1,25-dihydroxyvitamin D3 downregulates aromatase expression and inflammatory cytokines in human macrophages

B. Villaggio¹, S. Soldano², M. Cuto³

¹Research Laboratory of Nephrology, and ²Research Laboratory and Academic Unit of Clinical Rheumatology, Department of Internal Medicine, University of Genova, Genova, Italy.

Abstract

Objective

Vitamin D deficiency seems to be involved in the development and severity of autoimmune/inflammatory diseases such as rheumatoid arthritis (RA). To evaluate the influence of calcitriol (1,25-dihydroxyvitamin D3, 1,25(OH)₂D₃) on aromatase expression in cultures of human macrophages, as a new target for vitamin D cell modulation and pro-inflammatory cytokine production.

Methods

Cultures of human monocytic THP-1 cells were activated to macrophages and treated for 24 hours with 1,25(OH)₂D₃ (10⁻⁸M), 17β-estradiol (E₂, 10⁻⁸M) both alone and in combination, in order to evaluate the effects on the intracrine estrogen metabolism. Untreated human macrophages were used as controls (basal). P450-aromatase synthesis was evaluated by immunocytochemistry (ICC) and western blot analysis (WB). The expression of P450-aromatase gene (CYP19A1) was investigated by real-time PCR (RT-PCR). Macrophage pro-inflammatory cytokines IL1-β, IL-6 and TNF-α were evaluated by ELISA and WB.

Results

In E₂ untreated condition, 1,25(OH)₂D₃ reduced P450-aromatase synthesis and CYP19A1 gene expression in cultured cells. Moreover, pro-inflammatory cytokine production (IL1-β, IL-6 and TNF-α) was significantly reduced by 1,25(OH)₂D₃ treatment (p<0.001 vs. basal for all cytokines). However, 1,25(OH)₂D₃ was found to significantly downregulate the E₂-mediated increase in P450-aromatase synthesis and gene expression (p<0.001 for both vs. E₂-treated macrophages), as well as the production of all pro-inflammatory cytokines (p<0.001 vs. E₂-treated cells).

Conclusion

Our data suggest that 1,25(OH)₂D₃ may downregulate the pro-inflammatory cytokine production in human activated macrophages by significantly decreasing the aromatase activity, especially in presence of an estrogenic milieu such as in the RA synovial tissue.

Key words

1,25-dihydroxyvitamin-D3, aromatase, macrophages, 17β-estradiol
Introduction
The incidence of autoimmune rheumatic diseases is higher in female than in male patients, and gender-linked differences in sex hormone serum levels and their peripheral metabolites play an important role in the neuroendocrine immune/inflammatory response, particularly on monocye/macrophage cells (1). Interestingly, in the synovial tissue of rheumatoid arthritis (RA) patients, macrophages are involved in the peripheral sex hormone metabolism that implicate the local action of the P450-aromatase (encoded by the CYP19A1 gene): in fact oestrogens induce rapid physiological changes in aromatase activity and their local concentration can be rapidly altered in inflammatory tissues (2, 3). Moreover, the aromatase activity is increased together with the oestrogen/androgen ratio in the RA synovial tissue/fluids of both sexes and recent studies showed that calcitriol (1,25-dihydroxyvitamin D$_3$; 1,25(OH)$_2$D$_3$) significantly decreased the aromatase expression in human cancer cells (BCa cells) by acting as a selective aromatase modulator (4, 5).

Epidemiological evidence indicates a significant association between vitamin D deficiency and an increased incidence of autoimmune diseases and the recent finding of the vitamin D receptors (VDR) on the immune cells (i.e. monocytes/macrophages, dendritic cells, Th1 and Th17 cells, B cells), together with their ability to produce 1,25(OH)$_2$D$_3$ in intracrine manner, clearly suggested that vitamin D could have immunoregulatory properties (1, 6). Recently, a functional synergy between 1,25(OH)$_2$D$_3$ and 17β-oestradiol (E$_2$) by inducing an anti-inflammatory effect on self-reactive T-cells and macrophages was observed in multiple sclerosis patients (7).

As known, in RA patients, very low serum levels of vitamin D associated with higher disease activity were observed and it was also shown that the absence of VDR signalling causes a pro-inflammatory monocyte phenotype associated with increased inflammation, cartilage damage and bone erosions (8). Therefore, this study evaluated the effects of 1,25(OH)$_2$D$_3$ on aromatase expression and pro-inflammatory cytokine production in cultures of human macrophages, which are cells fully involved in immune/inflammatory responses (especially in RA synovitis).

Materials and methods

Materials
Human monocyctic THP-1 cell line derived macrophage was obtained from the American Type Culture Collection. 1,25(OH)$_2$D$_3$ and E$_2$ were obtained from Sigma Aldrich (St. Louis, MO, USA); RPMI-1640 cell culture medium, foetal bovine serum (FBS) and penicillin-streptomycin from Cambrex (Cambrex Bio Science, VE, Belgium). Primary antibodies to P450-aromatase, IL-1β, IL-6 and TNF-α were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used for immuno-cytochemistry (ICC) and western blot analysis (WB). The immunoprecipitation (IP) buffer was purchased from Sigma-Aldrich and the enhanced chemiluminescence system (ECL) for WB from Amersham (Amersham S.r.l, Milan, Italy). Enzyme immunoassay (ELISA) kit for IL-1β, IL-6, TNF-α was purchased from R&D Systems DuoSet (Minneapolis, MN).

The real-time PCR (RT-PCR) system was purchased from Eppendorf (Eppendorf Srl Milan, Italy). RNA extraction was performed by Qiagen system (Qiagen spa, Milan, Italy); the reverse transcription was obtained using the Super Script II system by Invitrogen (Invitrogen, UK). Primers for beta-actin (NM_001101), CYP19A1 (NM_000103) (P450 aromatase related gene) were obtained by PrimerDesign (PrimerDesign UK). The real-time PCR reaction was performed using Real MasterMix SYBR Green detection system (Eppendorf).

Cell cultures
Human THP-1 (1x10$^6$ cells/well) were activated to macrophages with 500 U/ml of interferon-gamma (IFN-gamma, Sigma-Aldrich) in RPMI-1640 medium without red phenol supplemented with 5% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin. Before starting with the experiments, a dose-screening of 1,25(OH)$_2$D$_3$ was per-
formed (data not shown) and the concentration of $10^{-8}$ M was selected, in accordance to previous study (9). Then, human macrophages were treated with 1,25(OH)$_2$D$_3$ ($10^{-8}$ M), E$_2$ ($10^{-8}$ M) alone and in combination for 24 hours. Untreated human macrophages were used as controls (basal). All experiments were done in triplicate.

**Immunocytochemistry (ICC) and image analysis**

Human macrophages (8x10$^4$ cells/ml) were spotted on the glass slide and incubated with primary antibody to human P450-aromatase (dilution 1:100) and subsequently with the biotin-streptavidin HRP system.

The slides were analysed by Leica Q500MC image analysis system (Leica, Cambridge, UK) for quantification of % positive area as pixel for μm$^2$. The data were obtained by comparison with negative control intra-assay.

**Western blot (WB) analysis**

Cells were lysed in IP buffer (20mM Tris-HCl, 150mM NaCl, 1mM phenylmethylsulfonylfluoride, 5 mg/ml aprotenin, 0.5% Nonidet P-40) for 1 hour at 4°C and the lysates were centrifuged for 10 min at 13,000 rpm. The cell culture supernatant was collected and stored at -80°C. Thereafter, for every condition 20 μg of protein were separated by SDS-PAGE gel electrophoresis and transferred into Hybond-C-nitrocellulose membrane. The membranes were incubated with primary antibodies to human P450 aromatase, IL1β, IL-6, TNF-α and actin (dilution 1:200) in DPBS at room temperature and were washed for 1 hour in 0.05% DPBS/Tween 20, pH 7.4. Finally, the membranes were incubated with horseradish peroxidase-labelled polyclonal IgG antibody (SC-2020; Santa Cruz Biotechnology) (dilution 1:5,000) in DPBS for 1 hour at room temperature. After washing in DPBS, the proteins were detected using ECL.

**Enzyme immunoassay (ELISA)**

Cytokines (IL1β, IL-6, TNF-α) were quantified by ELISA kit. Optical densities were evaluated in an automatic ELISA plate reader at 492 nm. The cytokine concentrations were calculated over the standard curve obtained with different concentrations of human recombinant IL1β, IL-6 and TNF-α. The results were shown as pg/mL.

**Real-Time PCR analysis (RT-PCR)**

Total RNA was isolated using the RNA extraction system by Qiagen. First-strand cDNA was synthesised from RNA using the SuperScript II reverse transcriptase system. For each condition 1 μg of total RNA in 20 μL reaction mix of SuperScript II was used.

The RT-PCR was performed using the SYBER Green detection system and the Realplex (Eppendorf). Briefly, 2 μL cDNA was loaded in triplicate with the Syber Green mix (1x) and the 10 μM primer mix for beta-actin and CYP19A1, respectively.

Relative expression of the target genes was obtained by the comparative threshold ($\Delta\Delta$Ct) method using the basal condition as calibrator and actin as housekeeping gene (10). For each PCR amplification the melting curve was obtained confirming the specificity of the SYBR Green assay.

**Statistical analysis**

The data are given as histogram of mean ± standard deviation (SD) using Tukey-Kramer multiple comparisons non-parametric post-test (ANOVA system) and box plots, in which the group medians were compared by non-parametric Dunn’s test. All data analysis was obtained from series of different experiments in triplicate. Probability values (p-values) lower than 0.05 were considered statistically significant and shown in figure legends.
Results

The ICC analysis showed that P450-aromatase synthesis was slightly decreased by 1,25(OH)\(_2\)D\(_3\) vs. basal, whereas E\(_2\) significantly increased the aromatase production (p<0.001 vs. basal). Interestingly, 1,25(OH)\(_2\)D\(_3\) significantly reduced the E\(_2\)-mediated increase in P450-aromatase synthesis (p<0.001 vs. E\(_2\)-treated cells) (Fig. 1).

To confirm the data observed by ICC, the WB analysis was performed and the results showed that E\(_2\) increased P450-aromatase synthesis vs. basal. Interestingly, 1,25(OH)\(_2\)D\(_3\) reduced the production of P450-aromatase vs. basal and it was able to reduce the increase in P450-aromatase synthesis induced by E\(_2\) (Fig. 2A).

Therefore, in order to clarify if P450-aromatase was regulated at transcriptional level by 1,25(OH)\(_2\)D\(_3\) and E\(_2\), the CYP19A1 gene expression was analysed by RT-PCR. The results showed that E\(_2\) was able to increase the CYP19A1 gene expression (p<0.001 vs. basal) whereas, interestingly, 1,25(OH)\(_2\)D\(_3\) significantly downregulated the E\(_2\)-induced increase in CYP19A1 gene expression (p<0.001 vs. E\(_2\)-treated cells) (Fig. 2B). The analysis of pro-inflammatory cytokines showed that 1,25(OH)\(_2\)D\(_3\) significantly reduced IL-1\(\beta\), IL-6 and TNF-\(\alpha\) level at 24 hours of treatment in the cell culture supernatant by ELISA (p<0.001 vs. basal for all cytokines) (Table I). Moreover, the capability of 1,25(OH)\(_2\)D\(_3\) to significantly downregulate all the investigated pro-inflammatory cytokines was shown also in presence of E\(_2\) (p<0.001 1,25(OH)\(_2\)D\(_3\)/E\(_2\) vs. E\(_2\)-treated macrophages for IL-1\(\beta\), IL-6, TNF-\(\alpha\)) (Table I). The results were confirmed by WB analysis (Fig. 3).

Discussion

The present in vitro study showed that the treatment with 1,25(OH)\(_2\)D\(_3\) induced a decrease in P450-aromatase synthesis and relative gene expression in activated human macrophages. The significant downregulation of aromatase expression exerted by 1,25(OH)\(_2\)D\(_3\) in presence of E\(_2\) interestingly suggests its possible down-regulatory effects on synovial tissue hyperplasia/inflammation and might indicate P450-aromatase as a target for 1,25(OH)\(_2\)D\(_3\), which might act as a tissue selective aromatase modulator (2, 11). The aromatase enzyme complex catalyses oestrogen synthesis from androgenic precursor and its overexpression leads to the altered testosterone: oestrogen ratio, which is associated to immune/inflammatory reactions in both sexes, supporting an important role exerted by the aromatase in the pathophysiology of autoimmune rheumatic diseases, such as at the level of synovial cells in RA (2).
Oestrogens play important roles through their peripheral metabolites in autoimmune rheumatic diseases and the elevated endogenous oestrogen levels due to the P450-aromatase over-expression (related to chronic inflammation and malignancy like breast cancer), seem to link aromatase/oestrogen tissue increase also to the prostate cancer growth hence supporting a possible therapeutic role of aromatase inhibition (5). The important link between oestrogen and aromatase was also supported by this study where it was shown that E2 significantly upregulated both P450-aromatase protein synthesis and gene expression.

A previous study showed that IFN-gamma and 1,25(OH)2D3 induce on THP-1 cells distinct patterns of cell surface antigen expression, cytokine production, and responsiveness to contact with activated T cells (9).

Interestingly, a significant dose-related decrease in IL-6 synthesis by mixed cultures of OA synoviocytes (fibroblasts 37% / macrophages 26%) following treatment with 1,25(OH)2D3 was recently found (RH Straub et al., personal communication).

The present study might suggest that the significant downregulation exerted by 1,25(OH)2D3 on E2-mediated increase in P450-aromatase synthesis and expression should have therapeutic implications at least on RA synovial tissue hyperplasia and inflammation, as also confirmed by the significant downregulation of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in human macrophages. The presence of VDR and intracrine synthesis of 1,25(OH)2D3 in monocytes/macrophages clearly support important interactions between vitamin D and the immune system (12). Taken together these results suggest possible anti-inflammatory and immunosuppressive intracrine actions exerted by 1,25(OH)2D3 in tissues rich in monocytes/macrophages (i.e. RA synovial tissue) and might support new implications for D hormone (replacement) treatment at least in immune/inflammatory conditions characterised by its reduced serum levels (i.e. RA) (13-15).

Further (ongoing) experiments are addressed to understand the effects of vitamin D on the modulation of pro-inflammatory cytokines and P450-aromatase expression as well as to understand the involved intracellular mechanisms of vitamin D action by investigating the intracellular signalling modulation (i.e. ERK/MAPK pathway), in primary cultures of monocytes/macrophages from RA patients and linkings with growing clinical applications (16, 17).

References