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

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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$(OH)_2D_3$) 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$(OH)_2D_3$ (10^{-8}M), 17β-estradiol (E_2, 10^{-8}M) 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 E_2 untreated condition, 1,25$(OH)_2D_3$ 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$(OH)_2D_3$ treatment ($p<0.001$ vs. basal for all cytokines). However, 1,25$(OH)_2D_3$ was found to significantly downregulate the E_2-mediated increase in P450-aromatase synthesis and gene expression ($p<0.001$ for both vs. E_2-treated macrophages), as well as the production of all pro-inflammatory cytokines ($p<0.001$ vs. E_2-treated cells).

Conclusion

Our data suggest that 1,25$(OH)_2D_3$ 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

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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 monocyte/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; $1,25(OH)_2D_3$) 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 $1,25(OH)_2D_3$ in intracrine manner, clearly suggested that vitamin D could have immunoregulatory properties (1, 6). Recently, a functional synergy between $1,25(OH)_2D_3$ and 17β-estradiol (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 $1,25(OH)_2D_3$ 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 monocytic THP-1 cell line derived macrophage was obtained from the American Type Culture Collection. $1,25(OH)_2D_3$ 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 immunocytochemistry (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 $1,25(OH)_2D_3$ was per-

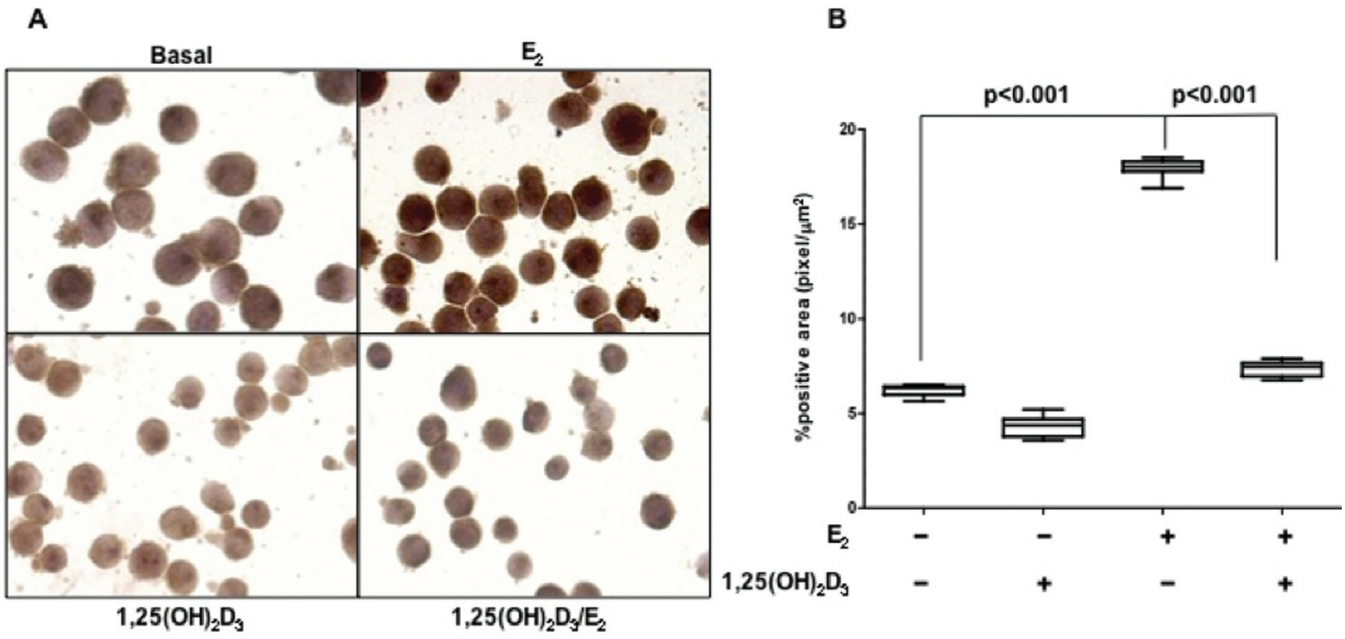

Fig. 1. P450 aromatase synthesis in cultured human macrophages. **(A)** Evaluation of human P450-aromatase synthesis by immunocytochemistry (ICC) staining (magnification 40x) and **(B)** related image analysis in cultures of human macrophages untreated (basal) and treated with 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), 17β-estradiol (E_2) alone and in combination ($1,25(OH)_2D_3/E_2$).

formed (data not shown) and the concentration of 10^{-8} M was selected, in according to previous study (9). Then, human macrophages were treated with $1,25(OH)_2D_3$ (10^{-8} M), E2 (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-streptoavidin HRP system.

The slides were analysed by Leica Q500MC image analysis system (Leica, Cambridge, UK) for quantisation 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 aprotinin, 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 ($\Delta\Delta C_t$) 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 $1,25(OH)_2D_3$ *vs*. basal, whereas E_2 significantly increased the aromatase production ($p<0.001$ vs. basal). Interestingly, $1,25(OH)_2D_3$ 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, $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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, $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ significantly reduced IL-1β, IL-6 and TNF-α level at 24 hours of treatment in the cell culture surnatant by ELISA ($p<0.001$ *vs*. basal for all cytokines) (Table I). Moreover, the capability of $1,25(OH)_2D_3$ to significantly downregulate all the investigated pro-inflammatory cytokines was shown also in presence of E_2 ($p<0.001$ $1,25(OH)_2D_3/E_2$ vs. E_2-treated macrophages for IL-1β, IL-6, TNF-α) (Table I). The results were confirmed by WB analysis (Fig. 3).

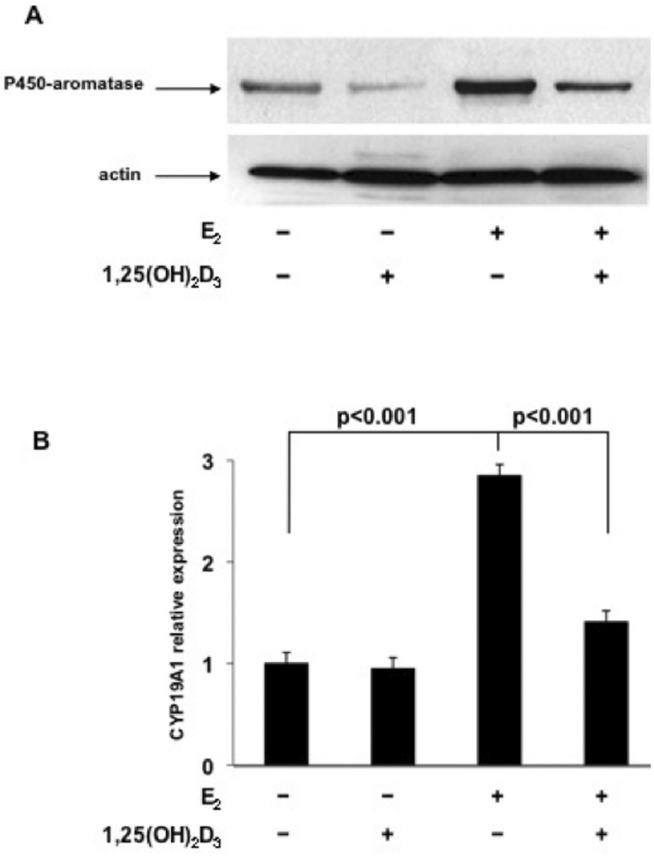

Fig. 2. P450 aromatase synthesis and gene expression in cultured human macrophages. **(A)** Evaluation of human P450-aromatase synthesis by Western blotting (WB) in cultures of human macrophages untreated (basal) and treated with 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), 17β-estradiol (E_2) alone and in combination ($1,25(OH)_2D_3/E_2$). **(B)** Evaluation of CYP19A1 mRNA expression by real-time PCR in cultures of human macrophages untreated (basal) and treated with 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), 17β-estradiol (E_2) alone and in combination ($1,25(OH)_2D_3/E_2$). The data of RT-PCR are expressed as relative expression of the target gene (CYP19A1) compared to the calibrator sample (basal).

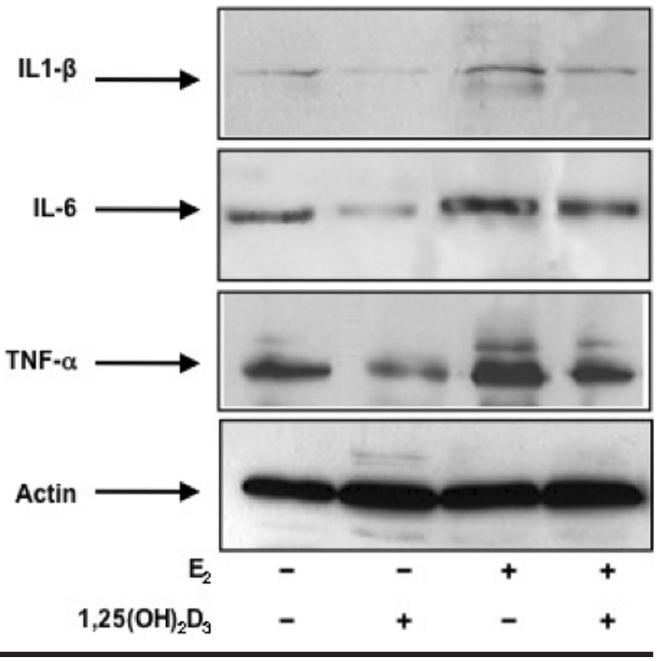

Fig. 3. Cytokine expression in cultured human macrophages. Evaluation of IL-1β, IL-6 and TNF-α by Western Blotting (WB) analysis in cultures of human macrophage untreated (basal) and treated with 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$), 17β-estradiol (E_2) alone and in combination ($1,25(OH)_2D_3/E_2$).

Discussion

The present *in vitro* study showed that the treatment with $1,25(OH)_2D_3$ induced a decrease in P450-aromatase synthesis and relative gene expression in activated human macrophages. The significant downregulation of aromatase expression exerted by $1,25(OH)_2D_3$ in presence of E_2 interestingly suggests its possible downregulatory effects on synovial tissue hyperplasia/inflammation and might indicate P450-aromatase as a target for $1,25(OH)_2D_3$, 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).

Table I. Cytokine production in cell culture surnatant from human macrophages.

Cytokines	Basal	$1,25(OH)_2D_3$		E_2	$1,25(OH)_2D_3/E_2$	
	pg/mL	pg/mL	*p*-value	pg/mL	pg/mL	*p*-value
IL1-β	1,250 ± 79	640 ± 105	$p<0.001$ *vs*. basal	1,450 ± 86	1,220 ± 135	$p<0.001$ *vs*. E2
IL-6	2,200 ± 161	1635 ± 125	$p<0.001$ *vs*. basal	2,100 ± 155	1,670 ± 67	$p<0.001$ *vs*. E2
TNF-α	1,400 ± 151	980 ± 122	$p<0.001$ *vs*. basal	1,540 ± 132	1,200 ± 140	$p<0.001$ *vs*. E2

Immunoenzymatic assay (ELISA) of IL-1β, IL-6 and TNF-α in cell culture surnatants from human macrophage untreated (basal) and treated with 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$), 17β-estradiol (E_2) alone and in combination ($1,25(OH)_2D_3/E_2$).
All assays were performed in triplicate; the values expressed as mean±SD.

Oestrogens play important roles through their peripheral metabolites in autoimmune rheumatic diseases and the elevated endogenous oestrogen levels due to the P450-aromatase overexpression (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 E_2 significantly upregulated both P450-aromatase protein synthesis and gene expression.

A previous study showed that IFN-gamma and $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ was recently found (RH Straub *et al*., personal communication).

The present study might suggest that the significant downregulation exerted by $1,25(OH)_2D_3$ on E_2-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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 monocyte/macrophages from RA patients and linkings with growing clinical applications (16, 17).

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