Unique Vitamin D Analogue for the Inhibition of NFkB in Treatment of Osteoarthritis

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ABSTRACT

Osteoarthritis (OA) is a leading cause of chronic disability in the United States, and the current methods for treatment are limited. Breakdown of structural collagen in articular cartilage by matrix metalloproteinases (MMPs), especially MMP-1 and MMP-13, is significantly increased in OA. Furthermore, it has been shown that mice deficient in MMP-13 are resistant to surgical induction of OA. The objective of this study was to analyze the efficacy of 1,25-dihydroxycholecalciferol (the active form of vitamin D3) and 20-hydroxycholecalciferol (a non-calcemic analogue of vitamin D3) to inhibit TNF-α activation of the MMP-1 and MMP-13 mediated collagen breakdown processes as a therapeutic intervention for inhibiting or preventing the progression of OA. Porcine chondrocytes were pre-incubated with varying concentrations of 1,25(OH)2D3 or 20(OH)D3, followed by TNF-α stimulation for 24 hours. Cells were then extracted for RNA and assayed for expression of anabolic and catabolic genes including MMP-1 and MMP-13 using quantitative PCR. The results demonstrated that after stimulation with TNF-α alone, MMP-1 and MMP-13 expressions were upregulated over 142-fold and 106-fold, respectively, in comparison to the non-stimulated control. Treatment with 10 nM 1,25(OH)2D3 and the 20(OH)D3 analog reduced MMP-13 expression by 62% and 60%, respectively, compared to TNF-α stimulation alone. Similar trends were also seen in expression levels of MMP-1, with reductions as high as 58% for 1,25(OH)2D3 and 40% for 20(OH)D3, at treatment concentrations of 10 nM. Overall, the data provides strong support for the efficacy of both 1,25(OH)2D3 and 20(OH)D3 as interventional agents of MMP expression in response to TNF-α; since MMPs are known to be important mediators in the development and progression of OA, our data also provide promise for these molecules as potential therapeutic agents in the treatment of OA. Particularly exciting was the parallel performance shown between 1,25(OH)2D3 and 20(OH)D3, as therapy with the latter could allow treatment without eliciting hypercalcemia.
INTRODUCTION

Osteoarthritis (OA) is the most prevalent form of arthritis and causes severe joint pain and disability in over 27 million Americans. It is also one of the leading causes of chronic disability in the United States, and for most states in the U.S., the annual cost of arthritis is more than 1% of the gross domestic product.¹ The disease results from a combination of trauma, stress, and aging, and leads to decreased work productivity, compromised quality of life, joint replacement, and excessive socioeconomic costs. Public health data shows that the prevalence of OA is expected to rise in the next two decades, and nearly 1 in 2 people will develop symptomatic knee OA within their lifetime.²

Currently, there is no cure for OA, and the standards of treatment are mostly limited to pain management, anti-inflammatory drugs, physical therapy, and eventual joint replacement.³ Previous data support a pathological mechanism for OA development via activation of the nuclear factor-κB (NFκB) pathway,³ leading to downstream expression of matrix metalloproteinases (MMPs), the primary enzymes involved in the degradation of the fibrillar matrix of cartilage in osteoarthritic joints.⁴⁵ Particularly important in OA are the collagensases MMP-1 and MMP-13, with chondrocytes surrounding OA lesions expressing higher levels of MMP-1 and MMP-13 compared to normal chondrocytes.⁶ Studies with animal models have shown that mice deficient in MMP-13 are resistant to surgically induced OA.⁶,⁷ Thus, inhibition or targeting of the NFκB pathway in areas of OA lesions could reduce or prevent the articular cartilage damage associated with OA. A schematic diagram of the NFκB pathway utilized in this study is provided in Figure 1.

Recent reports have shown that 1,25-dihydroxyvitamin D (1,25(OH)₂D₃), the active form of vitamin D, downregulates a variety of genes by blocking NFκB activation.⁸⁹ Although the exact molecular mechanism of this inhibition remains unknown, these reports⁸⁹ have suggested that 1,25(OH)₂D₃ blocks NFκB p65 nuclear translocation by either increasing IB levels or stabilizing the IB protein, the most critical step in regulating NFκB. Recent data⁹,¹⁰ have also shown that 1,25(OH)₂D₃-vitamin D receptor (VDR) attenuates NFκB activation via direct interaction between the VDR protein and the IKK subunit, therefore preventing formation of the IKK complex and subsequent phosphorylation of IB.

Figure 1. Schematic diagram of the NFB pathway. This diagram shows the TNF- stimulated NFB pathway, resulting in increased production of MMP.
The effects of 1,25(OH)₂D₃ also play an important role in the formation of the skin barrier, intestinal absorption of calcium, and immune function; it also has a wide variety of attenuating effects on cancer, proliferative diseases, and inflammatory skin diseases. However, at pharmacological dosages required to treat these conditions, it is well known that 1,25(OH)₂D₃ causes hypercalcemia, which limits its clinical utility.¹¹,¹² For this reason, various analogs of 1,25 (OH)₂D₃ have been the focus of much research. Recently, a new pathway has been identified in the metabolism of vitamin D that is catalyzed by cytochrome P450scC (CYP11A1) to yield 20α-hydroxyvitamin D₃ (20(OH)D₃) and several other P450scC derived metabolites.¹⁰,¹²,¹³ It has been shown that these vitamin D derivatives are equally as potent as 1,25(OH)₂D₃ in their antiproliferative, antiblastic, and antineoplastic properties, and several of these compounds do not exhibit effects of hypercalcemia, even at doses far above the therapeutic limits of 1,25 (OH)₂D₃.¹²,¹⁴ In particular, the 20(OH)D₃ analog of vitamin D has been shown to exhibit similar potency in inhibiting NFκB.¹²,¹⁴

In this study, our primary objective was to evaluate the ability of 1,25(OH)₂D₃ and 20(OH)D₃ to ameliorate the production of MMP-1 and MMP-13 by articular chondrocytes in vitro when stimulated with tumor necrosis factor-(TNF-α), a catabolic cytokine known to stimulate the NFκB pathway and the expression of MMP-1 and MMP-13. This study was carried out using primary chondrocytes harvested from porcine articular cartilage of the knee. We hypothesized that both soluble 1,25(OH)₂D₃ and 20(OH)D₃ would attenuate the expression of these MMPs in response to TNF-α. Gene expression was determined by quantitative PCR. In the process of the investigation, we also sought to evaluate the effects of these therapies on gene expression of several important extracellular matrix proteins including type I collagen and the cartilage-specific extracellular matrix proteins, type II collagen and aggrecan. Expression of the EPAS1 gene, a previously reported downstream target of NFB and transcriptional activator of MMP-13 expression, was also assessed.

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**METHODS**

**Sources of Tissues**

The primary chondrocytes used in this investigation were aseptically harvested from the articular cartilage of the medial and lateral femoral condyles of young domestic pigs ranging from 25 to 30 kilograms. Only healthy cartilage was taken for cell culture. Using sterile instruments, the knee joints were first exposed, followed by removal of surrounding muscles, tendons, and ligaments. Both the anterior and posterior cruciate ligaments were then cut in a transverse plane in order to fully open the joint space. Using scalpels and forceps, the articular cartilage was removed from the condyles in thin sections by cutting just beneath the surface in a direction that paralleled the natural curvature of the condyles. All tissues were taken from the knees of healthy pigs freshly sacrificed for other experiments according to approved protocols and experimental procedures of the University of Tennessee Health Science Center.

**Cell Culture**

The chondrocytes were isolated by 1-2 hours digestion at 37°C in 0.05% Pronase (Boehringer Mannheim, Mannheim, Germany), followed by overnight digestion in at 37°C in 0.2% collagenase (Worthington Biologicals, Lakewood, NJ) using modified F-12K medium (Invitrogen, Grand Island, NY) with 5% fetal calf serum (FCS, Atlanta Biologicals, GA). The cells were then plated at 15,000 cells/cm². Cells were cul-
tured at 37°C in a humidified atmosphere of 5% CO2 in air, and in F-12H media supplemented with 10% FCS, streptomycin (50 μg/mL; Invitrogen), penicillin G (50 IU/mL; Invitrogen), L-glutamine (2mM; Invitrogen), and ascorbic acid (50μg/mL; Invitrogen). The medium was changed every other day until the cells were confluent. For 48 hours directly prior to treatment, the media was supplemented with 1% charcoal stripped FCS rather than 10% FCS. The purpose of the charcoal stripped FCS was to remove endogenous vitamin D present in the fetal calf serum.

**Cell Treatment**

For the treatments, the cells were pre-incubated in new media with 1% stripped FCS with varying concentrations of 1,25(OH)2D3 (Sigma-Aldrich 17936 1α,25-Dihydroxyvitamin D3) or 20(OH)D3 (chemically synthesized as described by Slominski et al.)12,13 for two hours prior to stimulation with TNF-α (R&D Systems, Minneapolis, MN). The vitamin D was dissolved in 100% ethanol before dilution in culture medium. The highest concentration of ethanol ever used in the incubation media was 1%. The concentrations used for the 1,25(OH)2D3 and 20(OH)D3, ranged from 10⁻¹¹ to 10⁻⁸ M in 10-fold incremental dilutions. After the two hour pre-incubation period, the cells were stimulated with 5 ng/mL TNF-α for 24 hours. Cells left untreated or treated with TNF-α alone were used as controls; all treatments were performed in triplicate. After the 24 hour incubation period, the supernatants were removed and stored, and the cellular contents were extracted using Trizol (Invitrogen Life Technologies, Carlsbad, CA) for mRNA isolation and quantified for gene expression by real-time reverse transcription polymerase chain reaction (RT-PCR).

**Cell Viability**

For counting and general microscopic observations, isolated chondrocytes were stained with 0.4% trypan blue dye and counted under light microscopy using a 0.1 mm deep hemocytometer (Reichert, Buffalo, NY).

**Quantitative RT-PCR**

RNA was extracted from the cells with Trizol reagent according to the manufacturer's protocol. To measure target gene expression, we used an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) for RT-PCR with custom designed primers and fluorescently-labeled oligonucleotide probes specific for porcine genes; specifically, porcine collagen types I and II, aggrecan, EPAS1 (Life Technologies #4351372), MMP-1, MMP-13, and the housekeeping gene, -actin (as described by Cho et al.)16 Primers and probes were designed using annotated sequences from the porcine database and Applied Biosystems Primer Express software. According to the manufacturer's protocol,17,18 the cycle threshold (Ct) values were measured and the relative transcription levels were calculated. The data was plotted as a relevant expression calculated as 2⁻⁰.2Ct, where the cycle threshold is the beginning of the logarithmic amplification of the probe set, and Ct is the difference of the target gene Ct subtracted from the house-keeping gene Ct. Data was then calculated as 2 (the increase in probe signal generated with each cycle) to the negative exponential value of Ct and plotted as a relative change to either controls or the TNF-α stimulated group.

**Statistics**

All experiments were performed independently at least three times. Microsoft Excel with student t-test and analysis of variance were used to determine statistical significance. A p-value of less than 0.05 was considered statistically significant. A one-way ANOVA test was performed for analysis of cell viability.
RESULTS

Articular chondrocytes showed no change in cell viability after treatment with the various concentrations of 1,25(OH)₂D₃ and 20(OH)D₃ (highest concentration used is shown in the figure), nor was there any difference between the cells stimulated with TNF-α with or without 1% ethanol. There were no statistically significant differences in cell viability between groups, and the average cell viability among the groups was greater than 92% (Figure 2).

![Figure 2](image_url)

**Figure 2.** Effects on Cell Viability. Chondrocyte viability shown by trypan blue staining after a 24 hour incubation with the various compounds listed. As the figure indicates, there was no significant effect on cell viability between the treated groups when compared to the control group.

In order to investigate the therapeutic effect of 1,25(OH)₂D₃ and 20(OH)D₃ in inhibiting the NFB pathway, these compounds were added to articular chondrocytes stimulated with the cytokine TNF-α. The positive control group was treated with TNF-α and 1% ethanol, which was used as the vehicle for both forms of soluble vitamin D. A preliminary study⁶ that evaluated the dose response of the TNF-α used in our investigation showed increases in both MMP-1 gene expression and protein production with various concentrations of TNF-α. For this study, we used the lowest effective dose of TNF-α (5 ng/mL) required to show a significant response.⁶ Primary cultures that were not treated with TNF-α were cultured in the same manner and served as a reference (control) group for comparison of mRNA levels for these cytokine-treated groups.

**Promotion of Catabolism in Articular Chondrocytes by TNF-α**

Data comparing control groups to those with TNF-α stimulation and TNF-α with 1% ethanol stimulation for each of the genes evaluated is shown in Figure 3. Stimulation with TNF-α and 1% ethanol showed no significant differences in gene expression versus that seen with TNF-α alone for any of the evaluated genes. In all groups subjected to stimulation with TNF-α, the data confirmed the catabolic nature of this cytokine in articular cartilage. MMP-1 and MMP-13 gene expression was upregulated over 142-fold and 106-fold, respectively, in comparison to the non-stimulated control groups (Figure 3A). Aggrecan, type I collagen, and type II collagen all showed significant decreases in expression when treated with TNF-α, in comparison to the control groups (Figure 3B). Expression of the EPAS1 gene, shown by previous studies¹⁵ as a downstream target of NFB and a transcriptional activator of MMP-13 expression, was also significantly increased with TNF-α stimulation.

**Comparative Effects of 1,25(OH)₂-D₃ and 20(OH)-D₃ on MMP Expression**

Among the genes evaluated in this investigation, effects on gene expression were similar for both 1,25(OH)₂D₃ and 20(OH)D₃ treated groups. The most significant decrease in expression as a result of the two treatments occurred with MMP-13. Reductions as great as 62% at 10 nM were seen in the 1,25(OH)₂D₃ trials and 60% reductions at 10 nM were noted in the 20(OH)D₃ trials as compared to the group stimulated with TNF-α and 1% ethanol (Figure 4A).
Figure 3. Expression of various genes in chondrocytes after treatment with TNF-α, TNF-α (5 ng/mL), TNF-α+ EtOH (5 ng/mL TNF-α and 1% ethanol), or control (no stimulus). Expression of all genes is listed as fold change relative to the control group, which was given a value of 1. A) Changes in catabolic gene expression of MMP-1 and MMP-13; B) Changes in gene expression of type I collagen, type II collagen, aggrecan, and EPAS1.

Figure 4. Change in expression of MMP collagenases when treated with vitamin D. Articular chondrocytes were treated for 24 hr with various concentrations of 1,25(OH)₂D₃ and 20(OH)D₃ and TNF-α. Data is shown as a fold change relative to the TNF-treated group (5 ng/mL TNF-α + 1% EtOH), which was given a value of 1. A) MMP-13 gene expression; B) MMP-1 gene expression.

* indicates p value of <0.05

For MMP-1, parallels were also seen between 1,25(OH)₂D₃ and 20(OH)D₃ groups, though the decreases were not as notable as the decreases in MMP-13 expression. Analysis by RT-PCR demonstrated that MMP-1 expression induced with TNF-α was significantly reduced at concentrations of 1 nM and 10 nM of 1,25(OH)₂D₃, and significantly reduced expression occurred in groups treated with 20(OH)D₃ at 10 nM. At treatment concentrations of 10 nM, reductions as high as 58% and 40% were seen in both the 1,25(OH)₂D₃ and 20(OH)D₃ treatment groups, respectively (Figure 4B).
**Anabolic Changes in Gene Expression of Treated Articular Chondrocytes**

The data also demonstrated that treatment with 1,25(OH)_{2}D_{3} and 20(OH)D_{3} did not show any significant anabolic changes in type I collagen, type II collagen, or aggrecan in comparison to stimulation with TNF-α (Figure 5). In fact, type II collagen actually displayed a decrease in expression at all concentrations of both 1,25(OH)_{2}D_{3} and 20(OH)D_{3} (Figure 5B). Aggrecan displayed a statistically significant decrease at 10 nM in the 1,25(OH)_{2}D_{3} group (Figure 5C). Interestingly, regardless of therapeutic concentration, expression in the *EPAS1* gene was shown to have no significant change following treatment with 1,25(OH)_{2}D_{3} or 20(OH)D_{3} (Figure 5D).

**DISCUSSION**

The overall findings of this investigation support the ability of both 1,25(OH)_{2}D_{3} and 20(OH)D_{3} to downregulate the expression of MMP-1 and MMP-13 in articular chondrocytes in response to stimulation with TNF-α. Although nearly 60% reductions in MMP-1 and MMP-13 expression were seen with both treatments, their levels still remained 30-60 fold higher than the control group. In evaluating the therapeutic sig-
nificance of these reductions, it is important to note that the amount of inflammatory cytokine used in this experiment is actually much greater than the levels seen in OA and closer to the circulating levels seen in rheumatoid arthritis. This likely relates to the slower course of joint disability seen in OA. Therefore, the significance of the treatments on NFκB activation by TNF-α supports that 1,25(OH)₂D₃ and 20(OH)D₃ may be even more effective in the less aggressive OA. Since MMP-1 and MMP-13 are known to be important mediators in the development and progression of OA, our data lend support for both 1,25(OH)₂D₃ and 20(OH)D₃ as potential therapeutic agents for the treatment of OA. Particularly exciting was the effectiveness shown by the 20(OH)D₃ analog, which is non-calcemic and therefore does not affect serum calcium levels. A caveat, however, exists for therapy with 1,25(OH)₂D₃, the most effective therapeutic dose for 1,25(OH)₂D₃ was shown at 10 nM, similar to the 20(OH)D₃ analog. Since normal serum levels of 1,25(OH)₂D₃ are approximately 10⁻¹⁰ M, this dosage is 100-fold greater than normal physiological levels of 1,25(OH)₂D₃ in humans and would cause toxicity. We hope to circumvent this therapeutic dilemma by developing an alternative delivery system for the 1,25(OH)₂D₃ by which the drug will be loaded into nano-scale liposomes bound to type II collagen-specific antibodies and directed to specific areas of OA lesions. We have previously shown that these nanosomes are capable of selectively targeting areas of cartilage damage with exposed type II collagen. It is hoped that the use of 1,25(OH)₂D₃ in conjunction with these nanosomes will result in a new therapy effective in targeting specific areas and preventing the onset of OA with minimal adverse effects. Although an oral form of the non-calcemic 20(OH)D₃ analog would be preferred over the injectable nanosomes, we believe that the 1,25(OH)₂D₃ loaded into nano-scale liposomes will provide a shorter time period for FDA approval, as nanosomes are currently being used clinically for other applications.

In the process of this experiment, we also sought to evaluate the effects that these treatments had on the gene expression of several important matrix proteins present in articular cartilage. Unfortunately, the findings of this investigation did not provide any support for the ability of 1,25(OH)₂D₃ or 20(OH)D₃ to promote any anabolic effects on type I collagen, type II collagen, or aggrecan expression. In fact, treatment with each vitamin D isofom displayed a decrease in type II collagen transcription. This decrease in anabolic gene expression could be due to an inhibitory influence of vitamin D on the collagen expression driven by the TGF-β present in the FCS of the media. Vitamin D treatment of lung fibroblasts has previously been reported to prevent the upregulation of fibronectin and collagen in TGF-β treated fibroblasts. Together, the findings are consistent with the ability of 1,25(OH)₂D₃ and 20(OH)D₃ to help prevent catabolic pathways involved in OA, but they do not seem to offer any therapeutic benefit in promoting anabolic pathways needed to increase synthesis of extracellular matrix proteins.

Biological therapies that enhance the production of proteoglycans and collagens could be an effective way to promote and restore the integrity of the extracellular matrix in damaged cartilage. In recent years, many growth factors involved in skeletal development have been identified, and some recombinant growth factors and cytokine antagonists are already being used clinically for the treatment of musculoskeletal conditions. Transforming growth factor 1 (TGF-β1) and bone morphogenetic protein-2 (BMP-2) are some of the current growth factors studied that have been shown to stimulate cell proliferation and extracellular matrix synthesis. Future studies are needed in order to evaluate the therapeutic efficacy of TGF-β1 and BMP-2 in promoting the anabolic pathways required for restoration of the extracellular matrix in OA chondrocytes. The synergistic effect of the non-calcemic 20(OH)D₃ analog, or the nanosome-packaged 1,25
(OH)_2D_3, with these growth factors could potentially establish a new paradigm in the treatment of OA.

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REFERENCES


