Adhesion of human endometrium to the epithelial lining and extracellular matrix of amnion in vitro: an electron microscopic study

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One of the first steps in the pathogenesis of endometriosis is the attachment of the endometrium to the peritoneal lining. Since the peritoneum is extremely fragile and hard to obtain, amnion has been used as an in-vitro model to study adhesion. Scanning and transmission electron microscopy was applied to evaluate the adhesion of endometrial cells isolated in the proliferative and secretory phases of the menstrual cycle. Endometrial fragments obtained in either phase of the cycle were able to adhere to the extracellular matrix of the amnion. Fragments from proliferative phase endometrium showed active spreading and growth over the matrix surface, whereas fragments from secretory phase endometrium did not. Fragments from proliferative as well as secretory phase endometrium were able to adhere to the epithelial side of the amnion, but only at locations where the amniotic epithelium was damaged or partly absent. These observations indicate that the basement membrane and extracellular matrix provide a suitable substrate for endometrial cell attachment and growth and that endometrial cell adhesion occurs preferentially to subepithelial structures, whereas an intact epithelium prevents the adhesion of endometrial fragments to the amnion.

Key words: adhesion/amnion/endometriosis/endometrium/human/model/peritoneum

Introduction

Endometriosis is defined by the presence of endometrial tissue, glands with associated stroma, at locations outside the uterus. To explain the pathophysiological events that lead to endometriosis, Sampson (1940) has proposed the reflux implantation theory. This theory postulates that menstrual effluent retrogradely shed into the peritoneal cavity contains viable endometrium that may transplant to ectopic sites. It has been observed that shedded menstrual endometrium is viable (Keettel and Stein, 1951) and that blood (Blumenkranz et al., 1981) and viable endometrial cells are present in peritoneal fluid (Kruitwagen et al., 1991). These findings, together with the clinical observation of a high prevalence of endometriosis in adolescent girls with congenital menstrual outflow obstruction (Sanfilippo et al., 1986), support Sampson’s implantation theory.

A prerequisite for endometriosis to occur is the adhesion of endometrial cells or fragments to the peritoneum followed by implantation and growth. Endometrial fragments recovered from peritoneal fluid express integrin and cadherin cell adhesion molecules (Van der Linden et al., 1994), suggesting that these fragments are capable of establishing cell–cell or cell–extracellular matrix contacts with the peritoneal lining. However, the exact mechanism of the interaction between endometrial cells and peritoneum still remains to be elucidated.

Recently, we used the amnion as a model for peritoneum (Van der Linden et al., 1998). Amnion resembles peritoneum with respect to its general structure, consisting of an epithelial lining, basement membrane and extracellular matrix. Furthermore, both membranes show a similar expression of cytokeratins in the epithelial lining and of various extracellular matrix components (Van der Linden et al., 1996). Using this model, endometrial fragments were found to adhere to the matrix side of the amnion but not to the epithelial side (Van der Linden et al., 1996), suggesting that an intact mesothelium may prevent the endometrium from adhering to the peritoneum.

In this study, we aimed to characterize further the amnion and the adhesion of endometrial fragments obtained in the early proliferative phase of the menstrual cycle, to the epithelial and extracellular matrix side of the amnion using light and electron microscopy. Evidence is accumulating to suggest that a progesterone dominant environment results in a reversible regression of endometriosis (Lindsay, 1995). Therefore, we included endometrial fragments obtained in the secretory phase.

Materials and methods

Endometrium

Endometrium was collected using a Probet endometrial sampling device (Gynetics, Oisterwijk, The Netherlands) from volunteers who agreed to donate endometrium by informed consent. Samples were collected on days 7 or 8 and on day 21 of the menstrual cycle. After collection, the endometrium was rinsed once in complete medium, the endometrium was rinsed once in complete medium and then sequentially filtered through a 400 µm stainless steel sieve (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), a 100 µm nylon filter (Micronic, BV, Zwijndrecht, The Netherlands), and 100 µm nylon filter (Micronic, BV, Zwijndrecht, The Netherlands), and then centrifuged and the CM replaced by CM containing 0.25% collagenase I (ICN Biomedicals BV, Zoetermeer, The Netherlands) supplemented with 10% fetal calf serum, L-glutamine and 100 IU/ml penicillin and 100 µg/ml streptomycin (Life Technologies BV, Breda, The Netherlands) and subsequently minced into small pieces. The suspension was centrifuged and the CM replaced by CM containing 0.25% collagenase I (ICN Biomedicals BV, Zoetermeer, The Netherlands). The tissue was digested for 30–45 min at 37°C and then sequentially filtered through a 400 µm stainless steel sieve (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), a 100 µm nylon filter (Micronic, BV, Zoetermeer, The Netherlands) and then centrifuged and the CM replaced by CM containing 0.25% collagenase I (ICN Biomedicals BV, Zoetermeer, The Netherlands) and then centrifuged and the CM replaced by CM containing 0.25% collagenase I (ICN Biomedicals BV, Zoetermeer, The Netherlands).
Lelystad, the Netherlands) and a 30 μm polyamid filter (Stokvis & Smits, IJmuiden, The Netherlands). The endometrial fragments retained on the 100 and 30 μm filters were pooled, washed and seeded onto the amnion.

Figure 1. A light micrograph of a 1 μm section of Epon embedded amnion showing the different layers. E, epithelium; dE, degenerated epithelium; BM, basement membrane; CC compact connective tissue layer; F, fibroblast layer; LC, loose connective tissue layer (original magnification ×250, scale bar = 40 μm).

Figure 2. Ultrastructure of the amniotic epithelium. Scanning electron micrographs of amniotic epithelial cells from (A) PBS-stored and (B) fresh membranes (original magnification ×1600, scale bar = 10 μm). Transmission electron micrographs of (C) PBS-stored (original magnification ×5750, scale bar = 2 μm) and (D) fresh amnion (original magnification ×4350, scale bar = 2 μm). BM, basement membrane; MV, microvilli, B, blebbing.

Amnion
Fresh human placentas were obtained at the time of normal term delivery. The amnion reflectum was separated from the chorion and prepared in two different ways. The membrane was rinsed and stored at 4°C in phosphate buffered saline (PBS) for several days prior to use, or rinsed in PBS, placed in CM and used the same day. Sections of the membrane were divided into two parts. Both parts were fixed between two metal rings (Van der Linden et al., 1997), one oriented with the epithelial side upwards, the other with the matrix side upwards. Endometrial fragments were layered on top of the exposed side of the membrane and were incubated overnight at 37°C. Following incubation, the membranes were processed for electron microscopic analysis.

Electron microscopy
After incubation, the amnion samples with the endometrial fragments attached were rinsed thoroughly in PBS, mounted on a piece of cork and fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4). Prior to processing for scanning (SEM) and transmission (TEM) electron microscopy, each membrane was divided into two parts, both containing adhered endometrial fragments. Following fixation, one part of each membrane specimen was dehydrated in alcohol, critical point dried, sputtered with gold and viewed in a Philips 505 scanning electron microscope. The other halves were prepared for transmission...
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**Results**

**Structure of the amnion**

The amnion consists of several different layers that can be easily distinguished: the amniotic epithelium, the basement membrane, a compact connective tissue layer, a fibroblast layer, and a layer of loose connective tissue (Figure 1). The amniotic epithelium appears to be intact, but some isolated degenerating epithelial cells are present (Figure 1).

**Preparation of the amnion**

As observed with SEM, the morphology of the amniotic epithelial cells resulting from both procedures appeared normal (Figure 2A,B). However, many epithelial cells of PBS-stored amnions showed cytoplasmic extrusions (blebbing), whereas very little blebbing was observed on the fresh membranes. Generally, one extrusion per cell was observed located in the centre. TEM showed that the intracellular integrity of the amniotic epithelial cells from fresh amnion specimens appears to be preserved (Figure 2D), whereas no intact intracellular structures could be identified in the amnion specimens that were stored in PBS (Figure 2C). The morphology of the extracellular matrix was not affected by the preparation procedures.

**Adherence of endometrial fragments**

Most cells in the fragments have an intact intracellular ultrastructure after isolation and culture (Figure 3A). Endometrial epithelial as well as stromal cells were observed to adhere to the extracellular matrix (Figure 3A,B).

Endometrial fragments of cycle days 7 and 8 which were layered on the matrix side of freshly prepared amnions had adhered and spread out over the matrix surface, resulting in a monolayer (Figure 3C). When adhering to the extracellular matrix side, cycle day 21 fragments showed very little spreading after attachment (Figure 3D).

When seeded on the epithelial side of freshly prepared
amnions, fragments isolated on cycle days 7 and 8 adhered (Figure 4A), but only to locations where the epithelial lining was either damaged or partly absent (Figure 4A,B). No adherence occurred to intact epithelial cells of the amnion. The adhesive behaviour of endometrial fragments isolated on cycle day 21 was similar to that observed for endometrial fragments obtained from the proliferative phase.

Endometrial fragments layered on the extracellular matrix side of amnions that were stored in PBS for several days, also adhered and spread out over the matrix surface (Figure 5A). When layered on the epithelial side of the amnion, fragments also adhered (Figure 5B). Figure 5C shows an endometrial cell extending towards the basement membrane between two degenerating amniotic epithelial cells. In contrast to fresh amnion, direct cell–cell contact was observed between the endometrial fragments and the apical part of appositioned degenerated amniotic epithelial cells of PBS-stored amnion (Figure 5D).

Discussion
The mechanism of adherence of endometrial cells to the peritoneal lining is still unknown. In order to study these early events, we have developed an in-vitro model using amnion membranes (Van der Linden et al., 1996). Amnion is easy to obtain and has characteristics that are similar to those of peritoneum (Van der Linden et al., 1996).

Depending on the location on the amnion, two subtypes of epithelial cells can be distinguished. At the amnion reflectum (opposite the placental side) the epithelial cells are cuboidal, whereas the amnion at the side of the placenta contains columnar epithelial cells (results not shown). In this study, the amnion reflectum was used because its structure closely resembles that of peritoneum. In addition, the columnar epithelial cells at the placental side are easily damaged by handling.

Storage of the amnion membranes for an extended period of time in PBS at 4°C has detrimental effects on the intracellular ultrastructure of the amniotic epithelial cells (as observed with TEM), although the cell surface morphology of the amniotic epithelium remains intact. With the exception of the blebbing, the cell surface morphology of the PBS-stored amniotic epithelium remains intact. The cytoplasmic extrusions observed on the PBS-stored amnions are probably not the result of secretory activity or other normal cellular functions, because the blebs were not present on the fresh amnions. Instead, the blebbing may be a defence mechanism in the volume-compromised cells because of the hypothermic inactivation of the sodium–potassium membrane pump as a result of storage in PBS at 4°C (Hidalgo et al., 1996).

In contrast to amnion epithelial cells from fresh membranes,
epithelial cells from stored membranes were able to interact with endometrial fragments. Products released by, or present on membranes of the degenerating amniotic epithelial cells may facilitate the adhesion of endometrial cells. Alternatively, cell surface changes may allow cell adhesion molecules of endometrial fragments to establish links with the cytoskeletal remnants of amniotic epithelial cells. Because of the damaging effect of storage in PBS on amniotic epithelial cells, we performed the experiments with fresh amnion.

Endometrial fragments from both the proliferative and the secretory phase of the menstrual cycle adhered to the amnion. This interaction is probably established by integrins and cadherins present on the endometrial cells (Tabibzadeh, 1992; Lessey et al., 1994; Aplin et al., 1996). However, the subsequent spreading on the extracellular matrix of the fragments from secretory endometrium was restricted as compared to that of proliferative endometrium. Others showed that endometrial tissue obtained in the proliferative phase of the cycle, exposed to oestrogen and progesterone during 24 h and injected i.p. into nude mice, resulted in diminished formation of ectopic lesions in this model, when compared to fragments that have been exposed to oestrogen alone (Bruner et al., 1997). Therefore, the growth of endometrium that adheres to the peritoneum is suppressed in a progesterone dominated environment, suggesting that the proliferative phase would be more conducive to ectopic growth of menstrual endometrium than the secretory phase.

The attachment and extensive spreading of fragments from proliferative endometrium on the matrix side of the amnion suggests that the extracellular matrix is a suitable substrate to support endometrial cell spreading and growth. We also observed that adhesion preferentially occurs to subepithelial layers, i.e. to the exposed basement membrane or extracellular matrix. What remains to be determined, however, is whether the observed damage to the amniotic epithelial cells at the sites of attachment is a result of enzyme activity, such as proteases (Casslen et al., 1992; Martelli et al., 1992; Rodgers et al., 1994), originating from the endometrium or is the result...
of selective adhesion of endometrial fragments to sites where the epithelial lining has been traumatized. The finding that damaged amniotic epithelial cells are observed in areas where no endometrial fragments are present would support the latter hypothesis.

The mesothelium covering the peritoneum is very thin and vulnerable. Mechanical challenges to the serosa due to surgical procedures, antibodies, necrosis, residual blood, bacteria, inflammatory cells, toxins and physical and chemical injury can lead to tissue damage (Lorenz et al., 1997) and may explain the high frequency of occurrence of microscopic endometriosis in random biopsies of visually normal peritoneum (Vasquez et al., 1984; Murphy et al., 1986; Nisolle et al., 1990). Recovery of damaged mesothelium requires ~1 week (Zederfeldt et al., 1997), and would therefore allow ample opportunity for endometrial cell attachment. The presence of factors such as TGFβ1 (Oosterlynck et al., 1994) released by macrophages that are attracted to the site of injury, may facilitate the cell–cell or cell–matrix interactions (Dou et al., 1997). In cancer research, the preventive role of intact peritoneum in ectopic cell adhesion has already been shown. Implantation of tumour cells injected i.p. into rats is rarely seen whenever the peritoneum is intact. However, massive adhesion and growth of tumour cells was observed within 2–24 h after the mesothelium was traumatized (Buck, 1973). Wherever attachment occurred in the non-traumatized animals, the mesothelial cells had rounded up and lost contact with each other, allowing the tumour cells to attach to the exposed basement membrane (Buck, 1973). Studies with endothelium have also shown preferential adhesion of i.v. injected tumour cells to traumatized areas (Agostino and Clifton, 1965). These observations support the contention that ectopic cells adhere preferentially to subepithelial structures suggesting that exposition of peritoneal subepithelial structures due to damaging challenges would increase the risk of developing endometriosis in all women with patent Fallopian tubes, rather than the mere presence of retrogradely shed endometrial cells.

In conclusion, we have shown using electron microscopy that the amnion from term placenta is suitable as a model to study the adhesion of endometrial fragments to the epithelial side and to the extracellular matrix side of the peritoneum. Our findings support the hypothesis that the mesothelial lining acts as a defensive barrier which prevents the adhesion of ectopic tissues (Van der Linden et al., 1996). The in-vivo hormonal environment may affect the growth rather than the adhesion of endometrial fragments on extracellular matrix.

Because endometrial biopsy fragments from proliferative and secretory endometrium are not representative of the endometrial cells which are present during menstruation in the peritoneal fluid, we are currently investigating the adhesive behaviour of antegrade shed endometrial cells collected with the ‘Keeper’ device (Koks et al., 1997), to the amnion.

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References


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