Vitamin D Inhibits Activities of Metalloproteinase-9/-13 in Articular Cartilage In Vivo and In Vitro

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Summary Low levels of serum vitamin D have been shown to accelerate progression of osteoarthritis. However, the role of vitamin D in articular cartilage degradation and osteoarthritis development is still unclear. This study investigated the effects of vitamin D on articular cartilage degradation by testing matrix metalloproteinase (MMPs) activities in articular cartilage using the rat vitamin D deficiency model at the animal level and rat articular chondrocytes at the cell level. The in vivo study showed vitamin D deficiency increased the expressions of MMP-9 and MMP-13 in rat articular cartilage, and the increase was inhibited by 1α,25(OH)2D3 supplementation. The increased production of MMP-9 and MMP-13 in the articular chondrocytes induced by tumor necrosis factor-α (TNF-α) or phorbol-12-myristate-13-acetate (PMA) was significantly suppressed by concomitant treatment with 1α,25(OH)2D3 in vitro. The increased level of C-telopeptide of type II collagen (CTX-II) induced by TNF-α or PMA was also significantly suppressed by concomitant treatment with 1α,25(OH)2D3 in vitro. Thus, vitamin D intake may inhibit MMP activities and take part in the process of articular cartilage degeneration and osteoarthritis progression.

Key Words vitamin D, matrix metalloproteinases, articular cartilage, chondrocytes, TNF-α, PMA

Vitamin D emerges as an important fat-soluble nutrient with multiple functions in cancers, autoimmune diseases, infectious diseases, and cardiovascular diseases (1). Conflicting evidences regarding the role of vitamin D in the pathogenesis of osteoarthritis (OA) still exist in the literature (2–9). Although some clinical studies have shown no significant correlation between serum vitamin D levels and joint space loss (2, 4), recent observations suggest that the incidence and prevalence of osteoarthritis and the joint space loss of osteoarthritis increase more pronouncedly in populations with low serum 25-(OH)D3 levels (3, 5). Studies also have shown that vitamin D deficiency is associated with an increased risk of incidence of hip OA (6) and progression of knee OA (7), and 1α,25(OH)2D3 administration might inhibit the progression of arthritis (8). An in vivo animal study demonstrated that vitamin D deficiency could aggravate cartilage erosion in virtue of ovariectomy, which was significantly reduced by 1α,25(OH)2D3 supplementation (9). The exact role of vitamin D in articular OA remains unclear and exploration of the possible mechanism is demanded.

Homeostasis of articular cartilage is disrupted by reduced anabolic and elevated catabolic turnover. Catabolic activities of OA chondrocytes are related to the elevated release of matrix metalloproteinases (MMPs), a family of proteolytic enzymes, which contribute to degradation of the extracellular matrix of cartilage and bone resorption (10–12). 1α,25(OH)2D3 administration could cause significantly decreased collagenase activity in growth plates (13, 14). An in vitro study has shown that vitamin D receptors (VDRs) might contribute to the complex regulation of MMP production from chondrocytes in articular cartilage (15). Whether vitamin D could regulate MMP activities in vivo might help better explain the correlation between vitamin D and OA. This study hypothesizes that vitamin D could affect MMPs and influence catabolic activities in articular cartilage and aims to investigate the effects of vitamin D deficiency in diets on MMP activities in articular cartilage in vivo, and the effects of vitamin D treatment on MMP activities and related type II collagen degradation in vivo and in vitro.

MATERIALS AND METHODS

Animals. Eighteen female Sprague Dawley rats, 6 mo of age, were randomly allocated into three groups: the control group (CTL, fed on the control diet containing 1% calcium, 0.65% phosphorous, and 1,000 IU/kg of vitamin D for 10 wk), the vitamin D deficient-diets group (VDD, fed on the vitamin D deficient diet containing 1% calcium, 0.65% phosphorous, and vitamin D lower than 50 IU/kg for 10 wk) and the treatment
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Fig. 1. Comparison of serum levels of 1α,25(OH)2D3 and weights in all groups. The 10-wk vitamin D deficient diets resulted in a significant decrease in serum 1α,25(OH)2D3 levels compared with the control diets (34.2 ± 1.8 pg/mL versus 33.5 ± 2.3 pg/mL). Error bars indicate SE.

group (TRE, 0.1 μg/kg/d 1α,25(OH)2D3 for 3 d after 10-wk vitamin D deficient-diet feeding). 1α,25(OH)2D3 (Selleckchem, Houston, USA) was dissolved in ethanol and diluted in corn oil and was administered orally with a gavage cannula 14 h after lights on (16). Ultraviolet-free light (Philips, Amsterdam, The Netherlands) was provided for vitamin-D deficient diets rats. All the rats were housed 3 per cage at 25˚C with a 12-h light/12-h dark cycle. All experiments were approved by the Animal Ethics Committee of Peking University, and were performed according to Guidelines for the Care and Use of Laboratory Animals.

Enzyme-linked immunosorbent assay (ELISA). The rats were fasted (>12 h) before blood collection. Blood was sampled before sacrifice for assessment of serum 1α,25(OH)2D3. The serum samples were stored at −80˚C until being assayed. Serum 1α,25(OH)2D3 levels were measured using a competitive binding assay kit (HCB, Ottawa, Canada), with a sensitivity range of 1.0 pg/mL. All ELISA results were calculated as pg/mL.

Histology and immunohistochemistry. After euthanization and careful dissection, the entire knee joints were isolated and fixed in 4% formaldehyde for 24 h followed by decalcification in 10% ethylene diamine tetraacetic acid (EDTA) for 4–5 wk. The decalcified knee joints were cleaved along the medial collateral ligament into two sections and embedded in paraffin. Coronal sections (5 μm thick) were cut across the medial ligament (17, 18).

Five-micrometer thick paraffin tissue sections were deparaffinized in xylene and rehydrated in a reverse-graded series of ethanol and distilled water. For antigen retrieval, tissue sections were treated with pepsin (ZSGB-BIO, Beijing, China) for 30 min at 37˚C, followed by three 5-min washes with water. After quenching of endogenous peroxidase and blocking of nonspecific binding, sections were incubated overnight at 4˚C with either anti-rat MMP-13 or MMP-9 (1:200; both from Abcam, Cambridge, UK). The color was developed by incubation in DAB (ZSGB-BIO). The sections were counter-stained with hematoxylin. All sections were semi-quantitatively analyzed using Image Pro Plus (Version 6.0, Media Cybernetics, Rockville, USA).

Zymography. The MMP Zymography assay kit (Applygen Technologies, Beijing, China) was used for gelatin zymography to observe activities of MMP-9. Articular cartilage was carefully cut using a surgical scalpel. The protein extracted from articular cartilage was diluted with 4× non-reducing sample buffer (0.25 M Tris base 2.5% sodium dodecyl sulphate (SDS), 12.5% glycerol, 0.1% bromophenol blue) and subjected to electrophoresis on a 0.1% SDS polyacrylamide gel copolymerized with gelatin (1 mg/mL) as the substrate. After electrophoresis, gels were incubated in zymogram renaturing buffer for 30 min with gentle shaking followed by zymogram developing buffer for 20 h at 37˚C. Then gels were stained with 0.5% Coomassie brilliant blue, and then destained with 10% acetic acid and 30% methanol buffer. A positive control was also electrophoresed.

Western-blot analysis. Total proteins from articular cartilage were fractionated by SDS–polyacrylamide gel electrophoresis. After transfer to a nitrocellulose membrane (Sangon, Shanghai, China), antibodies against MMP-13 were used. The membranes were blocked in 5% skimmed milk in phosphate buffer saline (PBS)-Tween 20 for 1 h at room temperature and incubated overnight at 4˚C with the primary antibodies. The expression of MMP-13 protein was detected by enhanced chemiluminescence using peroxidase-labelled secondary antibodies. The bands were assessed by densitometry.

Articular chondrocyte culture. Articular cartilage from newborn Sprague-Dawley rats was enzymically digested to provide chondrocyte culture as previously described (19). Cartilage tissue was sequentially digested and the cell suspension was transferred to 25 cm² culture flasks. Toluidine blue staining and collagen II immunostaining were used to confirm the chondrocytes. Existence of vitamin D receptors in rat chondrocytes was tested through immunohistochemistry. Passage two was harvested and seeded into 24-well culture dishes at 1.0×10⁵ cells/cm². Chondrocyte cultures were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) +10% (v/v) fetal calf serum (FCS). The medium was changed to serum free medium once until sub-confluency. Four wells of chondrocytes of each group were cultured under one of the following serum-free conditions: in DMEM only, in DMEM containing 10 nM 1α,25(OH)2D3, in DMEM-
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containing 20 ng/mL tumor necrosis factor-α (TNF-α) (Sino Biological, Beijing, China), in DMEM containing 10 ng/mL phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, St. Louis, MO), in DMEM containing 20 ng/mL TNF-α and 10 nm 1α,25(OH)2D3, or in DMEM containing 10 ng/mL PMA and 10 nm 1α,25(OH)2D3. The conditioned media were collected after 24 h and assayed for MMP-9, MMP-13 and C-telopeptide of type II collagen (CTX-II) using ELISA.

Statistical analysis. Statistical analysis was performed with SPSS (v16.0, SPSS Inc., Chicago, USA). All data are presented as the mean±SE. Differences were evaluated by one-way analysis of variance (ANOVA), followed by pairwise comparison of the selected groups using Fisher’s protected least significant difference test. *p* values less than 0.05 were considered significant.

**RESULTS**

**Serum 1α,25(OH)2D3 levels**

The rats’ body weights were not significantly different among any of the groups. The effects of vitamin D deficiency on the serum 1α,25(OH)2D3 levels were demonstrated by comparing the CTL, VDD and TRE groups in Fig. 1. The average serum 1α,25(OH)2D3 concentration in the VDD group was significantly lower than that of the CTL group (*p*<0.05). Serum 1α,25(OH)2D3 concentration in the TRE group was significantly higher than in the VDD group (*p*<0.05).

**Expressions of MMP-9 and MMP-13 in vivo**

The expressions of MMP-9 and MMP-13 in articular cartilage in all the groups were evaluated by immunohistochemistry assays, Western blot or zymography (Fig. 2). Weak staining with MMP-9 and MMP-13 was observed in the articular cartilage of the CTL rats. Vitamin D deficiency diets resulted in significant increases of MMP-9 and MMP-13 expressions compared with the
CTL rats. By contrast, 1α,25(OH)₂D₃ supplementation significantly decreased MMP-9 and MMP-13 expressions in articular cartilage compared with the VDD group.

**Expressions of MMP-9 and MMP-13 in vitro**

VDRs were present in rat articular chondrocytes (Fig. 3D). Both MMP-9 and MMP-13 activities in chondrocytes were increased significantly by either TNF-α or PMA stimulation. The increased expressions of MMP-9 and MMP-13 stimulated by TNF-α or PMA were both significantly suppressed by 1α,25(OH)₂D₃ treatment. For normal chondrocytes without any stimulation, 1α,25(OH)₂D₃ treatment had no significant effects on MMP-9 expression, while it significantly increased MMP-13 expression (Fig. 4).

**CTX-II release from in vitro chondrocytes**

CTX-II is an important OA biological marker and can reflect degradation of the articular cartilage. CTX-II release from the extracellular matrix was increased significantly by either TNF-α or PMA stimulation. The increased level of CTX-II stimulated by TNF-α or PMA was both significantly suppressed by 1α,25(OH)₂D₃ treatment. For normal chondrocytes without any stimulation, 1α,25(OH)₂D₃ treatment had no significant effects on the level of CTX-II (Fig. 4).

**DISCUSSION**

MMPs are a family of protein catabolic enzymes that are capable of degrading all of the components of the extracellular matrix (20). They play an important role in arthritic diseases such as rheumatoid arthritis and osteoarthritis, where inflammatory processes stimulate cartilage cells to produce higher activities of MMPs leading to tissue destruction, such as MMP-13 and MMP-9 (21–23). MMP-13, the predominant collagenase in the rat, has the ability to specifically cleave triple helical collagen (24). In addition, it has been reported that the mRNA expression of MMP-13 is regulated by 1α,25(OH)₂D₃ in rat growth plate chondrocytes (25). After MMP-13 initially cleaves triple helical collagen, MMP-9 (gelatinase B) is involved in collagen destruction and the expression of MMP-9 is increased in osteoarthritic cartilage (26). To better understand the role of vitamin D in OA articular cartilage, investigation of MMP activities, especially that of MMP-13 and MMP-9, is of great significance.

The results suggest that vitamin D deficiency might be detrimental to articular cartilage through its modulation of MMP activity. Using a rat vitamin D deficiency model, the in vivo study demonstrated up-regulation of MMPs in the articular chondrocytes under vitamin D deficiency and down-regulation of MMPs with 1α,25(OH)₂D₃ supplementation. The in vitro study demonstrated the inhibition of the increased MMPs induced by TNF-α or PMA under 1α,25(OH)₂D₃ treatment. Other studies showed that the 25-hydroxyvitamin D 1α-hydroxylase knockout (1α(OH)ase−/−) in mice...
has direct effects on the process of OA (27). Vitamin D deficiency might be detrimental to articular cartilage, and 1α,25(OH)2D3 supplementation might be an effective therapeutic way to limit articular arthritis through its modulation of MMP activity. For the in vivo study, a rat vitamin D deficiency model was used to imitate the status of vitamin D intake deficit which was proved by the decreased serum 1α,25(OH)2D3 levels during the study period compared with those of the control-diet group (28). It is worth noting that supplementation of vitamin D to vitamin D deficiency rats could only partly reverse the MMP increase. MMP activities couldn’t be decreased below that of the control rats. To the best of our knowledge, the present study is the first to elucidate the effects of vitamin D deficiency in diets on MMP activities in articular cartilage in vivo.

Increases of both MMP-9 and MMP-13 stimulated by TNF-α or PMA were inhibited by 1α,25(OH)2D3 treatment. The effect of vitamin D on MMPs depends on chondrocyte status. On normal chondrocytes, vitamin D had little effect. In contrast, vitamin D effectively regulated the osteoarthritic chondrocytes’ activities.

Interestingly, at the cell level, the results showed that production of MMP-9 by the ‘healthy’ chondrocytes without any stimulation was not affected significantly by 1α,25(OH)2D3 treatment, while MMP-13 production by the ‘healthy’ chondrocytes was significantly increased by 1α,25(OH)2D3 treatment. Chen et al.’s study also showed that without any stimulation, 1α,25(OH)2D3 activates MMP13 gene expression in chondrocytes (29). Tetlow and Woolley’s study showed that 1α,25(OH)2D3 alone had no effect on MMP production by rheumatoid synovial fibroblasts in monolayer culture, but the simultaneous addition of 1α,25(OH)2D3 with IL-1β reduced the expected stimulation of MMP-1, MMP-3 and MMP-9 by up to 50% (30). All the results demonstrated that the different status of chondrocytes might account for different responses to vitamin D treatment. It is worth noting that 1α,25(OH)2D3 treatment has no significant effects on the overall level of CTX-II. However, MMP-9, MMP-13 and CTX-II were affected by 1α,25(OH)2D3 treatment once the chondrocytes were stimulated by TNF-α or PMA. The mechanism by which chondrocytes with different status respond to 1α,25(OH)2D3 treatment is still unclear and might deserve further investigation.

Although MMP-9 and MMP-13 are prominent and well characterized MMPs in rats (24, 25), there are many other enzymes in this family. Testing the reaction of more MMPs to vitamin D in future studies may help to improve our understanding of MMP activities in the articular cartilage. In this study, the effect of vitamin D treatment could be observed within 3 d. A previous study showed that the effect of vitamin D treatment on the growth plate could be observed within 48 h (13). Although the aim of the study was not to explore the time-dependent manner of the 1α,25(OH)2D3 effect on articular cartilage, testing the reaction at different times in future studies may help to improve intervention in this regard.

It is worth pointing out that vitamin D has complex functions in the metabolism system. In order to best describe the comprehensive status of vitamin D in the body, calcium and phosphate, 25-hydroxyvitamin D, PTH and FGF23 are all ideal to be investigated. The current study focused more on the metabolism of articular cartilage and addressed more the levels of 1α,25(OH)2D3. To better evaluate status of vitamin D, more biochemical data will be included in a future study.

In conclusion, this study demonstrated that vitamin D deficiency might act as a stimulus for MMP production in normal articular cartilage, and vitamin D might act as an inhibitor of MMP increase in OA articular cartilage. Considering that vitamin D deficiency is pretty common and vitamin D administration might have good compliance, any putative effect of vitamin D on OA is significant. Further exploration of vitamin D chondroprotective potential in clinical settings seems warranted.

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