In-vitro adhesion of endometrium to autologous peritoneal membranes: effect of the cycle phase and the stage of endometriosis*

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BACKGROUND: Endometrium can adhere to autologous peritoneum. This study was undertaken to determine the effect of the menstrual cycle phase and the presence and stage of endometriosis on in-vitro adhesion of endometrium onto autologous peritoneum. METHODS: This was performed in an academic medical research centre. Sixty-seven subfertile women with a visually normal pelvis (n = 18) and with biopsy-proven endometriosis (n = 49) were included. Endometrial and peritoneal biopsies were obtained at laparoscopy during menstrual, follicular and luteal phase. Endometrium was cultured in vitro with autologous peritoneum, followed by fixation, paraffin embedding, serial sectioning, hematoxylin–eosin and immunohistochemical staining. Endometrial–peritoneal adhesion was evaluated using light microscopy. RESULTS: Endometrial–peritoneal adhesion was observed in ~80% of the adhesion assays and was not affected by the phase of the cycle, or by the presence and stage of endometriosis. The continuity of the mesothelial layer was disrupted at the attachment sites. Epithelialization was observed along the edges to integrate the endometrial implant. After adhesion, histological changes were observed within and below the implant. CONCLUSIONS: Endometrium obtained during menstrual, follicular or luteal phase appears to have a similar potential to implant in vitro on autologous peritoneum, and this adhesion process is not affected by the stage of endometriosis.

Key words: adhesion/endometriosis/endometrium/peritoneum/tissue culture

Introduction

The pathogenesis of endometriosis is not completely understood. According to the implantation theory (Sampson, 1927) endometriosis arises as a result of retrograde menstruation of endometrial fragments through the Fallopian tubes into the peritoneal cavity with subsequent implantation and growth of these endometrial cells onto the peritoneum. Although observations in the baboon model of endometriosis (D’Hooghe, 1997) support this hypothesis, fundamental research in women is lacking largely because the phenomenon of retrograde menstruation has not been studied in depth. Although it is widely accepted that retrograde menstruation occurs in women (Halme et al., 1984), precise studies concerning the mechanisms, quantity and quality of retrograde menstruation are not available. Similarly, mechanisms of endometrial implantation on the peritoneal surface are not completely understood. There are also no studies addressing whether such mechanisms may be different between women with and without endometriosis.

The interaction between endometrium and peritoneum in vitro has been studied in various models. Effects of cytokines on adhesion of endometrial cells to monolayered mesothelial cells have been reported (Zhang et al., 1993; Wild et al., 1994). Some investigators have promoted the amniotic membrane as an in-vitro model for peritoneum and concluded that mesothelium acts as a barrier to the attachment of ectopic endometrium, suggesting that peritoneal damage is required for the adhesion of endometrial fragments (Van der Linden et al., 1996; Groothuis et al., 1998). This finding was refuted by others who reported that endometrium can attach to the intact mesothelial surface of the peritoneum, suggesting that peritoneal damage is not required before endometrial implantation (Witz et al., 1999, 2001).

To the best of our knowledge, no data are available on how endometrial–peritoneal adhesion is affected by the phase of the menstrual cycle, or by the presence or absence of endometriosis. This may be important, since data in baboons indicate that the in-vivo intrapelvic implantation potential of menstrual endometrium is higher than the implantation potential of endometrium derived from either the follicular or


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Materials and methods

Patients, laparoscopies and tissue collection

Our study protocol was approved by the institutional review board of Gasthuisberg Hospital for the protection of human subjects. Informed consent was obtained from all patients before entry into this study.

A diagnostic laparoscopy for investigation of pain and/or infertility was performed in 67 reproductive age women (range 19–44 years). Endometriosis was found in 49 women and 18 women had a normal pelvis. The 49 patients with endometriosis had rAFS stage I (n = 26), rAFS stage II (n = 11), rAFS stage III (n = 6) and rAFS stage IV (n = 6) endometriosis, according to the revised system of the American Society of Reproductive Medicine, 1996 (American Society for Reproductive Medicine, 1997). Laparoscopies were performed during the luteal phase of the menstrual cycle in 32 women, during the follicular phase in 15 women and during menses in 17 women (Table I). Laparoscopy was also performed in three women with amenorrhoea (Table I). Two of these three amenorrhoeic women had endometriosis and had amenorrhoea induced with GnRH agonists for severe disease (rAFS stage IV). The third patient with amenorrhoea did not have endometriosis but a diagnosis of the polycystic ovarian syndrome and was not receiving hormonal therapy. During laparoscopy, biopsies from parietal peritoneum and endometrium were obtained from each patient. A circular peritoneal biopsy of ~2 cm² was surgically excised from the left suprapubic parietal peritoneum (above the rectosigmoid) by CO₂ laser (15 Watt superpulse, Sharplan 40 C; Laser Industries, Tel Aviv, Israel). During the laser excision, the biopsy was not touched at all, except for the laser beam directed at the edge of the biopsy, to prevent macroscopic or microscopic peritoneal damage. At the end of the excision, the biopsy was carefully held and removed with a grasping forceps at the margin (not in the centre) of the biopsy, again to prevent peritoneal damage by surgical manipulation. During removal from the excision site, the biopsy underwent a spontaneous folding process and was slowly and gently extracted through a 5 mm small suprapubic trocar. The average thickness of a peritoneal biopsy was ~2 mm (including subperitoneal fat). Endometrial samples were obtained by curettage. A portion of the biopsy was fixed for histological confirmation of the menstrual phase.

Cell culture

All tissues were immediately placed in phosphate-buffered saline (PBS) and transported to the laboratory. The peritoneal biopsy was handled with care to avoid abrasion of the mesothelial lining of the peritoneum. After collection, the biopsies were washed in PBS to remove debris and excess blood cells. Subperitoneal fat was carefully excised from the peritoneal biopsies. The peritoneal biopsy was layered with its extracellular side on a PBS-wetted Spongostan film (Ferrosan, Denmark) in a 5 cm Petri dish (VWR, Leuven, Belgium) to stabilize the biopsy during handling and changing of the culture media. The endometrial biopsy was mechanically fragmented with a scalpel into small pieces (1–2 mm²). All the endometrial fragments were placed on top of the mesothelial side of the peritoneal biopsy. The amount of endometrial fragments added to the peritoneal biopsy was not measured or quantified. No endometrial fragments were placed on top of the extracellular matrix of the peritoneal biopsy.

The explants were then cultured in Dulbecco’s modified Eagle’s minimal essential medium/F-12 supplemented with 10% fetal calf serum at 37°C in 5% CO₂. For each adhesion assay, autologous endometrium and peritoneum from the same patient were used. The peritoneal biopsies with the endometrial fragments were then cultured without any disturbance for 24–168 h (1×24 h, n = 1; 2×24 h, n = 14; 3×24 h, n = 6; 4×24 h, n = 23; 5×24 h, n = 12; 6×24 h, n = 6; 7×24 h, n = 5). Culture medium was changed every 48 h without touching the tissues. After incubation, the peritoneum with the endometrial fragments was repeatedly rinsed in PBS by carefully holding the explant with a forceps and gently agitating it in a culture dish filled with PBS. The tissue samples were then transferred to 4% buffered formaldehyde for 24 h.

Histology

After fixation, the endometrial–peritoneal explant was cut into three or four pieces that were embedded together in the same paraffin block. This was done to limit the number of sections needed to obtain a full picture of the endometrial–peritoneal adhesion process. Serial sections (5 µm) of the paraffin blocks were taken and mounted on 3-aminopropyltriethoxysilane-coated slides (Sigma Chemical Co., Bornem, Belgium). Morphology and adhesion of the endometrial fragments to the peritoneum was evaluated at periodic intervals following haematoxylin and eosin stain. Adhesion was assumed when uninterrupted contact between the epithelial side of the peritoneal biopsy and endometrial fragments was observed at light microscope level. Endometrial biopsies were histologically evaluated for dating the menstrual cycle phase (Noyes et al., 1950). All slides were reviewed by a senior expert in the histopathology of endometrium and endometriosis.

Table I. Distribution of patients according to the presence or absence of endometriosis and according to the menstrual cycle phase in which samples were obtained

<table>
<thead>
<tr>
<th></th>
<th>Normal pelvis</th>
<th>Endometriosis</th>
<th>AFS I</th>
<th>AFS II</th>
<th>AFS III</th>
<th>AFS IV</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menstrual</td>
<td>5</td>
<td>12</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>Follicular</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>–</td>
<td>15</td>
</tr>
<tr>
<td>Luteal</td>
<td>7</td>
<td>25</td>
<td>16</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>Amenorrhoe</td>
<td>1</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>49</td>
<td>26</td>
<td>11</td>
<td>6</td>
<td>6</td>
<td>67</td>
</tr>
</tbody>
</table>

For AFS classification, see American Society for Reproductive Medicine (1997).
In-vitro endometrial–peritoneal interaction

Figure 1. Percentage of adhesion according to the time of culture.

Figure 2. Percentage of adhesion according to the phase of the menstrual cycle and the stage of endometriosis (‘endo’).

For immunohistochemical staining with CD10, slides were dewaxed, rehydrated and boiled in 0.01 mol/l of citrate buffer (pH 6.0) for 30 min in a microwave oven. After endogenous peroxidase activity had been blocked, slides were incubated with CD10 (1/100, 30 min; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), supplemented with normal human serum (1/25). Peroxidase activity was detected using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) and slides were counterstained with Harris haematoxylin.

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Staining for cytokeratin included digestion with pepsin (Sigma) in HCl and incubation with a mixture of H2O2 and NaN3 in methanol (30 min), followed by local treatment with a 2% bovine serum albumin solution (15 min). Slides were then incubated with a mouse anti-cytokeratin (1/400, Clone MNF 116; Dako) antibody for 2 h at room temperature. The secondary antibody was peroxidase-conjugated goat anti-mouse IgG (1/100, 30 min; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), supplemented with normal human serum (1/25). Peroxidase activity was detected using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) and slides were counterstained with Harris haematoxylin.

Statistical analysis

Statistical analysis to detect significant differences between groups was performed with χ2-tests. P < 0.05 was accepted as significant.

Results

Endometrial–peritoneal adhesion: the phase of the menstrual cycle and the stage of endometriosis

After 3–7 days in culture, the percentage adhesion between endometrium and peritoneum varied between 80 and 100% (Figure 1). Adhesion was observed in 81% (54/67) of the assays. Endometrial–peritoneal adhesion was observed in 15/17 (88%), 14/15 (93%) and 24/32 (75%) of the experiments performed with endometrium derived from menstrual, follicular or luteal phase endometrium respectively (P = not significant) (Figure 2). Adhesion was not observed using endometrium from the two rAFS stage IV endometriosis patients who had amenorrhoea following hormonal therapy with GnRH agonists. In contrast, adhesion was observed using the endometrium of the PCOS patient with amenorrhoea who did not have endometriosis. Endometrial–peritoneal adhesion was observed in 16 of 18 (89%) patients with a normal pelvis and in 38 of 49 (78%) patients with endometriosis (Figure 2). No correlation was observed between endometrial–peritoneal adhesion and the stage of endometriosis as adhesion was found in 81% (21/26) of patients with rAFS endometriosis stage I, 73% (8/11) of patients with rAFS endometriosis stage II, 100% (6/6) of patients with rAFS endometriosis stage III patients and 50% (3/6) of patients with rAFS endometriosis stage IV disease (75% of cycling patients with rAFS endometriosis stage IV, after exclusion of the two patients with amenorrhoea due to GnRH agonist treatment).

Endometrial–peritoneal adhesion: histology of attachment and implantation

Using light microscopy, a physical continuity between endometrial tissue and peritoneal biopsy was observed. The area of endometrium adherent to peritoneum consisted primarily of cells morphologically identical to stromal cells. Stromal cells appeared to invade (Figure 3A) into the peritoneum with the formation of a multicellular layer under the mesothelium (Figure 3B). The cells adhering to the peritoneum stained positively for endometrial stromal cell marker CD10 (Figure 3C), and negatively for CD68 and CD45. Under the attached endometrial fragments, the continuity of the mesothelial layer was interrupted, although there may be a few residual mesothelial cells between the implant and the peritoneum. Along the margins of the implant, an intact layer of mesothelial cells was identified. A process of implant re-epithelialization could be observed: mesothelial cells from the peritoneum started growing over the endometrial implant (Figure 3D). Continuity between the flattened cells of the mesothelial epithelium of the peritoneum and the columnar cells of the glandular epithelium of the implant was visible (Figure 3E). Histological and structural changes were observed in and below the endometrial implants. Under the epithelialized layer of the implant, most of the stromal cells were necrotic and replaced by fine fibrillar debris, whereas the glands remained clearly recognizable (Figure 3F). This debris stained intensely positive with CD10, whereas the glandular epithelium did not show any staining for CD10 (Figure 3G). In the interstitial tissue of peritoneum, below the implant, elastoid (swollen and fragmented) degeneration of the collagen fibres was observed (Figure 3H).

Immunohistochemistry

Immunohistochemical staining was performed on 5 μm tissue sections to identify adhering cells using monoclonal antibodies directed against cytokeratin, endometrial stromal cell marker CD10 (Toki et al., 2002), histiocyte/macrophage marker CD68 (Kp1; Dako, Glostrup, Denmark) and leukocyte marker CD-45 (Dako). It is well known that mesothelial and endometrial epithelial cells, but not endometrial stromal cells, stain positively for cytokeratin.

Staining for cytokeratin included digestion with pepsin (Sigma) in HCl and incubation with a mixture of H2O2 and NaN3 in methanol (30 min), followed by local treatment with a 2% bovine serum albumin solution (15 min). Slides were then incubated with a mouse anti-cytokeratin (1/400, Clone MNF 116; Dako) antibody for 2 h at room temperature. The secondary antibody was peroxidase-conjugated goat anti-mouse IgG (1/100, 30 min; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), supplemented with normal human serum (1/25). Peroxidase activity was detected using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) and slides were counterstained with Harris haematoxylin.

Percentage of adhesion according to the phase of the menstrual cycle. (Figure 2).

Percentage of adhesion according to the time of culture. (Figure 1).
Figure 3. (legend on facing page)
Discussion

To our knowledge, this is the first study investigating autologous endometrial–peritoneal adhesion potential according to the menstrual cycle phase, the presence or absence of endometriosis and the stage of endometriosis.

Several in-vivo and in-vitro models have been developed to study the potential for endometrial–peritoneal interactions. The baboon has been proposed as an in-vivo culture model for the study of endometrial–peritoneal implantation (D’Hooghe et al., 1995). Nude mice were used in one study (Nisolle et al., 2000) to observe transplantation of human endometrium. In vitro, potential interactions have been studied using amnion membranes (Van der Linden et al., 1996; Groothuis et al., 1998) or biopsies of human peritoneum (Groothuis et al., 1999; Witz et al., 1999, 2001), but conclusions are controversial. One group suggested that an intact mesothelium may prevent endometrial fragments from adhering to the peritoneum (Van der Linden et al., 1996; Groothuis et al., 1998). Electron microscopy supported this hypothesis that the mesothelial lining acts as a barrier for adhesion (Groothuis et al., 1999). Using endometrial tissue isolated from antegrade shed menstrual effluent, this group confirmed their previous results that adhesion was exclusively seen at locations where the epithelium was damaged or absent (Koks et al., 1999). In contrast, another group (Witz et al., 1999, 2001) reported that endometrium can attach rapidly to the intact mesothelial surface of peritoneum.

In the present study, we used mechanically fragmented endometrium in an in-vitro model to determine whether endometrium could adhere to autologous peritoneal surfaces. In our experiments, the area of endometrium adherent to peritoneum consisted mainly of cells morphologically and immunohistochemically identified as stromal cells, suggesting that stromal cells may play an important role in in-vivo attachment. Our results are in accordance with those of other investigators (Witz et al., 1999), although they also reported that in addition to stromal cells, endometrial epithelium can also attach to mesothelium (Witz et al., 2001). Experiments in nude mice have also demonstrated that stromal cells are involved in the attachment process and glandular cells in the growth of endometriotic lesions (Nisolle et al., 2000).

Our results indicated that a high degree of endometrial–peritoneal adhesion (80–100%) occurred after culturing the explants for 48 h. Unfortunately, only one experiment was performed with an incubation period of 24 h, so we cannot exclude more rapid adhesion. Others have shown that endometrial adhesion occurs within 1 h and that transmesothelial invasion occurs within 18 h (Witz et al., 2001).

Endometrial–peritoneal adhesion was observed at several sites on the peritoneal surface. The number of adhering fragments was not counted since the amount of added endometrium was not quantified before the experiment. Furthermore, overestimation of the number of adhesion sites would have been unavoidable since three or four tissue explants were embedded in the same paraffin block.

Our histological results suggest that a dynamic process occurs at the endometrial–peritoneal interface. Invasion of stromal cells into the peritoneum occurred rapidly with the formation of a multicellular layer under the mesothelium. Below the area of attachment of endometrial fragments, no identifiable mesothelium was present, and at the margins, a re-epithelialization process rapidly integrated the implant into the peritoneal layer. Others (Witz et al., 1999) have also observed that intact mesothelium occurs up to the point of attachment. The re-epithelialization process by the mesothelial epithelium is continued into the glandular epithelium of the endometrial implant. Under this epithelialized layer, the endometrial stroma was replaced by a mixture of fine fibrillar and necrotic debris. Finally, in interstitial peritoneal tissue, well-structured collagen fibres disintegrated as a result of elastoid degeneration. Interestingly, these histological observations were made in several experiments using endometrium from different phases of the menstrual cycle, regardless of the presence or absence of endometriosis and the stage of endometriosis.

A prerequisite for the success of adhesion and structural changes in the implant is the presence of viable stromal cells in the endometrial biopsy. Sometimes, necrotic tissue was observed in the endometrial biopsy and/or in the peritoneum. This could prevent the re-epithelialization and the integration of the implant. Probably, necrotic tissue is also responsible for a negative result of the adhesion process and may explain why adhesion was not observed in 100% of our assays.

In our study, peritoneal tissues were collected with the use of a CO₂ laser instead of a scalpel. The advantage of laser dissection is that it is not necessary to damage the peritoneum during excision. Technically, the laser technique allows the surgical excision of the biopsy without touching the biopsy. Only at the end of the biopsy can the tissue be grasped with a forceps at the margin and gently extracted from the abdomen. In contrast, the usage of scissors and forceps, and/or electrocoagulation, during peritoneal biopsy involves more manipulation of, and more damage to, the peritoneal surface.

The effect and the presence of the stage of endometriosis on the adhesion process between autologous endometrium and
peritoneum has never been studied in detail. Most investigators only included endometrium from women without endometriosis who were undergoing surgery for benign conditions (Groothuis et al., 1999; Witz et al., 1999, 2001). In our study, adhesion assays were performed with endometrium from patients with and without endometriosis including women with minimal, mild, moderate and severe endometriosis. No correlation was observed between endometrial–peritoneal adhesion and the presence or stage of endometriosis. It is possible that the lack of statistically significant results regarding endometrial–peritoneal adhesion in vitro between patients with and without endometriosis can be explained by lack of power. Indeed, although our study contained considerably more patients ($n = 67$) than the number of subjects ($n = 3–15$) studied in previous reports (Groothuis et al., 1999; Witz et al., 1999, 2001), power analysis revealed that at least 27 patients with endometriosis and 27 patients without endometriosis would be needed ($\beta = 0.8$ and $\alpha < 0.05$) to test the hypothesis that luteal endometrial–peritoneal adhesion in vitro occurs significantly more frequently (80%) in women with endometriosis than in women without endometriosis (40%). However, the observation during the menstrual and follicular phase that adhesion occurred in 100% of all patients without endometriosis directly undermined our hypothesis and prompted us to stop the study at an earlier stage.

The effect of the different phases of the cycle on the adhesion process has never been reported. Other investigators have used proliferative (Groothuis et al., 1998, 1999; Witz et al., 1999, 2001) secretory (Groothuis et al., 1998; Witz et al., 1999, 2001) or menstrual endometrium (Van der Linden and Dunselman, 1996; Nisolle et al., 2000), but did not compare their results with respect to adhesion capacity to peritoneum. Our results indicated that endometrium obtained during the menstrual, luteal or follicular phase had similar potential to implant on autologous peritoneum suggesting that the adhesion of endometrium onto peritoneum is a universal process, independent of the cycle phase. In-vitro models, however, are limited by their descriptive and qualitative nature. A quantitative adhesion assay is needed to study in detail the interaction between peritoneum and endometrium. Using this in-vitro model, the in-vivo situation in women is mimicked, but we cannot exclude several shortcomings in comparison with the in-vivo situation. Firstly, endometrial tissue and cells obtained after a transcervical uterine biopsy are probably different from endometrial tissue and cells present in the peritoneal fluid in vivo. Secondly, the collection, handling and culture of peritoneum may alter the propensity of peritoneum to adhere to endometrium. Therefore, short-term in-vivo co-culture of menstrual endometrium, peritoneal fluid and peritoneum is needed and being developed in the baboon (D’Hooghe, 1997) to study the early endometrial–peritoneal interactions in a clinically and biologically meaningful way. Further studies are also needed to investigate the precise mechanisms involved in endometrial–peritoneal implantation events.

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References


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