihydroxyvitamin D3. However, the increase in ADAMTS-2 protein (1.4 fold) was not proportional to the increase in ADAMTS-2 mRNA (3 fold).

Ascorbate stimulates synthesis of type I procollagen at transcriptional and posttranscriptional levels (Chan *et al.*, 1990). Since we were interested in regulatory mechanisms by which ADAMTS-2 and type I procollagen are coordinately regulated, we examined whether production of ADAMTS-2 was also up-regulated by ascorbate treatment. Ascorbate alone did not appreciably increase levels of secreted ADAMTS-2 protein. However, when added simultaneously with 1,25-dihydroxyvitamin D3, it induced secreted ADAMTS-2 protein 1.9-fold (Fig. 4).

Discussion

An active metabolite of vitamin D, 1,25-dihydroxyvitamin D3, is known to increase accumulation of type I collagen fibrils in the extracellular matrix. Early studies with osteoblastic cells indicated that this steroid hormone stimulates type I procollagen synthesis (Beresford *et al.*, 1986; Franceschi *et al.*, 1988; Kojima *et al.*, 1997; Kurihara *et al.*, 1986). In this paper, we have demonstrated that 1,25-dihydroxyvitamin D3, in conjunction with ascorbate, also stimulates synthesis of ADAMTS-2 protein, the major PNP that processes type I procollagen. The coordinated induction of fibrillar procollagens and their processing enzymes is also accomplished by transforming growth factor-β1 (TGF-β1), prototype of the TGF-β superfamily. This growth factor increases the synthesis of type I procollagens, ADAMTS-2, BMP-1, and mTLD (Ignotz *et al.*, 1987; Lee *et al.*, 1997; Wang *et al.*, 2003), which leads to net increases in the accumulation of type I collagen fibrils. Interestingly, TGF-β1 and 1,25-dihydroxyvitamin D3 synergistically increase synthesis of type I collagen in MG-63 human osteosarcoma cells (Bonewald *et al.*, 1992), suggesting interaction between the two factors. Although Smad 3, a component of the TGF-β signaling pathway, has been shown to potentiate vitamin D receptor (VDR) function (Yanagisawa *et al.*, 1999), it still remains to be determined which factors mediate cross-talk between TGF-β1 and 1,25-dihydroxyvitamin D3 during collagen production.

Our results indicate that the regulation of ADAMTS-2 synthesis by 1,25-dihydroxyvitamin D3 is complex. In osteoblastic cells, 1,25-dihydroxyvitamin D3 treatment increased steady state levels of ADAMTS-2 mRNA about 3-fold. However, this induction of ADAMTS-2 mRNA was not paralleled by comparable increases in its cognate protein, suggesting the involvement of other factors in the translational or posttranslational regulation of ADAMTS-2 synthesis. Therefore, future studies are needed to elucidate the mechanism by which ascorbate potentiates 1,25-dihydroxyvitamin D3-mediated induction of the ADAMTS-2 protein.

Acknowledgements

This work was supported by the Seoul Research and

Business Development Program (10548).

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