Nimesulide, a COX-2 inhibitor, does not reduce lesion size or number in a nude mouse model of endometriosis


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BACKGROUND: Women with endometriosis have elevated levels of cyclooxygenase-2 (COX-2) in peritoneal macrophages and endometriotic tissue. Inhibition of COX-2 has been shown to reduce inflammation, angiogenesis and cellular proliferation. It may also downregulate aromatase activity in ectopic endometrial lesions. Ectopic endometrial establishment and growth are therefore likely to be suppressed in the presence of COX-2 inhibitors. We hypothesized that COX-2 inhibition would reduce the size and number of ectopic human endometrial lesions in a nude mouse model of endometriosis.

METHODS: The selective COX-2 inhibitor, nimesulide, was administered to estrogen-supplemented nude mice implanted with human endometrial tissue. Ten days after implantation, the number and size of ectopic endometrial lesions were evaluated and compared with lesions from a control group. Immunohistochemical assessment of vascular development and macrophage and myofibroblast infiltration in control and treated lesions was performed.

RESULTS: There was no difference in the number or size of ectopic endometrial lesions in control and nimesulide-treated nude mice. Nimesulide did not induce a visually identifiable difference in blood vessel development or macrophage or myofibroblast infiltration in nude mouse explants.

CONCLUSION: The hypothesized biological properties of COX-2 inhibition did not influence lesion number or size in the nude mouse model of endometriosis.

Key words: COX-2 inhibitor/endometriosis/mouse model/nimesulide/prostaglandin

Introduction

Endometriosis affects 10% of women of reproductive age, inducing symptoms of chronic pelvic pain, dysmenorrhoea, dyspareunia and subfertility (Strathy et al., 1982). Non-steroidal anti-inflammatory (NSAID) medication is a longstanding treatment for the symptomatic relief of endometriosis-associated pain (Mahutte and Arici, 2003). The effect of NSAIDs on endometriotic lesion size and number has not been evaluated in clinical trials due to the risks and morbidity associated with repetitive laparoscopy. We theorized that inhibition of the prostaglandin pathway was likely to reduce the survival and growth of endometrium at an ectopic site.

Cyclooxygenases (COX) regulate the rate-limiting conversion of arachidonic acid to prostaglandin H2 (PGH2) in the prostaglandin synthesis pathway (Smith et al., 1996). Unlike COX-1 that is constitutively expressed, COX-2 transcription is upregulated at sites of inflammation (Vane et al., 1994). COX-2 has been implicated in inflammatory, angiogenic, proliferative and estrogenic cellular processes (Dannenberg et al., 2001) and is likely to be influential in several of the processes that lead to ectopic endometrial development. Inflammatory mediators such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) upregulated COX-2 mRNA and protein expression in cultured peritoneal macrophages (Wu et al., 2002). These factors induced the phosphorylation of inhibitor-κB and promoted the nuclear translocation of nuclear factor-κB (NF-κB) in endometriotic stromal cells (Sakamoto et al., 2003). The NF-κB binding region of the COX-2 promoter gene was found to be essential for COX-2 promoter activity in endometrial stromal cells (Tamura et al., 2002a,b). Other signalling pathways may also participate in IL-1β-mediated COX-2 regulation in endometrial stromal cells as inhibitors of extracellularly-related kinases 1 and 2, protein kinase A (PKA) as well as NF-κB were shown to inhibit IL-1β-induced COX-2 upregulation (Tamura et al., 2002a). Both IL-1β (Tamura et al., 2002a) and PGE2 (the main prostaglandin product of the COX pathway) (Tamura et al., 2002b) acted post-transcriptionally to stabilize COX-2 transcripts promoting sustained COX-2 enzyme activity in an inflammatory environment.

Endometriosis is an inflammatory condition (Haney and Weinberg, 1988). PGE2 (Karck et al., 1996; Raiter-Tenenbaum et al., 1998), TNF-α and IL-1β (Mori et al., 1991; Keenan et al., 1995) are all elevated in peritoneal...
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macrophage-conditioned media or in the peritoneal fluid from women with endometriosis when compared with disease-free women. The peritoneal macrophages of women with endometriosis have higher levels of COX-2 mRNA and protein than those from women without the disease (Wu et al., 2002). COX-2 mRNA and protein are also present in higher amounts in ectopic endometrial lesions when compared with eutopic endometrium from women with endometriosis. Eutopic tissue from disease-free women had the lowest levels of COX-2 mRNA and protein (Ota et al., 2001; Chishima et al., 2002). We hypothesized that a reduction in prostaglandin levels secondary to COX-2 inhibition was likely to disrupt the inflammatory processes evident in women with endometriosis.

Endometriotic blood vessel formation may be disrupted by the restriction of COX-2 activity. Products of the COX pathway, PGE1 and PGE2 (Diaz-Flores et al., 1994; Form and Auerbach, 1983; Milne and Jabbour, 2003), and thromboxane A2 (Daniel et al., 1999; Nie et al., 2000) promote blood vessel formation in vitro and in vivo. Additionally, COX-2 inhibitors reduce the vascularity of in vivo angiogenesis (Masferrer et al., 2000) and tumorigenesis models (Tsujii et al., 1998; Dormond et al., 2001). NS-398, a specific COX-2 inhibitor, was shown to inhibit endothelial cell spreading and migration in vitro by suppressing αβ3 activation of the cdc42 and Rac GTPases as well as reduce fibroblast growth factor-mediated angiogenesis in vivo (Dormond et al., 2001).

There is evidence that COX-2 inhibitors also inhibit vascular endothelial growth factor-A (VEGF-A)-mediated angiogenesis PGE2 induced VEGF expression in synovial fibroblasts in vitro (Ben-Av et al., 1995). COX-2-overexpressing colonic cancer cells secreted high levels of VEGF-A (Tsujii et al., 1998) and a significant association between COX-2, VEGF and microvascular density was demonstrated in human head and neck cancer (Gallo et al., 2002). COX-2 (−/−) mice have abnormal implantation and decidualization associated with defective VEGF-A signalling and abnormal angiogenesis in the endometrium (Matsumoto et al., 2002). The importance of VEGF-A-mediated angiogenesis in ectopic endometrial lesion formation has been demonstrated in a nude mouse model of endometriosis (Hull et al., 2003). We postulated that inhibition of COX-2 would reduce VEGF-A-mediated angiogenesis in endometriotic lesion development resulting in a reduction in lesion number and size.

COX-2-mediated mechanisms also influence cellular proliferation and apoptosis in ectopic endometrium. PGE2 induced endometrial glandular epithelial cell proliferation in vitro (Jabbour and Boddy, 2003). Overexpression of COX-2 is associated with malignant proliferation (for a review see Gasparini et al., 2003) and is seen in endometrial cancer (Tong et al., 2000; Jabbour et al., 2001). Conversely epidemiological studies have shown that long-term use of non-selective NSAIDS reduces several tumour types in humans (Thun et al., 1991; Schreinemachers and Everson, 1994). Cell lines that overexpress COX-2 were resistant to apoptosis and had increased amounts of the survival factor Bcl-2 (Tsujii and DuBois, 1995). Bcl-2 has been identified at higher levels in ectopic endometrium when compared with eutopic endometrium from the same women (Jones et al., 1998). Additionally, eutopic endometrium from women with endometriosis had higher Bcl-2 levels and lower numbers of apoptotic cells when compared with normal controls (Meresman et al., 2000). We proposed that COX-2 inhibitors promote apoptosis and inhibit glandular proliferation in endometriotic tissue leading to a reduction in the size and number of lesions.

We chose the nude mouse model of endometriosis to test the hypothesis that inhibitors of COX-2 would disrupt several cellular processes involved in ectopic endometrial lesion formation causing a reduction in lesion size and number. In this model, human eutopic endometrium is implanted s.c. into T-cell-deficient estrogen-supplemented nude mice. The resulting lesions are morphologically and histologically similar to human disease (Bruner-Tran et al., 2002).

The selective COX-2 inhibitor nimesulide was used to ascertain if COX-2 inhibition reduces the size or number of ectopic endometrial lesions in the nude mouse model of endometriosis. Nimesulide inhibits COX-2 activity by 50% (IC50) at a 10-fold lower dose (1 μmol/l) than the IC50 for COX-1 (10 μmol/l) in human whole blood assays (FitzGerald et al., 2001). The dose used in this study was consistent with the maximum tolerated dose used in an in vivo rat model of endometriosis (Gupta et al., 1999). A pharmacokinetic profile for nimesulide in the nude mouse model was established to optimize the dosage interval. Two independent experiments were performed using the nude mouse model of endometriosis to determine the influence of nimesulide on ectopic endometrial lesion number and size.

Subjects and methods
Ethical approval for the conduct of this study was obtained from the Cambridge Local Research Ethics Committee. All animal procedures were fully licensed under the Animal (Scientific Procedures) Act 1986 by the Home Office of the UK.

Recruitment of volunteers
Two female recruits aged 28 and 35 years gave informed consent to participate in this study. Both women had regular menstrual cycles (25 and 28 days), were not taking medications and were undergoing a therapeutic operative procedure for American Fertility Society stage III–IV endometriosis. Eutopic endometrium was obtained by pipelle suction curettage (Endocell, Wallach Surgical Devices Inc, Orange, CT) while the volunteers were anaesthetized. The secretory phase endometrium was collected on day 16 and 27, respectively, of the participant’s menstrual cycle. The cycle phase of both biopsies was histologically confirmed using Noyes criteria (Noyes et al., 1950).

Endometrial biopsies were immediately placed in pre-warmed phenol-red free Dulbecco’s modified Eagle’s medium (DMEM)/F12 culture medium (Sigma-Aldrich Co. Ltd, Poole, UK).

Explant cultures
This method has been described previously (Bruner et al., 1997). Endometrial biopsies were washed with DMEM/F12 serum-free medium and sectioned into 2 mm3 fragments. Groups of 18 endometrial fragments were separated within tissue culture inserts. Each
Haematoxylin and eosin staining was performed on all lesions. Tissue was fixed and the rest were snap-frozen and stored at −80°C. Lesions were measured in three dimensions with callipers and a tissue volume estimate was calculated by multiplying the three dimensions. If a mouse had more than one lesion, a single lesion was selected, usually the largest, for histological examination. Lesions were measured in a non-blinded fashion with callipers using the mouse on mouse reagent kit.

Preparation of nude mice

Athymic female nude mice were purchased from Charles River, Manston, UK. The animals were housed in individually ventilated cages. The animal room was maintained at between 19 and 23°C with a 12 h light/12 h dark cycle, and mice were provided with a standard diet and water ad libitum. All procedures were performed in a laminar flow hood using sterile instruments. At 6 weeks of age, the mice were administered 0.10–0.15 ml of an anesthetic mixture of Hypnorm [fentanyl citrate (0.045 mg/ml) and fluanisone (1.43 mg/ml)] (Roche, Welwyn Garden City, UK) and midazolam (0.7 mg/ml; Jansen Pharmaceuticala, Beese, Belgium) s.c. before performing bilateral ovariotomies. At the same time, estradiol-releasing pellets (1.5 mg/60 day release) (Innovative Research, Sarasota, FL) were implanted s.c. in the right flank of the nude mice. There was a minimum 10 day post-operative recovery period.

In vivo injection of human endometrial tissue

Human endometrial tissue was washed with sterile serum-free DMEM/F12. Each nude mouse received a single injection of 18 endometrial fragments in 150 μl of serum-free DMEM/F12 after numbing the skin with ethylchloride (BDH Laboratory Supplies, Poole, UK). S.c. injections were performed at a ventral midline site using 1 ml syringes and 19 gauge needles.

Experiment 1

Sixteen mice were assigned randomly to either the nimesulide-treated or control group. One mouse in the nimesulide group had to be excluded from the study as an infection developed at the site of the estrogen pellet insertion although it had developed an ectopic endometrial lesion.

Nimesulide (Sigma-Aldrich) was dissolved in polyethylene glycol (PEG) 300 (Sigma-Aldrich) in 25% normal saline to provide a dose of 25 mg/kg/day in a total volume of 0.1 ml. PEG 300 in 25% normal saline was the pharmacological control. Nimesulide and vehicle was injected s.c. into excess skin at the scrub of each animal’s neck. The first dose was administered immediately after s.c. implantation of human endometrium. Subsequent doses were at 24 h intervals for 14 days.

Lesions were measured in a non-blinded fashion with callipers through the hairless nude mouse skin every 3 days. Fourteen days after endometrial tissue injection (day 14), mice were sacrificed between 0 and 20 h after the final dose of nimesulide or vehicle at specified time intervals. A pharmacokinetic profile for nimesulide was based on plasma samples taken at serial time intervals. Nimesulide plasma concentrations were determined by standard liquid chromatography and mass spectrometry techniques.

All mice developed at least one lesion. After dissection, all lesions were measured in three dimensions with calipers and a tissue volume estimate was calculated by multiplying the three dimensions. If a mouse had more than one lesion, the volume estimate of each lesion was added together to give an estimate of total tissue load per mouse. Half the lesions were formalin fixed and the rest were snap-frozen and stored at −80°C. Haematoxylin and eosin staining was performed on all lesions.

Experiment 2

Fourteen mice were assigned randomly to receive nimesulide (n = 7) or vehicle substance (n = 7). On the basis of the results from experiment 1, three parameters were changed. Nimesulide was given at the same 25 mg/kg dose but at 12 h intervals to optimize the drug delivery according to the pharmacokinetic estimates. Twice daily drug administration was only continued for 10 days because lesion size differences were apparent by this stage in the first experiment. Finally, to avoid investigator bias, the final lesion measurements in experiment 2 were performed in triplicate in a blinded manner.

All lesions were measured and volume estimates calculated in triplicate as above. The mean of the triplicate volume estimates for each lesion was used.

Statistical analysis

In the first experiment, the small data set precluded an assumption of normal distribution, therefore the size of lesions in the nimesulide and control groups was compared on the basis of rank using the non-parametric Mann–Whitney U test. The probability that a new lesion in the nimesulide group would be smaller than a new lesion in the control groups was calculated using the formula [probability = Mann–Whitney U statistic/(n of group 1) × (n of group 2)].

In the second experiment, the mean of triplicate measurement values was used to rank the data. The Mann–Whitney U test was again used to compare the two groups. A power calculation was performed to assess the number of lesions required in each group to see a statistically significantly (P = 0.05) difference in the means of 6 mm³ at a power of 80%.

Immunohistochemistry using formalin-fixed sections

Paraffin sections (5 μm) from nude mouse lesions were dewaxed and rehydrated followed by antigen retrieval with 0.1% trypsin (DIFCO Laboratories, Detroit, MI) in 0.1% CaCl₂ (BDH Laboratory Supplies). An avidin–biotin blocking kit (Vector Laboratories Inc., Peterborough, UK) was used to reduce background staining. COX-2 was detected using a monoclonal mouse antibody against human COX-2 (Zymed Laboratories Inc., San Francisco, CA). Mouse IgG (Sigma-Aldrich Co. Ltd) was used as the negative control. The mouse on mouse kit (Vector Laboratories Inc.) was used for non-specific background blocking and antibody detection.

After blocking with 20% goat serum (Sigma-Aldrich Co. Ltd) in 0.01% bovine serum albumin (BSA; Sigma-Aldrich Co. Ltd) and phosphate-buffered saline (PBS), mouse and human endothelial cells were identified using rabbit anti-von Willebrand factor (vWF) antibody (5.7 μg/ml) (Dako Corporation, Ely, UK). Non-specific rabbit immunoglobulins (5.7 μg/ml) (Dako Corporation) were used as a primary antibody control. Goat anti-rabbit immunoglobulin (7.5 μg/ml) (Vector Laboratories Inc.) and the Vectastain ABC system (Vector Laboratories Inc.) were used to detect the primary antibody.

A mouse monoclonal antibody against α-smooth muscle actin (α-SMA; Sigma-Aldrich Co. Ltd) (2.3 μg/ml) was used to identify myofibroblasts. An isotype-matched control (Sigma-Aldrich Co. Ltd) at the same concentration was used as a negative control. Non-specific background blocking and antibody detection was performed using the mouse on mouse reagent kit.

Murine F4/80-positive macrophages were identified in 5 μm acetone-fixed frozen sections. Non-specific antibody activity was blocked with 20% goat serum in 0.1% PBS/BSA. The sections were exposed to rat anti-mouse F4/80 (Serotec Inc., Raleigh, NC)
(10 μg/ml) or an isotype-matched negative control (Serotec Inc.) (10 μg/ml) in 2% goat serum/0.1% BSA/PBS overnight at 4°C. The sections were washed in 0.1% Tween (BDH Laboratory Supplies) in PBS, then the secondary biotinylated goat anti-rat antibody (Zymed Laboratories Inc.) (1:4000) in 0.5% Tween/PBS was applied for 1 h at 37°C. After washing in PBS, the Vectastain ABC kit was used to detect antibody staining. Diaminobenzidine (DAB; Sigma-Aldrich Co. Ltd) was used to visualize the antibody reactions. All sections were counterstained with Carazzi’s haematoxylin, then rehydrated and mounted in Depex (BDH Laboratory Supplies). Microscopy was performed with an Ultraphot microscope (Carl Zeiss, New York, NY).

Results

Anti-human COX-2 antibodies detected COX-2 enzyme predominantly in the glandular epithelium of the ectopic endometrial lesions. Light staining was identified in the stromal compartment. Anti-COX-2 antibody was defined immunohistochemically by positive staining of the glandular epithelium of human eutopic endometrium (Figure 1), whereas minimal COX-2 staining was detected in mouse uterine tissue.

Nimesulide treatment did not influence lesion formation in either experiment. If the data from both experiments are combined, 14 of 14 nimesulide-treated mice and 14 of 15 control group mice developed lesions (Figures 2 and 4). The only mouse without a lesion was not exposed to nimesulide. Four mice in the nimesulide group and one in the control group developed more than one lesion (2–4) that were generally smaller than single lesions.

The results of the first study showed no significant difference in volume estimate of tissue load between the nimesulide (total of 11 lesions from seven mice) and control groups (nine lesions from eight mice) ($P = 0.298$, Mann–Whitney U test) (Figure 2). The mean volume estimate of lesions per mouse from the control group mice was $15.9 \pm 6.5 \text{mm}^3$, whereas the mean volume estimate of lesions per mouse from the nimesulide group was $14.1 \pm 9.1 \text{mm}^3$. The probability that a new observation from the nimesulide group would be smaller than a new observation from the control group was 64%. It was possible that a true effect of nimesulide was not detected because the drug dosage was inappropriate.

Although the nimesulide pharmacokinetic profile measurements were only made in one mouse at each time point, the profile was consistent with those seen in other in vivo models and in humans. The $C_{\text{max}}$ was 8200 nM, the $T_{\text{max}}$ was 1 h and the $t_{1/2}$ was $\sim 3.5$ h (Figure 3). However, the free plasma concentration of nimesulide at 12 h was negligible. We therefore decided to perform a further experiment to determine if the trend towards smaller nimesulide lesions persisted when nimesulide was administered twice daily.

When nimesulide was administered at 12 h intervals in experiment 2, the ranked difference in the volume estimate of tissue load between the nimesulide-treated (eight lesions from seven mice) and control groups (six lesions from seven mice) was not significant ($P = 0.53$, Mann–Whitney U test) (Figure 4). The mean volume estimate of lesions per mouse in the control group was $24.1 \pm 17.9 \text{mm}^3$, whereas the mean volume estimate of lesions per mouse in the nimesulide group was $23.6 \pm 9.7 \text{mm}^3$. The probability that a new observation from the nimesulide group would be smaller than a lesion from the control group in this experiment was only 30%. A sample size calculation indicated that 138 mice in each group would be required to detect with 80% power a significant difference ($P = 0.05$) in volume of $6 \text{mm}^3$ between the control and treated group means.

Nimesulide-treated and untreated lesions could not be differentiated histologically. All lesions contained glands lined with pseudostratified epithelium and central cystic spaces. Granulation tissue was identified peripherally, and small foci of necrosis and inflammation were observed centrally in implants from both the nimesulide and control groups (Figure 5A and B).

Myofibroblasts (α-SMA) (Figure 5C and D) and macrophages (F4/80) (Figure 5E and F) were identified in all lesions. No gross differences in macrophage and myofibroblast infiltration were seen between treatment groups.

An antibody to vWF was used to identify the vasculature supplying both nimesulide and control lesions. All lesions had a clearly identifiable vascular supply and there were no striking differences observed between lesions from nimesulide-treated and control mice (Figure 5G and H).

Discussion

In this study, the COX-2 inhibitor nimesulide did not inhibit the formation or growth of ectopic human endometrial lesions in nude mice. These results suggest that COX-2 inhibitors are unlikely to influence the establishment or early progression of endometriosis in an estrogenic environment.

We explored several methodological reasons that could account for the negative results seen in this study. The human COX-2 enzyme was detected immunohistochemically in the glandular epithelium and to a lesser extent in the stroma of nude mouse lesions. This indicates that, like endometriosis, COX-2 is present in the relevant cells in this model system. Therefore, the nude mouse is suitable for investigation of the effect of nimesulide on ectopic endometrial growth.

Additionally, the dose of nimesulide used in the nude mouse model of endometriosis was sufficient to induce a pharmacological effect in rodents. When 25 mg/kg of nimesulide was administered s.c. to rats, carrageenin-induced paw inflammation was effectively reduced for the entire duration of the study (6 h) (Gupta et al., 1999). The serum levels of nimesulide that induced this anti-inflammatory effect were comparable with those seen in nude mice given the same 25 mg/kg dose of nimesulide. A much lower dose of nimesulide (5 mg/kg) was administered, 1 h prior to carrageenin injection in a rat pleurisy model of inflammation. A reduction in exudate volume and percentage of inflammatory cells was demonstrated in the rats that received nimesulide. PGE$_2$ levels were also reduced in this treatment group ($P < 0.001$), indicating that COX-2 inhibition was the mechanism of action of nimesulide’s in vivo effect (Gilroy et al., 1998). Several other in vivo studies have also demonstrated
a reduction in arachidonic acid metabolites after administration of nimesulide at lower doses than used in this study (for a review see Magni, 1993). Taken together, these studies indicate that the serum nimesulide levels that resulted from 12 hourly s.c. administration of 25 mg/kg of nimesulide were sufficient to inhibit COX-2 selectively in the nude mouse model of endometriosis.

The pharmacokinetic profile indicated that the maximum plasma concentration value \( C_{\text{max}} \) (18 mg/l) was achieved \( \sim 1 \text{ h} \) \( T_{\text{max}} \) after administration of 25 mg/kg/day nimesulide to nude mice. Healthy human volunteers given an oral dose of nimesulide (50–200 mg) tablets had pharmacokinetic profiles with a generally lower \( C_{\text{max}} \) (1.9–9.85 mg/l) and a slightly longer \( T_{\text{max}} \) (1.67–3.17 h) (Davis and Brogden, 1994). This difference is likely to reflect the s.c. administration and high dose chosen to ensure adequate nimesulide exposure in the nude mouse model. In our study, the \( t_{1/2} \) was 2–3 h, which is consistent with human profiles (1.9–4.5 h) (Davis and Brogden, 1994). Although the pharmacokinetic profile was only based on one plasma estimate at each time point, the results indicate that the dose of nimesulide administered to the nude mice was adequate to inhibit the COX-2 enzyme in vivo.

The whole blood assay is an in vitro measure of COX-2 selectivity. Thromboxane B2 production during blood coagulation reflects platelet COX-1 activity, whereas PGE$_2$...
production after lipopolysaccharide stimulation of whole blood is an index of macrophage COX-2 function (Brideau et al., 1996). The effect of nimesulide on these two parameters has been measured and the IC_{50} for COX-2 is 1 \mu mol/l, whereas the IC_{50} for COX-1 is 10 \mu mol/l in human whole blood assays (mean of four values from different studies) (FitzGerald and Patrono, 2001). The free plasma concentration of nimesulide in serum from nude mice is likely to have selectively inhibited COX-2 for almost the entire 12 h dosing interval. However, it is possible that COX-2 activity recovered at trough plasma concentrations and COX-1 inhibition occurred at peak plasma concentrations, just before and after nimesulide administration. Other highly selective COX-2 inhibitors may be more effective than nimesulide in reducing ectopic endometrial lesions in nude mice. However, the results of this study suggest that COX-2 inhibitors with a COX-2 selectivity profile like that of nimesulide (FitzGerald and Patrono, 2001) are unlikely to inhibit endometriotic lesion development.

Lesion formation and development occurred in the nude mouse despite the administration of a COX-2 inhibitor at a therapeutic dose. Researchers using a mouse ear inflammatory assay have shown that COX-2 knockout mice maintain an inflammatory response to arachidonic acid (Morham et al., 1995). One explanation is that prostaglandins produced by homeostatic COX-1 activity contribute to inflammation. In our study, COX-1 activity may have been sufficient to permit lesion development in the nude mouse model.

It is possible that prostaglandin production by COX-1-mediated pathways in macrophages and fibroblasts may have masked nimesulide’s therapeutic influence. In macrophages and fibroblasts that constitutively express COX-1, in vitro induction of COX-2 caused only a nominal 1.5- to 2-fold increase in PGH_{2} biosynthetic activity (Lee et al., 1992; Evett et al., 1993). Elevated levels of COX-1 mRNA have been detected in peritoneal macrophages harvested from women with severe endometriosis when compared with normal controls (Wu et al., 2002). We identified macrophages and myofibroblasts in both treated and untreated nude mouse lesions. Prostaglandin production in these cells may have been minimally affected by COX-2 inhibition due to high constitutive COX-1 activity.
Vascular disruption has been shown to influence lesion number and size in the nude mouse model of endometriosis (Hull et al., 2003). Nimesulide treatment did not alter these parameters, suggesting that products of COX-2 do not alter vessel formation. It is possible that the outcome measure of lesion size would not detect subtle changes in vessel development after nimesulide exposure. However, immunohistochemical identification of endothelial cells indicated that there were no differences in vascularity between treatment groups. It is therefore unlikely that COX-2 significantly influences ectopic endometrial vascular supply in the nude mouse model.

Any influence of nimesulide on cellular proliferation and apoptosis may not have been apparent as treatment was only for a 10 day period. Murine models evaluating nimesulide’s effect on fast-growing lung tumour cell growth were able to determine growth inhibition after 7 days (Sheng et al., 1997; Williams et al., 2001). In the nude model of endometriosis, a longer time frame may be required to detect inhibition of the slower growth of non-invasive endometrial cells. As nude

Figure 5. Minimal histological and immunohistochemical differences between control and nimesulide-treated nude mouse lesions. Haematoxillin and eosin staining of a control (A) and treated (B) lesion. F4/80 antigen-positive murine macrophages in a control (C) and treated (D) lesion. Untreated (E) and nimesulide-exposed (F) nude mouse lesions containing α-smooth muscle actin-positive myofibroblasts. Blood vessels in control (G) and treated (H) lesions stained with von Willebrand factor (scale bar, A, B, E and F, 150 μm; C, D, G and H, 100 μm).
mice recover T-cell-mediated rejection of foreign tissue after 12 weeks of age, it is not feasible to assess such possible long-term effects in this model of endometriosis.

Eutopic endometrium from women with endometriosis in the secretory phase of the cycle was used to model the endometriotic disease process, as theorized by Sampson, as closely as possible in nude mice. Some researchers have argued that progesterone exposure in the secretory phase of the cycle inhibits lesion formation in the nude mouse (Bruner et al., 1997). Others have found that ectopic endometrial lesion development was independent of cycle phase in SCID mice (Aoki et al., 1994). In this study, the large numbers of lesions that developed in nude mice after injection of secretory phase endometrium (day 16 and 23) support the assertion that endometrial cycle phase does not influence nude mouse lesion development.

The development of endometriosis is dependent upon an estrogenic environment (Bulun et al., 2002), and 17B-aromatase (Noble et al., 1996) is elevated in endometriotic lesions. A possible positive feedback exists between aromatase activity and prostaglandins because PGE2 upregulates aromatase activity and estradiol stimulates COX-2 and PGE2 production (Noble et al., 1997; Bulun et al., 2000). COX-2 inhibitors are likely to reduce the COX-2-induced aromatase activity that has been identified in ecticp endometrial lesions. At a local level, this inhibition may occur in human ectopic endometrium. In nude mice, however, the effect of this inhibition may be obscured by iatrogenically administered estrogen.

The results of this study suggest that treatment with COX-2 inhibitors such as nimesulide is unlikely to reduce the size or number of endometriotic lesions in women, if administered in a constant estrogenic environment. In a study of histological samples obtained from 130 women (Fagotti et al., 2004), COX-2 immunostaining was more common in ovarian endometrioma (78.5%) than in peritoneal implants (11%) and rectovaginal nodules (13.3%). It may be that COX-2 inhibitors prove to influence ovarian endometriosis more than disease at other sites. Intrapertoneal lesions may also have a greater response to COX-2 inhibitors than nude mouse lesions that are supplied by peritoneal blood vessels (Hull et al., 2003) but lie outside the peritoneal cavity.

COX-2 inhibitors may also prove beneficial in the treatment of endometriosis-related pain unrelated to disease progression. Symptomatic improvement has been described in women with dysmenorrhea (Pulkkinen, 1993) and women with endometriosis-related pelvic pain and dyspareunia (Corbellis et al., 2004) after treatment with COX-2 selective agents. Although COX-2 inhibitors have been shown to reduce gastrointestinal side effects significantly when used to treat rheumatoid arthritis, clinical trials have shown little improvement in disease progression when compared with non-selective COX inhibitors (Deeks et al., 2002).

In the nude mouse model of endometriosis, the COX-2 inhibitor nimesulide did not reduce lesion size or number, nor did it influence vascular development or macrophage and myofibroblast infiltration. Although COX-2 inhibitors may provide pain relief with fewer side effects than non-selective NSAIDs, the results of our study suggest that in an estrogenic environment, COX-2 inhibitors are unlikely to reduce ectopic endometrial tissue load in women.

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