

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

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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- β 1 (TGF- β 1), 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- β 1 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- β 1 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- β 1, 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 μ g/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 μ g of total RNA was reverse transcribed for 1 h

at 42°C in a 20 μ l reaction containing 100 pmol Poly(dT) and AccuPower RT premix (Bioneer, Daejeon, Korea). The resulting cDNA (1 μ l) 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'-TGGTCAAACTCAGTCTCCT-3'; antisense, 5'-TAATCCTCAAAGGGCAATAA-3'), human β -actin (sense, 5'-GGCGGACTATGACTTAGTTG-3'; antisense, 5'-AGACCAAAGCCCTTCATACA-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-Dihydroxyvitamin 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- α 1(I) chain], and COL3A1 [the gene of pro- α 1(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

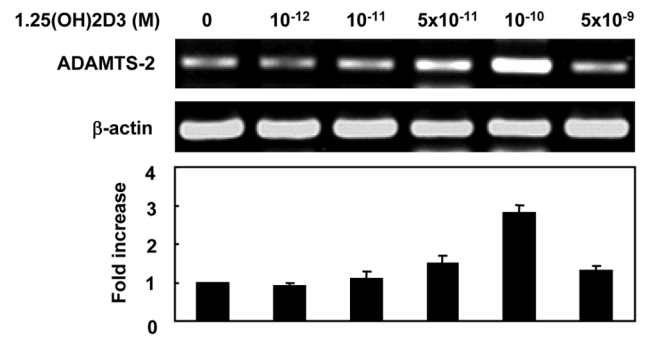


Fig. 2. 1,25-Dihydroxyvitamin D3 elevates steady state levels of ADAMTS-2 mRNA in a dose-dependent manner. MG63 cells were treated with increasing amounts of 1,25-dihydroxyvitamin D3 for 24 h. Total RNA was isolated from the cells and analyzed by RT-PCR for ADAMTS-2 (*top panel*) and β -actin (*middle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

first noticed at 6~12 h, with maximal induction at ~24 h post-treatment.

Levels of secreted ADAMTS-2 protein are increased by 1,25-dihydroxyvitamin 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-

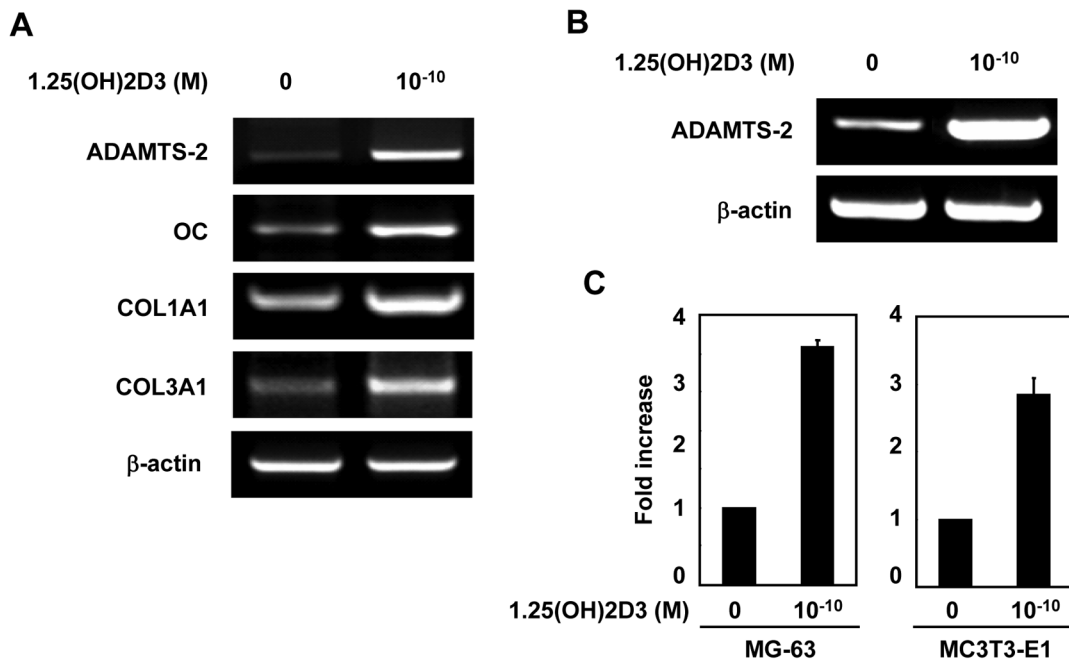


Fig. 1. 1,25-Dihydroxyvitamin D3 [1,25(OH)₂D₃] induces ADAMTS-2 mRNA in osteoblastic cells. Total RNA from MG-63 cells (A) and MC3T3-E1 cells (B), cultured in the absence and presence of 100 pM 1,25(OH)₂-vitamin D₃ for 24 h, was analyzed by RT-PCR for the expression of ADAMTS-2, osteocalcin (OC), COL1A1,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

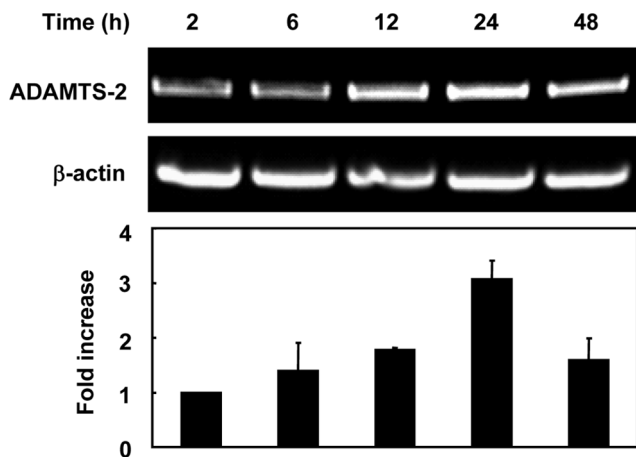


Fig. 3. 1,25-Dihydroxyvitamin D3 elevates steady state levels of ADAMTS-2 mRNA in a time-dependent manner. MG63 cells were treated with 100 pM 1,25-dihydroxyvitamin D3 for varying amounts of time. Total RNA was isolated from the cells and analyzed by RT-PCR for ADAMTS-2 (*top panel*) and β -actin (*middle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

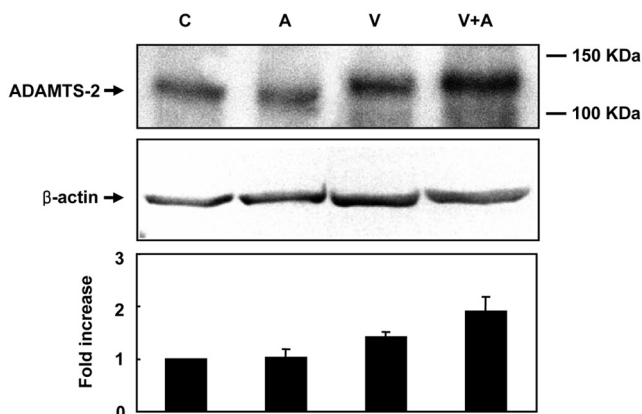


Fig. 4. Induction of secreted ADAMTS-2 protein by 1,25-dihydroxyvitamin D3 and ascorbate. Media from MG-63 cell culture were examined by Western blot analysis using peptide antibody 1435 which is directed against sequences in the ADAMTS-2 protease domain (Wang *et al.*, 2003). Lanes C, C+A, V, and V+A are from untreated control cultures or from cultures treated with ascorbate, 1,25-dihydroxyvitamin D3, or ascorbate 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 \pm 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- β 1 (TGF- β 1), prototype of the TGF- β superfamily. This growth factor increases the synthesis of type I procollagens, ADAMTS-2, BMP-1, and mTLD (Igotz *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- β 1 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- β 1 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.

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