Induction of endometriosis in the marmoset monkey (Callithrix jacchus)

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Endometriosis is an estrogen-dependent gynaecological disease associated with pain and infertility, which occurs in humans and menstruating primates. In this study, the marmoset monkey (Callithrix jacchus), which is a non-menstruating primate with high circulating estrogen levels, was used to test firstly the hypothesis that endometriosis is based on uterine shedding into the peritoneal cavity, secondly to study the pathogenesis of endometriosis due to its estrogenic situation. Female marmoset monkeys (n = 29) were exposed to two different experimental procedures (non-invasive versus invasive) for intrapelvic placement of endometrial cells by uterine flushing over an experimental period of 2–3 years. First endometriotic foci were detected by colour Doppler ultrasound at the bladder, the uterus and the ovaries at the earliest after 4 months of either treatments. However, invasive induction was more effective in terms of the time-course of induction and the number of resulting endometriotic foci. The analysis of the endometriotic foci by histology, immunohistochemistry and molecular techniques allowed a division into two distinct groups: an initial developing stage occurred, which under further treatment led to the second stage of established endometriosis. Both procedures showed a treatment-dependent increase of vascular supply to the endometriotic foci over the experimental period. The invasive method induced the final established stage of endometriosis more rapidly, with the expression of steroid receptors, aromatase, 17βHSD1 and CD10. Altogether, 72% of the treated marmoset monkeys developed endometriosis under our endometrial reflux protocols. Our data support the theory that endometriosis can be induced artificially in a non-menstruating primate (C. jacchus) by endometrial shedding into the peritoneal cavity. Because the marmoset is a primate with very high peripheral estrogen levels, this offers an interesting model for studying the pathogenesis of this estrogen-dependent disease, as well as for therapeutic impacts on enzymes involved in steroid metabolism.

Key words: CD10/endometriosis/enzymes of steroid metabolism/marmoset monkey/reflux of endometrial cells

Introduction

Endometriosis is not only a painful chronic disease, but also one of the most common causes of infertility, affecting more than 10% of premenopausal women (Giudice and Kao, 2004). Endometriosis occurs not only in humans, but also in those non-human primates with menstrual cycles, and is defined as a condition in which tissue histology similar to endometrium is found outside the uterine cavity. The exact pathophysiology of this disease remains unclear. Different theories are being discussed, though the most favoured hypothesis so far is the reflux of menstrual endometrium through the fallopian tubes into the pelvic cavity and its subsequent implantation at ectopic sites (Sampson, 1927). In order to study this disease, different animal models, involving primates (Sillé et al., 1996; D’Hooghe and Debrock, 2002; Fazleabas, 2005) or rodents (Nisolle et al., 2000; Defrère et al., 2005) have been used, each with special benefits, but also limitations. The marmoset monkey, represents a new endometriosis model, with features of both primates and rodents owing to their taxonomy and high peripheral estrogen levels.

Endometriosis is an estrogen-dependent disease, which does not occur before puberty and is rare after the menopause. Estrogen synthesis within endometriotic foci is increased due to aberrantly high local levels of aromatase (ARO) and 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1), accompanied by reduced expression of the estradiol activating enzyme 17βHSD2 (Zeitoun et al., 1998). These higher local estrogen concentrations induce in turn the production of prostaglandin E2, which then stimulates further ARO activity (Noble et al., 1997). Consequently, this vicious circle leads to additional estrogen production. Currently therapeutic approaches are still inadequate, mainly because the aetiology and pathogenesis of endometriosis and its estrogenic regulation are still unclear. The marmoset monkey is a primate with very high peripheral estrogen levels compared with human and other primate species, and naturally shows no signs of endometriosis. It therefore offers an interesting primate model to study the aetiology and pathogenesis of endometriosis, especially its local estrogenic regulation. Likewise the hypothesis of retrograde endometrial cell reflux can be readily tested in this non-menstruating primate. A further advantage of the marmoset monkey for experimental work is its small size, its being a non-seasonal breeder with short generation intervals, and other close endocrine similarities to the human situation (Hearn et al., 1978; Einspanier and Gore, 2005).
The aims of this study were (i) to induce endometriosis according to the theory of endometrial reflux and (ii) to characterize the developing endometriotic foci using different techniques in order to gain a better understanding of the pathogenesis of endometriosis.

Materials and methods

Animals

Female marmoset monkeys (n = 29, aged between 2 and 10 years) with well-documented family history were housed together with male partners under previously described conditions (Einspanier and Hodges, 1994). All females were similar in weight (380–470 g) at the beginning of the treatment. The nutrition of the monkeys was based on marmoset pellets, and seasonal fruits and vegetables.

For ovarian cycle analysis, females were bled (100μl/animal) on a regular basis for progestrone detection (Einspanier and Hodges, 1994) throughout the treatment period. A luteolytic dosage of prostaglandin F2α (Pf) was administered between days 10 and 14 of the luteal phase to prevent pregnancy and allow new follicle development. Ovarian and uterine cycle activities as well as blood flow in these tissues were analysed by colour Doppler analysis (GE Medical Systems, Sollingen, Germany) using a 10–15 MHz probe. Before the induction of endometriosis, ultrasound and colour Doppler scanning were used to ascertain the reproductive health of the animals. Anatomical integrity of the reproductive organs (determined by two-dimensional measurement of ovaries, uterus and cervix) as well as regular cyclic activity (defined by the identification of ovarian follicles and corpora lutea respectively) were recorded. Characteristic blood flow patterns of the A. uterina, A. arcuata and A. ovarica as well as stromal blood flow in corpora lutea were documented. As an important location for the development of endometriosis in marmosets, the urinary bladder was recorded.

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The study was approved by the appropriate local animal ethics committees (Braunschweig and Berlin, Germany).

Experimental induction of endometriosis by endometrial reflux

Two methods were applied during the late secretory phase to mimic endometrial reflux: I. non-invasive (n = 14 primates) and II. invasive induction (n = 15 primates). The non-invasive method was carried out under general anaesthesia (ketamine/xylazin injection or isoflurane inhalation) using a modified flushing set placed into the cervix or uterus, as described previously (Thomson et al., 1994). Sterile medium (M199, Invitrogen GmbH, Karlsruhe, Germany) in a total volume of 2 ml was flushed through the uterus into the abdominal cavity via the oviducts. Fluid accumulation in the abdominal cavity was monitored by ultrasound examination. This non-invasive induction was repeated every second cycle over a period of at least 8 months. Eight of the marmoset monkeys used for the non-invasive induction procedure were subsequently treated by the invasive induction procedure, because they had failed to show any signs of endometriosis after 8 months of treatment using the non-invasive method.

Invasive induction was performed by laparotomy under isoflurane anaesthesia (ketamine/xylazin injection or isoflurane inhalation) using a modified flushing procedure to mimic the normal abortion procedure at the beginning of the treatment (Einspanier et al., 1994). After the induction of endometriosis, animals were scanned monthly by colour Doppler in the abdominal region and possible changes in blood flow patterns or anatomy were recorded.

Histology and Immunohistochemistry

Collected tissue was kept in 4% buffered paraformaldehyde for 3 h before being embedded in paraffin. Sections of 5 μm were prepared and deparaffinized for histological and immunohistochemical analysis. Several sections from each focus were stained with methylene blue as well as haematoxylin and eosin. After staining, the flushed uterine cells from two monkeys were recovered from the abdomen, then placed on a glass slide, air-dried and briefly fixed in alcohol, before staining with methylene blue for histological analysis. Sections were processed for immunohistochemistry using the DAKO Envision protocol.

Molecular biology

For RNA extraction deep-frozen tissue samples were homogenized in RLT lysis buffer (Qiagen, Hilden, Germany) containing 1% [v/v] β-Mercaptoethanol using a rotor-stator homogenizer (IKA GmbH, Staufen, Germany). Cells stored in RNAlater (Qiagen) were diluted with 1 volume PBS (PAZ Laboratory GmbH, Colbe, Germany) to decrease the density of RNAlater, enabling pellets to be formed. These cell pellets were homogenized in RLT lysis buffer containing 1% [v/v] β-mercaptoethanol using a 20-gauge syringe. Tissue or cell lysates were then extracted using the RNeasy Mini Kit following the manufacturer’s protocol. Because of the small sample sizes only 20 μl instead of 30–50 μl, RNase-free water was used for eluting the RNA from the RNeasy silica-gel membrane. Quality of Callithrix RNA was determined after electrophoresis on 1% (w/v) formaldehyde-containing agarose gel followed by ethidium bromide staining. A volume of up to 11 μl total RNA eluate in a 20 μl reaction volume was reverse transcribed to obtain cDNA using SuperScript II reverse transcriptase (Invitrogen) and oligo(dT)12–18 primers (Hermann GbR, Freiburg, Germany) according to the manufacturer’s protocol. Before using the cDNA as a template for PCR, the RNA was removed by a Ribonuclease H digestion (Promega GmbH, Mannheim, Germany). Marmoset-specific PCR fragments encoding ER, PR, ARO, 17βHSD1, 2, 7 and RPS26 were amplified using

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Oligonucleotide primers for marmoset mRNA transcript of ARO, ER, PR, 17β-HSD1, 17β-HSD2, 17β-HSD7, Aromatase and RPS26

<table>
<thead>
<tr>
<th>Transcript</th>
<th>GenBank</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatase (ARO)</td>
<td>AY034779</td>
<td>5′-ACA ACT CGG CCC CTC TTT AT-3′</td>
<td>5′-AGG AGC TGC AAT CAG CAT TT-3′</td>
<td>498</td>
</tr>
<tr>
<td>Estrogen receptor α (ER)</td>
<td>X03635 (human)</td>
<td>5′-ATG ACC ATG ACC CTC AC-3′</td>
<td>5′-CGG AGA CAC GCT GTT GAG T-3′</td>
<td>315</td>
</tr>
<tr>
<td>Progesterone receptor (PR)</td>
<td>Z86038</td>
<td>5′-GTA TTC CAA ATG AAA GCC AAG C-3′</td>
<td>5′-AAC CAA TTG CCT TGA TGA GC-3′</td>
<td>591</td>
</tr>
<tr>
<td>17β-Hydroxysteroid dehydrogenase type 1 (17β-HSD1)</td>
<td>AF272013</td>
<td>5′-GCC CTG CAC TTG GCC GTA CG-3′</td>
<td>5′-GGC CTG CAG CAT CCG CAC AG-3′</td>
<td>330</td>
</tr>
<tr>
<td>17β-Hydroxysteroid dehydrogenase type 2 (17β-HSD2)</td>
<td>AF325910 (human)</td>
<td>5′-CTG TGG ATC AGA AGG CAG TC-3′</td>
<td>5′-GAT GAG CCA TAA GAT GCC AG-3′</td>
<td>469</td>
</tr>
<tr>
<td>17β-Hydroxysteroid dehydrogenase type 7 (17β-HSD7)</td>
<td>AF263468</td>
<td>5′-GGA ACT GGA GCC TCT CCT CT-3′</td>
<td>5′-TGG TCA ATG CTG TAC CTG GA-3′</td>
<td>233</td>
</tr>
<tr>
<td>Human ribosomal protein S26 (RPS26)</td>
<td>U41448</td>
<td>5′-AAT GGT CGT GCC AAA AAG GGC-3′</td>
<td>5′-TTA CAT GGG CTG TGG TGG GGG-3′</td>
<td>327</td>
</tr>
</tbody>
</table>

InViTaQ DNA-polymerase, while Pfu-polymerase (Fermentas GmbH, St. Leon-Rot, Germany) was used for 17β-HSD1, see Table I. The RPS26 is used as a housekeeping gene control and the PCR was performed for 35 cycles. All other PCR reactions were performed for 35 cycles at the optimized annealing temperature of each primer pair. PCR product each of 10 μl mixed with 2 μl of 6x Loading Dye Solution (Fermentas, St.Leon, Germany) was run on 1.5% agarose gels containing 0.3 μg/ml ethidium bromide. For sizing of PCR products, the GeneRuler™ 100 bp DNA Ladder Plus (Fermentas) was used. All reactions were performed three times for each RNA-preparation.

Data Analysis

Immunoreactive progesterone concentrations were calculated after logit-log transformations of their respective standard curves. Animal data were analysed for significance difference by Students t-test.

For evaluation of ultrasound data, endometriotic foci in their different stages of development are designated EM (endometriotic foci as seen only in B-mode), BF (focal increase in blood flow), EM+BF (endometriotic foci as seen in B-Mode associated with focal increase in blood flow). Increase in blood flow is measured by its velocity and documented as 4.3 m/s or 5.7 m/s according to the specific features of the ultrasound machine.

Two distinct phases of endometriosis were defined by histological analysis, which were classified by an independent observer using similar staging as described by Sillem et al. (1996). Staging according to the American Fertility Society for women was not suitable.

Results

In this study, 29 female marmoset monkeys aged more than 2 years with different breeding histories were exposed to two different experimental procedures to mimic retrograde endometrial reflux over an experimental period of 2–3 years, see Table II. Based on the duration and effectiveness of induction, two groups of endometriosis were observed based on histological analysis: (i) developing and (ii) established endometriosis, whereby the established endometriosis appears to represent a progression from the developing phase. In total, from the 14 primates with non-invasive induction, 6 showed endometriosis as confirmed by colour Doppler analysis, histology and immunohistochemistry. The blood flow and the number of these endometriotic foci were not significantly increased throughout the further treatment period (Table II); this could be due to tissue sample collection throughout each laparotomy. Eight animals did not show any signs of endometriosis during the treatment period of 2 years, so that a subsequent invasive induction was performed on these monkeys.

With invasive induction of endometriosis, first signs of increased blood flow at bladder and uterus was documented after 4 months of treatment in some animals (n = 4). This was confirmed by histology as a developing stage of endometriosis (Figure 1). After 7 months of treatment, endometriosis was described in 13 of the 15 primates, using colour Doppler, histology, immunohistochemistry and RT–PCR as diagnostic tools. The number of endometriotic foci increased and established stages of endometriosis had developed in almost all cases (Figure 2). Although after 18 months of treatment the overall number of foci did not significantly increase as compared with 7 months of

Table I. Oligonucleotide primers for marmoset mRNA transcript of ARO, ER, PR, 17β-HSD1, 2 & 7 and RPS26

Table II. General animal data on both treatments throughout an experimental period of 18 months

<table>
<thead>
<tr>
<th></th>
<th>Non-invasive method</th>
<th>Invasive method</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>14</td>
<td>15 (8)*</td>
</tr>
<tr>
<td>Age (years)</td>
<td>5.6 ± 1.2</td>
<td>6.3 ± 1.4</td>
</tr>
<tr>
<td>Number of animals with different breeding experience</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Number of animals with increased blood flow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>After 4 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Uterus</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Ovaries</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>After 7 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Uterus</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Ovaries</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>After 18 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>6</td>
<td>13 (2)*</td>
</tr>
<tr>
<td>Uterus</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Ovaries</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Total number of endometriotic foci visible by laparotomy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>After 4 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>n.s.</td>
<td>4</td>
</tr>
<tr>
<td>Uterus</td>
<td>n.s.</td>
<td>2</td>
</tr>
<tr>
<td>Ovaries</td>
<td>n.s.</td>
<td>–</td>
</tr>
<tr>
<td>After 7 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>11</td>
<td>25</td>
</tr>
<tr>
<td>Uterus</td>
<td>9</td>
<td>19</td>
</tr>
<tr>
<td>Ovaries</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>After 18 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>11</td>
<td>28 (4)*</td>
</tr>
<tr>
<td>Uterus</td>
<td>9</td>
<td>19 (3)*</td>
</tr>
<tr>
<td>Ovaries</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Number of animals without signs of endometriotic foci visible by laparotomy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>After 24 months</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

n.s.: no surgery performed.
*Animals treated with the invasive method, after having been treated with non-invasive method.
treatment, this could be due to the repeated sample collection by laparotomy. Two animals showed clinical symptoms in terms of irregular ovarian cycles after 7–10 months of treatment (Figure 3a).

As mentioned above, eight monkeys subjected to non-invasive induction failed to show any signs of endometriosis, and were subsequently treated by the invasive method. Here, the first signs of endometriosis became visible in two of the eight animals after 4–6 months of invasive treatment (Table II, animals listed in brackets under the invasive method).

Altogether, 21 of the 29 marmoset monkeys developed endometriosis of two distinct stages: developing and established endometriosis, whereas, eight monkeys have so far not shown any signs of endometriosis under both treatments after an experimental period of 24 months. Inspection by laparotomy demonstrated that all lesions, independent of treatment, were found at either bladder, uterus or ovary (Table II). The colour from these endometriotic foci ranged from reddish to opaque; however, there was no correlation with either treatment type or duration. Increased peritoneal fluid was observed in animals with histological established endometriosis. It was not observed in animals before induction.

The application of the invasive technique seemed to be more effective in terms of both grade of resulting endometriosis and time. When using the non-invasive technique mainly only the developing stage of endometriosis was achieved, as confirmed by histology. In contrast, when using the invasive technique mainly the established stage of endometriosis developed during the experimental period.

One female with proven endometriotic foci \((n = 5)\) was ovariectomized to assess the role of ovarian estrogen. This animal showed declining blood flow of the endometriotic foci after 1 month. Laparotomy, carried out 3 months later, demonstrated a decline in the number of endometriotic foci \((n = 2)\) and their size.

**Endocrine parameters of ovarian function**

The cycle lengths of almost all induced primates were physiologically normal throughout the experimental period, as represented by animal W7 (Figure 3a). The \(\text{PGF}_{2\alpha}\) application at mid-luteal phase was used to test ovarian function, by inducing luteolysis to prevent pregnancy, and to cause the onset of new follicle development. Progesterone levels were in the same range as reported in the literature (Hearn et al., 1978). Therefore, in most animals, ovarian function does not appear to be impaired by the treatment. However, two animals with many established endometriotic foci showed irregular cycles (Figure 3a) and the occurrence of ovarian cysts at around 1.5 years after induction (Figure 3b). PGF \(_{2\alpha}\) application for luteal regression was ineffective in these animals, indicating that normal ovarian function was disturbed.

**Colour Doppler analysis and in situ localization of endometriotic foci**

Structural changes visible at the ovaries, the uterus and the urinary bladder were analysed using colour Doppler ultrasonography. With the exception of two animals, the females showed normal ovarian activity in terms of growing follicles and formation of corpora lutea as confirmed by ultrasound examination. Colour Doppler ultrasound was also used to analyse increased angiogenesis, which is reported to be common around endometriotic foci (Nothnick, 2001). First signs of increased blood flow were predominantly detected around the bladder, the ovaries and/or the uterus after an average of 4–7 months of treatment. Further treatments caused an increase in blood flow velocity.
in the same locations as previously detected, namely at the bladder (Figure 4a), the ovaries and the uterus (Figure 4b). However, the pulsatility index (PI), which has proved a useful way of expressing blood flow impedance in women, was not applicable for this approach, because of the small size of the organs. After several flushings and colour Doppler analysis of areas with increased blood flow, these areas were then collected during laparotomy (Figure 5) and examined by histology, immunohistochemistry as well as RT–PCR (Figures 1 and 2; Figures 6 and 7). Usually endometriotic foci were located superficially at the bladder (Figure 5a), the uterus (Figure 5b) and the ovaries, whereas some lesions showed penetration of the tunica muscularis. No lesions have so far been detected at the intestinal tract or peritoneum of the abdominal wall. In established endometriosis, endometriotic foci could be seen as small ‘cysts’ which were filled with fluid (Figure 5a) and which were associated with increased blood flow. Eight monkeys so far have not shown any signs of endometriotic foci after using both methods of induction.

**Histology and immunohistochemistry**

Both flushing methods induced endometriosis, however, resulting in a different grade and time course. Two groups were distinguished based on histology: developing endometriosis (no glands, but surrounding stroma) and established endometriosis (glands and surrounding stroma). These groups were also confirmed by immunohistochemical markers. Developing endometriosis was found in all animals as a first stage. Further treatment and time led to the more advanced phenotype of established endometriosis.

The histological analysis of the tissue biopsies confirmed the presence of endometrial glands and stroma in the collected samples induced by both methods of flushing uterine cells out of the uterine cavity. However, stage-dependent differences were apparent in the examined samples, based on the induction method applied and the duration of induction, or the susceptibility of the individual animals to treatment. Different stages ranging from developing (Figure 1) to established stage (Figure 2) of endometriosis could be detected by histology. Similar to the data published by Sillem et al. (1996) for the cynomolgus monkey, the suggestive stage represented mainly stromal cells and macrophages, whereas the proven stages showed glands with stromal cells. As an example for the development of endometriotic foci, the marmoset monkey W19 had been followed sequentially (Figures 1 and 2). Few endometriotic foci were detected after the first invasive induction in this monkey by laparotomy. The histological picture confirmed developing endometriosis (Figure 1a) represented by blood cells, macrophages and stromal cells after 5 months treatment. Using CD10, a marker for endometriosis (Groisman and Meir, 2003; Oliva, 2004), a strong positive signal could be observed in this early stage of endometriosis (=established; Figure 1b). In contrast, only very weak expression of steroid receptors (Figure 1c and e), ARO or 17βHSD1 (Figure 1d and f) could be detected by immunohistochemistry. However, further treatments induced a proven endometriosis in the same marmoset monkey W19 after 15 months treatment, as shown in Figure 2. Some of the endometriotic foci, which were identified as ‘cysts’ in the colour Doppler examination, were of similar histological appearance to proven endometriosis. CD10 (Figure 2b), steroid receptors (Figure 2c and e) as well as steroidoenzymes like ARO (Figure 2f) and 17βHSD1 (Figure 2d) were expressed. Most striking was the up-regulation of 17βHSD1 and ARO in established endometriotic foci, whereas analysis of early endometriotic foci resulted in weak expression of both steroid receptors, 17βHSD1 and ARO.

**RT–PCR analysis**

The different stages of endometriosis, which have been described by histology and immunohistochemistry, were also confirmed by RT–PCR analysis, as shown in Figure 6. Endometrial cells, as well as endometriotic cells, were collected throughout invasive induction, because little is known about qualitative alterations in the uterine endometrium from patients with endometriosis. Estrogen und progesterone receptor transcripts were detectable in all collected endometrial samples and in all stages of endometriotic foci. ARO gene expression, which is up-regulated in endometriosis patients, was only present in endometriotic foci of the primates W4 and W7, which were flushed five (W4) or three times (W7). The other samples showed no ARO expression, including the eutopic endometrial sample from W4, collected at the same time as the endometriotic foci.

Transcripts for 17βHSD1, the enzyme necessary for converting estrone to estradiol as the most active estrogen, could also be detected in animal W4. This was the animal with the most advanced endometriosis based on histology and molecular biology, and additionally indicated irregular cycles and ovarian dysfunction. Weaker signals were present for endometrial cells from animals W5 and W16, which were collected throughout the second invasive flushing procedure. In contrast, transcripts of the estradiol inactivating enzyme 17βHSD2 could only be faintly detected in the sample with uterine cells from animal W16. Endometrial cells from W20 were collected throughout
the first flushing procedure, thus reflecting the situation before induction of endometriosis.

In Figure 7, the chronological development of endometrial foci from two animals (W2, W7) was examined. After two-times invasive induction of animal W3, part of the endometrial foci (W3a) was collected and showed weak expression for steroid receptors and 17βHSD7. After a further four months and after the third invasive induction, expression of ER and 17βHSD7 was present in the same endometrial foci (W3b). 17βHSD7 is also involved in estradiol production and is an enzyme related to cancer and endometriosis, similar as 17βHSD1 (Breitling et al., 2001). The histology of W3 could be described as developing endometriosis.

Endometrial foci from animal W7 were collected throughout two different time points; the first foci collected showed expression of steroid receptors and 17βHSD7 in the PCR and in the histology described as developing endometriosis. Later sampling of the same endometrial foci (W7d) confirmed now by histology an established endometriosis with strong expression of ARO, 17βHSD1, 17βHSD7 and ER. Two other endometrial foci (W7b, W7c), which were collected at the same time point, were in the histological stage of developing endometriosis with expression of ER (W7b, W7c) and 17βHSD7 (W7c).

Discussion

Using the non-menstruating primate (Callithrix jacchus) we were able to induce endometriosis by invasive as well as by non-invasive uterine flushing into the abdomen cavity, based on the hypothesis of endometrial reflux (Sampson theory). Two distinct stages of endometriosis could be described on the basis of histology, associated with the duration of induction, though also with individual animal variation. The
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induced endometriotic foci were mainly located at the bladder, the uterus and the ovary, and could be confirmed by histology, immunohistochemistry and RT–PCR. The establishment of endometriosis could also be followed using the non-invasive technique of colour Doppler ultrasound examination. In general, first signs of developing endometriotic foci were observed at the earliest after 4 months of treatment, whereas proven endometriosis was observed only after about 18 months of treatment in 72% (21 of a total number of 29) of the marmoset monkeys.

As opposed to the baboon (D’Hooghe et al., 1996) and to the rhesus monkey (Hadfield et al., 1997), spontaneous endometriosis has never previously been described for the marmoset monkey. Abdominal laparotomy of at least 250 female marmoset monkeys (2–12 years of age) with and without breeding experience at the German Primate Center Göttingen (1990–2002) confirmed that no signs of endometriosis could be detected in the marmoset monkey (A. Einspanier, personal communication). Also, no viable endometrial cells have ever been reported in the peritoneal cavity during the cycle of the marmoset monkey, as have been described for the human situation during the non menstrual phase (proliferative) of the cycle (Bartosik et al., 1986). Thus, the pathogenesis of endometriosis through retrograde endometrial reflux is strongly supported by our present results obtained by manipulation of a non-menstruating primate. These further confirm earlier results obtained, for example, in baboons by ligation of the cervix (D’Hooghe, 1997), as well as in humans with outlet obstruction to menstrual flow (Nunley and Kitchin, 1980).

Although retrograde menstruation is reported in 70–90% of all women (Blumenkrantz et al., 1981) and 62% of baboons (D’Hooghe et al., 1996; D’Hooghe and Debrock, 2002), endometriosis is diagnosed in only 10% of the former and 25% of the latter. Interestingly, in our study 72% of the marmoset monkeys developed endometriosis under our induction protocol within a time frame of 2–3 years. Endometriosis was induced in this study in the late stage of the cycle, when the endometrium is in the secretory phase and of edema-like structure. However, we cannot exclude the possibility that menstruation-related factors could have a much stronger effect, and are responsible for the differences between the induced endometriosis cases. The eight monkeys which have so far not shown any signs of endometriosis were young (2–4 years of age), breeding-inexperienced, and two of them were close relatives. However, endometriosis could also be established in other young and breeding-inexperienced marmosets in this study. Although the invasive method induced endometriosis of different stages more effectively in terms of time and frequency, no clear association is evident between the severity of the resulting endometriosis and the treatment method or its duration. The different reactions of the individual animals can probably be understood in the light of the complex pathogenesis of endometriosis, and need further examination. So far there is no information as to whether the susceptibility to induction of endometriosis is inherited in marmoset monkeys, whereas a familial aggregation has been shown for humans (Simpson et al., 1980; Moen and Magnus, 1993; Hadfield et al., 1997; Kennedy et al., 2001; Stefansson et al., 2002; Treloar et al., 2002).

Figure 6. RT–PCR products of ER, PR ARO, 17βHSD1 & 2 and RPS26 from endometrial foci from two animals (W3 and W7) throughout different time points. Collection of endometrial foci occurred: W3a: two times of invasive induction (September 04), W3b: three times of invasive induction (January 05), W7a: three times of invasive induction (November 04), W7b–d: fourth time of invasive induction (January 05); W7a and W7b were collected from the identical endometrial foci, just chronologically collected.

Figure 7. RT–PCR products of ER, PR ARO, 17βHSD1, 2 & 7 and RPS26 from endometrial foci from different marmoset monkeys (W16, W20, W4 and W5) as well as developing (W1) and established endometriotic foci (W4 and W7) of different marmoset monkeys subjected to 1.5% agarose gel electrophoresis. Uterine cells collected throughout second (W16 and W5) or fifth flushing (W4) and uterine foci during second flushing (W1), third (W7) and fifth flushing (W4). Steroid receptors (ER and PR) are expressed in all samples, whereas ARO is just detectable in histological established endometriotic foci (W4, W7). 17βHSD1 is only present in uterine foci from well-established animal W4; however, uterine cells collected throughout the same stages showed also positive signal for 17βHSD1.
Recently, a strong family factor has also been reported in theses macaques (Zondervan et al., 2004), further supporting a genetic predisposition to the disorder. It seems very likely that duration of treatment, age and breeding experience and/or immunological situation may have an effect on the success rate for the induction of endometriosis.

Symptoms for endometriosis are based amongst other things on chronic pelvic pain and infertility. However, there is a poor correlation between the severity of the disease (size and number of lesions) and the human patients’ complaints (The American Fertility Society, 1985; Bedaiwy and Falcone, 2004). The same appears to be true also for primates: pain behaviour is not observed, and only two marmoset monkeys with induced endometriosis showed clinical symptoms, such as changes in ovarian cycle length and ovarian abnormalities. Nevertheless, all treated monkeys with established endometriosis did show a fluid-filled peritoneum, which had not been already present at the first flushing. Histological analysis of all such endometriotic foci showed well-established endometriosis with strong ARO, 17βHSD1 and a decreased 17βHSD2 expression. Whether other animals with established endometriosis will show clinical symptoms in terms of infertility at a later age cannot yet be answered, and await new breeding experiments.

In humans, endometriosis is mainly located not only on the pelvic peritoneum, but also on the ovaries and on the rectovaginal septum, whereas bladder endometriosis seems to be relatively rare (Nisolle et al., 1997). There seems to be a species-specific location, for example in baboons, lesions were never found on the ovaries (D’Hooghe et al., 1996). In our monkey model, endometriosis was mainly located at the bladder, the uterus and the ovaries. However, this may be due to their different posture and manner of locomotion. The mechanism of adherence of refluxed endometrial cells to the peritoneum is still unclear. In our primate model, endometrial cells from the late secretory phase were attached to the intact abdominal peritoneum is still unclear. In our primate model, endometriotic cells mainly located at the bladder, the uterus and the ovaries. However, the mechanism of adherence of refluxed endometrial cells to the peritoneum is still unclear. In our primate model, endometriosis was mainly located at the bladder, the uterus and the ovaries. Nevertheless, all treated monkeys with established endometriosis showed a fluid-filled peritoneum, which had not been already present at the first flushing. Histological analysis of all such endometriotic foci showed well-established endometriosis with strong ARO, 17βHSD1 and a decreased 17βHSD2 expression. Whether other animals with established endometriosis will show clinical symptoms in terms of infertility at a later age cannot yet be answered, and await new breeding experiments.

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The marmoset monkey has very high estradiol levels compared with the human or non-human primate situation (Hearn et al., 1978), without showing any signs of endometriosis naturally, endometriosis could be induced by retrograde flushing of uterine cells. However, the foci of established endometriosis were reduced in size and number, following a decrease of the endogenous ovarian production of estrogens as a result of ovariecotmy. Endometriotic foci have high local estradiol biosynthesis with low estrogen inactivation compared with the endometrium from unaffected primates; however, even eutopic endometrial cells from affected monkeys show initial responses to the changes in estrogen production. Taken together, the marmoset monkey offers a useful and interesting model with which to study the pathogenesis of endometriosis, and its relationship to local and central estrogen metabolism.

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References


Endometriosis in primates


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