The selective vitamin D receptor agonist, elocalcitol, reduces endometriosis development in a mouse model by inhibiting peritoneal inflammation

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BACKGROUND: Endometriosis, which is characterized by the growth of endometrial tissue at ectopic locations as well as vascular development and inflammation, is still an unmet clinical need since an optimal drug that allows for both pain and infertility management does not exist. Since both the eutopic and the ectopic endometrium express the vitamin D receptor (VDR), and VDR agonists are endowed with anti-proliferative and anti-inflammatory properties, we evaluated the effect of elocalcitol, a VDR agonist with low calcemic liability, in a mouse model of experimentally induced endometriosis.

METHODS AND RESULTS: Endometriosis was induced by injection of syngeneic endometrial tissue fragments into adult Balb/c female mice. After having confirmed by immunohistochemistry that endometriotic lesions developing in mice expressed VDR, the mice were administered with elocalcitol (100 μg/kg) or vehicle orally, once a day, for various durations of time. In this model, elocalcitol was able to reduce total lesion weight up to 70% upon treatment for 1 week before and 2 weeks after disease induction. Interestingly, a therapeutic effect was also observed on already established lesions. Elocalcitol was shown to reduce the capacity of mouse endometrial cells to adhere to collagen. In addition in treated mice, a decreased state of peritoneal inflammation was demonstrated by the inhibition of macrophage recruitment and inflammatory cytokine secretion.

CONCLUSIONS: The VDR agonist elocalcitol inhibits lesion development in a validated mouse model of endometriosis, and exerts a protective effect on both the implantation and organization of transferred endometrial tissue. These preliminary data in mice provide a sound rationale for further testing in primate models and eventually in humans.

Key words: endometriosis / vitamin D receptor agonist / inflammation

Introduction

Endometriosis is a common disease characterized by the persistence and growth of vascularized endometrial tissue at ectopic sites, typically the pelvis, and associated with pelvic pain and infertility (Kyama et al., 2003; Giudice and Kao, 2004; Nap et al., 2004). The development of the disease in the pelvis is attributed to the attachment and the survival of endometrial cells in the peritoneal cavity, and progressive invasion of the peritoneum with neoangiogenesis leading to the spreading of the disease. A frequent histological feature of endometriosis is...
represented by signs of inflammation. Increased levels of acute inflammatory cytokines are likely to enhance the adhesion of shed endometrial fragments onto peritoneal surfaces while proteolytic metalloproteinases may further promote implantation of the fragments. RANTES and interleukin (IL)-8 attract natural killer cells and macrophages that are typical of endometriosis. Moreover, nuclear factor-κB (NF-κB) appears to be constitutively activated in endometriotic cells (Guo, 2007). Thus, in patients with endometriosis, inflammatory and immune responses, angiogenesis and apoptosis are altered in favour of the survival and replenishment of endometriotic tissue (Bulu, 2009).

The human cycling endometrium has been shown to express the vitamin D receptor (VDR) (Vigano et al., 2006), and a few studies have demonstrated the functional role of vitamin D₃ in female reproduction (Kwiecinski et al., 1989; Yoshizawa et al., 1997; Shand et al., 2010). The VDR is a nuclear ligand-inducible transcription factor that regulates, in complex with hormonally active vitamin D, the expression of more than 900 genes involved in a wide array of physiological functions including calcium homeostasis, growth control, differentiation and apoptosis of many cell types, regulation of immune responses and angiogenesis (Carlberg, 2003; Holick, 2004; Nagpal et al., 2005). The discovery of VDR expression in most cell types of the immune system prompted a number of studies investigating the capacity of VDR agonists to modulate immune responses (Adorini, 2002). VDR agonists were found to be selective inhibitors of Th1 cell development (Mattner et al., 2000; Adorini, 2005) and to inhibit Th1-type cytokines (Alroy et al., 1995; Cippitelli and Santoni, 1998). Moreover, in addition to exerting direct effects on T cell activation, VDR agonists modulate the function of dendritic cells and monocytes—macrophages leading them to acquire tolerogenic properties. It has been shown that 1α,25(OH)₂D₃, the active hormonal form of vitamin D₃, induces the differentiation of myeloid progenitors into macrophages (Koeffler et al., 1984), exerts strong suppressive effects on interferon-γ-stimulated macrophages (Helming et al., 2005) and mediates human anti-microbial responses (Liu et al., 2006). Several immunomodulatory effects could be mediated by the capacity of VDR agonists to inhibit the nuclear factor NF-κB (Griffin et al., 2003). In addition, the inhibition of leukocyte infiltration into inflammatory sites by treatment with VDR agonists is associated with their capacity to inhibit chemokine production by cells in the target organ via the inhibition of NF-κB activation (Giarratana et al., 2004).

Based on their immunoregulatory and anti-inflammatory properties, VDR agonists have so far found therapeutic application in different indications, mainly osteoporosis, secondary hyperparathyroidism and psoriasis (Adorini and Penna, 2008). However, the pleiotropic anti-inflammatory effects exerted by VDR agonists might be beneficial in other pathological conditions characterized by chronic inflammation (Holick, 2007).

The main aim of this study was to determine the effect of oral administration of elocalcitol (1α-fluoro-25-hydroxy-1,23E-diene-26,27-bishomo-20-epi-cholecalciferol), a VDR agonist with low calcemic liability and well-defined anti-proliferative and anti-inflammatory properties (Nagpal et al., 2005; Adorini et al., 2007), on the development of endometriotic lesions in a mouse model of endometriosis. With this aim, we have further validated the mouse model for reproducibility and ability to recapitulate the biological behaviour of human endometriosis. Endometriotic lesions developing in mice were then assessed for VDR expression and the effects of elocalcitol on endometriosis initiation and propagation were evaluated. Finally, the molecular mechanisms of these effects were investigated: (i) by testing elocalcitol treatment on in vitro adhesion of endometrial cells to collagen as a critical step reflecting the ability of endometrial cells to adhere and invade surrounding tissues; and (ii) by evaluating inflammatory cytokines and macrophage recruitment in the peritoneum of elocalcitol-treated mice.

**Materials and Methods**

**Treatments**

The VDR agonist elocalcitol was provided by Dr Milan Uskokovic (BioXell Inc., Nutley, NJ, USA). The compound was reconstituted with 100% ethanol and stored at −80 °C in an oxygen-free atmosphere in the dark. The ethanol stock of elocalcitol was dissolved in Miglyol 812 vehicle (caprylic/capric triglyceride) at the appropriate concentration. Control mice received the vehicle containing the same amount of ethanol. Drug (or vehicle) treatment was carried out by daily oral gavage after weighing the mouse (8–10 animals per treatment group). Serum calcium levels were measured with a commercially available colorimetric assay (Sentinel Diagnostics, Milan, Italy), according to the manufacturer’s instructions.

**Validation of the endometriosis model**

All procedures were performed in the animal facility of the San Raffaele Scientific Institute (Milan, Italy) in accordance with European Union guidelines and with the approval of the Institutional Animal Care and Use Committee of our Institution. Eight-week-old female mice were obtained from Charles River Laboratories (Calco, Como, Italy) and kept under controlled conditions. All animals were allowed to have at least 1 week of acclimatization to this environment before surgery. We used 8–10 animals per experimental group for each independent experiment. The mouse model of endometriosis was adapted from the model described (Somigliana et al., 1999). Briefly, donor mice were injected with 17β-estradiol (AMSA, Rome, Italy; 3 μg/mouse) and 1 week later were sacrificed, and the uterus was removed, the two horns were isolated, the myometrium was removed by scraping and the remaining endometrial tissue was reduced to small fragments with scissors. The fragments derived from the isolated uterine horns were weighed and resuspended in saline with ampicillin (1 mg/ml) and then half of the preparation was injected into the peritoneum of each of two recipient mice with a syringe (Day 0). Based on the protocol to be used, mice were sacrificed at specific time points by administration of a lethal dose of anaesthetic, their abdomen was opened and lesion presence was evaluated by an operator blinded to the different conditions. Lesions could be identified as translucent or group superfical lesions mainly found on the abdominal wall, on the epiploon and around the uterus. Deeply infiltrating lesions were never observed in this model. In some cases, lesions resembling human chocolate cysts were found. Of the total 350 mice with experimentally induced endometriosis analysed in this study, 30 presented necrotic lesions and were excluded from the analysis. The lesions were heterogeneous in number, shape, size and fluid content. Therefore, in order to provide a standard quantitative evaluation of the overall extent of endometriosis, the dry weight of all lesions was used as the parameter to evaluate the disease extent (Somigliana et al., 1999). In previous reports (Somigliana et al., 1999; Rossi et al., 2000; Bacci et al., 2009), we have validated and improved this mouse model. While in the original model, endometriotic lesion development and extent had been assessed by the
measurement of both dry weight and surface area, we have subsequently demonstrated that the two measures did strongly correlate (Somigliana et al., 1999). Therefore, we have decided to limit the evaluation to the dry weight assessment. We have also established that no differences in gross and microscopic appearance or in the number of endometriotic lesions were observed between C57BL/6 and BALB/c mice (Somigliana et al., 1999). Therefore, our choice to use one or the other strain for the different kinds of experiments was mostly based on practical reasons (availability and easiness to handle). Finally, ovarectomization of the recipient mice (Somigliana et al., 1999) was considered not to be a necessary procedure, because we have demonstrated that in mice, endometriosi s progression was dependent on the functional and structural integrity of the ovaries independent of the cycle phase and that estrogen-supplemented mice showed only a slight but not significant increase in lesion size compared with non-supplemented ones (Rossi et al., 2000).

Therefore, in all subsequent experiments, we decided to eliminate ovariectomy and estrogen supplementation of the recipient mice while maintaining the estrogen supplementation of donor mice (Basci et al., 2009).

In the present study, the mouse model was further validated with three different strategies:

(i) By removing the uterus from transgenic C57BL/6 mice expressing enhanced green fluorescent protein (GFP) under the human ubiquitin C promoter [C57BL76-Tg(UBC-GFP)30Scha/J; The Jackson Laboratory, Bar Harbor, ME, USA], and therefore expressing GFP in all tissues, and by transferring the endometrial fragments to C57BL/6 wild-type mice. When mice were sacrificed 2 weeks after endometrial transfer, endometriotic lesions of donor origin were easily identifiable as green fluorescent under UV light of a dissection microscope. This step allowed precise visualization of the tissue origin of the endometriotic lesions.

(ii) By treating recipient animals with a GnRH analogue (GnRHa, Enantone; Takeda, Rome, Italy) to block estrogen-induced proliferation of endometriotic lesions. This step allowed us to mirror the human condition induced by a hypoestrogenic state that inhibits endometriosis development. Briefly, Enantone (1 mg/kg i.m.) was administered to recipient mice 1 week before and on the day of the operation. Endometriotic lesion development was measured after 2 weeks as described above. At the time of lesion harvesting, blood samples were obtained from the carotid artery and serum estradiol levels were measured with ELISA kits (Estradiol ELISA, Cayman Chemical Co., Ann Arbor, MI, USA).

(iii) By showing its high reproducibility. Thirty couples of paired animals (Groups A and B) were each injected with fragments from one of the two uterine horns from estrogen-treated donor mice and overall lesion weight was evaluated in each group after 2 weeks. This step allowed us to verify the variability of lesion development between the mice pairs.

**Experimental design**

Starting from 4 h after the endometrial injections, paired Balb/c mice that have received the uterine horns from the same donor were administered with elocalcitol (100 μg/kg) in miglyol or miglyol alone orally, once a day, 5 days a week for 2 weeks (from Days 0 to 4 and from Days 7 to 12) (Protocol 1, see cartoon in Fig. 2A). In addition to this treatment protocol, we further investigated elocalcitol therapeutic potential on the prevention of lesion establishment and on the regression of endometriotic lesions by treating the mice with elocalcitol (100 μg/kg) in miglyol or miglyol alone orally, once a day, according to the following protocols: Protocol 2, from Days −7 to −3; Protocol 3, from Days −7 to −3, from Days 0 to 4 and from Days 7 to 12; Protocol 4, from Days 2 to 4 and from Days 7 to 12; Protocol 5, from Days 14 to 18 and from Days 21 to 25.

Lesions were evaluated on Day 14 in the case of Protocols 1–4 and on Day 28 in the case of Protocol 5. The dry weight of all lesions isolated from each elocalcitol-treated mouse was assessed and compared with that of miglyol-treated mice.

**Histology and immunohistochemistry**

In order to perform histological analysis, endometriotic lesions were removed from the peritoneum of mice and immediately fixed in 4% paraformaldehyde and cryopreserved in 10–30% sucrose and then in OCT embedded tissue blocks. Five-micrometre sections were stained with haematoxylin–eosin to detect the histological structure. For immunohistochemical analysis, tissues cut into 5 μm sections were placed on pre-treated slides and processed in 0.01 M sodium citrate buffer (pH 6.0) in a microwave for 10 min at the maximum power for antigen retrieval. Slides were then incubated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity and then washed in phosphate-buffered saline (PBS). After appropriate blocking, the slides were then incubated with a rabbit polyclonal antibody against mouse estrogen receptor (ER)-α (Santa Cruz clone MC-20) or rabbit polyclonal immunoglobulin G (IgG; ab27472, Abcam, Cambridge, UK) as a control, overnight at 4°C followed by an anti-rabbit Vectastain ABC kit (Vector Laboratories, Vinci, Italy) for 1 h at room temperature. Positive immunostaining was revealed with Nova Red substrate (Vector Laboratories) and nuclei counterstained with Mayer haematoxylin. VDR staining was performed with an anti-VDR polyclonal antibody (PAI-7/L), Thermo Scientific, Fremont, CA, USA) or rabbit polyclonal IgG (ab27472, Abcam) as a control, followed by an anti-rabbit HRP Polymer Mach4 detection kit (Biocare Medical, Concord, CA, USA), and nuclei were counterstained with Mayer haematoxylin. Positive controls for ER-α and VDR are performed with mouse eutopic endometrium and mouse skin, respectively. Slides were examined under a Nikon Eclipse 55i microscope (Nikon, Tokyo, Japan), objective ×20/0.75, eyepiece ×10. Images were captured with a Digital Sight DS-5 M digital camera (Nikon) using Lucia G software (Laboratory Imaging, Prague, Czech Republic).

**Cell adhesion assay**

Paired animals that had received endometrial fragments from the same donor animals were treated with elocalcitol (100 μg/kg) in miglyol or miglyol alone orally once a day, for 2 days. Then, the animals were sacrificed and uterus horns were removed. The myometrium was removed by scraping with a scalpel blade and the remaining endometrial tissue was reduced to small fragments with scissors. Polystyrene 96-well plates (Euroclone, Pero, Italy) were coated with 50 μl/well of 8 mg/ml extracellular matrix (ECM) gel from E-H-S. mouse sarcoma, Sigma, Milan, Italy) and left uncovered in a laminar flow hood overnight to allow evaporation. The plates were then rinsed with PBS and used for the adhesion assays. Endometrial tissue fragments obtained as described above, were washed three times with PBS, trypsinized, resuspended in Dulbecco’s modified Eagle’s medium (DMEM)/0.1% fatty acid-free bovine serum albumin and were then seeded into 200 μl medium at a density of 2 × 10⁴/ml on ECM. After 30 min or 1 h of incubation at 37°C, the wells were gently rinsed three times with PBS to remove unattached cells. The remaining cells in 96-well plates were reacted with CyQUANT™ cell proliferation kit (Life Technologies, Invitrogen, Monza, Italy). Fluorescence measurements were made using a microplate reader with excitation at 485 nm and emission detection at 530 nm. Adhesion data are expressed as mean fluorescence ± SEM of one representative experiment out of three independent experiments, measured in triplicate samples.
Isolation of peritoneal macrophages

Mouse peritoneal cells were elicited by an intraperitoneal injection of 4 ml of PBS containing 2 mM EDTA into mice with experimentally induced endometriosis treated for 2 weeks with elocalcitol in miglyol or miglyol alone. Peritoneal exudate cells, obtained from pooled peritoneal fluids from five mice, containing lymphocytes and macrophages, were washed twice and resuspended in ice-cold RPMI-1640/glutamax, 5% fetal bovine serum (FBS), pen/strep and sodium pyruvate. Cell viability, verified by trypan blue exclusion, was typically >98%. No significant difference in the total number of cells elicited was found in different animals within the same treatment group. The cells were then seeded in flat-bottomed 96-well plates at 1 x 10⁶ cells/ml in a final volume of 200 μl. The cells were incubated at 37°C for 2 h to adhere, and non-adherent cells were removed by repeated washing with RPMI 1640 medium. Cells were stained with allophycocyanin-conjugated anti-CD11b antibody (BD Biosciences, Bucinascio, Italy) and phycoerythrin-conjugated anti-F4-80 antibody (R&D Systems, Abingdon, UK) and analysed by flow cytometry to confirm that they were isolated in fact macrophages (data not shown).

ELISA quantification of cytokines

Cytokines were quantified in the peritoneal fluids (pools from five mice) that were freshly collected from treated and untreated mice. In the supernatants of peritoneal macrophages isolated from treated and untreated mice stimulated with medium alone (48 h) or lipopolysaccharide (LPS, Sigma, St Louis, MO, USA) (100 ng/ml) (24 h). At the end of the incubation, culture supernatants were collected and cytokines were quantified using specific ELISA assays (R&D Systems). All ELISA determinations were performed in triplicate on the undiluted samples.

Statistics

Differences between groups were compared by Student’s paired t-test or the Kruskal–Wallis test as appropriate. A probability value of <0.05 was considered as statistically significant.

Results

The mouse model of endometriosis recapitulates the histomorphological features and biological behaviour of human endometriosis

The model used in the present study was adapted from the one described by us (Somigliana et al., 1999) and further validated (Rossi et al., 2000; Bacci et al., 2009). Donor mouse were administered with 17β-estradiol for 1 week and then sacrificed, and the two uterine horns were fragmented and injected into the peritoneum of two recipient mice. Recipient mice were sacrificed and their abdomen was opened to check for lesion presence at the times indicated for each treatment protocol. Of the 350 animals challenged with syngeneic uterine horn, 92% showed evidence of active (non-necrotic) peritoneal endometriosis at the time of abdominal inspection. This implies that less than 10% of the animals failed to develop the disease.

On gross examination, lesions consisted of pink to tanned isolated or multicystic nodules bulging from and loosely attached to the serosal coat. Evidence of neovascularization was observed on their surface in the form of a rich vascular net, as described previously (Somigliana et al., 1999). Similar to humans, lesions were influenced by the effect of gravity and were mainly found on the anterior abdominal wall, and also on the omentum, the posterior abdominal wall and around the uterus.

Results from the validation phase were the following:

(i) In the endometriosis model in which the uterine samples were derived from donor mice expressing GFP (C57BL/6-GFP), the endometriotic lesions found in the recipient mice were easily detectable as green fluorescent under UV light (Fig. 1A, I–II). In this model, we confirmed that all the lesions formed in the peritoneal cavity of the recipient mice were of donor origin but interestingly surrounded by a rim of GFP-negative stromal tissue of recipient origin (Fig. 1A, III).

(ii) Estrogen-dependent lesion growth was reduced by the administration of a GnRHa in recipient mice. As shown in Fig. 1B, the GnRHα Enantone (leuprorelin acetate, depot formulation) at 1 mg/kg injected i.m. 1 week before and on the day of surgery was shown to reduce endometriotic lesion weight by ~70%.

(iii) The comparison of the lesion weight obtained in 30 couples of paired animals (Groups A and B) injected with fragments from one of the two uterine horns from estrogen-treated donor mice showed a very similar amount of endometriotic tissues in the two groups of mice (Fig. 1C). This suggests that the variability of the disease development is minimal between the pairs.

To better characterize the lesions, we performed histological analysis on representative mice showing a clear cystic organization. Haematoxylin–eosin staining showed a tissue organization typical of human endometriotic lesions with endometrioid glandular epithelium accompanied by endometrial-type stroma and rarely some smooth muscle fibres surrounding the cysts (Fig. 1D, I–IV). Immunostaining with mouse ER-α and anti-VDR antibodies confirmed that the lesions analysed expressed ER-α (Fig. 1D, V and VI) as well as VDR (Fig. 1D, VIII–IX).

Treatment with elocalcitol reduces the total weight of endometriotic lesions

Balb/c mice were used for the efficacy experiments in this study since this strain is prone to endometriotic lesion development and because of an easier handling compared with the C57BL/6 mice. The two uterine horns from a donor Balb/c mouse were fragmented and injected into the peritoneum of two recipient Balb/c mice, one of which was treated with elocalcitol (100 µg/kg) in miglyol orally, once a day, 5 days a week for 2 weeks, and the other was treated with miglyol alone. After the treatment with elocalcitol or miglyol, all mice appeared healthy with no obvious adverse effects. Body weight was measured daily and no significant changes were observed between treated and untreated animals. Lesions were collected and quantified 14 days after endometriosis induction (Fig. 2A). There was no association between the occurrence of sporadic necrotic lesions and the type of treatment. The total weight of the lesions isolated from mice treated with elocalcitol (100 µg/kg) was significantly lower than the total lesion weight of miglyol-treated mice (mean ± SEM lesion weight: 8.45 ± 1.039 versus 3.53 ± 0.540 mg in miglyol- and elocalcitol-treated animals, respectively; Fig. 2B). Estradiol levels in peripheral blood of miglyol- and elocalcitol-treated mice were measured and no difference was found (0.748 ± 0.0791 versus 0.827 ± 0.0797 ng/ml in miglyol- and elocalcitol-treated mice, respectively).
The observed effect of elocalcitol in reducing endometriotic lesion weight was dose-dependent: when different doses of elocalcitol ranging from 0.1 to 300 μg/kg were administered, we observed no effect for the 0.1 and 10 μg/kg doses and 24 ± 20, 50 ± 14, 66 ± 14 and 68 ± 12 per cent reductions for the 30, 100, 200 and 300 μg/kg doses, respectively (Fig. 2C).

We further investigated elocalcitol therapeutic potential on the prevention of lesion establishment and on the regression of endometriotic lesions (Fig. 3A and B). In one set of experiments, we treated mice daily for 5 days before endometriosis induction with elocalcitol 100 μg/kg and we observed a partial reduction in the total lesion weight of 40 ± 22%. When mice were treated from Days 0 to 4 and from Days 7 to 12, we observed a 48 ± 13% reduction. The maximal reduction in lesion size was observed upon treatment of animals from Days −7 to −3, from Days 0 to 4 and from Days 7 to 12 (73 ± 9% of reduction). Interestingly, some efficacy was still detectable when elocalcitol treatment was initiated 2 days after endometriosis induction when adhesion of transplanted endometrial fragments to the peritoneum had already taken place, and continued from Days 2 to 4 and from Days 7 to 12. Under these conditions, a significant reduction in lesion weight was observed when mice were sacrificed at Day 14 after endometriosis induction (35 ± 10%). More importantly, a therapeutic effect was also observed when elocalcitol treatment was initiated 14 days after endometriosis induction and continued from Days 14 to 18 and from Days 21 to 25 until sacrifice at Day 28 (34 ± 14%), demonstrating the potential efficacy of elocalcitol to treat already established lesions.

**Elocalcitol reduces adhesion of endometrial cells**

We tested the effect of elocalcitol on the adhesive properties of endometrial cells to collagen since this function characterizes the plasticity of the endometrial tissue and reflects the ability of endometrial cells to adhere and invade surrounding tissues (Sharpe-Timms, 2001). To address this point, we set up in vitro adhesion assays with mouse endometrial stromal cells. Briefly, endometrial stromal cells were purified from a pool of five mice treated with 100 μg/kg elocalcitol or miglyol for 2 days. Adhesion on collagen-coated plates was tested after 30 min and 1 h. In vivo treatment of mice with elocalcitol was able to dramatically reduce the number of adherent cells (Fig. 4).

**Elocalcitol reduces the peritoneal inflammatory response**

Elocalcitol has well-defined anti-proliferative and anti-inflammatory effects. Since the inflammatory response mediated by peritoneal

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**Figure 1** Validation of the mouse model of experimentally induced endometriosis. (A) GFP-positive lesions can be identified as translucid isolated or grouped cysts, fluorescent under UV light. (I) and (II) show GFP-positive lesions in the abdominal cavity, in vivo visualized under a dissection microscope, while (III) shows a section of the same lesion observed under a fluorescence microscope at ×10 magnification. The boundary between the endometriotic lesion and tissues of the recipient mice is evident. Note that while the lesion derives from the donor mouse, the tissue surrounding the fluorescent cyst is derived from the recipient mouse as it is GFP-negative. (B) Treatment with a GnRHα 1 week before and on the day of the operation blocks endometriotic lesion development. The mean ± SEM of the total weight of the lesions collected at 2 weeks after surgery from 10 mouse pairs either vehicle- or GnRHα-treated. Differences between groups were compared by Student’s t-test *P<0.01. (C) Thirty couples of paired animals (Groups A and B) were each injected with fragments from one of the two uterine horns from estrogen-treated donor mice and lesion weights were evaluated after 2 weeks. The mean ± SEM of total weight of the lesions collected from 30 mouse pairs is reported. Differences between groups were compared by Student’s t-test. (D) Frozen sections stained with haematoxylin and eosin of one representative lesion obtained from a mouse with experimentally induced endometriosis (I–II) and from a lesion derived from a woman with endometriosis (III–IV). Immunohistochemical staining with an anti-ER-α antibody of one representative lesion removed from a mouse with experimentally induced endometriosis (ER-α, V–VI). Immunohistochemical staining with anti-VDR antibody of one representative lesion removed from a mouse with experimentally induced endometriosis (VDR, VII–IX). Stainings with rabbit polyclonal IgG were performed as controls for ER-α and VDR (VII and X, respectively). (I, III, V and VIII) Magnification ×40; (II and IV) magnification ×100; (VI, VII, IX and X) magnification ×200.
macrophage is thought to sustain the progression of endometriosis in humans (Kyama et al., 2003), we analysed the peritoneal inflammatory response in mice treated with elocalcitol for 2 weeks after endometrial transfer. Elocalcitol was able to inhibit macrophage recruitment in the peritoneum (Fig. 5A; $10^{5} \pm 1334 \times 10^{3}$ versus $6342 \times 10^{3} \pm 1243 \times 10^{3}$ total peritoneal macrophages in miglyol- and elocalcitol-treated mice, respectively). Levels of IL-1α and IL-1β were found to be reduced in the freshly collected peritoneal fluids of elocalcitol-treated mice (Fig. 5A). Peritoneal macrophages obtained from pools of five elocalcitol- or five miglyol-treated mice were purified by adhesion to plastic and kept in culture for 48 h. Macrophage-conditioned medium were analysed by ELISA assays for TNF-α, IL-1α, IL-1β, IL-6, IL-10, macrophage inflammatory protein (MIP)-2 and vascular endothelial growth factor (VEGF). The inflammatory cytokines were reduced in the supernatants from peritoneal macrophages in vitro cultured for 48 h (Fig. 5B). Similar results were observed after 72 h in culture (data not shown). VEGF and MIP-2 levels were almost not changed after 48 h of culture and only slightly reduced by elocalcitol treatment after 72 h of culture (data not shown). However, treatment with elocalcitol did not affect viability and function of peritoneal macrophages since the same macrophages were still capable of secreting pro-inflammatory cytokines and chemokines when stimulated in vitro with LPS (Fig. 5C).

Figure 2 Elocalcitol treatment significantly reduces the total lesion weight. (A) Estradiol-treated female Balb/c mice were sacrificed and the uteri removed and split. Endometrial tissue was mechanically disrupted before intraperitoneal injection into an experimental/control mouse pair. Four hours after endometriosis induction, pairs of recipient mice receiving matched uterine horns were orally treated with elocalcitol 100 μg/kg or miglyol alone, once a day, 5 days a week for 2 weeks. After 2 weeks, mice were given a lethal dose of anaesthetic and their abdomen was open to check for lesion presence. The lesions were carefully removed, let dry and weighted. (B) Lesion weight of individual animals. The difference between elocalcitol- and miglyol-treated groups is statistically significant ($**P < 0.001$, $***P < 0.0001$). Data are expressed as mean ± SEM of the total lesion weight from three independent experiments with eight experimental/control mouse pairs. Differences between groups were compared by Student’s t-test. (C) Dose–response of treatment with six different doses of elocalcitol. Results represent the mean per cent values ± SEM of lesion weight reduction in eight mice treated with each dose of elocalcitol compared with miglyol alone. Differences between groups were compared by the Kruskal–Wallis test ($P < 0.001$).

Figure 3 Effect of different protocols of administration of elocalcitol on endometriotic lesion development. (A) Estradiol-treated female Balb/c mice were sacrificed and the uteri removed and split. Endometrial tissue was mechanically disrupted before intraperitoneal injection into an experimental/control mouse pair. One mouse of the pair was injected with miglyol and the other with 100 μg/kg of elocalcitol according to the indicated protocols: Protocol 1, from Days 0 to 4 and from Days 7 to 12; Protocol 2, from Days −7 to −3; Protocol 3, from Days −7 to −3, from Days 0 to 4 and from Days 7 to 12; Protocol 4, from Days 2 to 4 and from Days 7 to 12; Protocol 5, from Days 14 to 18 and from Days 21 to 25. Lesions were evaluated 14 days after endometrial tissue injection in the case of Protocols 1–4 and 28 days after endometrial tissue injection in the case of Protocol 5. (B) Effects of different treatment protocols on total lesion weight. Results represent mean per cent values ± SEM of lesion weight reduction in eight mice per protocol group compared with miglyol: Protocol 1 (black bar, 48% reduction), Protocol 2 (white bar, 40% reduction), Protocol 3 (grey bar, 73% of reduction), Protocol 4 (diagonal dashed bar, 35% reduction) and Protocol 5 (horizontal dashed bar, 34% reduction). The difference between elocalcitol- and miglyol-treated groups is statistically significant ($*P < 0.001; ***P < 0.0001$). Differences between groups were compared by the Kruskal–Wallis test.
Discussion

Currently, endometriosis is still an unmet clinical need since an optimal drug that allows for both pain management and continued attempts to conceive does not exist. The current medical treatment modalities are only somewhat effective in relieving pain, often with relatively short-term effects. In addition, they have many undesirable and sometimes severe side effects that may prohibit the long-term management that is needed for endometriosis. The continuous use of combined oral contraceptives, although perhaps exhibiting the best cost-effectiveness, is still far from optimal. Consequently, more efficacious therapeutics, preferably with improved safety and cost profiles, are surely needed (Guo et al., 2009). An ideal medical treatment would eliminate endometriotic lesions, prevent recurrence and not impede ovulation.

Here, we show that the VDR agonist elocalcitol inhibits lesion development in a validated mouse model of endometriosis. The maximal effect (73% lesion weight reduction) was observed upon oral daily administration of elocalcitol for 3 weeks starting 1 week before the endometriosis induction, suggesting that elocalcitol exerts a protective effect on both the implantation and organization of transferred endometrial tissue. Since this maximal efficacy was observed for the longest drug exposure, we cannot exclude a dose-dependent effect of elocalcitol on lesion development. However, exposure to elocalcitol for only 1 week before disease induction was as efficacious as exposure for 2 weeks after disease induction (40% inhibition versus 35%), suggesting that the total dose of drug received was not the only factor responsible for the observed therapeutic effect.

Progress in endometriosis research is quite limited by the lack of a valuable and promptly available model of the disease (Somigliana et al., 1999). Endometriosis occurs spontaneously only in primates, which happen to be the only animals with cyclic menstrual periods and retrograde menses (Tirado-González et al., 2010). Though spontaneous endometriosis in non-human primates probably constitutes the most suitable model to study this disease, its low incidence and slow progression have encouraged the development of experimental methods to induce the formation of lesions in these animals. However, both in the context of spontaneous or induced disease, ethical issues and the high costs associated with primate manipulation represent limitations associated with these models, especially for studies aimed at assessing the efficacy of novel candidate drugs.
these reasons, animal models based on laboratory mice have been widely used for endometriosis research. Heterologous models based on the xenotransplantation of human endometrial tissue into immuno-deficient mice are used mainly when specific effects or functions on human endometrium need to be evaluated. However, since the immune response is altered and reduced in these models, they are unable to replicate the immune changes and responses that occur at the endometriosis implantation sites in humans. Moreover, they cannot be used to test drugs that target components of the immune system itself (Tirado-González et al., 2010). Homologous models can be based on surgical transplantation of a uterine square in the peritoneal cavity or, alternatively, as in our case, endometriotic lesions are directly induced by intraperitoneal injection of endometrial fragments from donor to recipient mice. This latter model can be very useful for two reasons: (i) it allows to evaluation of both tissue implantation and lesion progression, and (ii) since donor and recipient mice are syngeneic, it permits testing of novel therapeutic interventions which are able to act on the peritoneal immune response and inflammatory inflammation associated with the disease.

In our model, murine endometriotic implants show a histopathology similar to that observed in human endometriotic lesions and the model recapitulates the histomorphology of the human disease. Indeed, all lesions consisted of endometrial glands and stroma often appearing with a cystic structure, and they were all characterized by an intense neovascularization and by expression of ERα, and could be dramatically reduced by treatment with GnRH analogues. Moreover, further to these characteristic features, novel observations could be derived from the model. For instance, it is known that a fibromuscular tissue often differentiates around the nodules but its origin is still unclear (van Kaam et al., 2008). The GFP mouse experiments reported herein indicate that while the lesion structure was of donor origin, the fibromuscular tissue surrounding the nodules was derived from the recipient mice, thus supporting the idea that a reaction of the local environment to the presence of ectopic endometrium represents an intrinsic property of the disease. On the other hand, the model presents some limitations. First, the injection of donor endometrial fragments into the peritoneum of receiving animals leads to the development of lesions that are highly heterogeneous in shape, size and liquid content. For this reason, the measurement of width of the lesions represents a very approximate and poorly reproducible readout leaving dry weight as the only reliable and reproducible outcome measure. Dry weight is a function of lesion size, and endometriotic and fibrotic tissue content but it does not take into account the parenchymal component. Secondly, since the model does not recapitulate the menstrual shedding of the endometrium, it does not mimic the ovarian disease and it is not adequate to evaluate symptoms. Indeed, the efficacy of the drug on pain relief has not been explored in this study. For these reasons, further preclinical evaluation in a baboon model should be taken into consideration before advancing elocalcitol into clinical development (D’Hooghe et al., 2009).

The mechanisms potentially involved in the therapeutic effect of elocalcitol are at least two.

(i) Inhibition of the capacity of endometrial cells to adhere to a collagen substrate. Tissue invasion involved in endometriosis development is a complex process constituted by different subsequent interdependent phases and controlled by complicated cross-talk mechanisms between cells and the extracellular microenvironment. The first phase is represented by the attachment to the extracellular matrix, a step governed by adhesion molecules, most notably receptors of the integrin family (Gentili et al., 2007), and shown to be inhibited by elocalcitol. (ii) Inhibition of the inflammatory response. In fact, elocalcitol has been shown to reduce both macrophage number and macrophage-associated inflammatory cytokine secretion in the peritoneum of mice with endometriosis. Whether the observed anti-inflammatory effect mainly derives from the inefficient recruitment of macrophages into the peritoneal cavity or rather from the decreased cytokine secretion remains difficult to establish but available data from the present study and from previous reports (Adorini et al., 2007) support both these mechanisms. Certainly, the prompt functional response of peritoneal macrophages to LPS stimulation strongly suggests that the anti-inflammatory effect of elocalcitol is only transitory and does not permanently affect the ability of macrophages to respond to strong inflammatory stimuli in vivo.

Macrophages play a critical role, via their ability to guide tissue regeneration, in the growth of tumours and in diseases characterized by persistent tissue remodelling and inflammation (Allavena et al., 2008; Brunelli and Rovere-Querini, 2008). Several lines of evidence suggest that macrophages are locally activated and not only trapped in ectopic endometrial lesions (Lebovic et al., 2001; Minici et al., 2008). For example, the NF-κB transcription factor is activated in macrophages from endometriosis patients (Lousse et al., 2008), and responsive genes that determine macrophage functions have been shown to undergo transactivation, supporting angiogenesis and tissue remodelling (Timmer and Nizet, 2008). Accordingly, the in vivo inhibition of NF-κB interferes with the growth of experimentally induced endometriotic lesions (Gonzalez-Ramos et al., 2008). We have recently demonstrated that ectopic lesions depend on peritoneal macrophages for their growth and vascularization (Bacci et al., 2009). In the same mouse model used herein, the depletion of macrophages by means of clodronate liposomes or monoclonal antibodies led to an altered lesion organization and development. Here we show that IL-1α and IL-1β, important regulators of the inflammatory NF-κB pathway, present at high levels in the peritoneal fluid of women with endometriosis, are down-regulated in the peritoneal fluids of elocalcitol-treated mice, therefore supporting the anti-inflammatory effect of elocalcitol.

Based on the results of this study, a possible translation into the clinical setting would be to administer elocalcitol during the perimenstrual and menstrual phase of the cycle. In this phase, all the potential activities of the compound (inhibition of inflammation, inhibition of endometrial cell adhesion, inhibition of lesion organization) could be exerted with the maximal efficacy. Therefore, the drug might be useful in preventing disease development in subjects at high risk of disease recurrence. This is of particular clinical relevance considering that endometriosis recurrence rate is estimated to be around 20% at 2 years and 40–50% at 5 years (Guo et al., 2009). Endometriosis has a distinctive tendency to recur after conservative surgery (Shakiba et al., 2008; Guo et al., 2009). The consequences of endometriosis relapse on reproductive performance may be particularly detrimental owing to peritoneal and gonadal damage caused by both recurrent disease and repeated surgery. Therefore, a medical therapy constitutes an important alternative or complement to surgery (Vercellini et al., 2011).
Any benefit of VDR agonists needs to be balanced against the risk of side effects and toxicity. While hormonal medical therapies suppress ovarian function therefore dismissing the option of a normal and safe pregnancy during treatment, VDR agonists show no unfavourable effect on ovarian, endometrial and also bone function. Moreover, elocalcitol has been shown to have minimal effects on calcium homeostasis and to be endowed with a favourable safety profile, as recently demonstrated in a clinical trial in patients with benign prostatic hyperplasia (Colli et al., 2006).

The realistic objective to suppress, rather than eliminate implant growth, can be achieved with an anti-inflammatory drug such as elocalcitol that may represent a safe treatment in limiting the growth of pre-existing lesions and treating recurrences. Further experiments using primate models as well as clinical trials will be helpful in evaluating the therapeutic potential of elocalcitol in women with endometriosis.

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Authors’ roles
M.M. performed analysis of the data, supervised the in vivo studies and performed the statistical analysis. P.V. contributed to the preparation of the manuscript. D.G. performed the adhesion and chemotaxis assays. B.C. and E.C. performed all in vivo studies. P.D.L. performed the cytokine assays. A.M. performed the immunohistochemical analysis. M.C. contributed to the preparation of the manuscript. E.S. supervised the in vivo studies and contributed to the preparation of the manuscript. P.P.-B. conceived the scientific idea, supervised the project and prepared the manuscript.

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Conflict of interest
The authors have no financial, personal or competing interests.

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