Tissue integrity is essential for ectopic implantation of human endometrium in the chicken chorioallantoic membrane

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BACKGROUND: Not all women with patent tubes develop clinically manifest endometriosis. Quality and quantity of endometrium in retrograde menstruation may be the determining factor in the development of the disease. We hypothesize that retrograde shedding of endometrial fragments with preserved integrity facilitates implantation of endometrium in ectopic locations, resulting in endometriotic lesion development. We evaluate the impact of tissue integrity on the success of endometriosis-like lesion development in the chicken embryo chorioallantoic membrane (CAM) model. METHODS: Menstrual and non-menstrual (cyclic) endometrium were collected by biopsy, and either minced or enzymatically dispersed. Spontaneously shed menstrual effluent was collected by a menstrual cup, and cells and tissue were isolated. We evaluated whether infiltration or lesion formation in the CAM occurred after transplantation of endometrium onto the CAM. RESULTS: Transplantation of biopsied menstrual and cyclic endometrium fragments, and of endometrium fragments >1 mm³ isolated from menstrual effluent, resulted in lesion formation. Transplantation of endometrial cells isolated from menstrual effluent did not lead to lesion formation. After transplantation of digested biopsied cyclic endometrium, infiltration in the CAM but no lesions were observed. CONCLUSION: In the CAM assay, integrity of tissue architecture determines success of implantation of human endometrium in ectopic locations.

Key words: chorioallantoic membrane model/ectopic implantation/endometriosis/menstrual endometrium/tissue integrity

Introduction

According to Sampson’s hypothesis on the pathogenesis of endometriosis, viable, spontaneously shed endometrial tissue arrives in the abdominal cavity and implants in the peritoneum (Sampson, 1927). It is not clear how endometriotic lesions come into existence after the adhesion of endometrium to the peritoneum. Transplantation of endometrium, biopsied during the non-menstrual phase of the cycle (cyclic endometrium) results in the development of endometriosis-like lesions in the chicken embryo chorioallantoic membrane (CAM), a model that has been used to study tumour transplantation and invasion (Murphy, 1912; Leighton, 1964; Scher et al., 1976). These endometriosis-like lesions consist of human stromal and glandular cells, as confirmed by in-situ hybridization for human chromosome 1 (Maas et al., 2001).

Since menstrual endometrium enters the abdominal cavity and is supposedly ‘the seed’ that develops into endometriotic lesions, menstrual endometrium is the most appropriate tissue to study regarding the early pathogenesis of endometriosis (Groothuis, 1999). Until now, menstrual or endometrial characteristics responsible for the ectopic implantation of endometrium are not clear. Shedding of a sufficient amount of endometrium with preserved integrity may facilitate implantation of endometrium in an ectopic location. This hypothesis is supported by the association of increased amounts of retrograde menstruation with a higher risk of developing endometriosis (Sanfilippo et al., 1986; Darrow et al., 1993; D’Hooghe et al., 1994). Moreover, Sillem et al. demonstrated in the cynomolgus monkey model that collagenase digestion of endometrial tissue fragments prior to transplantation reduces the ability to implant (Sillem et al., 1996).

In the present study, the CAM model was used to evaluate the impact of endometrial tissue integrity on endometriosis-like lesion formation.

Materials and methods

Study design

The following human endometrium preparations were transplanted onto the CAM: (i) biopsied cyclic endometrium, (ii) biopsied...
menstrual endometrium, (iii) cells and tissue fragments isolated from spontaneously shed menstrual effluent, and (iv) enzymatically dispersed endometrium. To account for the effects of tissue handling and manipulation of endometrium, the impact of storage of endometrium in the Keeper (a vaginal cup used for the collection of spontaneously shed menstrual effluent) and of tissue handling on the potential to form lesions was evaluated. For these controls the following were transplanted onto the CAM, (v) biopsied menstrual endometrium stored in the Keeper, and (vi) biopsied menstrual endometrium stored in the Keeper and processed as under (iii).

Infiltration and endometriosis-like lesion formation were evaluated. In addition, proliferation in endometrium prior to and after transplantation onto the CAM was studied.

**Tissue handling**

**Biopsied endometrium**

Endometrium was collected by biopsy from 12 women with normal ovulatory cycles, undergoing laparoscopy for benign conditions. An Endobiops endometrium sampling device (Gynotec, Malden, The Netherlands) was used. Endometrium was collected during the non-menstrual phase of the cycle (n = 6, cyclic endometrium) or during menstruation (n = 6, menstrual endometrium). After collection, endometrium was placed in serum-free medium composed of Dulbecco’s modified Eagle’s medium/Ham’s F-12 supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B, and stripped of blood. The endometrial tissue was carefully dissected into uniform pieces of 1–2 mm³ and transplanted onto the CAM (Maas et al., 2001). To monitor infiltration and lesion development after transplantation of biopsied menstrual endometrium, CAM were embedded in paraffin after 24, 48 and 72 h.

**Isolation of endometrium from menstrual effluent**

Menstrual effluent was collected by 10 volunteers with regular menstrual cycles. These women had no history of endometriosis and did not use oral contraceptives. Collection took place in a menstrual cup for 2–3 h during day 1 or day 2 of menstruation. The soft natural rubber cup, shaped like a cone (‘Keeper’, Den Haag, The Netherlands), was inserted into the upper vagina with the opening enclosing the cervix. Immediately after collection, the menstrual effluent was brought to the laboratory in a sterile plastic container (Koks et al., 1997).

Menstrual effluent was resuspended in serum-free medium, layered on a Ficoll-Paque gradient (Sigma–Aldrich Chemie BV, Zwijndrecht, The Netherlands) and centrifuged at 1200 g for 30 min at 4°C. The interphase containing endometrial and inflammatory cells was collected and washed twice in routine medium. The pellet was resuspended in 20–30 µl of serum-free medium and transferred onto the CAM.

In case endometrium fragments >1 mm³ could be identified in the menstrual effluent just below the interphase after the Ficoll-Paque gradient centrifugation, these fragments were picked up from the suspension with a forceps and transplanted onto the CAM.

**Collagenase digestion of cyclic endometrium**

In order to evaluate the effect of destruction of tissue integrity on the ability to form endometriosis-like lesions, collagenase digestion of endometrium was performed. After collection of biopsied cyclic endometrium (n = 8), endometrium was rinsed in serum-free medium and minced into small pieces. The suspension was centrifuged, and the medium was replaced by medium containing 0.25% collagenase type I (ICN Biomedicals BV, Zoetermeer, The Netherlands) and 0.1% trypsin (Gibco BRL). The tissue was digested for 15–20 min at 37°C and filtered through a 400 µm stainless steel sieve (Sigma–Aldrich Chemie BV, Zwijndrecht, The Netherlands). The cells were pelleted and washed once in serum-free medium. The pellet, consisting of collagenase-digested glandular and stromal cells, was resuspended in 20–30 µl of serum-free medium and transplanted onto the CAM.

**Biopsied menstrual endometrium stored in the Keeper**

To evaluate whether tissue collection in the Keeper affects the ability to form lesions, biopsied menstrual endometrium (n = 4) was stored in a small amount of serum-free medium in a Keeper for 2–3 h at 37°C. Subsequently, the endometrial tissue was stripped of blood and carefully sectioned into uniform pieces of 1–2 mm³ and transplanted onto the CAM.

**Biopsied menstrual endometrium stored in the Keeper and processed as menstrual effluent**

To evaluate whether laboratory procedures necessary to isolate endometrial cells and tissue from spontaneously shed menstrual effluent affect lesion formation, biopsied menstrual endometrium (n = 4) was kept in a Keeper at 37°C for 2–3 h in a small amount of serum-free medium, and subsequently processed in the same way as menstrual effluent. After processing, endometrial tissue fragments were transplanted onto the CAM.

Of each endometrium sample collected for this study, one tissue fragment was fixed in formalin immediately after collection and embedded in paraffin. Paraffin sections (4 µm) were cut and either stained with haematoxylin and eosin (HE) for histological evaluation or stored for later immunohistochemical analysis.

The use of human tissue for this study was approved by the Institutional Review Board of the academisch ziekenhuis Maastricht, and all women participating in the study gave their written informed consent.

**CAM model**

Fertilized eggs of Lohman-selected White Leghorns were incubated for 3 days at 37°C, 55% relative air humidity, while being rotated hourly. At day 3 of incubation, a rectangular window (1 × 1.5 cm) was made in the eggshell. A total of 2 ml of albumen was withdrawn using a 21G needle, through the large blunt edge of the egg. The window was covered with Scotch tape to prevent dehydration. The eggs were replaced in the incubator without rotation until day 8–11 of incubation.

The CAM is an impenetrable barrier to invasive cells unless it has been traumatized by removing the upper peridermal part of the double epithelial layer, leaving the basal layer intact. Therefore, just before the transplantation of endometrium a small part of the CAM was gently traumatized by laying a 1 cm² wide strip of sterile ether-extracted lens paper onto the surface of the epithelium and then removing it immediately (Armstrong et al., 1982; Maas et al., 2001).

Endometrial tissue was transplanted onto the CAM. Following transplantation, the window was covered again and the egg was placed back in the incubator. After incubating for 24, 48 and 72 h, the transplanted tissue including the surrounding CAM was excised, fixed in formalin and embedded in paraffin. Paraffin sections were cut and either stained with HE for histological evaluation or stored on slides for later immunohistochemical analysis.

**Immunohistochemistry**

Proliferation was evaluated by immunohistochemistry using a mouse monoclonal antibody against the proliferation marker Ki67 (MIB-1, 31
Epithelium was stained using a mouse monoclonal antibody against pan-cytokeratin (Clone MNF 116, 1:100; Dako, Glostrup, Denmark).

In short, paraffin sections were deparaffinized by incubation with xylene for 2–3 min and rehydrated in alcohol series. Endogenous peroxidase activity was blocked by incubation with 0.25% hydrogen peroxide in methanol for 20 min. Sections were rinsed three times in phosphate-buffered saline (PBS) and were heated to 95°C in citrate buffer (pH 6.0) for 20 min in preparation for incubation with the MIB-1 antibody, or digested in 0.1% pepsin in 0.1 N HCl for 30 min in preparation for incubation with the pan-cytokeratin antibody. After rinsing again in PBS, sections were incubated overnight at 4°C with the primary antibody.

After three PBS rinses, sections were exposed to Envision anti-mouse (Dako, Glostrup, Denmark) for 30 min. After rinsing in PBS, antibody binding was visualized with 3',3'-diaminobenzidine. Sections were washed and counterstained with haematoxylin, washed again, dehydrated and mounted for light microscopy.

**Results**

### Lesion development in biopsied menstrual endometrium

Twenty-four hours after transplantation of biopsied menstrual endometrium, endometrial cells were observed in direct contact with the CAM mesenchyme (Figure 1A and B). 48 h after transplantation, lesions were observed consisting of endometrial glands and heterogeneous stroma, with blood vessels containing nucleated erythrocytes in close proximity of the edge of the lesions (Figure 1C). 72 h after transplantation, organized lesions were observed in the CAM mesenchyme, with intact glands and surrounding endometrial stroma, mimicking normal endometrium and endometriotic lesions. Nucleated erythrocytes containing vessels were present within the lesions (Figure 1D).

### Proliferation

Prior to transplantation, Ki67 positive cells were present both in menstrual and in cyclic endometrium (Figure 2A). 72 h after transplantation, Ki67 staining was almost absent (Figure 2B).

### Impact of tissue integrity on lesion formation

The abilities of the various cell and tissue preparations to infiltrate the CAM and to form endometriosis-like lesions 72 h...
Transplantation of biopsied cyclic and biopsied menstrual endometrium resulted in infiltration in 74 and 78% and in formation of endometriosis-like lesions in 68 and 67% of CAM respectively. Transplantation of endometrial cell suspensions isolated from spontaneously shed menstrual effluent collected by the Keeper resulted neither in infiltration nor in lesion formation in the CAM. In 4/10 menstrual effluent samples, intact tissue fragments with a size >1 mm³ were present in menstrual effluent and could be picked up from the suspension with a forceps. These fragments were composed of intact glands and stromal tissue as confirmed by immunohistochemical staining for cytokeratin and vimentin respectively (data not shown). After transplantation onto the CAM they were able to infiltrate in 63% and to form lesions in 44% of CAM. Transplantation of endometrium after collagenase digestion resulted in infiltration in 53%, but did not result in lesion formation in the CAM.

After transplantation of biopsied menstrual endometrium that had been stored in the Keeper, or had been processed in the same way as menstrual effluent, infiltration and lesion formation in the CAM were still observed.

Discussion

Lesion development

In this study we have shown that biopsied menstrual endometrium and endometrial tissue fragments isolated from spontaneously shed menstrual effluent are able to form endometriosis-like lesions in the CAM model, in a similar fashion to biopsied cyclic endometrium. 24 h after transplantation, direct contact between the endometrium and the CAM mesenchyme was observed, and after 72 h complete lesions were present in the CAM.

These observations imply that lesions originate from endometrial cells that have migrated into the CAM mesenchyme. Alternatively, lesions may develop from rapidly proliferating endometrial cells. Contrary to what we expected, proliferation marker Ki67 is hardly expressed in the lesions while it is expressed in the endometrium prior to transplantation. This suggests a minor role for cell proliferation in the organization of lesions. These findings are in accordance with results of other authors, who reported a significantly reduced proliferation activity in the epithelium of ectopic lesions as compared with the eutopic endometrium (Jones et al., 1995; Scotti et al., 2000). Therefore, it is likely that the ability of infiltrated endometrial cells to rebuild the original tissue structure is responsible for lesion formation. However, we cannot exclude the possibility that hormonal or other environmental factors in the body which are absent in the CAM may play a role in lesion formation as well.

Impact of tissue integrity on lesion formation

Transplantation of biopsied cyclic and menstrual endometrium results in infiltration and endometriosis-like lesions, whereas transplantation of single endometrial cells isolated from

Table I. Impact of tissue integrity on infiltration and endometriosis-like lesion formation in the chorioallantoic membrane (CAM)

<table>
<thead>
<tr>
<th>Endometrium</th>
<th>No. of CAM</th>
<th>Infiltration</th>
<th>Lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biopsied cyclic endometrium</td>
<td>19</td>
<td>14 (74)</td>
<td>13 (68)</td>
</tr>
<tr>
<td>Biopsied menstrual endometrium</td>
<td>18</td>
<td>14 (78)</td>
<td>12 (67)</td>
</tr>
<tr>
<td>Endometrial cells isolated from menstrual effluent</td>
<td>50</td>
<td>0 (0)*</td>
<td>0 (0)*</td>
</tr>
<tr>
<td>collected in Keeper</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue fragments in menstrual effluent collected in Keeper</td>
<td>16</td>
<td>10 (63)</td>
<td>7 (44)**</td>
</tr>
<tr>
<td>Collagenase digested biopsied cyclic endometrium</td>
<td>45</td>
<td>24 (53)**</td>
<td>1 (2)*</td>
</tr>
<tr>
<td>Biopsied menstrual endometrium, stored in Keeper</td>
<td>13</td>
<td>10 (77)</td>
<td>9 (69)</td>
</tr>
<tr>
<td>Biopsied menstrual endometrium, stored in Keeper</td>
<td>11</td>
<td>6 (55)</td>
<td>4 (36)**</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.
*P < 0.01, **P < 0.1 compared with biopsied cyclic endometrium.
spontaneously shed menstrual effluent does not. This suggests that the tissue architecture of biopsied endometrium, which consists of organized glandular epithelium and stroma, is pivotal for the ability of endometrium to infiltrate and to form lesions.

During menstruation, endometrial tissue is exposed to high levels of matrix-degrading enzymes (Marbax et al., 1995; Koks et al., 2000). In most cases, menstrual effluent consists of single endometrial glandular and stromal cells instead of intact tissue fragments. These endometrial cells adhere easily to the basement membrane of amnion and peritoneum (Koks et al., 1999), but they are not able to infiltrate and develop endometriosis-like lesions in the CAM. Apparently, single endometrial cells lack the essential mutual contact in which glandular structures are intact and surrounding stromal cells are present, and consequently lack the ability to form lesions. In 4/10 menstrual effluent samples in this study, intact endometrial fragments >1 mm³ could be retrieved from the menstrual effluent. These fragments, composed of intact glandular structures and surrounding stromal cells, were able to induce endometriosis-like lesions in the CAM, whereas single cells from the same menstrual effluent samples were not. Compared with biopsied endometrium, the number of lesions formed was slightly reduced, suggesting a diminished capacity of shed fragments of endometrium to form lesions, most likely as a result of the tissue degradation process which has been initiated at menstruation. Therefore, it is tempting to suggest that women who shed intact endometrial fragments in their menstrual effluent are more prone to develop endometriosis.

The capacity to form lesions disappeared once biopsied cyclic endometrium was digested by collagenase prior to transplantation. This is in accordance with the findings of Sillem et al. in the cynomolgus monkey model. These authors found that enzymatic treatment of endometrial tissue reduced the development of endometriotic lesions (Sillem et al., 1996).

The observation that transplantation of endometrial cells isolated from menstrual effluent does not result in lesion formation may alternatively be a consequence of the contact of menstrual effluent with the Keeper, or of laboratory procedures necessary to isolate endometrial cells and fragments from menstrual effluent. For this reason we have exposed biopsied menstrual endometrium to storage in the Keeper, and to the same laboratory procedures that were used for isolation of endometrial cells and tissue from menstrual effluent. Lesion formation was not affected after storage of biopsied menstrual endometrium in the Keeper. When biopsied menstrual endometrium was processed in the same way as shed menstrual effluent, lesion formation was slightly reduced. For this reason, we consider it safe to argue that laboratory procedures used to isolate endometrial cells and tissue from menstrual effluent prior to transplantation are not responsible for the complete lack of lesion formation after transplantation of cells isolated from menstrual effluent.

In conclusion, we have shown that biopsied menstrual endometrium is able to develop into endometriosis-like lesions in the CAM model. Spontaneously shed menstrual endometrium will also form lesions if tissue fragments are of sufficient size, assuring preservation of tissue architecture and interaction between epithelial glands and the stromal compart-

ment. These findings indicate that tissue integrity is crucial for the development of endometriotic lesions.

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