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Effects of a selective cyclooxygenase-2 inhibitor on endometrial epithelial cells from patients with endometriosis

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BACKGROUND: Celecoxib, a selective cyclooxygenase (COX)-2 inhibitor, also has anti-proliferative properties and pro-apoptotic effects on different *in vivo* and *in vitro* models, two actions that may be efficacious in therapy for endometriosis. We evaluated the effects of celecoxib on apoptosis and proliferation, and vascular endothelial growth factor (VEGF) production and COX-2 expression and activity in endometrial epithelial cells (EECs). METHODS AND RESULTS: Thirty-two endometriosis and 13 control women were included in the study. EECs from eutopic endometrium and control biopsies were cultured with different doses of celecoxib. Celecoxib at 50, 75 and 100 μM (versus vehicle control) inhibited EEC proliferation in cultures from controls ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively) and patients with endometriosis ($P < 0.05$, $P < 0.01$ and $P < 0.01$), as assessed by ^{3}H-thymidine uptake. Celecoxib at 50, 75 and 100 μM induced apoptosis in EEC from controls ($P < 0.05$, $P < 0.001$ and $P < 0.001$) and patients with endometriosis ($P < 0.001$, $P < 0.001$ and $P < 0.01$), as revealed by the Acridine Orange–Ethidium Bromide technique. Western blot analysis showed that celecoxib was effective at increasing COX-2 protein at 100 μM in EEC from endometriosis patients ($P < 0.05$). In EEC from endometriosis patients, celecoxib at 25, 50 and 100 μM was also effective in reducing COX-2 activity, reflected in the reduction of prostaglandin E_2 (PGE_2) synthesis ($P < 0.001$), and VEGF secretion ($P < 0.001$; $P < 0.05$ and $P < 0.001$), assessed by enzyme-linked immunosorbent assay. Exogenous PGE_2 did not reverse celecoxib-induced growth inhibition. CONCLUSIONS: This study suggests a direct effect of celecoxib on reduction of endometrial growth and supports further research on selective COX-2 inhibition as a novel therapeutic modality in endometriosis.

Keywords: endometriosis; cyclooxygenase-2 inhibitor; eutopic endometrium; apoptosis; cell proliferation

Introduction

Endometriosis is one of the most common benign gynaecological diseases in women of reproductive age and is defined as the presence and proliferation of endometrial glands and stroma outside the uterine cavity. This disease is estrogen-dependent and its medical treatment is principally aimed at down-regulating the ovarian estrogen production by the application of GnRH agonists, progestins, androgenic agents or oral contraceptives either in a continuous or cyclic fashion. Unfortunately, these treatments are often associated with side effects and high recurrence rates (Valle and Sciarra, 2003; Mihalyi *et al*., 2006). There is a real need for more effective approaches to the prevention and treatment of endometriosis. Cyclooxygenase (COX)-2 inhibitors show great promise in this respect (Ebert *et al*., 2005).

COX-1 and COX-2 are isoenzymes that execute the synthesis of prostaglandins (PGs). While COX-1 is constitutively expressed in tissue, COX-2 is inducible and up-regulated in pathways of pain and inflammation (Vane *et al*., 1994; Ebert *et al*., 2005). Non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely used therapeutic agents in the treatment of pain and inflammation. Unlike regular NSAIDs, COX-2 selective inhibitors do not inhibit COX-1 and thus show promise as drugs that spare the gastrointestinal system. Celecoxib is a potent COX-2 inhibitor being developed for the treatment of rheumatoid arthritis and osteoarthritis (Chun and Surh, 2004).

Consistent data suggest that COX-2 inhibitors can reduce cancer risk in patients, can prevent tumorigenesis and suppress established tumour growth in animals (Bundred and Barnes, 2005). In addition, COX-2-specific inhibitors have the ability to block cell growth and induce apoptosis and cell cycle arrest in various tumour cell lines (Kundu *et al*., 2002; Basu *et al*., 2005). Also, it has been shown that COX-2 inhibitors such as celecoxib are potent inhibitors of angiogenesis both *in vitro* and *in vivo* (Gately and Li, 2004; Basu *et al*., 2005).

COX-2 overexpression was detected in eutopic and ectopic endometrium from patients with endometriosis (Ota *et al*., 2001; Matsuzaki *et al*., 2004b). Furthermore, large amounts of PGs have been found in peritoneal fluid from endometriosis patients and endometriotic lesions (De Leon *et al*., 1986). It has been shown that PGE_2 overproduction leads to excessive estradiol formation and that estradiol and cytokines (interleukin-1β and tumour necrosis factor-α), which are increased in endometriosis, induce COX-2 (Attar and Bulun, 2006). In addition, the COX-2/vascular endothelial growth factor (VEGF) association has been shown in human samples of non-small cell lung cancer (Gately and Li, 2004). PGE_2 increases VEGF production (Gately and Li, 2004) and then VEGF stimulates COX-2 expression in endothelial cells (Tamura *et al*., 2006). This pathway establishes a positive feedback loop in favour of high concentrations of estrogen, PGE_2 and VEGF in endometriosis (Bulun *et al*., 2002). Based on these data, we can speculate that inhibition of COX-2 would be a promising therapy toward controlling the growth of the endometriotic lesion.

In endometriosis research, recent animal studies demonstrated that treatment with COX-2 inhibitors prevents the implantation of endometrium to ectopic sites (Matsuzaki *et al*., 2004a) and induces the regression of endometrial explants in rats (Dogan *et al*., 2004) and mice (Ozawa *et al*., 2006). However, the mechanisms underlying this regression remain unclear.

To examine the potential application of celecoxib, a potent COX-2 inhibitor, in the treatment of endometriosis, we investigated the effects of this drug on cell proliferation and apoptosis of cultured human endometrial epithelial cells (EECs). In addition, our study evaluated the effect of celecoxib on *in vitro* VEGF and PGE_2 secretion, as well as on COX-2 expression in EECs from patients with endometriosis.

Materials and Methods

Subjects

A total of 45 patients who underwent diagnostic laparoscopy for infertility participated in this study: 32 with untreated endometriosis (Stages I and II) and 13 controls. Control subjects were infertile women without endometriosis, with tubal factor infertility or unexplained infertility, undergoing diagnostic laparoscopy. Male factor infertility was discarded. To avoid false negatives, only patients who did not complain of pelvic pain were considered for the control group. All patients were infertile, showed regular menstrual cycles and had not received any hormonal medical treatment for the last 6 months. Staging of the disease was performed according to the Revised American Society for Reproductive Medicine Classification (ASRM, 1997). Further confirmation of the disease was performed by histologic documentation. Biopsy specimens of eutopic endometrium were obtained from all subjects in the proliferative phase, as described previously (Meresman *et al*., 2000). Our experience indicates that there is a more efficient recovery of epithelial cells in the proliferative phase than in the secretory phase of the cycle and other authors have observed the same (Bongso *et al*., 1988). The biopsies were taken from the anterior uterine wall toward the fundal area.

This study was approved by the Ethics and Research Committee of the Biology and Experimental Medicine Institute of Buenos Aires, Argentina, and all subjects included in the study signed informed consents.

Isolation and culture of EECs

EECs were obtained from eutopic endometrial biopsies of endometriosis patients and controls. The tissue was immediately placed into culture medium and processed within 60 min of collection. Epithelial cells were enzymatically separated and isolated by successive centrifugation, and primary cultures were established for *in vitro* studies using the method described previously (Meresman *et al*., 2003a,b). Briefly, the explant was minced, washed and placed in basic medium (Dulbecco's modified Eagle's medium F-12, Gibco, Paisley, UK) containing 100 IU/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml amphotericin B (Gibco) with 0.5 mg/ml collagenase (type I, Gibco). After a 2-h incubation at 37°C in an atmosphere of 5% CO_2, the resulting suspension was centrifuged at $100 \times g$ for 5 min. The pellet containing glands was re-suspended in nutrient medium and spun again at $100 \times g$ for 5 min. The final pellet mainly contained epithelial cells. Any stromal cells remaining with the glands were further separated by selective adherence to plastic tissue culture dishes for 2 h. The enriched epithelial cells were cultured with media enriched with 10% fetal bovine serum (FBS; Gibco) and were grown to sub-confluence (70–80%) at 37°C for 48 h before the experiments. In all cases, each biopsy was utilized to run one or more experiments and there was no pooling of biopsies.

It has been previously shown that this method guarantees purity of epithelial cells in culture (Meresman *et al*., 2003a,b). Briefly, after 48-h culture, EECs were fixed in 100% methanol for 20 min and stained by indirect immunofluorescence using mouse monoclonal anti-cytokeratin 56 kDa (reacts with cytokeratin polypeptides 1, 2, 5, 6, 7, 8, 11, 14, 16, 17, 18; Serotec Ltd, Oxford, UK) followed by anti-immunoglobulin(Ig)-fluorescein isothiocyanate (Dako Ltd, Cambridge, UK) as secondary antibody. Cultures incubated without the primary antibody were included as controls in all experiments. Slides were viewed on a microscope equipped with fluorescence and differential interference contrast optics. Identical evaluation was also done on endometrial stromal cells.

The broad-spectrum cytokeratin antibody produced clear labelling in the epithelial cells. No staining of stromal cells was observed with this anti-cytokeratin antibody and no fluorescent labelling was observed in control cultures incubated with the secondary antibody alone. The purity of culture was 85–90% for epithelial cells as determined by immunofluorescent staining.

Cell proliferation assay

Fifty thousand EECs per well were plated in 96-microwell plates and incubated with 10% FBS medium. After a 48-h incubation, the cells were washed and celecoxib (Pfizer, Caguas, USA) 10, 20, 25, 40, 50, 75 and 100 μM was added to supplemented 2.5% FBS medium. The cells were incubated with the agent for 48 additional hours, at which time the cells had reached 70% confluence. Vehicle control cells consisted of EECs incubated with 2.5% FBS medium with 1 μl dimethylsulphoxide (D5879, Sigma, St Louis, MO, USA) per ml of medium, the vehicle in which celecoxib was reconstituted.

Twenty-four hours before harvesting, 1 μCi ^{3}H-thymidine (Nen, Dupont, Boston, MA, USA) was added to each microwell and DNA synthesis was assessed by ^{3}H-thymidine incorporation using a liquid scintillation counter (Meresman *et al*., 2003a).

In order to determine whether added PGE_2 could counteract the growth inhibitory effect of celecoxib, EECs were treated with celecoxib (50 μM) and 200–2000 pg/ml PGE_2 and incubated for 48 h before determining ^{3}H-thymidine incorporation, as mentioned above. The dose of celecoxib used in this assay was chosen based

on the lowest concentration that had a significant effect on inhibiting cell proliferation.

Apoptosis assay

A total of 120 000 EECs per well were plated in Lab-Tek 8-well culture chambers (Nalge Nunc, Naperville, IL, USA). After 48-h incubation with 10% FBS medium, the cells were washed and cultured with 2.5% FBS medium. Cells were incubated with the addition of celecoxib at 10, 20, 25, 40, 50, 75 and 100 μM or vehicle control. The concentrations in the study were based on our titrations assessing apoptosis with celecoxib for the EECs and from several published studies on other cells (Jendrossek *et al*., 2003; Basu *et al*., 2005). The cells were incubated for 24 additional hours and the percentage of apoptotic cells was assessed by the Acridine Orange–Ethidium Bromide (AO–EB) technique. AO is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into double-stranded nucleic acid (DNA) or red fluorescence if bound to single-stranded nucleic acid (RNA). EB is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA (Baskic *et al*., 2006). It is specific for apoptotic forms of cell death and does not significantly label cells undergoing necrotic death provoked by injury (Abrams *et al*., 1993; Ribble *et al*., 2005). After addition of the AO (4 μg/ml)–EB (4 μg/ml) mix, the cells were viewed using a fluorescence microscope by two observers. There was no significant difference in the results obtained between the two observers ($P = 0.66$).

Detection of COX-2 by immunocytochemistry

A total of 120 000 EECs per well were plated in Lab-Tek 8-well culture chambers (Nalge Nunc). After a 48-h incubation with 10% FBS medium, the cells were washed and incubated for an additional 24-h period with celecoxib at 25, 40, 50, 75 and 100 μM in medium supplemented with 2.5% FBS or vehicle control. Following treatments, chamber slides were fixed and standard procedures for immunocytochemistry were performed. Cells were incubated overnight with rabbit polyclonal anti-human COX-2 antibody (1:25, sc-1747, Santa Cruz, Santa Cruz Biotechnology Inc., CA, USA) at 4°C. After that, cells were treated for 60 min with the corresponding biotinylated secondary antibody and later with streptavidine conjugated to horseradish peroxidase (LSAB+System, Dako).

Binding was visualized by incubating slides with diaminobenzidine and lightly counterstained with haematoxylin. As a negative control, Ig of the same Ig class and concentration as the primary antibody was used. The negative control showed absence of specific staining. COX-2-positive cells were identified by the presence of brown cellular reactivity.

Evaluation of COX-2 expression by western blot

A total of 10^6 EECs per well were plated in 6-well plates. After incubation for 48 h with 10% FBS medium, the cells were washed and incubated for an additional 24-h period with celecoxib at 25, 40, 50, 75 and 100 μM in medium supplemented with 2.5% FBS or vehicle control. Following treatment, cells were lysed by scraping in chilled lysis buffer supplemented with protease inhibitors (P8340, Sigma). After freeze thawing, the lysate was centrifuged at 15 000 × *g* for 10 min at 4°C and protein concentrations in the supernatant were measured by the Bradford assay (Bradford, 1976). Equal protein samples (20 μg) were electrophoresed through a 10% sodium dodecyl sulphate–polyacrylamide gel. The separated proteins were transferred to nitrocellulose membranes, blocked for 1 h in 5% low-fat powdered milk and incubated with the rabbit-polyclonal anti-human COX-2 antibody (1:200, sc-1747, Santa Cruz) at 4°C overnight. After overnight incubation, membranes were incubated with a goat peroxidase-conjugated anti-rabbit IgG secondary antibody (1:1000, A4914, Sigma) at room temperature for 1 h. Protein bands were visualized by incubating the membranes with an enhanced chemiluminescence reagent (Perkin Elmer Life Science, Boston, MA, USA) followed by exposure to Kodak X-Omat AR films. The levels of protein were compared and analysed by densitometric studies using Scion Image for Windows (Scion Corporation, Worman's Mill, CT, USA). Consistency of protein loading was validated with β-actin loaded as control, using a mouse-monoclonal anti-human β-actin antibody (1:2000, Abcam, Cambridge, UK) as primary antibody and an anti-mouse IgG as secondary antibody (1:1000, HAF007, R&D Systems, Minneapolis, MN, USA).

Measurement of VEGF and PGE_2 by ELISA

EEC cultures were left undisturbed at 37°C for 48 h. Subsequently, the cells were washed and incubated with basic medium supplemented with 2.5% FBS for an additional 24 h after the addition of celecoxib at 25, 50 and 100 μM or vehicle control. Cells were prepared for assays as described above. The cell culture supernatant was removed from each well and centrifuged for 5 min at 2000 × *g* to pellet any floating cells. Supernatants not assayed immediately were frozen at −80°C. The conditioned medium was collected and assayed for VEGF and PGE_2 using commercial enzyme-linked immunosorbent assay (ELISA) kits (Cytelisa, Human VEGF kit, EL-V, Cytimmune Sciences Inc., MD, USA, and Correlate-EIA, PGE_2 Enzyme Inmunoassay Kit, Assay Designs, Ann Arbor, MI, USA). The sensitivity level for the VEGF ELISA was 26.6 pg/ml and for the PGE_2 ELISA was 13.4 pg/ml. The intra-assay variability for VEGF and PGE_2 was ±7.3 and 8.9%, respectively, while the inter-assay variability was ±10.5 and 3.0% for VEGF and PGE_2, respectively. All samples were assessed in duplicate. Results were expressed as percentage of vehicle control cells ± SEM.

Statistics

Statistical comparisons were performed by Kruskal–Wallis non-parametric analysis of variance test, followed by Dunn's multiple comparison test. Regardless of the statistical test, only a *P*-value ≤0.05 was considered significant.

Results

Effects of celecoxib on EEC proliferation

The effects of different concentrations of celecoxib on EEC proliferation are displayed in Fig. 1. We found that in EEC cultures from controls, celecoxib at low concentrations (10, 20, 25 and 40 μM) had no effect on basal DNA synthesis. In contrast, after exposure to 50, 75 and 100 μM of celecoxib, the EECs showed a significantly lower degree of cell proliferation ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, versus vehicle control cell proliferation).

In EEC cultures from endometriosis patients, ^{3}H-thymidine uptake was significantly down-regulated by celecoxib at high concentrations: 50, 75 and 100 μM ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively, versus vehicle control cell proliferation). Lower concentrations of celecoxib (10, 20, 25 and 40 μM) had no significant effect on basal cell proliferation.

Effects of celecoxib on EEC apoptosis

The effects of different concentrations of celecoxib on EEC apoptosis are displayed in Fig. 2. Celecoxib 50, 75 and

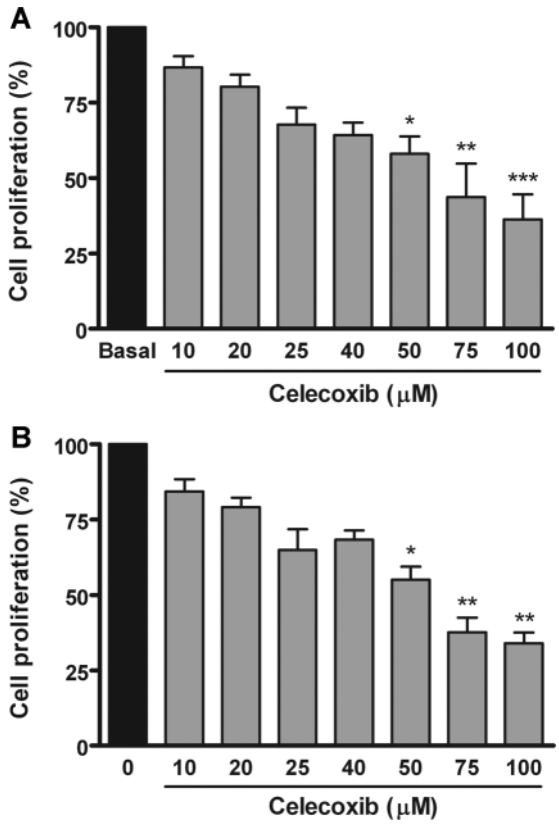

Figure 1: Effects of celecoxib on cell proliferation in endometrial cell cultures from control subjects and patients with endometriosis. Endometrial epithelial cell cultures from control women (**A**) and patients with endometriosis (**B**) treated with different doses of celecoxib or vehicle were assessed for cell proliferation by the ^{3}H-thymidine uptake assay. Values $\pm$ SEM are expressed as percentage of vehicle control cell proliferation set as 100%. $n = 5$ for control women and $n = 5$ for endometriosis patients. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle control.

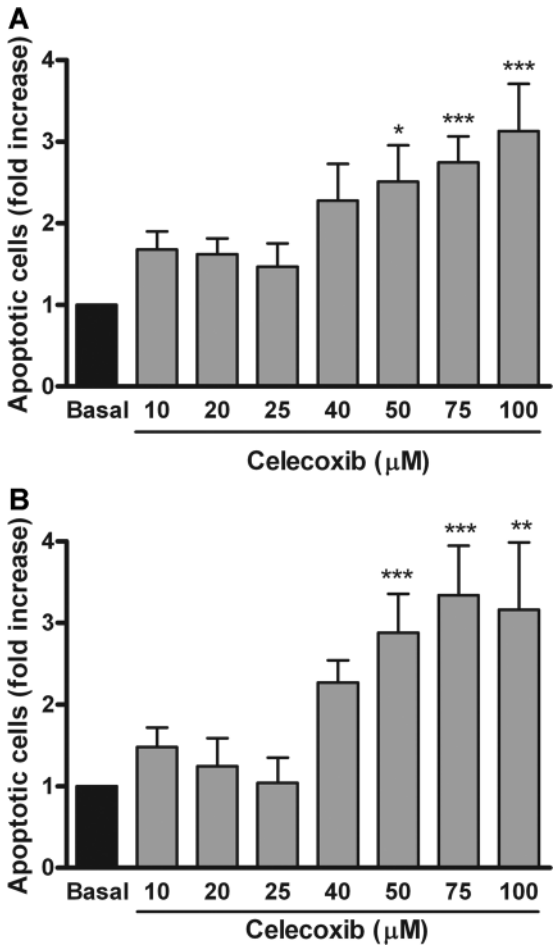

Figure 2: Effects of celecoxib on apoptosis in endometrial cell cultures from control subjects and patients with endometriosis. Endometrial epithelial cell cultures from control women (**A**) and patients with endometriosis (**B**) treated with different doses of celecoxib or vehicle were assessed for apoptosis by the AO–EB technique. Values $\pm$ SEM are expressed as fold increase of vehicle control apoptosis set as 1. $n = 8$ for control women and $n = 8$ for endometriosis patients. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle control.

100 μM showed a significant effect on endometrial growth, enhancing apoptosis in endometrial cell cultures from controls ($P < 0.05$, $P < 0.001$ and $P < 0.001$, respectively, versus vehicle control apoptosis). In contrast, celecoxib 10, 20, 25 and 40 μM had no effect on basal apoptosis.

As well, in EECs from patients with endometriosis, celecoxib 50, 75 and 100 μM showed a significant effect enhancing the apoptosis levels ($P < 0.001$, $P < 0.001$ and $P < 0.01$, respectively, versus vehicle control apoptosis). In contrast, celecoxib 10, 20, 25 and 40 μM had no significant effect on basal apoptosis.

In this study and in previous works performed in our laboratory (Meresman *et al*., 2003a; Bilotas *et al*., 2007), we found no significant differences in the degrees of cell proliferation and in the percentage of apoptotic cells between endometrial cell cultures from endometriosis patients and controls. Taking all these results into account, we decided to utilize only cultures from patients with endometriosis to evaluate the experiments that follow.

Effect of celecoxib on COX-2 expression

The effect of different doses of celecoxib on the expression of COX-2 in EECs in culture from patients with endometriosis was studied by immunocytochemistry. COX-2 expression was observed in the cytoplasm of EECs treated with celecoxib (25 and 50 μM) as well as in vehicle control cells (Fig. 3A).

To assess whether there were variations in the level of COX-2 protein after exposure to different doses of celecoxib (25, 40, 50, 75 and 100 μM), COX-2 expression was analysed by western blot. The pattern observed was similar to the one seen by immunocytochemistry. COX-2 protein was observed in celecoxib-treated EECs and in vehicle control cells, showing a significant increase after treatment with 100 μM of celecoxib ($P < 0.05$) (Fig. 3B).

Effect of celecoxib on PGE_2 synthesis

COX-2 activity was evaluated by measuring the levels of PGE_2 released into the conditioned media by EECs in culture treated with different doses of celecoxib for 24 h. Fig. 4 shows that the concentrations of celecoxib tested (25, 50 and 100 μM) significantly reduced the levels of PGE_2 in supernatants ($P < 0.001$ versus vehicle control).

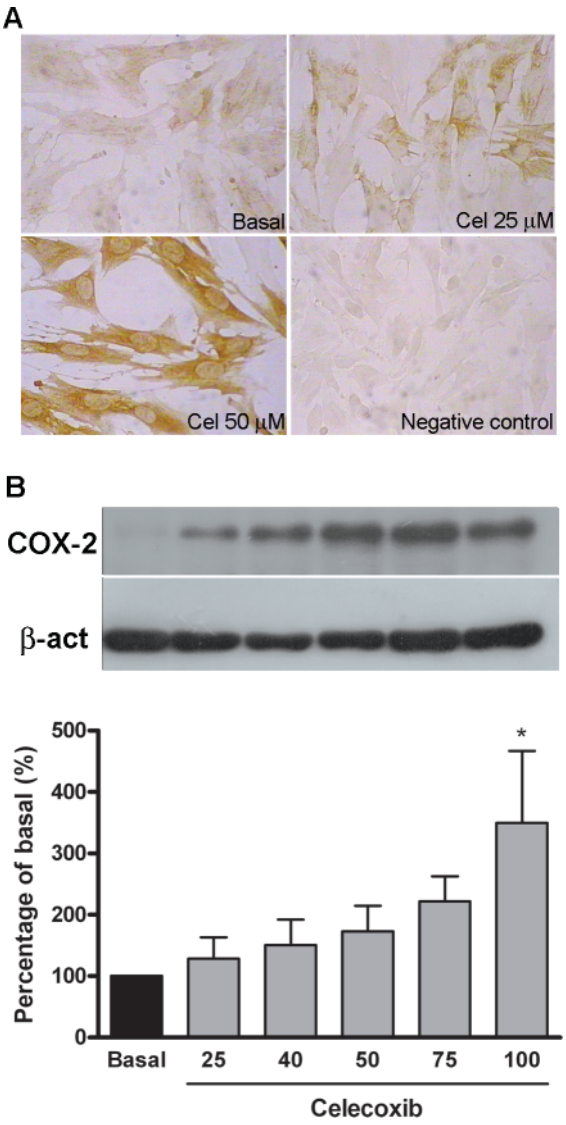

Figure 3: (**A**) Immunocytochemistry of cyclooxygenase (COX)-2 expression in endometrial epithelial cell (EEC) cultures from subjects with endometriosis after treatment with celecoxib. EECs from patients with endometriosis were plated and incubated in cell chamber slides with vehicle control conditions or with celecoxib 25 or 50 μM. In negative control, normal rabbit IgG was used instead of primary antibody. Magnification: ×400. (**B**) Effects of celecoxib on COX-2 expression in endometrial cell cultures from subjects with endometriosis. Western blot analysis was performed on cell homogenates from EEC cultures from patients with endometriosis after treatment with 25, 40, 50, 75 and 100 μM celecoxib or vehicle control. A representative blot is presented in the upper panel. The lower panel shows quantification results for COX-2 expressed as a percentage of vehicle control values ± SEM. Consistency of protein loading was validated with β-actin loaded as control. $n = 10$ $^{*}P < 0.05$ versus vehicle control.

Effect of celecoxib on VEGF secretion

Fig. 5 shows the effect of celecoxib on the secretion of the pro-angiogenic factor VEGF by EECs in culture treated with different doses of celecoxib for 24 h. The concentrations of celecoxib tested (25, 50 and 100 μM) induced a significant reduction in VEGF secretion compared with vehicle control ($P < 0.001$, $P < 0.05$ and $P < 0.001$, respectively).

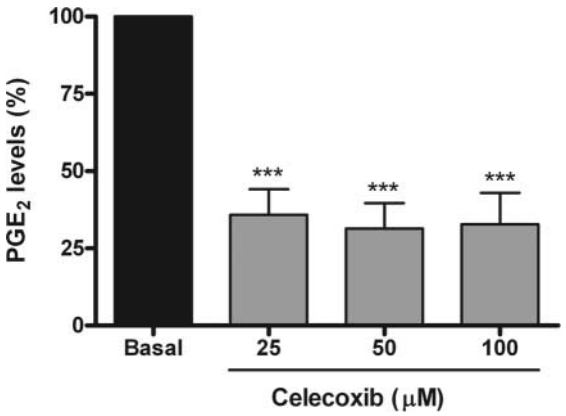

Figure 4: Effects of celecoxib on cyclooxygenase (COX)-2 activity in endometrial cell cultures from patients with endometriosis. COX-2 activity was studied measuring prostaglandin E_2 (PGE_2) levels in conditioned media of endometrial epithelial cells from patients with endometriosis treated with different concentrations of celecoxib or vehicle. Enzyme immunoassay was performed in duplicate for each sample, bars represent media ± SEM. Values are expressed as percentage of vehicle control PGE_2 levels set as 100%. $n = 9$ $^{***}P < 0.001$ versus vehicle control PGE_2 levels.

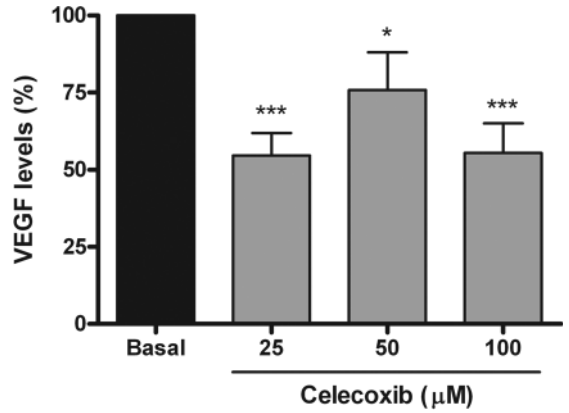

Figure 5: Effects of celecoxib on vascular endothelial growth factor (VEGF) levels in endometrial cell cultures from patients with endometriosis. VEGF levels in conditioned media of endometrial epithelial cells from patients with endometriosis treated with different concentrations of celecoxib or vehicle. Enzyme immunoassay was performed in duplicate for each sample, bars represent media ± SEM. Values are expressed as percentage of vehicle control VEGF levels set as 100%. $n = 13$ $^{*}P < 0.05$, $^{***}P < 0.001$ versus vehicle control VEGF levels.

Effect of the addition of celecoxib and PGE_2 on EEC proliferation

Since celecoxib caused growth inhibition and inhibited PGE_2 secretion, we speculated that the addition of exogenous PGE_2 to celecoxib-stimulated EECs in culture might abrogate the effect of celecoxib on cell proliferation. Therefore, to determine whether celecoxib-induced growth inhibition could be reversed by exogenous PGE_2, EECs from patients with endometriosis were stimulated simultaneously with 50 μM celecoxib and PGE_2 in different doses. Growth inhibition induced by 50 μM celecoxib could not be restored by addition of exogenous PGE_2 ($P > 0.05$ versus EECs stimulated with celecoxib alone), thereby suggesting that celecoxib-induced

growth inhibition in these cells may be independent of PGE_2 levels. Results are displayed in Fig. 6.

Discussion

Current medical therapies for endometriosis, which are usually aimed at reducing the endogenous steroid production, are not effective in at least half of these patients, and their adverse effect profiles constitute a significant problem (Rice, 2002; Mihalyi *et al*., 2006). Given the inefficiency of the available treatments, developing novel therapeutic strategies may be useful to improve our ability to eliminate endometriotic lesions and to prevent the recurrence of the disease.

In this study, epithelial cell cultures of eutopic human endometrium from endometriosis patients were used in a model to evaluate cell proliferation, apoptosis, COX-2 expression and VEGF and PGE_2 secretion in response to the addition of the COX-2 inhibitor celecoxib. Although the cells employed in this investigation were not derived from endometriotic implants and their *in vitro* response may not be identical, the rationale for the use of eutopic endometrial cells in short-term culture as a model for endometriosis research has been documented previously (Surrey and Halme, 1990). Furthermore, the epithelial fraction was the one studied since it has been seen that COX-2 is preferentially expressed in this cell type rather than in stromal cells (Ota *et al*., 2001).

Endometriosis is associated with increased COX-2 expression in eutopic and ectopic endometrium (Ota *et al*., 2001; Matsuzaki *et al*., 2004b). It has also been shown that PG levels are elevated in serum and peritoneal fluid from endometriosis patients (De Leon *et al*., 1986; Gazvani and Templeton, 2002). Inhibition of COX by conventional NSAIDs or selective COX-2 inhibitors may therefore lead to relief of pain associated with endometriosis (Nasir and Bope, 2004).

Recently, multiple studies have suggested that the administration of a COX-2 inhibitor reduces tumour development by suppressing proliferation and angiogenesis as well as by increasing apoptosis (Elder *et al*., 2000; Leahy *et al*., 2002; Basu *et al*., 2005; Grosch *et al*., 2006). However, the effect of COX-2 inhibitors on the endometrial cell growth in endometriosis is still unknown. The present study clearly demonstrates that in EECs from patients with endometriosis and controls, treatment with the selective COX-2 inhibitor, celecoxib, effectively reduces cell proliferation and induces cell death. We observed that celecoxib-induced apoptosis in EECs in culture revealed by the AO–EB technique. Staining of apoptotic cells with fluorescent dyes such as AO and EB is considered a correct method for evaluating the changed nuclear morphology. Recently, Baskic *et al*. compared several methods for detecting apoptosis and necrosis in human leukocytes. The authors conclude that both fluorescence methods, AO and EB, provided reliable and reproducible results and distinguished clearly between subpopulations of apoptotic cells (Baskic *et al*., 2006).

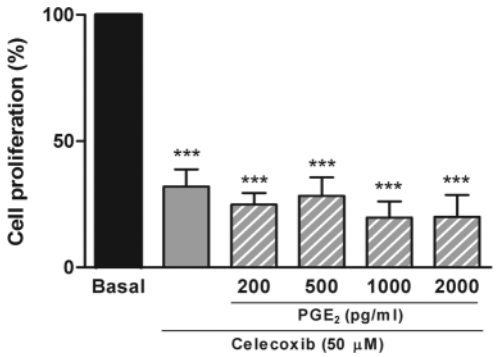

Figure 6: Effects of PGE_2 addition to celecoxib-stimulated endometrial epithelial cell (EEC) cultures from endometriosis patients. EEC cultures from patients with endometriosis treated with celecoxib (50 μM) and different concentrations of PGE_2 or vehicle were assessed for cell proliferation by the ^{3}H-thymidine uptake assay. Values ± SEM are expressed as percentage of vehicle control cell proliferation set as 100%. $n = 9$ $^{***}P < 0.001$ versus vehicle control.

Celecoxib-induced apoptosis has previously been reported by other authors. Basu *et al*. (2005) and Jendrossek *et al*. (2003) described that treatment with celecoxib enhanced apoptosis in human breast cancer cells and Jurkat T cells, respectively. There is evidence that the intrinsic pathway is implicated in the activation of apoptosis after celecoxib treatment (Jendrossek *et al*., 2003; Dandekar *et al*., 2005), yet other authors have reported activation of the extrinsic pathway (Kim *et al*., 2004; Liu *et al*., 2004). Furthermore, the doses of celecoxib that significantly induced apoptosis in the present study are clinically relevant since studies conducted by other authors reveal that the concentration of COX-2 inhibitors in serum of treated patients varies from 20 to 100 μM (Munkarah *et al*., 2003; Basu *et al*., 2005). Nevertheless, there are discrepancies in this regard. Other authors state that the plasma concentration corresponds to 3 μM when patients were treated with daily doses of 400 mg of celecoxib (Maier *et al*., 2004; Grosch *et al*., 2006). According to these investigators, the differences found in COX-2 inhibitor concentration needed to observe anti-proliferative effects relies on the fact that, in order to induce regression of tumour growth in patients, several weeks of treatment are needed while, *in vitro*, the anti-proliferative effects are seen in a few hours (Maier *et al*., 2005).

Our findings are consistent with previous results from other authors who found that COX-2 inhibitors were successful at inhibiting tumour growth in different cell lines (Wu *et al*., 2004; Basu *et al*., 2005). Nevertheless, provision of exogenous PGE_2 did not reverse celecoxib-induced growth inhibition in EECs. This suggests that celecoxib-induced growth inhibition in EECs is independent of PGE_2. Corroborating our findings are previous reports that conclude that the addition of PGE_2 had no effect on growth inhibition induced by COX-2 inhibitors (Elder *et al*., 2000; Basu *et al*., 2005).

Additionally, celecoxib treatment reduces PGE_2 secretion by EECs from patients with endometriosis. PGE_2 is the major PG produced by EECs, and the production of secreted PGE_2 is therefore an appropriate measure of COX activity. A concentration of 25 μM of celecoxib was effective at inhibiting PGE_2 secretion, however, under these conditions, growth inhibition was not observed. The reduction of PGE_2 production induced by 25 μM celecoxib in EECs without the induction of growth inhibition supports the proposal that the growth

arrest induced by higher concentrations (50–100 μM) of celecoxib is not simply due to reduced PGE_2 secretion. Similar results were obtained by Grosch *et al.* (2001) and Han *et al.* (2004) who speculated that the mechanisms involved in the inhibition of cell proliferation due to celecoxib treatment are independent of COX-2 activity and expression, since the concentrations needed to induce cell cycle arrest and inhibit proliferation are higher than those needed to inhibit COX-2 activity. In agreement, it has been reported that celecoxib induces apoptosis through mechanisms independent of COX-2 in cancer cell lines (Jendrossek *et al.*, 2003; Lai *et al.*, 2003; Grosch *et al.*, 2006), and it can also induce apoptosis in cells deficient in COX-2 (Grosch *et al.*, 2001; Pang *et al.*, 2007). Further studies are needed to confirm whether the inhibitory effect triggered by celecoxib is independent of COX-2 activity.

In addition to growth inhibition and COX-2 activity reduction, we observed that COX-2 protein is significantly increased in EECs from endometriosis patients treated with 100 μM celecoxib. Also, the western blot analysis for COX-2 protein showed a clear but not statistically significant increase after treatment with 25–75 μM celecoxib (Fig. 3B). The mechanism for this is not known, however, it has been suggested that COX-2-dependent products may inhibit COX-2 expression in a negative feedback loop: here, celecoxib treatment inhibited COX-2 activity, shown by reduced PGE_2 secretion, perhaps resulting in removal of negative feedback and COX-2 induction. There are similar data on COX-2 inhibitor treatment leading to strong up-regulation of COX-2 protein expression in several reports (Elder *et al.*, 2000; Levitt *et al.*, 2004; Basu *et al.*, 2005).

The establishment, maintenance and growth of endometriosis are dependent in part on the development of new blood vessels and it is known that the VEGF family is critical in the process of angiogenesis. Activity of COX-2 results in an increased VEGF expression (Li *et al.*, 2002). In addition, it has been shown that patients with endometriosis have a greater concentration of VEGF in peritoneal fluid compared with controls (Mahnke *et al.*, 2000). Treating rats with rofecoxib induced a decrease in the explant size accompanied by a decrease in peritoneal fluid VEGF levels (Dogan *et al.*, 2004). In our study, VEGF levels were significantly lower in the celecoxib-treated EECs in comparison to vehicle control cells. Down-regulation of eicosanoid production by COX-2 inhibitors is reported widely as a mechanism underlying their antiangiogenic activity (Gately and Li, 2004).

To the best of our knowledge, this is the first *in vitro* study suggesting a direct effect of celecoxib COX-2 inhibitor on the reduction of endometrial growth. Nevertheless, in animal models for endometriosis, it was shown that celecoxib prevented the establishment of new endometriotic lesions and the growth of already established ones (Matsuzaki *et al.*, 2004a).

With the development of COX-2-specific inhibitors, better-tolerated therapeutic options are available. In humans, only one report has been published and suggested that the use of COX-2-specific inhibitors was effective in the management of pelvic pain associated with endometriosis (Cobellis *et al.*, 2004).

The role of COX-2 in endometrial growth inhibition continues to be under investigation, and the data in our study support further research on selective COX-2 inhibition as a novel therapeutic modality in endometriosis.

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