Cycling and early pregnant endometrium as a site of regulated expression of the vitamin D system

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Abstract

In addition to its calcitropic function, the secosteroid 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), has potent anti-proliferative/immunomodulatory effects on various tissues. Consistently, the enzyme that catalyzes the synthesis of 1,25(OH)₂D₃, 1 α -hydroxylase (1 α -OHase) and the vitamin D receptor have a widespread tissue distribution. Among site-specific functions, the hormone has been suggested to be involved in uterine physiology. However, molecular analysis of the vitamin D system in normal endometrium throughout the menstrual cycle as well as its regulation in the context of endometrial physiological and pathological events have received very limited attention. Thus, we have studied expression, localization and regulation of 1 α -OHase in human cycling and early pregnant endometrium. The capacity for 1 α -hydroxylation and the presence of vitamin D receptor in endometrial cells have also been evaluated. The functional significance of these findings has been tested by evaluating gene expression of the catabolic enzyme, vitamin D 24-hydroxylase, and of the adhesion protein, osteopontin. Finally, to verify any potential dysfunction of the vitamin D system in endometriosis, a reproductive disease characterized by immune-mediated anomalies, we have analyzed expression of 1 α -OHase in both eutopic and ectopic endometrium of affected patients. Results obtained showed that the active form of the 1 α -OHase gene was expressed in human endometrial stromal cells independent of the cycle phase but with a significant increase in early pregnant decidua. A similar profile was observed for the protein, which was abundantly expressed in the cytoplasm of both endometrial stroma and epithelial glands. Both cycling and early pregnant endometrial cells also expressed the vitamin D receptor. In the same cells, 1 α -OHase mRNA levels were significantly stimulated by the pro-inflammatory cytokine interleukin (IL)-1 β (50 and 500 pg/ml) while addition of the active form of the hormone could modulate both *CYP24* and osteopontin gene expression. The 1 α -OHase gene was also expressed in ectopic endometrium and its levels were increased in proliferative phase cultures derived from patients with endometriosis. Human cycling endometrium may be included among the extrarenal sites able to synthesize vitamin D. The IL-1 β -mediated induction of 1 α -OHase gene and the hormonal modulation of osteopontin support a role for the hormone in the immunological mechanisms underlying uterine function. Abnormalities of this system are present in endometriosis.

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Introduction

Vitamin D is a major regulator of mineral ion homeostasis by facilitating calcium and phosphate uptake in the gut and by modulating bone cell development and action (Kumar 1980, Jones *et al.* 1998). However, the most potent metabolite of vitamin D, the secosteroid 1,25-dihydroxyvitamin D (1,25(OH)₂D₃), has also been demonstrated to affect a wide range of functions not immediately linked to calcium homeostasis, suggesting a much broader physiological impact of the hormone in the body than originally envisioned (Zehnder *et al.* 2001). Specifically, 1,25(OH)₂D₃ has been shown to exert anti-proliferative and immunosuppressive

effects on several cell types (Peehl *et al.* 1994, Lemire *et al.* 1995, Muller & Bendtzen 1996, Long & Santos 1999). The importance of 1,25(OH)₂D₃ as a pleiotropic modulator of tissue functions is strengthened by the presence of key components of vitamin D metabolism at different sites. The cognate nuclear receptor for 1,25(OH)₂D₃ (VDR) is ubiquitous in proliferating cells (Hewison *et al.* 2000) and the mitochondrial cytochrome P450 enzyme 25-hydroxyvitamin D₃-1 α -hydroxylase (1 α -OHase), which catalyzes the synthesis of 1,25(OH)₂D₃ from its precursor 25-hydroxyvitamin D₃, is expressed in the kidney and in several extrarenal tissues (Zehnder *et al.* 2001, 2002b). The widespread co-expression of VDR and 1 α -OHase emphasizes a

putative role for $1,25(\text{OH})_2\text{D}_3$ as an autocrine/paracrine agent with diverse physiological functions (Hewison *et al.* 2000).

In the late 1980s and early 1990s, particular attention was directed towards the potential significance of the vitamin D endocrine system in human reproductive processes. Increased circulating levels of $1,25(\text{OH})_2\text{D}_3$ were observed during estroprogestins treatment as well as during human gestation (Kumar 1980, Hartwell *et al.* 1990, Salle *et al.* 2000). Moreover, uterine and placental cells were shown to synthesize $1,25(\text{OH})_2\text{D}_3$ (Acker *et al.* 1982, Delvin *et al.* 1985, Kachkache *et al.* 1993) and a single report demonstrated elevated serum levels of the hormone in a specific pathological condition of the reproductive system, endometriosis (Hartwell *et al.* 1990), which is characterized by immune system anomalies (Vignali *et al.* 2002).

In the last years, interest in this specific topic has been greatly increased for two reasons: (i) the development of mice deficient in VDR or $1\alpha\text{-OHase}$ has revealed that these animals show impaired ovarian folliculogenesis and uterine hypoplasia (Yoshizawa *et al.* 1997, Kinuta *et al.* 2000, Panda *et al.* 2001) and (ii) vitamin D seems to influence the reproductive system not only at the ovarian level but also at the endometrial level. Indeed, some of the genes recently identified to be uniquely regulated at the site of embryo implantation, for instance calbindin-D9k in the mouse and the homeobox (*Hox*) *A10* gene in humans, are vitamin D-dependent proteins (Rots *et al.* 1998, Nie *et al.* 2000, Salamonsen *et al.* 2002, Du *et al.* 2005).

To further investigate the significance of local $1,25(\text{OH})_2\text{D}_3$ production in the human uterus, in the present study we have analyzed endometrial expression of $1\alpha\text{-OHase}$ at both the mRNA and protein levels during the menstrual cycle and in early pregnancy. Local regulation of the enzyme by factors physiologically present at the fetal-maternal interface has also been addressed. Furthermore, we have investigated whether endometrial $1\alpha\text{-OHase}$ expression could effectively result in the synthesis of the hormone from its precursor $25(\text{OH})\text{D}_3$, verified the presence of the hormone receptor in endometrium and analyzed the functional effects of these results by evaluating the induction of specific target genes. Finally, to confirm the potential role of the vitamin D system in endometriosis, we have evaluated $1\alpha\text{-OHase}$ expression in both eutopic and ectopic endometrium of affected patients.

Materials and methods

Reagents

Progesterone and $25(\text{OH})\text{D}_3$ were purchased from Sigma (Milano, Italy). Human recombinant interleukin (IL)- 1β and tumor necrosis factor- α (TNF- α) were

obtained from Amersham Biosciences (Amersham, Cologno Monzese, Italy). Culture medium consisted of Ham's F-10 (Sigma, Italy) supplemented with 2 mM L-glutamine (Sigma, Italy), antibiotics, fungizone and 10% heat-inactivated FCS (Sigma, Italy). Collagenase A was purchased from Roche (Milano, Italy) and hyaluronidase from Sigma (Mountain View, CA, USA). All reagents for real-time quantitative PCR were from Applied Biosystems (Foster City, CA, USA). Primers for $1\alpha\text{-OHase}$ and hypoxanthine phosphoribosyl-transferase-1 (HPRT-1) used for qualitative PCR analysis were from Sigma. A conventional RIA kit for the quantitative measurement of $1,25(\text{OH})_2\text{D}_3$ was obtained from DiaSorin (Stillwater, MN, USA). Monoclonal antibody to vitamin D receptor was from Alexis Biochemicals (Lausen, Switzerland), antibody to β -actin was from Sigma, antiserum to $1\alpha\text{-OHase}$ was from The Binding Site (Birmingham, UK) and anti-CD45-FITC/CD14-PE was from Becton Dickinson.

Sample collection

Human samples were obtained from women who attended the endoscopic surgical service of the II Department of Obstetrics and Gynecology of the University of Milan to undergo gynecological laparoscopy for unexplained infertility, pelvic pain or benign ovarian pathology. Women with previous autoimmune, neoplastic, hepatic or thyroid disorders were excluded from the study. All subjects were younger than 40, had regular menstrual cycles and none had received hormones for at least 3 months.

Samples of uterine endometrium were obtained from 77 women. At laparoscopic visualization, 27 had evidence of endometriosis that was staged, according to the Revised American Fertility Society Classification (1997) (American Society for Reproductive Medicine 1997), as minimal (stage I) in 4 cases, mild (stage II) in 5 cases, moderate (stage III) in 11 cases and severe (stage IV) in 7 cases. In the 50 patients in whom endometriosis was not diagnosed, laparoscopic examination demonstrated normal pelvic organs in 6 cases, pelvic adhesions in 7 cases, benign ovarian pathology in 22 cases and benign uterine pathology in 15 cases. Among patients with endometriosis 14 were in the proliferative and 13 in the secretory phase of the cycle, while among controls 22 were in the proliferative and 28 in the secretory phase. Dating was based on the last menstrual period and on histological examination of the samples.

Endometriotic samples from peritoneal lesions and endometriotic cysts were excised from nine patients with severe endometriosis at operation and immediately frozen at -80°C .

Decidual tissues were obtained from 38 healthy women undergoing elective termination of normal pregnancies between 8 and 13 weeks of gestation. The

operative method used was cervical dilatation followed by vacuum extraction of the products of conception. After a careful aspiration of all the trophoblastic material, a biopsy curette was used to obtain decidual tissue.

Patients were informed in detail that serum or tissue samples would be used for research purposes and they gave a written consent. Approval for this study was granted by the local Human Institutional Investigation Committee.

Cell culture

Establishment of stromal cell monolayers from normal endometrial tissue has been described in detail in previous studies (Viganò *et al.* 1993). Diffuse and strong cytoplasmatic immunostaining for vimentin was demonstrated in nearly all (90%) cultured endometrial stromal cells. Cytofluorimetric analysis showed that leukocyte contamination (CD45-positive cells) of our cultures was less than 2%. Briefly, tissue was minced and digested in 10 ml Ham's F-10 containing 0.2% collagenase. Single stromal cells were separated from epithelium by differential sedimentation at unity gravity and selective adhesion to tissue culture dishes. Decidual tissue was minced thoroughly between two scalpels and digested for 1 h in HAM'S F-10 with 0.1% collagenase and 0.2% hyaluronidase. Decidual cells were then separated from dead cells and red cells by Ficoll-Hypaque density gradient. The cells at the interface were plated and left at 37 °C overnight, then washed several times to remove non-adherent cells and debris. Endometrial or decidual cells were cultured to subconfluence in Ham's F-10 with 10% FCS and antibiotics in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. Subconfluence was reached after a minimum of 8 days during which culture medium was changed every other day. To evaluate the effects of different compounds on endometrial 1 α -OHase mRNA expression, endometrial stromal cells were cultured with 0.1% bovine serum albumin when stimulated for 24 h with and without IL-1 β or TNF- α and with charcoal-stripped calf serum when stimulated for up to 9 days with and without progesterone. To evaluate the effects of 1,25(OH)₂D₃ on endometrial vitamin D-24-hydroxylase gene (*CYP24*) and osteopontin mRNA expression, cells were cultured with 0.1% bovine serum albumin with and without different concentrations of the hormone for four hours.

Immunohistochemistry

Immunohistochemical analysis of 1 α -OHase expression was performed using a previously described method (Zehnder *et al.* 2002b). Briefly, tissues were formalin-fixed, paraffin-embedded, cut into 4- μ m sections and placed on pre-treated slides. Sections were dewaxed and

rehydrated through graded alcohol using standard procedures. They were processed in 0.01 M sodium citrate buffer (pH 6.0) in a microwave for 15 min at the maximum power for antigen retrieval. Slides were then incubated with 1% hydrogen peroxide in Tris-buffered saline, pH 7.6 to block endogenous peroxidase activity, and then washed in Tris. The slides were then incubated with 1 α -OHase antiserum (1:150) in 10% normal swine serum overnight at 4 °C. After rinsing with Tris-buffered saline for 15 min, donkey anti-sheep IgG peroxidase conjugated (1:200) was added to sections for 45 min. Staining was developed using 3,3'-diaminobenzidine (2.5 mg/ml) followed by counterstaining with Mayer's hematoxylin. Negative control sections included the omission of primary antibody, the use of primary antibody preadsorbed with a 100-fold excess of immunizing peptide or the analysis of the liver as a negative tissue (Zehnder *et al.* 2001).

Extraction of mRNA, qualitative RT-PCR analysis for 1 α -OHase and sequence analysis

Total RNA was extracted from cells and tissues using Trizol (Invitrogen Life Technology, Carlsbad, CA, USA). One microgram total RNA was transcribed into cDNA using 1 mM of each dNTP, 0.75 μ g random hexamer primers, 25 units RNase inhibitor, and 200 units reverse transcriptase (RT) (Promega Corporation, Madison, WI, USA) in a total volume of 25 μ l. cDNA was amplified with 500 nM 1 α -OHase-specific primers (upstream: 5' ATG ACC CAG ACC CTC AAG 3'; downstream: 5' GTC GCA GAC TAC GTT GTT CAG 3') using RedTaq DNA polymerase (Sigma) according to the following protocol: 94 °C for 5 min (1 cycle), 94 °C for 30 s, 59 °C for 30 s, 72 °C for 30 s (38 cycles), 72 °C for 5 min (1 cycle). The primers were designed to generate a fragment of 492 bp that spans two introns. In each experiment, a negative control was prepared using all reagents and substituting 1 μ l water for the reverse transcriptase. Integrity of RNA and absence of genomic contamination was assessed by amplification of HPRT-1 gene as previously described (Viganò *et al.* 2002). The specificity of the products generated by the indicated primers was verified by sequence analysis.

Evaluation of gene expression by real-time RT-quantitative PCR (RT-qPCR) analysis

One microgram total RNA was reverse transcribed for 2 h at 37 °C using the high-capacity cDNA archive kit. The ABI Prism 7900 sequence detection system (Applied Biosystems) was used for real-time RT-qPCR analysis using HPRT-1 as an endogenous control. Real-time PCR was performed using specific primers and probes for 1 α -OHase, osteopontin and *CYP24* target genes

(Assays-on-Demand Gene Expression Products, Applied Biosystems). Validation experiments were performed using the 1:2 diluted templates. Reaction conditions included 10 µl 2 × TaqMan Universal PCR Master Mix, 1 µl primers and probes mixture, 50 ng template cDNA and nuclease-free water on a 96-well reaction plate. The total reaction volume was 20 µl. The cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C followed by 1 min at 60 °C. The data were analyzed using the comparative Ct method, where Ct is the cycle number at which fluorescence first exceeds the threshold. The Δ cycle threshold (Δ Ct) values from each sample were obtained by subtracting the values for the reference gene from the sample Ct. For each experimental sample the $2^{-\Delta\text{Ct}}$ has been calculated and data have been graphically indicated as relative expression.

Western blot for VDR and 1 α -OHase

Samples of endometrial stromal and decidual cell cultures were treated with a lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 10 µg/ml leupeptin and 10 µg/ml aprotinin. Lysates were subsequently centrifuged at 13 000 *g* for 30 min and the supernatant was collected for protein analysis. Sample protein concentration was determined using a commercial protein assay kit (BCA Protein Assay Kit, Pierce Biotechnology, Rockford, IL, USA). Proteins resolved by SDS-PAGE were transferred to Hybond ECL nitrocellulose membranes (Amersham, Italy). For VDR detection, after brief washing in TBST (25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1% Tween 20), the membrane was blocked with 5% skim milk plus 5% BSA/TBST overnight at 4 °C. All subsequent steps were performed at room temperature. The membrane was incubated for 3 h with 11 µg anti-VDR antibody diluted in 25 ml 5% skim milk plus 5% BSA/TBST. After one hour washing with TBST, membranes were incubated for 1 h with peroxidase-conjugated anti-rat IgG (Sigma). For 1 α -OHase detection, the membrane was blocked with 5% skim milk plus 5% BSA in TBST for 2 h at room temperature. The membrane was incubated overnight with anti-1 α -OHase antibody diluted 1:500 in 5% skim milk plus 5% BSA/TBST. After 1 h washing with TBST, membranes were incubated for 1 h with peroxidase-conjugated anti-sheep IgG (Sigma). For protein load control, anti- β -actin mouse monoclonal antibodies were used. Anti-mouse IgG secondary antibody was used at 1:5000 dilution. Bound antibodies were visualized by chemiluminescence. Control experiments were included where primary antibody was omitted or primary antibody was preabsorbed with a 100-fold excess of immunizing peptide.

Measurement of 1,25(OH) $_2$ D $_3$ production

Endometrial cells were seeded (5×10^5 cells/well/ml) in a 6-well plate. After washing with serum-free medium, cells were incubated with different concentrations of 25(OH)D $_3$ solubilized in absolute ethanol (0.1% final concentration) in 1% FCS culture medium for 24 h. The conditioned medium and cell monolayers were harvested. The quantitative detection of 1,25(OH) $_2$ D $_3$ levels was performed using a commercially available RIA kit with an intra-assay coefficient of variation of 6.8–11.3%, an interassay coefficient of variation of 11.2–14.6% and a sensitivity of <2 pg/ml. The concentration of 1,25(OH) $_2$ D $_3$ levels was expressed as pg/ml.

Statistical analysis

Differences between groups were compared, as appropriate, by unpaired Student's *t*-test, analysis of variance (ANOVA) and Fisher protected least significant difference-test as post-test. Probability <0.05 was considered to be statistically significant.

Results

Expression of 1 α -OHase and vitamin D receptor in normal endometrium

Normal endometrial stromal cells obtained from samples in different phases of the menstrual cycle and early pregnant decidual cells were first evaluated for 1 α -OHase mRNA expression by qualitative RT-PCR. During cell isolation and culture, a great effort was directed towards the complete elimination of immune cells as potential contaminants. Only cell populations 98% free of CD45- and CD14-positive cells as evaluated by phenotypic analysis were included in the study. PCR products were consistently detected in all samples analyzed (Fig. 1A). Estimated and actual size of the PCR products was 492 bp. The identity of the amplified products with the primer-defined 1 α -OHase DNA sequence was confirmed by sequence analysis (data not shown). Quantification of 1 α -OHase mRNA levels in early pregnant decidual cells and in endometrial cells in different phases of the cycle was performed by real-time RT-qPCR analysis. Results of the experiments performed in normal endometrial stromal cells derived from $n=28$ different tissues ($n=13$ and $n=15$ in the proliferative and secretory phases respectively) and decidual cells obtained from $n=25$ different samples indicated that levels of 1 α -OHase mRNA were similar in endometrial stromal cells independent of the phase of the cycle but were significantly increased in decidual cells ($P<0.05$) (Fig. 1B). Western blot analysis showed that the observed differences in 1 α -OHase mRNA levels were maintained at the protein level. A single western blot species (56 kDa) that corresponded to

Figure 1 Expression of 1α -OHase in normal cycling endometrial stromal cells and early pregnant decidua. (A) Qualitative RT-PCR of representative cases of proliferative phase endometrial stromal cells (lane 1), secretory phase endometrial stromal cells (lane 2) and early pregnant decidua (lane 3). Size marker is shown in the first left lane of the gel (MW). Lane 4 shows the negative control performed by substituting 1 μ l water for the RNA. (B) Real time-qPCR analysis of 1α -OHase mRNA in cultured cells derived from $n=13$ proliferative phase endometrial samples (PR), $n=15$ secretory phase endometrial samples (SE) and $n=25$ decidua samples. Data are presented as mean $\pm$ S.E.M. 1α -OHase relative expression. * $P<0.05$ versus decidua cells. (C) Western blot profile of total homogenate of endometrial and decidua cells. Three representative cases of proliferative phase endometrial stromal cells (lane 1), secretory phase endometrial stromal cells (lane 2) and early pregnant decidua cells (lane 3) are shown. Lane 4 represents a negative control in which primary antibody was preabsorbed with a 100-fold excess of immunizing peptide. Detection of β -actin was used for protein load control.

the reported renal 1α -OHase (Fig. 1C) was detected in both proliferative ($n=3$) and secretory ($n=3$) phase endometrial stromal cells and in first trimester decidua cells ($n=4$). Densitometric analysis of western blots showed an increase of about 40% in 1α -OHase expression in decidua versus normal cycling endometria of both phases.

To determine the cellular localization of 1α -OHase, its expression was also evaluated by immunohistochemistry. Comparable immunostaining for 1α -OHase was present in the endometrium of both proliferative and secretory phases (Fig. 2C,D). The protein was abundantly expressed in the cytoplasm of both stromal and epithelial layers with a diffuse distribution. As relative high levels of 1α -OHase protein have previously been shown in first trimester decidua (Zehnder *et al.* 2002b) this tissue was used as the positive control (Fig. 2A,B). Immunopositive cells were diffusely distributed throughout the decidua and had no special relationship with the blood vessels. Staining was absent in the negative controls (Fig. 2E,F).

To evaluate whether the cycling endometrium could also be a target of $1,25(\text{OH})_2\text{D}_3$ action, the presence of nuclear receptor VDR protein was investigated in proliferative ($n=3$) and secretory ($n=3$) phase endometrial stromal cells by western blot analysis. Decidua cells ($n=4$) have previously been shown to express the vitamin D receptor and were used as a positive control (Zehnder *et al.* 2002b). Rat antibody to human VDR reacted with both endometrial and decidua samples resulting in a band of 54 kDa (Fig. 3).

Regulation of 1α -OHase gene expression in normal endometrial stromal cells

We also investigated transcriptional regulation of 1α -OHase expression in endometrial stromal cells by IL- 1β ($n=6$), TNF- α ($n=6$) or progesterone ($n=6$) using real time RT-qPCR. IL- 1β , at concentrations of 50 and 500 pg/ml, significantly increased 1α -OHase expression by 44% and 130% respectively, while TNF- α at concentrations of 0.1 ng/ml and 1 ng/ml could not elicit a significant increase (Fig. 4). Incubation for up to 9 days with progesterone had no effect on endometrial 1α -OHase mRNA levels (data not shown). Similar experiments could not be performed in epithelial endometrial cells given the difficulty in sub-culturing this particular cell population (Viganò *et al.* 1993). Thus, no conclusions can be inferred for the regulation of 1α -OHase expression in glandular cells.

Production of $1,25(\text{OH})_2\text{D}_3$ and effect of the hormone on specific target genes in endometrial and decidua cells

Measurement of $1,25(\text{OH})_2\text{D}_3$ levels in the supernatant of endometrial stromal cells treated with $25(\text{OH})\text{D}_3$

Figure 2 Cellular localization of 1α -OHase in human cycling endometrium and early pregnant decidua as evaluated by immunohistochemistry. The presence of immunoreactive 1α -OHase is indicated by the brown staining in the cytoplasm of both epithelial and stromal cells of endometrial biopsies collected during the proliferative (C) and the secretory (D) phase of the menstrual cycle. Similarly, early pregnant decidua show 1α -OHase immunostaining (A, low magnification; B, higher magnification). The negative controls for specificity of 1α -OHase expression were performed by preabsorbing the antiserum with 100-fold excess of immunizing peptide (E) or testing a negative tissue such as the liver (F). (Original magnification: $\times 100$ for A and E and $\times 200$ for B, C, D and F.)

resulted in a constitutive and dose-dependent production of the hormone, thus indicating that endometrium represents a site of local conversion from the precursor to the active form (Fig. 5A). Levels of produced $1,25(\text{OH})_2\text{D}_3$ were similar to those detected in other recognized extrarenal sites of production (Fritsche *et al.* 2003).

The functional consequences of $1,25(\text{OH})_2\text{D}_3$ production and the presence of VDR were tested by evaluating the expression level of two target genes, *CYP24* and

osteopontin. *CYP24* is one of the most potent $1,25(\text{OH})_2\text{D}_3$ -responding genes and its protein product is responsible for the hydroxylation reaction that deactivates the biologically active vitamin D sterol. Osteopontin is an adhesion molecule with roles in implantation and decidualization (Johnson *et al.* 2003) and is regulated by vitamin D in different tissues (Christakos *et al.* 2003). Levels of *CYP24* mRNA were negligible in unstimulated endometrial cells and were detectable but still very low in unstimulated decidual

Figure 3 Expression of VDR in endometrial stromal cells and early pregnant decidual cells as evaluated by Western blot. Three representative cases of proliferative phase endometrial stromal cells (lane 1), secretory phase endometrial stromal cells (lane 2) and early pregnant decidual cells (lane 3) are shown. Lane 4 represents a negative control in which primary antibody was preabsorbed with a 100-fold excess of immunizing peptide.

cells. A significant and strong increase was observed in both types of culture after addition of $1,25(\text{OH})_2\text{D}_3$ for 4 h (Fig. 5B). Similar to that reported for other fibroblast-like cells (Tashiro *et al.* 2004), in endometrial cells transcription levels went up to about 2000-fold for a $1,25(\text{OH})_2\text{D}_3$ concentration of 1000 nM; in decidual cells this increase was still very strong. Cells were also tested for osteopontin expression, which in the basal condition, as previously reported by von Wolff *et al.* (2004), was significantly increased in decidual cells when compared with endometrial stroma. Addition of $1,25(\text{OH})_2\text{D}_3$ for 4 h at a concentration of 1000 nM significantly stimulated osteopontin expression levels by about 60–70% in both types of culture (Fig. 5C).

Figure 4 Effect of IL-1 β and TNF- α treatment on 1α -OHase gene expression as evaluated by real-time RT-qPCR analysis. Messenger RNA levels were evaluated in endometrial stromal cells treated with and without different concentrations of the cytokines for 24 h. Data from $n=6$ experiments were analyzed by real time qPCR and presented as mean $\pm$ S.E.M. relative expression. * $P < 0.05$ vs corresponding unstimulated controls.

Expression of 1α -OHase in eutopic and ectopic endometrium from patients with endometriosis

Comparison of mRNA levels for 1α -OHase in eutopic endometrial samples of patients with ($n=14$ and $n=13$ in the proliferative and secretory phases respectively) and without endometriosis by real time RT-qPCR demonstrated a significant increase for proliferative phase cultures derived from patients affected by the disease ($P < 0.05$) (Fig. 6A). Western blot analysis confirmed that these differences were maintained at the protein level (Fig. 6B).

Three biopsies of endometriotic peritoneal lesions and nine biopsies of endometriotic cysts were tested for 1α -OHase mRNA expression by qualitative RT-PCR. Amplified DNA products were consistently detected in all samples analyzed (Fig. 6C).

Discussion

Recent findings have emphasized the potential role of $1,25(\text{OH})_2\text{D}_3$ in decidual physiology (Zehnder *et al.* 2002b). To the best of our knowledge, this is the first study that has investigated the vitamin D system in human cycling endometrium. The presence of the enzyme that catalyzes the synthesis of the active form of vitamin D in cycling endometrium and its up-regulation in first trimester decidua, support the possibility that this hormone might be involved in some mechanisms of pregnancy establishment or maintenance.

Even more intriguing in this context is the observation that IL-1 β caused a strong increase in 1α -OHase mRNA expression in endometrial stromal cells. IL-1 β is thought to actively participate in the synchronized cooperation between the endometrium and the preimplanting embryo under the influence of steroid hormones, both in mice and humans (Lindhard *et al.* 2002). In humans, secretion of embryonic IL-1 β seems to be the first response of the blastocyst to the receptive endometrium, stimulating a second wave of cytokines essential for attachment of the blastocyst (Lindhard *et al.* 2002). We have herein demonstrated that 1α -OHase is among those genes transcriptionally modulated by IL-1 β at the endometrial level. Interestingly, in contrast to what has been observed for macrophages and endothelial cells (Pryke *et al.* 1990, Zehnder *et al.* 2002a), the mechanism of cytokine-induced 1α -OHase activity at the endometrial level seems to rely mostly on IL-1 β . Indeed, from the results of this study, the TNF- α -mediated effect on endometrial 1α -OHase expression is not as potent as that obtained with very low concentrations of IL-1 β .

The effect of vitamin D in the uterus may be exerted either via the regulation of specific target genes (Du *et al.* 2005) or through the well established immunomodulatory effects of the hormone (Lemire *et al.* 1995). Among the target genes there are calbindins and the *HoxA10*

gene which are critical for implantation in mice and in humans respectively (Benson *et al.* 1996, Taylor *et al.* 1998, Nie *et al.* 2000, Salamonsen *et al.* 2002, Du *et al.* 2005) and are known to be transcriptionally regulated by vitamin D in different cell types (Rots *et al.* 1998, Nie *et al.* 2000, Du *et al.* 2005). We have herein identified osteopontin as another possible target gene regulated by vitamin D. Osteopontin is an acidic member of the small integrin-binding ligand *N*-linked glycoprotein family of extracellular matrix proteins/cytokines. This versatile protein is a major constituent of the uterine-placental microenvironment and exerts its influence as a component of the endometrial gland secretion and as a gene product expressed by uterine stroma contributing to a decidualization-like transformation that correlates with the degree of conceptus invasiveness (Johnson *et al.* 2003). While 1,25(OH)₂D₃ has been shown to induce the gene in some tissues, this is the first demonstration of this effect in endometrial and decidual cells, giving further support for a role of the hormone as a local paracrine signal.

If we consider a possible action of 1,25(OH)₂D₃ as a natural regulator of the immune system, it is important to note that these effects can be viewed in the context of the local immune responses supposed to be critical for the development of a normal pregnancy. Indeed, in a successful pregnancy, the cytokine profile is thought to be shifted away from cell-mediated (T helper (Th)1-type) responses towards humoral immunity (Th2-type) (Hill *et al.* 1995, Viganò *et al.* 2002). Vitamin D promotes the shift away from Th1-type responses and favours a Th2-type immunity by inhibiting the secretion of IL-12, IL-2, TNF and γ -interferon by T cells, macrophages and dendritic cells (Limire *et al.* 1995, Muller & Bendtzen 1996, D'Ambrosio *et al.* 1998, Long & Santos 1999).

In the present study, we have also demonstrated expression of 1 α -OHase by ectopic endometriotic implants and the presence of higher levels in eutopic cells of women affected by the disease. As for many other molecules found to be aberrantly present in

Figure 5 Synthesis of 1,25(OH)₂D₃ in endometrial cells and transcriptional effects on specific target genes. (A) 1,25(OH)₂D₃ local synthesis from 25(OH)D₃ was measured by an RIA assay. Endometrial stromal cells were cultured for 24 h in 1% FCS culture medium in the presence or absence of 25(OH)D₃. (B) Real time RT-qPCR analysis of *Cyp24* mRNA in cultures derived from *n*=3 endometrial samples and *n*=5 decidual samples. Cells were treated with and without different concentrations of the hormone for 4 h. Data are presented as mean $\pm$ S.E.M. *Cyp24* relative expression. **P*<0.05 versus corresponding unstimulated samples. (C) Real time RT-qPCR analysis of osteopontin mRNA in cultures derived from *n*=4 endometrial samples and *n*=4 decidual samples. Cells were treated with and without different concentrations of the hormone for 4 h. Data are presented as mean $\pm$ S.E.M. osteopontin relative expression. **P*<0.05 versus corresponding unstimulated or indicated samples.

Figure 6 Expression of 1 α -OHase in eutopic endometrial stromal cells from women with and without endometriosis and in ectopic tissues. (A) Real time RT-qPCR analysis of 1 α -OHase mRNA in cultured cells derived from $n=28$ eutopic endometrial samples from disease-free women and $n=27$ eutopic samples from affected women. Data are presented as mean $\pm$ S.E.M. 1 α -OHase relative expression. * $P < 0.05$ versus corresponding secretory phase samples. PR, proliferative phase; SE, secretory phase. (B) Western blot profile of total homogenate of eutopic endometrial cells. Representative cases of proliferative (lane 1) and secretory (lane 2) phase eutopic endometrial cells from disease-free women and proliferative (lane 3) and secretory (lane 4) phase eutopic endometrial cells from affected women are shown. Lane 5 represents a negative control in which primary antibody was preabsorbed with a 100-fold excess of immunizing peptide. Detection of β -actin was used for protein load control. (C) Analysis of 1 α -OHase mRNA by qualitative RT-PCR in eutopic endometrial stromal cells of women with endometriosis (lanes 1–2, proliferative and secretory phases of the cycle respectively), in two biopsies of endometriotic peritoneal lesions (lanes 3–4) and in one biopsy of endometriotic cysts (lane 5). Lane 6 is the negative control prepared using all reagents and substituting 1 μ l water for the reverse transcriptase. Size marker is shown on the left side of the gel (MW).

endometriosis, it is difficult to clarify whether the increased endometrial 1 α -OHase mRNA levels in the proliferative phase represent a primary event or a consequence of the disease. It might be hypothesized that a secondary endometrial response to the peritoneal inflammatory reactions, immediately following menstruation, is responsible for this increase. Alternatively, endometrium from women affected might be constitutively more able to produce vitamin D. In both cases, the hormone, mostly for its ability to modulate cytokine production and inflammatory mediators (D'Ambrosio *et al.* 1998) may influence the functional activities of specific immune populations, such as natural killer cells that, in these women, are characterized by peculiar features (Vignali *et al.* 2002).

In conclusion, the results of this study support the following observations: (i) human cycling endometrium may be included among those sites capable of extrarenal synthesis and action of vitamin D; (ii) the enzyme 1 α -OHase is expressed in human endometrial stromal cells independently of the phase of the menstrual cycle but its expression is up-regulated in early pregnant versus cycling endometrium; (iii) IL-1 β is a potent inducer of endometrial 1 α -OHase mRNA expression; (iv) endometrium also expresses the vitamin D receptor; (v) in endometrial and decidual cells, the *Cyp24* gene, whose product catalyzes the first step of the degradation pathway of 1,25(OH) $_2$ D $_3$, is strongly induced transcriptionally by the hormone; (vi) the osteopontin gene is a target of vitamin D action in both cycling and early pregnant endometrium; (vii) in endometriosis patients,

the gene coding for 1 α -OHase is expressed also in ectopic endometrium and its expression is enhanced in eutopic endometrium during the proliferative phase.

Taken together, these results confirm the necessity to further investigate the functional role of the vitamin D system at the endometrial level.

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