Vitamin D Attenuation of Inflammatory Cytokine Production by Gingival Fibroblasts

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Abstract

**Background and Objective:** Vitamin D [1,25(OH)2D3] (1,25D3) maintains healthy osseous tissue, stimulates production of the antimicrobial peptide cathelicidin, and also has anti-inflammatory effects, but it can cause hypercalcemia. Evidence links diminished serum levels of 1,25D3 with increased gingival inflammation. Periodontitis progression is associated with increased local production of inflammatory mediators by immune cells and gingival fibroblasts. These include interleukin (IL)-6, a regulator of osteoclastic bone resorption, and the neutrophil chemoattractant IL-8, both regulated by signaling pathways, including NF-κB and MAPK/AP-1. **Objectives:** Determine effects of 1,25D3 or a non-calcemic analog [20(OH)D3] (20D3) on IL-1β-stimulated IL-6 and IL-8 production, and NF-κB and MAPK/AP-1 activation, by human gingival fibroblasts.

**Materials and Methods:** Human gingival fibroblasts were incubated ± IL-1β, with or without exposure to 1,25D3 or 20D3. IL-6 and IL-8 in culture supernatants were measured by ELISA. NF-κB (p65) and AP-1 (phospho-cJun) and were measured in nuclear extracts via binding to specific oligonucleotides. Data were analyzed using ANOVA and Scheffe’s F procedure for post hoc comparisons.

**Results:** IL-1β-stimulated IL-6 and IL-8 levels were both significantly inhibited (40-60%) by 1,25D3, but not 20D3 (0-15% inhibition, not statistically significant). Both
1,25D3 and 20D3 significantly and similarly inhibited IL-1β-stimulated nuclear levels of p65 and phospho-cJun.

**Conclusion:** Reduction of the activation of NF-κB and AP-1 is not alone able to strongly inhibit IL-1β stimulated IL-6 and IL-8 gene expression. 1,25D3 but not 20D3 may affect some of the many other factors/processes/pathways that in turn regulate the expression of these genes. However, the results suggest that topical application of ligands of the vitamin D receptor may be useful in the local treatment of periodontitis while reducing adverse systemic effects.
Introduction

Vitamin D has been traditionally recognized as a central regulator of calcium and phosphorus homeostasis, and accordingly, bone metabolism and density (1, 2). Vitamin D, in the form of vitamin D³, is obtained from dietary sources or synthesized in the skin following exposure to ultraviolet rays (3-7). It is then metabolized in the liver to form 25-hydroxyvitamin D³ (25(OH)D³), the main circulating metabolite of vitamin D, and is further altered in the kidney to form, which 1α,25-dihydroxyvitamin D³ (1,25(OH)₂D³) (1,25D³), the biologically active metabolite of vitamin D (8).

Several reports have shown a significant association between periodontal health and the intake of vitamin D and calcium (9, 10). Osteoporosis and loss of bone mass contribute to periodontal disease susceptibility (11). Dietary supplementation of vitamin D and calcium may improve periodontal health, increase bone density, and inhibit alveolar bone resorption (12, 13). Studies demonstrate that calcium and vitamin D supplementation may reduce the severity of periodontal disease when used above the daily recommended dose of 800-1,000 IU (14), and that response to periodontal surgery might be partially dependent upon the patient’s baseline 25(OH)D³ status (15). Other studies and a recent review by Stein et al. suggest that vitamin D also appears to have a “perio-protective role” in maintaining the periodontium, by regulating both the innate and adaptive immune response. (16, 17).
Identification of vitamin D receptors (VDRs) as well as 25(OH)D3 activating enzymes in cell types not associated with mineralized tissue, such as human gingival fibroblasts and periodontal ligament cells in the periodontal soft tissue, implies that 1,25D3 also has a broader physiologic function, both in the periodontium and beyond (8). Vitamin D deficiency may place subjects at a heightened risk for not only low mineral bone density/osteoporosis and osteopenia, but also infectious and chronic inflammatory diseases including periodontitis (18-20). Pharmacologic doses of vitamin D that might be used in prevention or treatment of diseases, however, can lead to hypercalcemia. Slominski et al. have formulated a vitamin D analog, 20D3, that can activate VDRs, without inducing hypercalcemia (21).

Fibroblasts, including human gingival fibroblasts (HGF) and human periodontal ligament cells (HPDLC), are the predominant cells in the periodontium and have been shown to play an important role in both the remodeling of healthy gingival tissue and in the pathogenic mechanisms associated with periodontal diseases (22, 23). HGF, stimulated by bacterial LPS or IL-1, produce large quantities of inflammatory cytokines such as IL-6 and IL-8, and are critical for sustaining inflammation in periodontal disease via production of these mediators (22,24,25).

There has been little reported in the literature concerning the regulatory effects of 1,25D3 or its derivatives upon HGF. However, one recent study showed that vitamin D may potentially inhibit periodontal inflammation induced by Porphyromonas
*gingivalis (P. gingivalis)* partly by decreasing the IL-8 expression in HPDLCs (26). Also, a recent article demonstrated that fibroblast cultures derived from human nasal polyps displayed diminished production of inflammatory mediators following treatment with 1,25D3 derivatives (27). Furthermore, two studies utilizing murine embryonic fibroblasts suggested that VDRs contributed to moderating the inflammatory environment via their inhibition of NF-kappa B activation (28, 29). Thus, there is some evidence to suggest that 1,25D3 may regulate the production of inflammatory mediators from fibroblasts derived from different tissues and species. The aim of this study was to evaluate the role of 1,25D3 on attenuating inflammatory cytokine production by gingival fibroblasts.
Material and Methods

Human Gingival Fibroblasts

Normal human gingival fibroblasts from a healthy patient with non-inflamed gingiva were derived from gingival explants using standard techniques in accordance with the UTHSC IRB protocol as described earlier (30). The cells were grown in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies Corp.; Grand Island NY), supplemented with 10% (v/v) newborn calf serum (Life Technologies) and 100 μg/ml gentamicin (Sigma-Aldrich; St. Louis, MO) (termed growth medium) at 37ºC in a humidified atmosphere of 5% CO₂ in air, and passaged by brief treatment with trypsin (0.25%) (Life Technologies).

Vitamin D, Vitamin D Analog, IL-1β

1,25D3 and 20D3 were provided by Dr. Andrzej Slominski (University of Alabama at Birmingham). The reagents were solubilized in ethanol (5 μg/vial) and stored at (-80°C). Stock solutions were prepared in ethanol at 100 μM. Human recombinant IL-1β was obtained from R&D Systems (Minneapolis, MN).

Cell Viability and Membrane Integrity

Effects of 1,25D3, 20D3, and the solvent for both (ethanol) on cell viability were assessed by determining their effects on the ability of the cells to cleave the tetrazolium salt (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)
(MTT) to a formazan dye, using a kit from Boehringer Mannheim Corp. (Indianapolis, IN). Individual wells of 96-well microtiter tissue culture plates (Becton Dickinson Labware; Franklin Lakes, NJ) were seeded with $2.5 \times 10^4$ cells in growth medium. The cells were incubated overnight at $37^\circ$C. The medium was then removed, the cells were washed with phosphate-buffered saline (PBS), and serum-free medium supplemented with 100 µg/ml gentamicin (DMEM-gent), containing 1,25D3 or 20D3 (0.1-100 nM; final concentration of ethanol was 1%), or ethanol (0.01 – 10%; control medium was DMEM-gent), and incubated for 1, 3, or 6d before the addition of MTT (0.5 mg/ml). After exposure to the chemicals, MTT was added to the cells at a final concentration of 0.5 mg/ml and incubated for 4 hr at $37^\circ$C. Purple formazan crystals produced from the MTT by metabolically active cells and were solubilized by overnight exposure to a solubilization solution provided in the kit, at $37^\circ$C. Absorbance was read at 540 nm using a microtiter plate spectrophotometer (SPECTROstarNano; BMG Labtech GmbH, Offenburg, Germany).

Results. Results were expressed as % control ($A_{540nm}$ in cells exposed to DMEM-gent only).

Cytotoxicity caused by 1,25D3 or 20D3 leading to plasma membrane damage was measured using the Cytotoxicity Detection Kit (LDH) (Roche Diagnostics Corp.; Indianapolis, IN). Lactate dehydrogenase (LDH), a stable cytoplasmic enzyme found in all cells, is quickly released upon damage to the plasma membrane. Individual wells of 96-well microtiter tissue culture plates were seeded with $2.5 \times 10^4$ cells in DMEM-gent + 1% NCS and cultured for 24 hr at $37^\circ$C. (A low serum concentration
was used to reduce background absorbance because animal sera contain various levels of LDH). The medium was removed, the cells were washed with PBS, and DMEM-gent containing 1,25D3 or 20D3 (0.01 – 10 nM; final concentration of ethanol was 1%) was added to the wells. To determine maximum LDH release (high control), some cells were solubilized with a final concentration of 1% (w/v) Triton X-100 (Boehringer Mannheim). Spontaneous LDH release (low control) was determined by incubating the cells with serum-free DMEM-gent containing 1% ethanol. The cells were exposed to the test materials for 3 hr, a time frame in which plasma membrane damage can be specifically detected (31). Cell-free supernatants were removed and transferred to clean 96-well plates. LDH activity was assayed in the supernatants by a reaction in which the tetrazolium salt, INT, was reduced to a red formazan salt. Absorbance was read at 490 nm using a microtiterplate spectrophotometer. Results were expressed as % cytotoxicity [(experimental value-low control/high control-low control) x 100].

**Effect of 1,25D3 and 20D3 on Constitutive and IL-1β-stimulated IL-6 and IL-8 Production**

Constitutive and IL-1β-stimulated IL-6 and IL-8 levels, and the effects of 1,25D3 and 20D3 were measured in cell-conditioned media using commercially available ELISA kits (R&D Systems). Previous work in our laboratory has shown that human gingival fibroblasts constitutively produce both of these cytokines (32). To obtain conditioned media, the cells were seeded at 7.5 x 10⁴ cells in 24-well plates (Corning, Inc.; Corning, NY) in growth medium and cultured overnight at 37°C. The
growth medium was then removed, and the wells washed once with PBS and then
serum-free medium with or without non-toxic concentrations of 1,25D3 or 20D3
(0.001 – 10 nM) were added. (Stock solutions were made so that the final ethanol
concentration in these solutions was 1%; control medium was DMEM-gent
containing 1% ethanol). Previous work in this laboratory has shown that normal
human gingival fibroblast IL-6 and IL-8 production are stimulated by IL-1β (33-35).
In some experiments, the cells were pre-incubated with 1,25D3 or 20D3 (0.001 – 10
nM) for 2 or 24 hr before addition of IL-1β (0.01 nM), or were added concurrently
with IL-1β. In other experiments the cells were pre-incubated with IL-1β (0.01 nM)
for 2, 6, or 24 hr, before the addition of 1,25D3 or 20D3 (0.1 – 10 nM). After
incubation for 24 hr, the cell supernatants were harvested and assayed for IL-6 and
IL-8, per the procedure outlined in the kit.

**Effect of NDM on IL-1β-stimulated NF-κB and AP-1 Activation**

The inhibitory effects of 1,25D3 or 20D3 on IL-1β-stimulated NF-κB and AP-1
activation were tested using the TransAm™ NF-κB p65 Transcription Factor Assay
Kit and the TransAm™ AP-1 c-Jun Transcription Factor Assay Kits, respectively
(Active Motif North America; Carlsbad, CA). The NF-κB kit contains a 96-well plate
to which an oligonucleotide containing the NF-κB consensus binding site has been
immobilized. The primary antibody used to detect p65 recognizes an epitope that is
accessible only when NF-κB is activated, which frees its subunits such as p65 and
allows them to bind to this target consensus DNA sequence on the plate. Therefore
this assay is a reliable measure of the inhibition of NF-κB activation. Similarly, the
AP-1 phospho-c-Jun kit uses an oligonucleotide to which AP-1 binds. The antibody used to detect phospho-c-Jun binds to an epitope accessible only when phospho-c-Jun is bound to the DNA oligonucleotide bound to the plate. JNK and phosphorylation of c-Jun and its dimerization appear to be essential for cytokine-induced AP-1 activation (36). For this reason the AP-1 c-Jun assay is a reliable measure of the inhibition of AP-1 activation.

Fibroblasts were seeded at 1.5 x 10^6 cells/well of 6 well plates (Corning) in growth medium and incubated overnight. The wells were washed once with PBS, and serum-free DMEM-gent ± 1,25D3 or 20D3 (10 nM; final concentration of ethanol was 1%) was added and incubated for 2h at 37°C. Then IL-1β (final concentration 0.01nM) was added to some wells, and the cells were incubated for 60 or 90 min at 37°C. At the end of the incubation period, the media were aspirated and the cells were washed with ice-cold PBS containing phosphatase inhibitors (Nuclear Extract Kit, Active Motif North America; Carlsbad CA). Then the cells were removed by scraping using a sterile cell scraper (Corning) in ice-cold PBS/phosphatase inhibitors. The suspension was transferred to a pre-chilled 15ml centrifuge tube (Corning) and centrifuged for 5 min at 500 rpm and 4°C. The supernatant was discarded and the cell pellet was re-suspended in hypotonic buffer (Active Motif) and transferred to a pre-chilled microcentrifuge tube (Sarstedt Inc.; Newton, NC). After incubating for 15 min on ice, detergent was added and the tubes were vortexed at the highest setting for 10 sec. The tubes were centrifuged for 30 sec at 14000 x g and 4°C. The nuclear pellets were re-suspended in complete lysis buffer
(Active Motif), vortexed as above for 10 sec, then incubated for 30 min on ice, on a rocking platform. The tubes were vortexed 30 sec as above and centrifuged for 10 min at 14000 x g at 4°C. The supernatants (nuclear extracts) were transferred to pre-chilled microcentrifuge tubes and stored at -80°C. Nuclear fractions were assayed for p65 and phospho-cJun using the kits described above.

**Statistical Analysis**

All experiments were performed with triplicate samples. Data was expressed as mean ± standard deviation and analyzed using a one-way analysis of variance (ANOVA) and Scheffe’s F procedure for post hoc comparisons, using StatView® software.
Results

Cytotoxicity and Viability

Short-term exposure (3 hours) of the cells to 1,25D3 or 20D3 did not cause significant membrane damage at any concentration (data not shown). On the other hand, long-term exposure to 1,25D3 or 20D3 did result in a decrease in cell viability (Figure 1A,B). A 3 or 6 day exposure to 1,25D3 resulted in a 10-40% decrease in cell viability, at concentrations of 0.01-100nM. Similarly, there was a 10-60% decrease in viability of cells exposed to 20D3, also at the same concentrations. However, a 1 day exposure to either 1,25D3 or 20D3, at concentrations \( \leq 10nM \), did not cause a significant decrease in cell viability, and subsequent experiments used these conditions.

Effect of 1,25D3 or 20D3 on Constitutive and IL-1β-stimulated IL-6 and IL-8 Production

1,25D3 (1 and 10 nM only) significantly decreased constitutive IL-6 and IL-8 production by ~50% and ~30% respectively (p<0.01). On the other hand, 20D3 had no inhibitory effect on IL-6 production and decreased IL-8 production by ~10-20% at higher concentrations (not statistically significant) (Figure 2A,B). 1,25D3 caused a maximum 40-60% decrease in IL-1β-stimulated IL-6 production (Figure 3A) at higher concentrations under all conditions testing effects of concurrent addition of IL-1β or pre-incubation with 1,25D3, but 20D3 had no significant effect,
although it did cause ~10% decreases at some concentrations (Figure 3B). Similarly, 1,25D3 at higher concentrations, but not 20D3 caused a 40-50% decrease in IL-8 production (Figure 4A,B), under all conditions tested. The analog caused non-significant decreases of ~5-15% in IL-1β-stimulated IL-8 levels.

1,25D3 but not 20D3 caused significant 50-60% and 30-40% decreases in IL-6 production when the cells were exposed to IL-1β for 2 hours or 6 hours, respectively, before the addition of vitamin D or the analog, compared to the control (Figure 5A,B). However, only the highest 1,25D3 concentration tested (10nM) significantly decreased IL-6 production when the cells were pre-incubated with IL-1β for 24 hr. Similarly, 1,25D3 but not 20D3 caused a 30-50% decrease in IL-8 production (Figure 6A,B), when added to the cells after pre-incubation with IL-1β for 2 hr. Furthermore, 1,25D3 caused 10-20% decreases in IL-8 levels when added to the cells 6 or 24 hours after IL-1β, which reached significance in only one instance (10 nM, 24-hr pre-incubation).

**Effect of 1,25D3 or 20D3 on IL-1β-stimulated Nuclear levels of NF-κB (p65) and AP-1 (phospho-cJun)**

At both time periods tested, IL-1β-stimulated nuclear p65 levels 3-3.5-fold over controls (Table). This was reduced approximately 10% (p≤0.02) by both 1,25D3 and 20D3, at both time periods. Very low nuclear levels of phospho-cJun were detected at 60 min after addition of IL-1β. At 90 min, there were much higher levels (stimulated by IL-1β 3-fold over control). 1,25D3 and 20D3 reduced nuclear levels
of phospho-cJun by 12% (p<0.0001) and 30% (p<0.0001), respectively.


**Discussion**

The destruction of periodontal tissues is enhanced by increased inflammation via many mediators, including IL-6 and IL-8. Vitamin D can regulate innate and adaptive immune responses and may reduce the severity of periodontal disease. Gingival fibroblasts are important contributors to inflammation in the periodontium, and in this study we have determined effects of 1,25D3 and the non calcemic analog 20D3 on human gingival fibroblast production of IL-6 and IL-8 in vitro.

In the present study, short term exposure of the fibroblasts to 1,25D3 or 20D3 did not cause membrane damage, but longer (>24 hr) exposure to concentrations >10 nM decreased cell viability. In contrast, Feng et al., working with human rheumatoid fibroblast-like synovicytes, reported no toxicity of 1,25D3 (0.1nM-100nM) after 48-hr exposure (37), and Nazzal et al. reported no effects on human gingival epithelial cell viability after 6-day exposure to 1,25D3 or 20D3 (0.1nM-1μM) (38). Furthermore, Rostkowska-Nadolska et al. investigated the effects of vitamin D derivatives (10μM-100μM) on the inhibition of IL-6 and IL-8 expression in human nasal polyp fibroblasts and also reported no toxicity (39). Cytotoxic effects may depend upon concentration, time of exposure, and the type of cell, as well as the particular cell line, reflecting interindividual differences in response.

Constitutive as well as IL-1β-stimulated IL-6 and IL-8 production by gingival fibroblasts was decreased by 1,25D3, but not significantly affected by 20D3. The
inhibitory effect of 1,25D3 on pro-inflammatory cytokine production is consistent with other studies. For example, Andrukhov et al. (40) found that 25(OH)D3 or 1,25D3 inhibited \textit{P. gingivalis}-stimulated human periodontal ligament cell production of IL-8, but not IL-6. However, Nebel et al. found that 1,25D3 inhibited IL-6 production by \textit{E. coli} LPS-stimulated human PDL cells by approximately 50\% (41). Our findings, as well as those of similar studies, suggest that 1,25D3 may be an important factor in the inflammatory response of cells in chronic inflammatory diseases such as periodontal disease, and that sufficient 1,25D3 may be able to more effectively regulate the inflammatory process. An epidemiological analysis of cross-sectional data from the National Health and Nutrition Examination Survey 2001 to 2004 by Jonsson et al. concluded that higher attachment levels and presence of more teeth were associated with participants who had sufficient levels of 1,25D3 (>20 ng/mL) compared to participants with levels below this threshold. Furthermore, the odds ratio of having moderate/severe periodontitis in participants who were 1,25D3-deficient was 1.19 compared to participants who were 1,25D3-sufficient (42).

In the present study, when cells were pre-incubated with IL-1β, 1,25D3, but 20D3, significantly decreased both IL-6 and IL-8 production, although the maximum inhibition in most instances seen was not as great as when cells were incubated with 1,25D3 before addition of IL-1β. Moreover, the longer the pre-incubation period with IL-1β, the less effective was 1,25D3 in reducing cytokine levels. This may suggest that in the presence of established inflammation, the effects of 1,25D3
may not be as great as its preventive effects. This supports the idea that adequate intake of vitamin D may aid in the prevention of periodontal disease following the reduction of gingival inflammation and local levels of inflammatory cytokines.

In gingival fibroblasts, IL-1β-stimulated control of IL-6 and IL-8 production is regulated largely via the NF-κB and AP-1 signaling pathways (43). In the present study, 1,25D3 reduced IL-1β-stimulated IL-6 and IL-8 protein levels by ~50%, but 20D3 had no or statistically non-significant effects. However, we found that both 1,25D3 and 20D3 caused partial, similar, and statistically significant reductions of IL-1β-stimulated nuclear p65 levels (~10%), and of IL-1β-stimulated nuclear AP-1 (phospho-cJun) levels (12 - ~ 30%). Regarding NF-κB, this could be due to effects on IκBα, which masks nuclear localization signals of NF-κB proteins and sequesters them in an inactive state in the cytoplasm. Both 1,25D3 and 20D3 inhibit NF-κB by stimulating IκBα expression, mRNA stability, and decreasing phosphorylation of IκBα, with subsequent p65 sequestration (in human keratinocytes and murine macrophages) (44,45). With respect to AP-1, 1,25D3 can dephosphorylate and inactivate/ p38 MAP kinase, via an increase in MKP1 and MKP5 (46-48), which can dephosphorylate p38 (as well as JNK) (49). Both p38 and JNK can activate AP-1 and subsequent IL-6 and IL-8 expression.

The biological effects of 1,25D3 and 20D3 appear to be mediated by binding to the vitamin D receptor (VDR) (50, 51). For example, VDR is directly involved in regulation of NF-κB, which suggests its role in inhibition of inflammation,
particularly in the presence of vitamin D ligands (52). While 1,25D3 caused much
greater reduction than 20D3 on protein levels of IL-6 and IL-8, their similar
inhibitory effects on nuclear levels of NF-κB (p65) and AP-1 (phospho-cJun)
transcription factors suggests that their reduced activation is not alone able to
strongly inhibit IL-6 and IL-8 gene expression in these cells. 1,25D3 may inhibit
other factors/processes affecting the expression of these genes, that 20D3 does not.
These may include cofactors that allow efficient recruitment of RNA polymerase and
the proteins that make up the pre-initiation complex; post-transcriptional
modifications of transcription factors that affect DNA binding (i.e. phosphorylation,
acetylation); transactivation; modifications of histones which play a factor in gene
regulation; mRNA stabilization; or activity of repressor proteins (53-55).
Interference with factors and processes such as these may be required for optimal
repression of IL-8 or IL-6 transcription and ultimately protein expression, and may
occur through the actions of 1,25D3 but not 20D3. This notion is consistent with a
report by Harant et al. (55), which suggested that vitamin D interference of DNA
binding in MRC-5 normal human fibroblasts was responsible for its inhibition of IL-
1β-stimulated IL-6 and IL-8 mRNA levels. Similar results were found in a study
using THP-1 human monocytic cells (56).

In addition to the NF-κB and AP-1 pathways, IL-1β-stimulated IL-6 and IL-8 can be
regulated by others, including C/EBP (NF-IL6); cAMP-dependent pathways; and
PKC-dependent pathways (57, 58). Moreover, PGE₂ regulates IL-6 production in
human gingival fibroblasts (58) and vitamin D3 can decrease PGE₂ production in
fibroblasts (59). It may be that 1,25D3 can inhibit some of these other pathways but the analog cannot, leading to the differences observed in IL-6 and IL-8 protein inhibition, despite similar inhibitory effects on NF-κB and AP-1 activation.

As suggested by Amano et al., topical application of VDR ligands may be useful in the local treatment of periodontitis while reducing adverse systemic effects (60). Our results suggest that 1,25D3 might be more useful than 20D3 analog in this regard, as far as reducing IL-1β-stimulated IL-6 and IL-8 production by local gingival fibroblasts is concerned. The hypercalcemic effects of 1,25D3 might be kept to a minimum because of its local administration, and it has been found that prolonged topical application of calcitriol (1,25D3) ointment in patients with chronic plaque psoriasis did not result in significant or long-lasting elevations in serum calcium levels (61). These findings provide the rationale for the topical application of vitamin D as an additional treatment option at specific sites exhibiting “active” disease during supportive periodontal therapy. Further large-scale, controlled studies are needed to clarify the mechanisms by which 1,25D3 attenuates the inflammatory effect of human gingival fibroblasts and establish the proper therapeutic dosage. The anti-inflammatory properties of vitamin D make it a valuable “perio-protective” agent that may be included in our clinical armamentarium. It also suggests that it may be beneficial to monitor the vitamin D levels of patients who might be more susceptible to periodontal disease.
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Legends for Figures

Figure 1. Effects of 1,25D3 or 20D3 on Cell Viability.
Fibroblasts were seeded at 2.5 x 10^4 cells/well in 96-well plates in growth medium. After overnight incubation at 37°C, the medium was removed, the wells were washed with PBS, and serum-free DMEM-gent containing 1,25D3 (A) or 20 D3 (B) (0.1 – 100 nM) was added and incubated for 1, 3, or 6 days. Control medium was DMEM-gent containing 1% ethanol. Cytotoxicity was determined by measuring effects on the ability of the cells to cleave the tetrazolium salt MTT to a colored formazan dye. Absorbance was read at 540 nm using a microtiter plate spectrophotometer. Results of multiple experiments with triplicate samples were expressed as mean % control (A_{540nm} in cells exposed to DMEM-gent only) ± standard deviation.

Figure 2. Effects of 1,25D3 or 20D3 on Constitutive IL-6 and IL-8 Production.
Fibroblasts were seeded at 7.5 x 10^4 cells in 24-well plates in growth medium and cultured overnight at 37°C. The medium was removed, the wells washed once with PBS, and then serum-free DMEM-gent with or without non-toxic concentrations of 1,25D3 or 20D3 (0.001 – 10 nM) were added. Control medium was DMEM-gent-1% EtOH. After incubation for 24 hr, the cell supernatants were harvested and assayed for IL-6 (A) and IL-8 (B) by ELISA. Results of multiple experiments with triplicate samples were expressed as
mean % Control [amount of IL-6 or IL-8 (pg/ml) produced by cells exposed DMEM-gent only, set at 100%] ± standard deviation.

**Figure 3. Effects of Pre-Incubation with 1,25D3 or 20D3 on IL-1β-Stimulated IL-6 Production.**

Fibroblasts were seeded at 7.5 x 10^4 cells in 24-well plates in growth medium and cultured overnight at 37°C. The medium was removed, the wells washed once with PBS and then serum-free DMEM-gent with or without non-toxic concentrations of 1,25D3 (A) or 20D3 (B) (0.001 – 10 nM) were added. Control medium was DMEM-gent-1% EtOH. The cells were incubated with 1,25D3 or 20D3 for 2 or 24 hr before addition of IL-1β (0.01 nM), or were added concurrently with IL-1β. After incubation for an additional 24 hr, the cell supernatants were harvested and assayed for IL-6 by ELISA. Results of multiple experiments with triplicate samples were expressed as mean % Control [amount of IL-6 (pg/ml) produced by cells exposed to IL-1β only, set at 100%] ± standard deviation. In (A): *p=0.045, **p=0.01, ***p<0.001, ****p<0.0001 vs. Control; in (B): *p=0.02 vs. Control.

**Figure 4. Effects of Pre-Incubation with 1,25D3 or 20D3 on IL-1β-Stimulated IL-8 Production.**

Fibroblasts were seeded at 7.5 x 10^4 cells in 24-well plates in growth medium and cultured overnight at 37°C. The medium was removed, the wells washed once with PBS and then serum-free DMEM-gent with or without non-toxic
concentrations of 1,25D3 (A) or 20D3 (B) (0.001 – 10 nM) were added. Control medium was DMEM-gent-1% EtOH. The cells were incubated with 1,25D3 or 20D3 for 2 or 24 hr before addition of IL-1β (0.01 nM), or were added concurrently with IL-1β. After incubation for an additional 24 hr, the cell supernatants were harvested and assayed for IL-8 by ELISA. Results of multiple experiments with triplicate samples were expressed as mean % Control [amount of IL-8 (pg/ml) produced by cells exposed to IL-1β only, set at 100%] ± standard deviation. *p<0.01; **p<0.001; ***p<0.0005 vs. Control.

Figure 5. Effects of Pre-Incubation with IL-1β before Addition of 1,25D3 or 20D3 on IL-6 Production.

Fibroblasts were seeded at 7.5 x 10^4 cells in 24-well plates in growth medium and cultured overnight at 37°C. The medium was removed, the wells washed once with PBS and then serum-free DMEM-gent with or without IL-1β was added. The cells were incubated with IL-1β for 2, 6, or 24 hr, before the addition of 1,25D3 (A) or 20D3 (B) (0.1 – 10 nM). After incubation for an additional 24 hr, the cell supernatants were harvested and assayed for IL-6. Results of multiple experiments with triplicate samples were expressed as mean % Control [amount of IL-6 (pg/ml) produced by cells exposed to IL-1β only, set at 100%] ± standard deviation.
Figure 6. Effects of Pre-Incubation with IL-1β before Addition of 1,25D3 or 20D3 on IL-8 Production.

Fibroblasts were seeded at 7.5 x 10^4 cells in 24-well plates in growth medium and cultured overnight at 37°C. The medium was removed, the wells washed once with PBS and then serum-free DMEM-gent with or without IL-1β was added. The cells were incubated with IL-1β for 2, 6, or 24 hr, before the addition of 1,25D3 (A) or 20D3 (B) (0.1 – 10 nM). After incubation for an additional 24 hr, the cell supernatants were harvested and assayed for IL-8. Results of multiple experiments with triplicate samples were expressed as mean % Control [amount of IL-8 (pg/ml) produced by cells exposed to IL-1β only, set at 100%] ± standard deviation.
Table

Effect of 1,25D3 or 20D3 on Nuclear Levels of IL-1β-stimulated p65 (NF-κB) and phospho-c-Jun (AP-1)

<table>
<thead>
<tr>
<th></th>
<th>p65 60 min</th>
<th>p65 90 min</th>
<th>p-cJun 60 min</th>
<th>p-cJun 90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.28* ±0.07</td>
<td>1.19 ±0.07</td>
<td>0.14 ±0.07</td>
<td>1.67 ±0.07</td>
</tr>
<tr>
<td>IL-1β</td>
<td>3.55a ±0.03</td>
<td>2.96a ±0.07</td>
<td>0.26 ±0.03</td>
<td>4.4a ±0.07</td>
</tr>
<tr>
<td>IL-1 + 1,25D3</td>
<td>3.2c ±0.07</td>
<td>2.65a ±0.02</td>
<td>0.203 ±0.07</td>
<td>3.88a ±0.02</td>
</tr>
<tr>
<td>IL-1β + 20D3</td>
<td>3.18 ±0.27</td>
<td>2.45b ±0.14</td>
<td>0.2 ±0.27</td>
<td>3.13a ±0.14</td>
</tr>
</tbody>
</table>

*Mean Abs. 450 nm ± standard deviation

a p<0.0001; b p≤0.002; c p≤0.02.

Values in red are significantly < those from cells exposed to IL-1β alone.
2A

IL-6 (% Control)

1,25 D3          20 D3

* p<0.01 vs. Control

2B

IL-8 (pg/ml)

1,25 D3          20 D3

* p<0.01 vs. Control