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Identification and Quantification of Dopamine Receptor 2 in Human Eutopic and Ectopic Endometrium: A Novel Molecular Target for Endometriosis Therapy[1]

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ABSTRACT

Previous studies in an experimental mouse model of endometriosis have shown that the dopamine agonist (DA) cabergoline (Cb2) reduces angiogenesis and endometriotic lesions, hypothetically binding to the dopamine receptor type-2 (DRD2). To date, this has not been described in human endometrium and/or endometriotic lesions. Thus, we aimed to investigate the presence of DRD2 in said tissues. Endometrium fragments were implanted in nude mice treated with different doses of Cb2. Polymerase chain reaction assays and immunohistochemistry were performed to analyze the gene and protein expressions (respectively) of DRD2, VEGF, and VEGF receptor-2 (KDR). In addition, lesions and endometrium from women with mild and severe endometriosis and endometrium from healthy women were collected to analyze their gene expression profile. In experimental endometriosis, DRD2 was expressed at gene and protein levels in all three groups. VEGF gene and protein expressions were significantly lower in lesions treated with Cb2 than in controls. KDR protein expression was significantly lower in experimental lesions treated with Cb2 than in controls. In eutopic endometria, there was a significant decrease in *DRD2* expression and an increase in *VEGF* in women with mild and severe endometriosis with respect to healthy patients. In endometriosis, *KDR* expression was significantly higher in red than in white and black lesions. *VEGF* expression was significantly lower in black than in red lesions. *DRD2* is present in the human eutopic and ectopic endometrium and is regulated by DA, which provides the rationale for pilot studies to explore its use in the treatment of endometriosis.

angiogenesis, cabergoline, dopamine, dopamine agonist, dopamine receptor 2, endometriosis, endometrium, KDR, VEGF

INTRODUCTION

The treatment of endometriosis is a real challenge to modern gynecology. It occurs in various clinical situations, and its recurrence is as high as 21.5% and 40%–50% after 2 and 5 yr, respectively [1]. Medical therapies to treat this condition are continuously under development. In the last two decades, most approaches have sought to decrease circulating estradiol levels and induce a hypoestrogenic milieu that reduces the severity of the disease and improves symptoms [2–4]. These therapies have largely been based on evidence of the estrogen dependency of the disease [5]. Unfortunately, they have only achieved partial success and can be applied only for a limited time because of considerable negative side effects.

At present, new strategies are underway to acquire a better understanding of the pathophysiology of the disorder [2–4]. One of these new approaches is to target the angiogenic process, whose presence in endometriotic implants is essential for their establishment and development [6]. It is known that the endometrium has angiogenic potential and that endometriotic lesions are larger in areas with a rich blood supply [7]. Moreover, proangiogenic factors, such as vascular endothelial growth factor (VEGF), hepatocyte growth factor, interleukin 8 (IL8), IL15, macrophage migration inhibitory factor, neutrophil-activating factor (growth-regulated gene-alpha), tumor necrosis factor alpha, erythropoietin, and angiogenin are more pronounced in the peritoneal fluid of women with endometriosis [8]. Even antiangiogenic modulators, such as adiponectin or the antiangiogenic chemokine interferon-γ-induced protein 10 (CXCL10), are weaker in the peritoneal fluid of these patients [8].

Several antiangiogenic drugs have been successfully employed to target the VEGF system in animal models of endometriosis [9, 10]. This strategy is based on the fact that increased amounts of VEGF are released by peritoneal macrophages in women with endometriosis [11] and on the positive correlation between the severity of the disease and the secretion of VEGF in peritoneal fluid [12–14].

Our group has developed a strategy to target VEGF in an experimental model of endometriosis. We focused our attention on the dopamine (Dp)/dopamine receptor 2 (DRD2) pathway, whose activation is implicated in the regulation of angiogenic events [15, 16] mediated by VEGF/VEGF receptor 2 (KDR) signaling [17]. In fact, administration of high doses of DRD2 agonists has been shown to simultaneously block tumor-related angiogenesis and vascular permeability in a mouse cancer model [18].

In previous studies by our group, the antiangiogenic effect of the dopamine agonist (DA) cabergoline (Cb2) has been demonstrated in a nude mouse model of endometriosis [19]. Animals were treated with different doses of Cb2 during a 2-wk period. After administration of DA, the percentage of active lesions significantly declined in Cb2-treated mice with respect to untreated mice. After a histological study, a lax stroma with lost cellularity and organization and a diminished gland

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TABLE 1. Distribution of animals and human endometrium tissue for mice peritoneum transplantation.

	Animals employed in morphological, immunohistochemical, and morphometric studies (n = 12)			Animals employed in molecular studies (n = 12)		
Donor[a]	Control group	Low dose group	High dose group	Control group	Low dose group	High dose group
Donor 1	1	1	1	1	1	1
Donor 2	1	1	1	1	1	1
Donor 3	1	1	1	1	1	1
Donor 4	1	1	1	1	1	1
Total no. of animals	4	4	4	4	4	4

[a] All tissue is from the prolifertive phase of the menstrual cycle.

component was observed in the lesions of Cb2-treated mice compared with those of controls. On a protein level, it was demonstrated that the proliferation index and KDR activity were significantly lower in the experimentally induced lesions from Cb2-treated animals. Laser scanning confocal microscopy revealed that the newly formed vessels were significantly lower in mice treated with Cb2 than in untreated animals. Molecular studies showed a significant decrease of pro/angiogenic factors (*VEGF* and *NOTCH4*) in the lesions of mice treated with DA [19].

To exert its action on endometriotic tissue, DA must bind with the dopamine receptor type-2 (DRD2). This receptor has not been described in the human endometrium and/or endometriotic lesions. Human term decidual tissue, obtained during vaginal delivery, was the first reproductive tissue in which DRD2 was described [20]. Alternatively, DA may exert its action through the inhibition of prolactin (PRL) secretion, a powerful angiogenic agent, by blood vessel receptors [21]. Interestingly, several reports have associated hyperprolactinemia with the presence of endometriosis [22, 23]. To throw light on the potential direct action of DA on endometriotic lesions, we have investigated the presence of DRD2 in human eutopic and ectopic endometria of women with different degrees of endometriosis. First, we explored this hypothesis in our established animal model of endometriosis by increasing the experiments at the molecular and protein levels. Second, we assessed the expression of *DRD2* in eutopic and ectopic endometria of patients with endometriosis and analyzed its relation with the VEGF system.

MATERIALS AND METHODS

This study was approved by our Institutional Review Board, and the informed consent of participants was obtained prior to biopsy collection. Similarly, all experimental procedures with animals were approved by the Animal Ethics Committee of the University of Valencia and were in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC), and with the Spanish Real Decreto of 10 October 2005 (RD 1201/2005).

Experimental Endometriosis

Human endometrial tissue. Informed consent was obtained prior to endometrial biopsy collection. Although names were kept confidential, patient age, cycle stage, and medication history were made available. Ovarian stimulation was initiated on Day 21 of the menstrual cycle with a gonadotropin-releasing hormone agonist (Synarel; SEID Laboratories, Barcelona, Spain). After menses, donors were stimulated with a dose of 200–225 IU of recombinant follicle-stimulating hormone (Gonal-F; Merck-Serono Laboratories, Barcelona, Spain) until follicles reached a diameter of 18 mm. At this point, 250 μg of recombinant human chorionic gonadotropin (Ovitrelle; Merck-Serono Laboratories) was administered, and ovum pickup was performed 36 h later. Employing a pipelle cannule, human endometria were obtained at ovum pickup from oocyte donors (n = 4, age range: 18–34 yr) with normal menstrual cycles and no history of endometriosis. Biopsies were placed in prewarmed sterile PBS solution at pH 7.4 and transported to the laboratory, where specimens were cut into pieces of approximately 2 × 3 mm. A part of each biopsy was fixed in 4% buffered formaldehyde and embedded in paraffin for histological confirmation of the proliferative phase using established criteria [24]. The remaining tissue was transplanted into peritonea of nude mice, as described in Table 1.

Animal model of endometriosis. The model of endometriosis was established as previously described [19]. Sixty-day-release sterile capsules containing 18 mg of 17β-estradiol (Innovative Research of America, Sarasota, FL) were placed s.c. in the necks of twenty-four 5-wk-old ovariectomized female nude mice (Harlan Ibérica S.L., Barcelona, Spain). Four days later, four fresh 2- to 3-mm human endometrium fragments were fixed in the peritoneum of each mouse using *n*-butyl-ester cyanoacrylate adhesive (3M Animal Care products). Human endometrial samples from each donor were employed in one animal from each experimental group, as described in Table 1. Three weeks after establishment of lesions, Cb2 (Pfizer Laboratories) diluted in a vehicle solution (1:6 alcohol in sterile water mixture) was administered orally by gavage at doses of 0 (control), 0.05 (low dose), and 0.1 (high dose) mg/kg per day for 14 days. These doses were selected based on previous studies [19, 25]. A total of 24 animals, divided into three experimental groups, were included in the study. Three weeks after implantation of endometrial tissue, and 2 weeks after Cb2 treatment, animals were killed by cervical dislocation, and their abdominal skin and peritoneum were opened to enable their visceral organs to be examined under a binocular microscope and the endometriotic implants evaluated. Implants with suspected endometriosis were counted and photographed. The size of two perpendicular diameters (D1, D2) was measured in implants to the nearest tenth of a millimeter using callipers. The cross-section area (CSA) was calculated for each lesion according to a previously described [26, 27] method for an ovoid ($D1 \times D2 \times \pi/4$), and the implants were dissected. Tissue was obtained for molecular analysis from 12 animals, and the remaining 12 underwent the same procedures for morphological, immunohistochemical, and morphometric studies.

TaqMan PCR assays in experimental endometriotic lesions. The gene expression profiles of *DRD2*, *VEGF*, and *KDR* were studied in the experimental lesions of control and Cb2-treated mice. Total RNA extracted from samples was purified using a RNA purification kit (Qiagen) and was submitted to DNase I treatment. The quantity and integrity of isolated total RNA were assessed with the RNA 6000 Nano LabChipw kit, using the Agilent 2100 Bioanalyzer (Agilent Technologies). Duplicate TaqMan PCR assays for each gene target were performed in cDNA samples. Predeveloped TaqMan PCR assays (PE Applied Biosystems) that recognize both human and mouse genes were employed to analyze the expression of *DRD2*, *VEGF*, and *KDR*, and the housekeeping gene 18S ribosomal RNA was used to normalize the target gene Ct values. Prior to assay selection, primer sequences were assessed by conducting a BLAST search of GenBank. Assays that showed a human-mouse homology of 90% were selected to analyze the gene expression profile of experimental lesions. A sample of the mouse peritoneum was included in the study as a control. The PCR conditions and expression of final results were as previously described [28]. The cDNA obtained from sarcoma 180 tumor cells (S-180) and human umbilical vein endothelial cells were employed as a negative and a positive control, respectively [18].

Light microscopic and immunohistochemistry studies. Implants were fixed in 4% buffered formalin, embedded in paraffin wax, and cut into 4-μm sections from the entire specimen. The sections were stained with Harris hematoxylin and eosin (Sigma) and were examined microscopically for the presence of histological hallmarks of endometriosis, such as endometrial glands and stroma. The effectiveness of this model has been evaluated in previous studies, reaching a 70.8% ± 5.2% recovery rate and 89.6% ± 5.7% of active lesions (composed of glands and stroma) [19].

DRD2, VEGF, and KDR protein expression was analyzed using immunohistochemistry techniques to establish whether or not they were

affected by Cb2 treatment. DRD2 expression was studied employing a rabbit anti-DRD2 antibody (Santa Cruz Biotechnology) at a 1:250 dilution. VEGF and KDR expression was studied employing a rabbit anti-VEGF antibody (Abcam, Cambridge, U.K.) at 1:500 dilution and a rabbit anti-KDR antibody (Cell Signaling Technology Inc.) at 1:250 dilution, incubated at 4°C overnight. Biotinylated goat anti-rabbit (Abcam) was employed as the secondary antibody. Mouse central nervous system was used as a positive control for the DRD2 immunoassay [29, 30], and breast carcinoma [31] was used as a positive control for VEGF and KDR immunoassays. Sections were deparaffinized and rehydrated through graded ethanol and were then rinsed in distilled water and treated with 0.3% H_2O_2 and 10% normal horse serum to block endogenous peroxidase and nonspecific binding, respectively. Antigen retrieval was performed by pressure cooker boiling for 3 min in 10 mmol/L citrate buffer (pH 6.0). The LSAB method (DakoCytomation, Glostrup, Denmark) was used, followed by detection with 3,3′-diaminobenzidine. For each section of tissue, a serial section was employed as a negative control. Immunostaining was analyzed by a morphometric study. Histology and immunohistochemistry analyses of experimental endometriotic lesions were evaluated in a blinded fashion by two pathologists specializing in gynecology and in handling of laboratory animals.

Morphometric studies. A morphometric study was carried out to quantify DRD2, VEGF, and KDR protein expression. Images of immunohistochemistry sections were captured with an Olympus BH2-UMA (Tokyo, Japan) optical microscope connected to a JVC/TK-1270 (JVC Corp., Yokohama, Japan) video camera and a computer-digitized plate. The images were analyzed by Image-Pro Plus 5.1 software (Media Cybernetics, Silver Spring, MD) to calculate the total microscopic area of each lesion and the positive immunostained areas of each sample.

The areas were expressed per square millimeter of each lesion sample. These values were measured in three noncontiguous sections in a blinded fashion. DRD2, VEGF, and KDR protein expression was determined with respect to the total area of tissue and expressed as labeling index (ratio of positively stained area:total area of the tissue).

Human Endometriosis Studies

Collection of endometrium tissue and endometriotic lesions. The study group consisted of 10 patients who fulfilled the following inclusion criteria: at least 2 yr of primary infertility; mild (n = 5, mean age: 35.6 ± 1.7 yr, range: 33–37 yr) and severe (n = 5; mean age: 35.8 ± 1.3 yr, range: 34–37 yr) endometriosis; regular cycles; and normal male factor. Endometriosis was diagnosed by laparoscopy and histologically confirmed and classified according to the American Society of Reproductive Medicine [32]. The control group was composed of healthy women (n = 5; mean age: 34.2 ± 4.1 yr, range: 27–37 yr) undergoing gynecological surgery for benign conditions (tubal sterilization). All patients were of reproductive age, with normal regular cycles and without other pathologies associated.

Lesions and endometrial tissue from endometriosis patients and endometria from the control group were collected during the follicular phase of a spontaneous menstrual cycle using laparoscopic procedures. The endometrial tissue was obtained employing a pipelle cannule. Endometriotic lesions were macroscopically and microscopically characterized as follows: active red lesions with numerous glands and stroma and an intense vascularization; inactive black lesions with fewer glands and stroma but displaying more fibrosis; and white lesions representing scar tissue with fibrosis [33]. None of the patients had received hormone therapy during the 6-mo period prior to surgery. Samples were washed in prewarmed PBS, homogenized in Trizol, and cryopreserved at −80°C until they were due to be processed and analyzed. One portion of each sample was fixed in formalin for histological confirmation of cycle stage and to determine the presence of endometrial glands and stroma in the lesions.

TaqMan PCR assays in human endometriotic tissue. The gene expression profile studies of *DRD2*, *VEGF*, and *KDR* in samples of human endometrium and endometriotic lesions were performed following the same procedure as that of the animal studies as described above.

Statistical Analysis

Statistical analyses were performed using GraphPad Instat V3.0 (GraphPad Software, San Diego, CA). Kruskal-Wallis was employed for the overall analysis of the data to determine whether or not they followed a normal distribution. Nonparametric Mann-Whitney and Dunn multiple comparison tests were carried out to compare individual means. Categorical data were expressed as number and percentage, and numerical data were expressed as mean ± SD, except when specified otherwise. In all cases, significance was considered to be $P < 0.05$.

RESULTS

Histological Evaluation of the Experimental Lesions

All mice were weighed before being killed, and this showed that body weight did not differ significantly among the animals (data not shown). After opening the peritoneal cavity, the visceral organs were examined and found to be healthy and normal in all of the mice.

The four human endometrium implants transplanted onto the peritoneal wall prior to Cb2 treatment were macroscopically observed in all mice (Fig. 1A), as in previous studies.

The CSA was calculated for each lesion, and the average of the measurements for each animal was obtained. No significant ($P > 0.05$) difference between control (27.3 ± 2.7 mm^2), low-dose (26.3 ± 3.1 mm^2), and high-dose (25.2 ± 3.4 mm^2) groups was detected.

Histologically, the experimental lesions presented hallmarks of human pathology, namely, endometrial glands and stroma and a well-preserved morphology. Human endometrial stroma surrounding glandular areas was easily differentiated from muscular-conjunctive murine tissue (Fig. 1B). As in previous studies, it was observed that lesions from control mice presented a rich cellular stroma and well-preserved structure (Fig. 1, C and D), whereas lesions from mice treated with low (Fig. 1, E and F) and high (Fig. 1, G and H) doses of Cb2 exhibited a lax stroma with a weak organization.

DRD2, VEGF, and KDR Gene and Protein Expression in Experimental Endometriosis

To understand more thoroughly the varying expression of DRD2 in endometriotic lesions, we employed this rodent model to administer a DA and observe subsequent changes in the explanted tissue. *DRD2* was expressed in all three groups of lesions (Fig. 2), and although an increase in *DRD2* expression was observed when Cb2 was administered, there were no statistical differences between the groups treated with low and high doses of Cb2 and controls. *VEGF* expression was significantly lower in lesions treated with low and high doses of Cb2 than in controls (Fig. 2). No difference was observed in the *KDR* expression of the groups treated with low and high doses of Cb2 compared with controls; however, treatment did produce a decrease in *KDR* expression (Fig. 2).

We studied DRD2, VEGF, and KDR protein expression employing immunohistochemistry and morphometry assays. DRD2 was expressed at the protein level in all three groups of lesions (Fig. 3A), but there were no statistical differences between the groups treated with low and high doses of Cb2 and controls (Fig. 3B). A decrease of VEGF and KDR staining in Cb2-treated groups with respect to controls was observed (Fig. 3A). Morphometric analysis revealed a significant reduction of VEGF expression in mice treated with low and high doses of Cb2 compared with the controls (Fig. 3B). Cb2 administration at low and high doses significantly decreased KDR expression with regard to that in controls (Fig. 3B).

DRD2, VEGF, and KDR Gene Expression in Eutopic Endometria and Endometriotic Lesions

Molecular studies were performed to expose the presence of and possible relationship between *DRD2*, *VEGF*, and *KDR* expression in the eutopic endometria of women with and without endometriosis and in peritoneal endometriotic lesions.

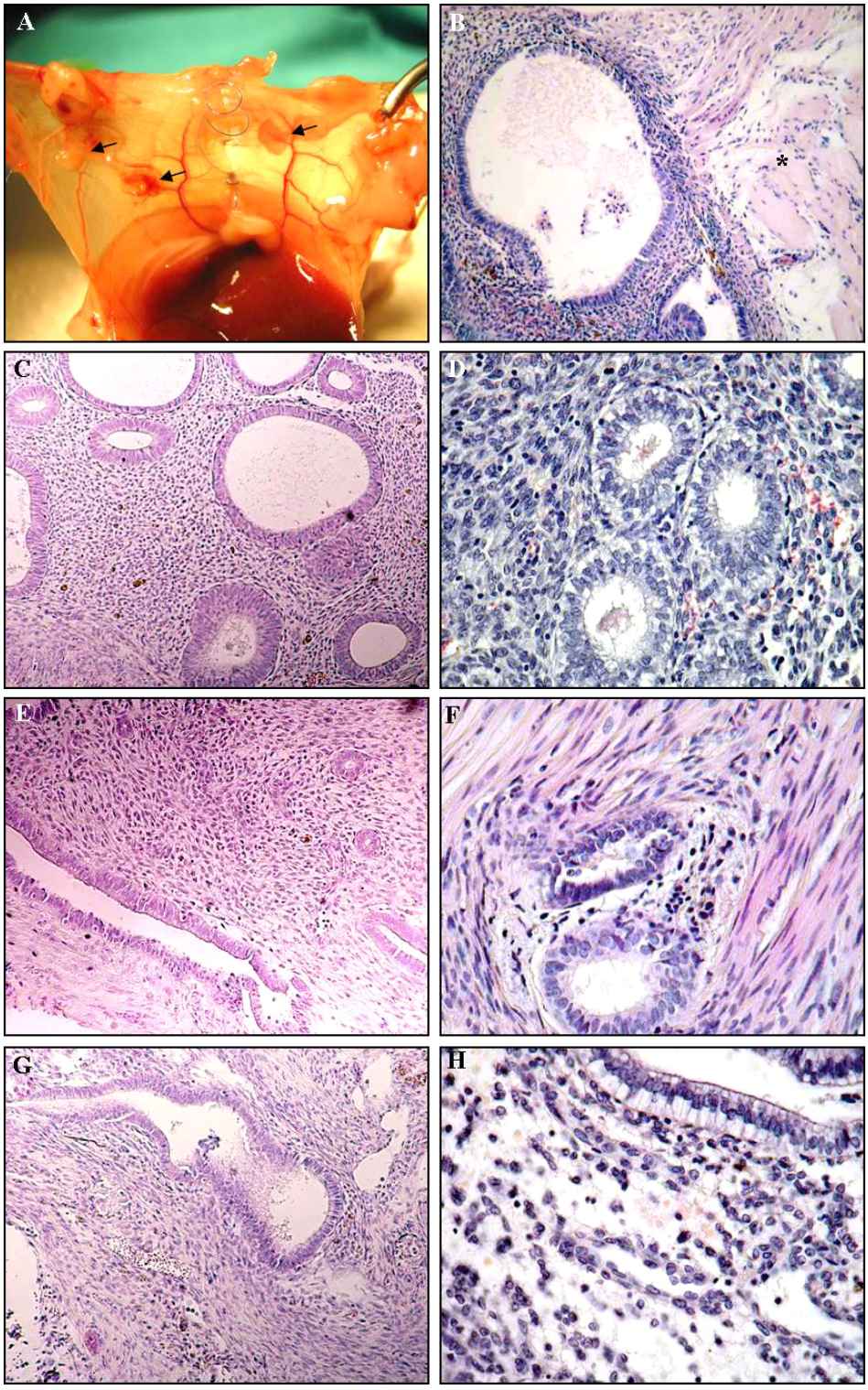

FIG. 1. Human endometrial implants (arrows) on the peritoneal wall of mice (**A**) were analyzed 3 wk after implantation and after 2 wk of treatment with Cb2 or vehicle, which confirmed the successful engraftment of endometrial tissue fragments. Microphotographs of the histological study show that implants presented a cellular stroma surrounding glandular areas that was easily differentiated from the muscular-conjunctive murine tissue (asterisk in **B**). The lesions in control mice (**C** and **D**) showed a higher cellular stroma and complete reorganization and structure compared with lesions in mice treated with low (**E** and **F**) and high (**G** and **H**) doses of Cb2, which presented a lax stroma with a lack of cellularity and organization. Hematoxylin-eosin stain; original magnifications ×40 (**B**, **C**, **E**, and **G**) and ×100 (**D**, **F**, and **H**). Sixteen samples relate to four samples per mouse and four mice per experimental group.

No significant difference ($P = 0.5039$) was found between the age of healthy control patients and patients with mild and severe endometriosis.

In eutopic endometria, there was a significantly lower *DRD2* expression in patients with mild and severe endometriosis than in healthy patients (Fig. 4A). The expression of *VEGF* in patients with mild and severe endometriosis was significantly higher than in healthy patients (Fig. 4B). *KDR* expression in patients with mild and severe endometriosis did not differ from that in healthy patients (Fig. 4C).

In endometriotic lesions, no significant difference was detected in *DRD2* expression according to type of lesion (Fig. 4D), but a higher expression was observed in black than in red lesions. There was also a significant decrease of *VEGF* expression in black lesions with respect to red lesions, but no significant difference was found between white and red lesions,

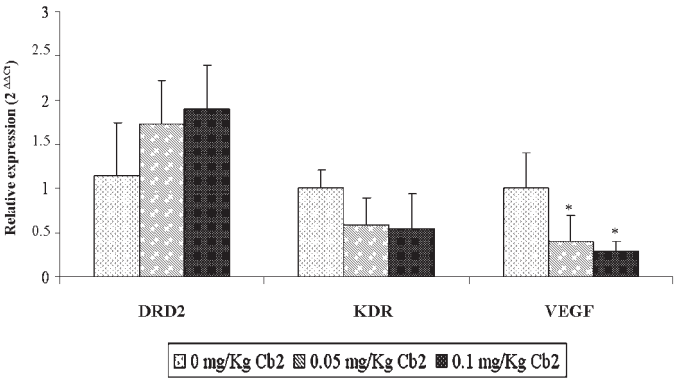

FIG. 2. Quantification of gene expression of *DRD2*, *VEGF*, and *KDR* in human endometrial implants in mice treated with low and high doses of Cb2 and untreated animals by TaqMan RT-PCR. A significant decrease in *VEGF* expression was observed in lesions treated with low and high doses of Cb2 compared with controls. No difference in the *DRD2* and *KDR* expression of the groups treated with low and high doses of Cb2 compared with controls was observed; however, treatment did produce an increase in *DRD2* expression and a decrease in *KDR* expression when Cb2 was administered (data expressed as mean ± SD). $*P < 0.05$. Sixteen samples relate to four samples per mouse and four mice per experimental group.

or between white and black lesions (Fig. 4E). In addition, a significantly higher expression of *KDR* was observed in red lesions than in white and black lesions (Fig. 4F).

DISCUSSION

The results of the present study show for the first time that DRD2 is present in human endometrial tissue in both eutopic endometria and endometriotic lesions. This finding, together with our previous observations of a direct and clear effect of DA on experimental endometriosis, open the way to further studies in humans to assess the potential efficacy of DA in the medical treatment of this disease.

Previous work by other groups has identified DRD2 in decidual tissue after delivery [20]. Moreover, indirect evidence suggests that dopamine is capable of stimulating nitric oxide release by human epithelial endometrial cells [34]. The role of the sympathetic system in the uterus was reported some time ago and has been related to the normal physiology of pregnancy and delivery and to pathological states [35], but a role in the endometrium, and specifically in the pathophysiology of endometriosis, has not been proposed until now.

In addition, we have classified human endometriotic lesions as red, black, and white, and we have analyzed differences between them [33]. As expected if earlier reports are

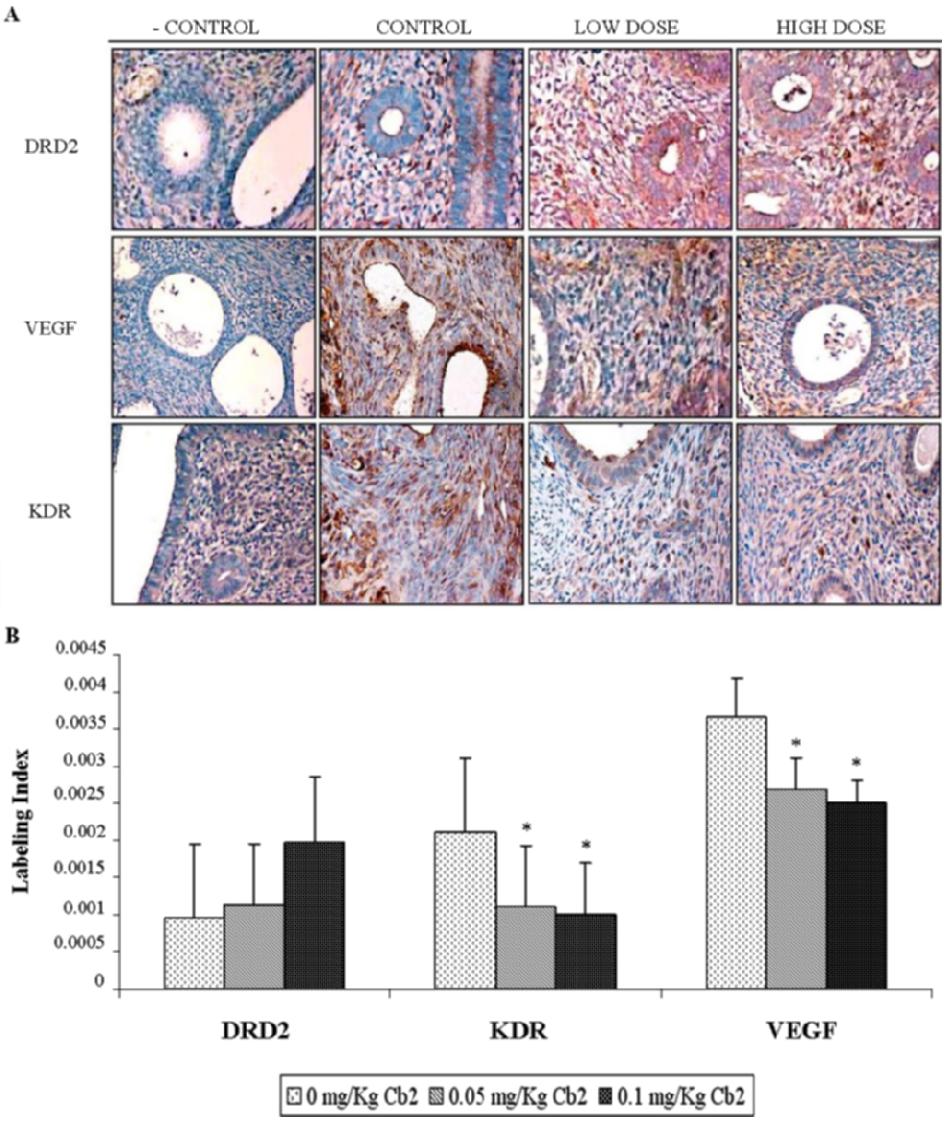

FIG. 3. **A**) DRD2, VEGF, and KDR protein expression study in experimental lesions by immunohistochemistry technique. Microphotographs show a higher level of VEGF and KDR staining in control lesions than in Cb2-treated lesions, whereas DRD2 staining was greater in Cb2-treated groups than in controls. **B**) Quantification of DRD2, VEGF, and KDR at protein level by morphometric analysis. The labeling index is the ratio of positively stained areas to the total area of the tissues. A significant decrease was observed in VEGF and KDR expression at protein level in the Cb2-treated groups versus controls ($*P < 0.05$). Data are expressed as mean ± SD (original magnification ×63). Sixteen samples relate to four samples per mouse and four mice per experimental group.

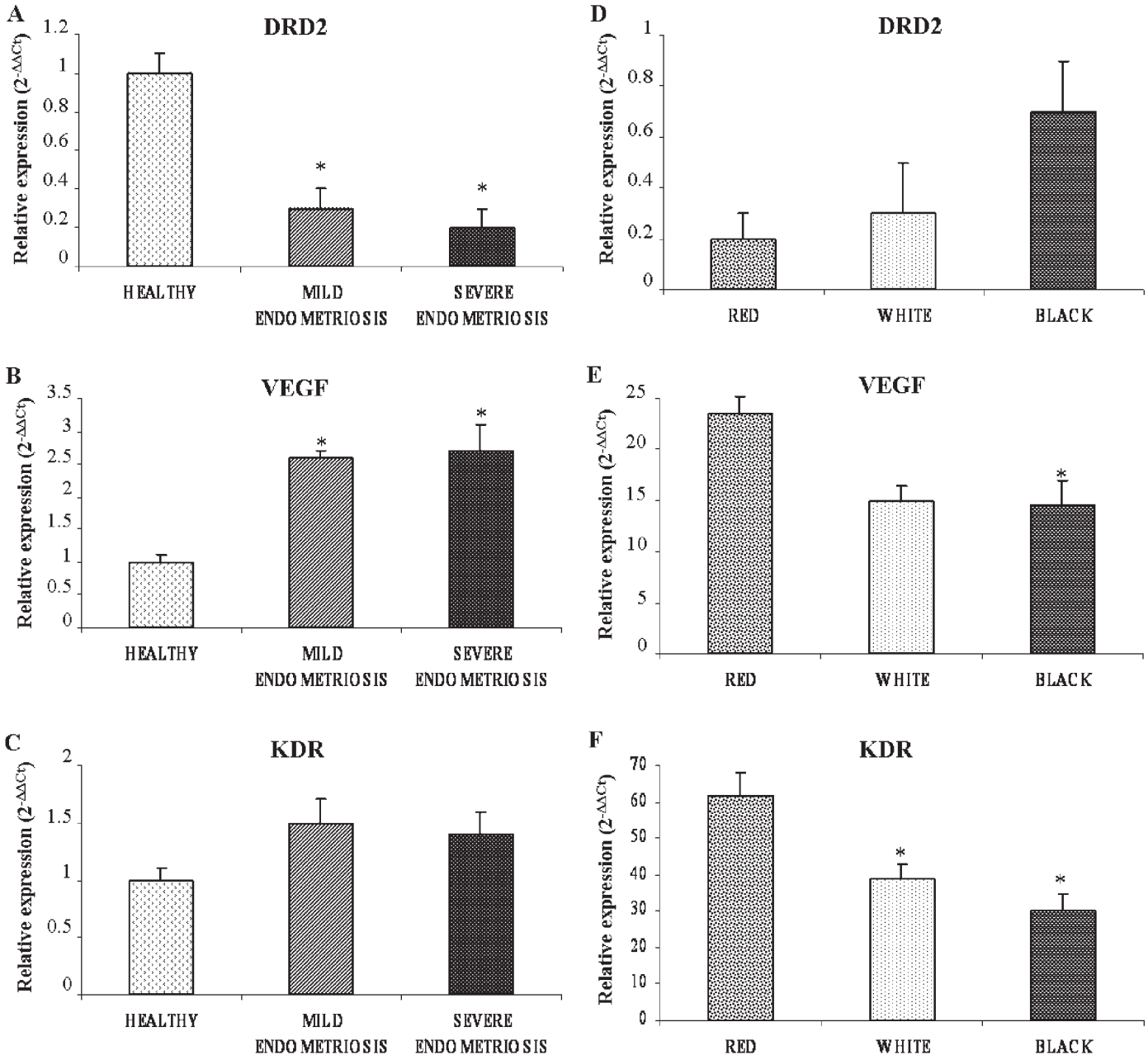

FIG. 4. Molecular studies to explore the presence and possible relationship between *DRD2* (**A**), *VEGF* (**B**), and *KDR* (**C**) gene expression in the eutopic endometrium of women with (n = 10) and without (n = 5) endometriosis. A significant decrease of *DRD2* expression in patients with mild and severe endometriosis compared with healthy patients was observed (**A**), whereas a significant increase of *VEGF* expression in patients with mild and severe endometriosis compared with healthy patients was detected (**B**). No significant difference in *KDR* expression in patients with mild and severe endometriosis compared with healthy patients was observed (**C**). Molecular analysis of *DRD2* (**D**), *VEGF* (**E**), and *KDR* (**F**) expression in peritoneal endometriotic lesions. No significant difference was detected in *DRD2* expression according to type of lesion (**D**), but an expression increase in black compared with red lesions was observed, whereas a significant decrease of *VEGF* expression in black lesions compared with red lesions was found. No significant difference in *VEGF* expression was detected between white and red lesions, or between white and black lesions (**E**). A significantly higher expression of *KDR* was observed in red lesions than in white and black lesions (**F**). Data are expressed as mean ± SD; $^*P < 0.05$.

considered [36, 37], *VEGF* expression was much higher in red lesions, which is a consequence of this being the most active and fastest-growing type of lesion. Similarly, *KDR* expression was also considerably higher in red lesions than in black and white lesions. Interestingly, we observed a trend toward a lower presence of *DRD2* as lesions became more active, which would initially rule out the use of DA to treat endometriosis. However, two aspects provide clarification of this apparent contradiction.

First, there is a negative correlation between VEGF levels and the expression of DRD2 [17, 38, 39]. Ablation of peripheral dopaminergic nerves in animals induces angiogenesis, vascular density, and hyperpermeability, as well as tumoral growth [17]. Additionally, knockout mice for DRD2 exhibit an enhanced angiogenesis and tumoral growth with respect to controls. This effect is associated with an increase in KDR phosphorylation, a critical step in angiogenesis [38, 39]. We know that the lesions described in the present study

represent different stages of the disease and that in women with endometriosis, lesions can regress or reappear in a proximal or distal area for no obvious reason. It is possible that the dopaminergic tone in each individual plays a fundamental role in the spontaneous changes observed in untreated patients with endometriosis. In fact, catecholamines may play important roles in the myometrium [35] and ovaries [40]. A similar phenomenon may occur in other reproductive tissues, such as eutopically or ectopically implanted endometria. Bearing all of this in mind, the hypothesis of an involvement of the dopaminergic system in the natural genesis of endometriosis should be explored further, and it may clarify why black lesions exhibit higher levels of *DRD2* than red ones.

Second, in the endometrial implants of our mouse model, we observed a trend toward an increased presence of DRD2 when higher doses of Cb2 were employed (Fig. 2). Thus, it is possible that DA treatment induces the expression of its type-2 receptor in different tissues, including the endometrium. This would allow Cb2 to bind to its receptors and would subsequently lead to the biological response of reduced angiogenesis and increased degeneration of the tissue. Indeed, we can speculate about a biological system in which this retroalimentation takes place.

To confirm the results obtained with the analysis of gene expression profiles in the endometriosis-like lesions after Cb2 treatment, protein levels were assessed by immunohistochemical and morphometric studies in the experimental endometriosis model. DRD2 protein expression in the endometriosislike lesions of human endometrial tissue origin followed the same pattern as the *DRD2* expression gene profile, with both tending to show an increased presence of DRD2 when higher doses of Cb2 were employed (Fig. 3). VEGF and KDR were underexpressed at protein levels when Cb2 was administered to the animals. These data provide further endorsement of the gene expression analyses performed in this study and of previous results obtained in molecular studies in which we observed a decrease in KDR phosphorylation with Cb2 and a shift toward a higher expression of antiangiogenic factors and a lower expression of proangiogenic molecules when Cb2 was employed following a significant decrease of new blood vessels in our experimental endometriosis model [19].

The present study is an extension of previous publications by our group on this topic [9]. For this reason, certain steps in the design of the study were avoided; this is the case for the morphological confirmation of endometriosislike lesions in the experimental animal model and inhibition of newly formed blood vessels after Cb2 treatment. We have previously shown that our model results in a recovery rate of 70.8% $\pm$ 5.2% in implanted tissue and 89.6% $\pm$ 5.7% in active lesions (composed of glands and stroma) [19]. The neoangiogenesis study, in which we employed immunofluorescence staining and laser scanning confocal microscopy, demonstrated that the formation of new blood vessels was significantly suppressed in endometriosislike lesions in mice treated with 0.05 mg/kg Cb2 (13.5% $\pm$ 1.1%) and 0.1 mg/kg Cb2 (10.8% $\pm$ 3.2%) in comparison with controls (0 mg/kg Cb2; 75.4% $\pm$ 1.6%) [19]. Similarly, the size of the samples obtained did not allow for the use of TaqMan PCR and immunohistochemistry in the analyses of human tissues from healthy patients and patients with endometriosis. We believe that the effects of DAs with respect to the prevention and/or treatment of endometriosis should be tested in pilot studies in nonhuman primate models for endometriosis [41], and later in humans.

To summarize, we describe the presence of DRD2 in human eutopic and ectopic endometria and demonstrate the regulation of these receptors when DAs are employed to target endometriotic lesions. Our results provide an impulse for further research into the role of steroid hormones in the regulation of these receptors during the menstrual cycle, and should encourage pilot studies in humans with the aim of exploring the potential of DAs as an alternative in the treatment of endometriosis.

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