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# 1,25(OH)$_2$D$_3$ and calcipotriol, its hypocalcemic analog, exert a long-lasting anti-inflammatory and anti-proliferative effect in synoviocytes cultured from patients with rheumatoid arthritis and osteoarthritis

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ABSTRACT

*Objectives:* We investigated the effects of 1,25-dihydroxy vitamin D$_3$ (1,25(OH)$_2$D$_3$), i.e. biologically active vitamin D and calcipotriol, a vitamin D analog, on growth and secretion of inflammatory mediators in synovial stromal cells (SSC) of patients with rheumatoid arthritis (RA) or osteoarthritis (OA).
*Methods:* Synovial stromal cells (SSC) isolated during knee prosthesis surgery from four patients with RA and four with OA were exposed to 1,25(OH)$_2$D$_3$ or calcipotriol with or without stimulation of cells with IL-1β or TNF-α. The proliferation of cells was studied by MTT assay. Levels of cytokines were analyzed by a magnetic bead–based multiplex assay (a panel of 27 important cytokines and IL-6 alone) and RT-PCR was used to validate the concentrations of the key cytokines secreted by SSC. The vitamin D receptor (VDR) was visualized by immunofluorescence in SSC and by immunohistochemistry in the synovial tissues of three RA and three OA patients.
*Results:* We detected intense staining for VDR in the synovial lining and vascular endothelium in tissue sections from all our RA and OA patients. Both 1,25(OH)$_2$D$_3$ and calcipotriol inhibited SSC proliferation for a prolonged time (up to 23 days with calcipotriol), but dexamethasone tended to increase SSC proliferation in a 4-day culture. 1,25(OH)$_2$D$_3$, calcipotriol and dexamethasone reduced the secretion of most inflammatory factors. Calcipotriol and dexamethasone additively reduced the secretions of IL-6, IFN-γ, basic FGF and VEGF in TNF-α stimulated SSC. The level of IL-6 was still diminished at 10 days after exposure, emphasizing the long-term impact of calcipotriol on SSC.
*Conclusions:* Exposure for 24–48 h to 1,25(OH)$_2$D$_3$ or calcipotriol causes a long-lasting inhibition of cell proliferation and cytokine production in SSC *in vitro*.



## 1. Introduction

Rheumatoid arthritis (RA) is characterized by an autoimmune-mediated inflammation of the synovium. Rheumatoid factor (RF) and antibodies against citrullinated peptides (ACPA) are recognized markers of seropositive RA, but their role in disease pathophysiology is still unknown [1]. The inflammatory activity in osteoarthritis (OA) is attributable to a non-autoimmune mediated mechanism. In both diseases, there is an excessive proliferation of synovial stromal cells (SSC), which secrete

*Abbreviations:* RA, rheumatoid arthritis; OA, osteoarthritis; SSC, synovial stromal cells; MTT, (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

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cytokines, chemokines and growth factors, ultimately contributing to cartilage and bone destruction.

1,25-Dihydroxy-vitamin-$D_3$ ($1,25(OH)_2D_3$) is the biologically active form of vitamin D, a vitamin that plays a major role in the regulation of calcium metabolism, cell growth, differentiation and apoptosis. $1,25(OH)_2D_3$ is a steroid hormone that elicits both rapid and slow cellular responses either by binding to the vitamin D receptor (VDR) [2] or to a distinct receptor [3]. $1,25(OH)_2D_3$ signaling inhibits the NF-κB, MAPK and JAK/stat pathways which have been intracellular targets for drug development in RA [4]. The VDR ultimately regulates thousands of genes by binding to either positive or negative VDREs (vitamin $D_3$ response element) in the promoters of these genes.

The final step of the biosynthesis of $1,25(OH)_2D_3$, catalyzed by 1α-hydroxylase, takes place in the proximal tubule of the kidney, as well as in macrophages and mesenchymal stem cells [5,6]. Vitamin D has been shown to be crucial for the function of the immune system i.e. $1,25(OH)_2D_3$ exerts many favorable effects on different parts of the innate and adaptive immune system [7]. A low serum $25(OH)D_3$ level has been associated with an earlier onset of rheumatoid arthritis and a more active disease [7,8]. In a mouse model of RA, a combination of VDR disruption and a TNF-α transgene led to the spontaneous development of arthritis in the animals, highlighting the importance of VDR in the suppression of inflammation at least in mice [9]. In addition, $1,25(OH)_2D_3$ has displayed antiproliferative/anti-cancer effects in many different cell lines [10].

Synthetic glucocorticoids (GC) have been traditionally used in the local or systemic treatment of RA due to their anti-inflammatory and anti-erosive properties in the inflamed joint. Although small and medium doses of GC prevent local erosions in cortical bone, the prolonged use of GC may accelerate the loss of trabecular bone in patients with established RA. Although an adequate vitamin D and calcium intake may alleviate this negative effect of GS therapy on trabecular bone, GC still trigger catabolic actions on muscle and skin and are responsible for many metabolic and cardiovascular adverse effects. Hence, there is a clear unmet need to develop alternatives to glucocorticoids in the treatment of RA and OA.

More than 3000 vitamin D analogs have been developed and tested in the treatment of cancer, hyperparathyroidism and hyperproliferative diseases, like psoriasis [11]. The hypercalcemic activity of calcipotriol is 100 times less than that of $1,25(OH)_2D_3$ [12] and its efficacy and safety in the local treatment of psoriasis have been documented [13]. We have now investigated 1,25 $(OH)_2D_3$ and calcipotriol as new potential candidates for the treatment of RA and OA using synovial stromal cells as our *in vitro* model.

## 2. Materials and methods

### 2.1. Subjects

Synovial tissue were collected from four patients with seropositive RA and four patients with OA that had undergone a total knee replacement therapy in Oulu University Hospital during the years 2012–2014. All RA patients fulfilled the American College of Rheumatology criteria for RA (1987) and were both ACPA and RF positive. The Ethics Committee of Oulu University provided an approving statement of the study protocol. All patients gave their informed consent for the use of sample material.

### 2.2. Cell cultures

Twelve 2–3 mm pieces of synovial tissue were digested with 4 ml of collagenase (100 U/ml) (Worthington) and 20 μl DNase (the final concentration of 50 U/ml) (Sigma-Aldrich) at 37 °C for 2–3 h and centrifuged. The pellet was suspended in complete media (α-MEM 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin) and cells were allowed to adhere overnight. The non-adherent cells were rinsed away and adherent cells were further cultured in the complete media. A half of the media was changed two times every week. All analyses were conducted between the third and fifth passages.

### 2.3. Reagents

$1,25(OH)_2D_3$ (Sigma-Aldrich) and calcipotriol (Santa Cruz Biochemistry) were dissolved in 99% ethanol. Recombinant human IL-1β/1F2 (R&D Systems) and dexamethasone (Sigma-Aldrich) were dissolved in sterile PBS and TNF-α (Sigma-Aldrich) in sterile PBS containing 0.1% BSA. $1,25(OH)_2D_3$ and calcipotriol were stored at −20 °C and cytokines at −80 °C. MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) was dissolved in sterile water and stored at −20 °C.

### 2.4. Measurement of cell proliferation

The MTT assay was used to evaluate the effect of $1,25(OH)_2D_3$, calcipotriol and dexamethasone on the proliferation of SSCs. Briefly, 1000 cells/well were seeded into 96-well plates in 6 parallel wells with 100 μl of the complete media and allowed to adhere overnight. The SSCs were stimulated with IL-1β (0.5 ng/ml) or TNF-α (3 ng/ml) and then simultaneously treated with different concentrations of $1,25(OH)_2D_3$ (0, 1, 10 and 100 nM) or calcipotriol (0, 1, 10 and 100 nM) and/or dexamethasone (0, 1 and 10 nM). Control cells were prepared in the same way using a saline or ethanol vehicle. The 23-day cultures were maintained in complete media containing TNF-α (3 ng/ml) in culture flasks. Four days before the MTT test, the cells in the flasks were detached with trypsin and re-divided so that there were 1000 cells/well in the 96-well plates. In the MTT test, the test media were removed and replaced with 100 μl of MTT solution at different time points (1, 3, 7, 10 and 23 days). After incubation at 37 °C for 4 h, the MTT solution was removed and 100 μl of dimethyl sulfoxide (DMSO, Sigma Aldrich) was added. After 10 min incubation at room temperature, the absorbance was measured with a plate reader (Victor$^2$, Perkin Elmer, Wallac) at 550 and 650 nm. Background signal (A650 nm) was subtracted from the 550 nm signal in the analyses.

### 2.5. Cell cycle analysis by flow cytometry

Flow cytometry for cell cycle distribution was performed using an LSRFortessa (BD Biosciences). Up to one million cells of four RA SSC and three OA SSC cultures were exposed to an ethanol vehicle or 10 nM of calcipotriol in a complete medium for 3 days. The cells were detached with trypsin digestion, washed three times with ice-cold PBS and fixed with ice-cold 75% ethanol at 4 °C overnight. The fixed cells were washed twice with ice-cold PBS and stained in propidium iodide (PI) buffer containing 100 mM sodium citrate, 0.1% Triton X-100, 0.2 mg/ml RNAase, and 50 μg/ml propidium iodide for 1 h at 4 °C. Cells were distributed to different phases according to the intensity of staining (the more DNA, the more staining) according to manufacturers' instructions.

### 2.6. Apoptosis analysis by flow cytometry

Apoptosis analyses for each SSC culture were conducted using an LSRFortessa (BD Biosciences) flow cytometer. FITC (fluorescein isothiocyanate)-Annexin V and propidium iodide (PI) kit (BD Biosciences) were used to distinguish early apoptotic and necrotic