Culture of menstrual endometrium with peritoneal explants and mesothelial monolayers confirms attachment to intact mesothelial cells*

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BACKGROUND: To evaluate adhesion of menstrual endometrium (ME) to intact peritoneal mesothelium.

METHODS: Explants of peritoneum were cultured for 1 h with ME (n = 6). Specimens were serially sectioned for haematoxylin and eosin stain and immunohistochemistry using an anti-cytokeratin antibody to label mesothelium. Confocal laser scanning microscopy (CLSM) was performed to identify an intact layer of mesothelial cells (MC) underlying sites of ME attachment. Also, ME and MC were labelled with Cell-Tracker® dyes. ME was cultured with mesothelial monolayers for 1 h (n = 10). Cultures were examined with differential interference contrast and CLSM. Optical sections were taken and a three-dimensional model was constructed. RESULTS: In the peritoneal explants, ME adhered to intact mesothelium. There was no evidence of transmesothelial invasion. CLSM of sections of the explants demonstrated an intact monolayer of cytokeratin positive cells below the sites of ME implantation. Cytokeratin negative and positive ME cells adhered to mesothelial cells. Likewise, the ME attached to cultured mesothelium. Orthogonal sections and three-dimensional reconstruction confirmed an intact monolayer of mesothelium underlying ME attachment sites. CONCLUSIONS: This study confirms that ME adheres rapidly to intact peritoneal mesothelium. Further studies are needed that characterize the mechanisms of ME adhesion to, and migration through, mesothelial cells.

Key words: cell adhesion/endometriosis/mesothelium/peritoneum

Introduction

Using explants of human peritoneum and mechanically dispersed proliferative and secretory phase endometrium, we have demonstrated that endometrial stromal cells (ESC) and endometrial epithelial cells (EEC) rapidly adhere to intact peritoneal mesothelium (Witz et al., 2001a). Using the same model, we have demonstrated that mesothelial invasion occurs by 18–24 h (Witz et al., 1999).

In a separate model, we have demonstrated adhesion of cultured ESC and EEC to cultured monolayers of human mesothelial cells. Confocal laser scanning microscopy (CLSM) was used to evaluate the process of endometrial cell attachment to mesothelial cells. Individual endometrial cells (both ESC and EEC) were followed over a 24 h period. Similar to the model using peritoneal explants and mechanically dispersed endometrium, we observed attachment to mesothelium within 1 h followed by rapid transmesothelial invasion (i.e. <24 h) (Witz et al., 2001b).

Some authors have questioned whether eutopic endometrium collected during the proliferative and secretory phase is the best tissue to model endometriosis (Redwine, 2001). Sampson’s theory regarding the pathogenesis of endometriosis proposes that menstrual endometrial (ME) tissue passes through the Fallopian tubes during menstruation then attaches and proliferates at ectopic sites in the peritoneal cavity (Sampson, 1927).

A previous study has evaluated the potential of antegrade shed ME (i.e. passed through the cervix) endometrium to adhere to the peritoneum. ME was found adherent to peritoneal explants only at locations where mesothelium was absent or damaged (Koks et al., 1999). However, the investigators cultured the endometrium and peritoneal biopsies overnight: a sufficient period of time to allow attachment and transmesothelial migration. The purpose of the present study was to confirm

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that menstrual shed endometrial cells have the capacity to adhere to peritoneal mesothelium. This study was performed to complement our previous studies using proliferative and secretory phase endometrium that have demonstrated adhesion of endometrial fragments to intact mesothelium. In addition, the ability of menstrual shed endometrium to bind to intact cultured, passaged mesothelial monolayers was evaluated.

Materials and methods
Approval for this study was obtained from the Institutional Review Board of the University of Texas Health Science Center at San Antonio.

Tissue collection
Peritoneum
Peritoneum from the anterior abdominal wall was collected from reproductive-age women (n = 6) without endometriosis who were undergoing surgery for benign conditions. The tissue was immediately placed in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma, St Louis, MO, USA) plus 10% defined fetal calf serum (FCS) (HyClone; Logan, UT, USA) and transported to the laboratory. Fat was carefully trimmed. Explants of peritoneum were cut to ~6×6 mm² and placed in 30 mm Petri dishes (Corning, Corning, NY, USA) with the mesothelium facing upward.

Menstrual endometrium
Antegraded ME was collected using a menstrual collection device (Instead® Feminine Protection Cup; Instead, Missoula, MT, USA). Volunteers were asked to wear the Instead® menstrual cup overnight on cycle day 1 or 2. The menstrual cup was placed in the vagina prior to going to sleep. Upon waking, the cup and its contents were placed into Cellgro® Complete Serum Free Medium (Mediatech, Herndon, VA, USA) and brought to the laboratory. Volunteers were asymptomatic, reproductive-age women.

Using aseptic technique, the menstrual cup was rinsed. Menstruum was centrifuged at 365 g for 3 min. Red blood cells were lysed osmotically by exposure to Tris-buffered (Sigma) ammonium chloride (Fischer, Pittsburgh, PA, USA) (140 mmol/l) for 5 min. The ME was then placed in DMEM plus 10% FCS and subsequently filtered through a 250 µm sieve to remove squamous epithelial cells. The tissue retained on the sieve remained in several large aggregates. The aggregates were mechanically dispersed with a scalpel and then by a series of gradually smaller hypodermic needles through 21 gauge.

Explant culture
Approximately 15 mg of endometrium (wet weight) was added to the culture immediately above the peritoneum. The explants were then cultured for 1 h. After 1 h, the explants of peritoneum were fixed and rinsed with phosphate-buffered (Sigma) ice-cold 4% formaldehyde (EM Sciences, Gibbstown, NJ, USA). The base of the explant was grasped with a fine tissue forceps beneath the mesothelial surface. The explants were rinsed and gently agitated in formaldehyde. Explants were then embedded in paraffin.

Histology and immunohistochemistry
Paraffin blocks were serially sectioned and examined at periodic intervals with haematoxylin and eosin stain (H&E). When an attached fragment of endometrium was encountered, 5 µm sections were taken through the explant until the entire fragment of attached endometrium was sampled.

To aid in identification of mesothelium beneath an endometrial implant and to differentiate menstrual epithelial and stromal cells, immunohistochemistry was performed using a mouse monoclonal antibody to cytokeratin 8 (clone 35BH11; Dako, Carpenteria, CA, USA) (La Rocca and Rheinwald, 1984). Endometrial epithelial cells expressed cytokeratin whereas stromal cells did not. Slides were then incubated with rhodamine-conjugated goat anti-mouse IgG (TRITC) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Negative controls were performed by substituting non-immune mouse immunoglobulin (Dako) for the primary antibody. Slides labelled with anti-cytokeratin antibodies were examined using CLSM.

Mesothelial cell culture
Mesothelial cell culture was performed as previously described (Witz et al., 1998; Dechaud et al., 2001). Briefly, mesothelial cells were enzymatically dispersed from sections of peritoneum using 0.1% collagenase, type 1 (Worthington Biomedical, Freehold, NJ, USA) and 0.05% DNase (Sigma). Cells were plated in 25 cm² tissue culture flasks and grown in Eagle’s minimum essential medium (D-valine modification) (Sigma) supplemented with epidermal growth factor (Invitrogen, Carlsbad, CA, USA) (20 ng/ml), 1-glutamine (Gibco, Grand Island, NJ, USA), 1% antibiotic/antimycotic (Gibco), HEPES buffer (Mediatech) and 10% fetal calf serum. When a subconfluent monolayer was obtained, cells were passed.

Following the first passage, mesothelial cells were also plated on 8-well chamber slides (Nunc, Naperville, IL, USA). Morphological assessment of the monolayer cultures was performed using H&E staining. Mesothelial cells were polygonal with irregular borders and had small nuclear:cytoplasmic ratios (Witz et al., 1998). In addition to assessment of morphology, purity of mesothelial monolayer cultures was determined by incubation with monoclonal antibodies to human cytokeratin (Oncogene Science, Uniondale, NY, USA), vimentin (Oncogene Science), CD45 (The Binding Site, San Diego, CA, USA), and von Willebrand factor (Dako, Carpenteria, CA, USA) (La Rocca and Rheinwald, 1984; Witz et al., 1998). Slides were incubated with biotinylated anti-mouse secondary antibody followed by avidin–bixin-peroxidase complex (Vectastain® Elite, Burlingame, CA, USA). The slides were then reacted with diaminobenzidine (DAB) (Pierce Endogen, Rockford IL, USA) and hydrogen peroxide yielding a brown reaction.

Cell labelling
Following the first or second passage, mesothelial cells were incubated with chloromethylbenzoylamidotetramethylrhodamine (CMTMR) (Molecular Probes, Eugene, OR, USA) (10–15 µmol/l) for 5 min. Cells were then plated in 30 mm Petri dishes with a human collagen IV-coated (Sigma) coverslip insert (Matek, Ashland, MA, USA). Cells were grown to near confluence (n = 10).

ME was collected and prepared as described above. Endometrial fragments were labelled with chloromethylfluorescein diacetate (CMFDA) (15 µmol/l) for 5 min. The ME was placed over the CMTMR-labelled mesothelial cells. Following 1 h of culture, the dishes were rinsed with cold formaldehyde. The Petri dishes were then inverted for 1 h in a bath of formaldehyde and non-adherent cells were allowed to separate from the coverslips by gravity sedimentation.

Confocal laser scanning microscopy
CLSM was performed using an Olympus IX70 inverted microscope and the Olympus Fluoview System (Nagano, Japan). Rhodamine was excited with a HeNe green laser (Melles Griot, Carlsbad, CA, USA) at 543 nm. Rhodamine emission was detected using a 560 nm long pass filter. Fluorescein was excited with an argon laser at 488 nm.
Digital images of 512/H11003

ME that was adherent to intact mesothelium. There was no evidence of transmesothelial invasion at any site of attachment. That is, an intact layer of mesothelium could be identified under the endometrial implants that were also circumscribed by an intact layer of mesothelium (Figure 1).

CLSM demonstrated an intact monolayer of cytokeratin positive cells below sites of ME attachment. In many areas underlying large ME fragments, a distinct layer of intact mesothelium was difficult to identify using H&E staining. When serial sections were examined, the resolution of the CLSM allowed for identification of a distinct layer of cytokeratin positive mesothelial cells underlying the site of ME attachment. In addition, cytokeratin labelling of epithelial cells differentiated menstrual EEC from ESC (Figure 2). There was no labelling of tissue when non-immune mouse immunoglobulin was substituted for the anti-cytokeratin primary antibody (data not shown).

Attachment to mesothelial monolayers

Mesothelial cells, grown in monolayers, were polygonal with irregular borders, and had small nuclear:cytoplasmic ratios (Witz et al., 1998) (Figure 3). Mesothelial cells expressed cytokeratin and vimentin, and did not express von Willebrand factor or CD45 (data not shown).

ME adhered to the mesothelial monolayers grown on collagen IV. Cell tracker dyes discriminated mesothelial cells from ME. By taking optical sections at 0.5 µm intervals, orthogonal sections were constructed. The orthogonal sections allowed examination of the cells in the X–Z and Y–Z planes. These sections confirmed an intact monolayer of mesothelium underlying ME attachment sites (Figure 4). Likewise, three-dimensional reconstruction demonstrated ME overlying uninterrupted mesothelium (Figure 5).

Discussion

Some investigators have postulated that mesothelial cells act as a barrier to attachment of ectopic cells to the peritoneum. In a recent study (Groothuis et al., 1999), proliferative phase endometrium from women without endometriosis was cultured with peritoneal explants cultured for 16 h. The explants were then examined with light microscopy and transmission electron microscopy (TEM). Adhesion of endometrial fragments was observed only at locations where the mesothelium was absent or damaged and the basement membrane was exposed. Groothuis et al. concluded that intact mesothelium constitutes a defence barrier that prevents adhesion of endometrial fragments. They hypothesized that trauma to the mesothelial lining is a prerequisite for endometrial cell adhesion. In contrast, studies in our laboratory refute this hypothesis.

Using light microscopy, CLSM and TEM, we have demonstrated adhesion of ESC and EEC to intact mesothelium within 1 h. In addition, we have demonstrated that transmesothelial migration of ESC and EEC occurs within 18–24 h (Witz et al., 1999, 2000, 2001).

Koks et al. reported an in-vitro study evaluating the ability of ME to adhere to amniotic membranes and peritoneal biopsies. After overnight culture, fragments of ME could be seen adherent to amnion and peritoneum. However, when the

Results

Peritoneal explants

All explants of peritoneum had ME endometrium attached after 1 h of culture. Serial sections stained with H&E revealed ME that was adherent to intact mesothelium. There was no

Figure 1. Haematoxylin and eosin stain demonstrating attachment of menstrual endometrium to intact mesothelium. Original magnifications: (A) ×100, bar = 50 µm; (B and C) ×250, bar = 20 µm.

(Melles Griot). Fluorescein emission was detected at 505–525 nm. Digital images of 512×512 pixels were recorded using a speed of 1.69 s/scan. Fluorescein and rhodamine emissions were pseudocolorized with green and red respectively. Images were processed for printing using Adobe Photoshop® (San Jose, CA, USA) on a Dell personal computer (Austin, TX, USA).

Explants were examined with a ×60 1.4 numeric aperture (N.A.) oil immersion objective. Simultaneous bright field images were recorded.

Mesothelial monolayers were examined following 1 h of culture with CLSM using a ×40 1.35 N.A. oil immersion objective and simultaneous bright field imaging using differential interference contrast (DIC). The attachment of menstrual shed endometrium was evaluated on regions of the coverslip where a confluent ‘island’ of mesothelium was identified. In these areas, optical sections were taken through the cells in the Z axis at 0.5 µm intervals. Orthogonal images were then created. A three-dimensional model was constructed using Axiovision® software (Carl Zeiss, Thornwood, NY, USA).
Mesothelial–menstrual endometrium adhesion

Figure 2. Confocal laser-scanning microscope image of menstrual endometrial cells attached to an intact layer of mesothelial cells labelled with anti-cytokeratin antibody. (A and B) Endometrial stromal (Str) cells, which do not express cytokeratin (arrows) can be seen attached to mesothelial cells. (C and D) Endometrial epithelial (Epi) cells expressing cytokeratin (arrows) can be seen attached to mesothelial cells. Original magnifications: (A) ×600, bar = 10 µm; (B) ×525, bar = 10 µm; (C) ×225, bar = 20 µm; (D) ×525, bar = 10 µm.

Figure 3. Differential interference contrast image of confluent mesothelial cell monolayer. Mesothelial cells were identified as squamoid polygonal cells. Original magnification: ×100, bar = 50 µm.

ME was layered on the epithelial side of amnion or peritoneum (i.e. on the side with amnioncytes and mesothelium), adhesion was only described at locations where the epithelium was damaged or absent (Koks et al., 1999). Further work by this group of investigators suggests that ME produces soluble factors that lead to mesothelial disruption and exposure of the underlying basement membrane. The authors conclude that this disruption of mesothelium is requisite for binding of ME (Koks et al., 2000; Demir-Weusten et al., 2000; Dunselman et al., 2001). The present study, using 1 h cultures, rebuts this conclusion. We have demonstrated that ME rapidly adheres to mesothelial cells. Similar to previous studies in our laboratory, the present study used human peritoneal explants to prove that adhesion is possible between mesothelial cells and endometrial cells (Witz et al., 1999, 2000). In addition, the present study also used mesothelial cells grown in monolayers to demonstrate this adhesion. These results suggest that peritoneal mesothelial monolayer cultures, along with CLSM, could reliably be used to evaluate the process of mesothelial cell adhesion. Taken together, the present study confirms previous work in our
Critical to Sampson’s theory is the viability of sloughed endometrial cells and the capacity to implant at ectopic sites (Sampson, 1927). The antegrade shed menstrual tissue collected in the present study consisted of single cells, small aggregates, and glands surrounded by fragments of stroma. These findings are similar to those of a previous study (Koks, et al., 1997). The viability of this ME and the ability to implant at ectopic locations is well established (Keettel and Stein, 1951; Ridley and Edwards, 1958).

Unfortunately, any in-vitro model of the early endometriotic lesion remains hypothetical, as there is no similar condition
the mesothelial cells begin to proliferate over the invading endometrium (Witz et al., 2000). Thus, the transition from attachment to invasion occurs too rapidly to permit observation of endometrial cell attachment to peritoneal mesothelium in vivo. Moreover, rapid invasion of endometrial cells through the mesothelium may explain why some investigators have failed to demonstrate adhesion of endometrium to intact mesothelium (Groothuis et al., 1999; Koks et al., 1999; Dunselman et al., 2001).

In the absence of an in-vitro 'gold standard', it seems prudent to establish a model that most closely resembles the theorized conditions of the peritoneum at the time of endometrial attachment. The majority of peritoneal endometriotic lesions are thought to arise from transtubal passage of ME and attachment to peritoneal surfaces (Witz, 1999). Ideally, menstrual effluent collected from the peritoneal cavity could be used for study. Unfortunately, routine collection of this tissue is not practical. On the other hand, antegrade shed menstruum would be expected to be a reasonable substitute. Further studies are needed that characterize the mechanism of ME–mesothelial cell adhesion and transmesothelial invasion. In addition, comparison of adhesiveness and invasiveness of menstrual endometrium from different groups of women (e.g. patients with and without endometriosis) deserves further evaluation. We believe that peritoneal explants and mesothelial monolayers, as used in the present study, are suitable to accomplish this goal.

**References**


**Figure 5.** Three-dimensional reconstruction performed on 0.5 μm optical sections collected in the Z-plane. The three-dimensional reconstruction demonstrates attachment of menstrual endometrium (green) to the surface of mesothelial cells (red).


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