CELL OF THE MUCOUS MEMBRANE OF THE FEMALE GENITAL TRACT IN CULTURE: A COMPARATIVE STUDY WITH REGARD TO THE HISTOGENESIS OF ENDOMETRIOSIS

GERTRUDE MUNGYER, WIM N. P. WILLEMSEN, RUNE ROLLAND, HANS M. VEMER, FRANS C. S. RAMAERKERS, PAUL H. K. JAP, AND LAMBERT G. POELS

Department of Cytology and Histology (G. M., P. H. K. J., L. G. P.), Department of Obstetrics and Gynecology (W. N. P. W., R. R., H. M. V.), and Department of Pathologic Anatomy (P. C. S. R.), University of Nijmegen, Geert Grooteplein Noord 21, 6500 HB, Nijmegen, Th Netherlands

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SUMMARY

Cellular elements from the mucous membrane of the uterus and oviducts and from peritoneal washings were cultured. The in vitro behavior of these cells was compared to elucidate the histogenesis of endometriosis and the role of various diagnostic procedures.

In 65% of the cultured material obtained by uterine-tubal flushing, proliferating cells of the uterine-tubal mucous membrane were present. Their morphology and behavior corresponded to those of cultured cells obtained by separate washing of the uterine cavity and the tubes, respectively, curedtted material, and biopsies of endometriosis lesions.

Epithelial and stromal cells were identified using phase contrast microscopy, electron microscopy, and immunohistochemical methods. These cell types did not occur in peritoneal washings before the flushing of uterus and tubes. It was therefore assumed that they were detached and transported to the pelvic cavity during the above-mentioned procedures. In view of their intensive proliferation they may form the basis in the development of nodules of endometriosis. This would support the implantation theory concerning the pathogenesis of endometriosis. Interactions between epithelial and mesothelial cells point to the possible role of the latter in encapsulating the endometrial elements.

Key words: endometrium; endometriosis; tissue culture; ultrastructure; intermediate filaments.

INTRODUCTION

Endometriosis is a condition in which tissue resembling the uterine mucous membrane occurs aberrantly in various locations inside or outside of the pelvic cavity. The histogenesis of the disease is unclear. One of the most important theories, the implantation theory of Sampson (18), assumes that endometrial tissues might reach the pelvic cavity at retrograde menstruation, settle on the lining mesothelium, proliferate, and form nodules of endometriosis. This disease has been found frequently in infertile patients, and a relationship between endometriosis and infertility has been postulated (7,11,21). Infertile patients often undergo diagnostic laparoscopy and hysterosalpingography whereby the oviducts are flushed by way of the uterine cavity to test the patency of the tubes. During this procedure endometrial cells might desquamate and be transported to the pelvic cavity, settle, proliferate, and form nodules of endometriosis. The aims of the present study were to investigate whether viable cells of the endometrium reach the pelvic cavity on flushing, to identify the cell types, and to examine their proliferative capacity. For these purposes the material contained in uterine-tubal flushes was investigated by means of tissue culture. The composition of the cell population, the identification of various cell types and their biological properties, as well as their mutual interactions were studied by light and electron microscopy and immunohistochemical methods; their properties and behavior were compared with cultured endometrial cells from various other sources.

MATERIALS AND METHODS

Source of cells. Cell suspensions were obtained from 85 pelvic washings before flushing the tubes; 115 preovulatory uterine-tubal flushes; 8 jet washes of the uterine cavity after ligation of the tubes; 6 tubal washings in patients with isthmic tubal occlusion (3) or with congenital aplasia of the uterus (3); and 30 ascitic fluids from patients with ovarian tumors or peritoneal metastases of other neoplasms.

Tissue fragments of endometriosis nodules from four patients and uterine curettage specimens from six premenopausal patients were cultured. The procedures of gynecological interventions were described in a previous paper (25).

Culture methods. The cell suspensions were collected under sterile conditions into culture medium consisting
### TABLE 1

<table>
<thead>
<tr>
<th>Keratin pKer</th>
<th>Cytokeratin18</th>
<th>Vimentin</th>
<th>OV-TL3</th>
<th>Factor VIII-Related Protein</th>
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<tr>
<td>Epithelium</td>
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<td>Stromal Cells</td>
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<tr>
<td>Mesothelial cells</td>
<td>+</td>
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<tr>
<td>Fibroblasts</td>
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*+/+-++ = Positive reaction; -- = negative reaction; ± = traces of reaction.

of RPMI 1640 with sodium bicarbonate and HEPES (Dutch modification, Gibco-Europe, Paisley, Scotland), 100 U/ml penicillin, and 100 µg/ml streptomycin. After centrifugation the cell pellet was resuspended in fresh medium supplemented with 20% fetal bovine serum. The suspensions were then incubated in plastic tissue culture flasks (Costar, Cambridge, MA) at 37°C in a humidified atmosphere of 5% CO₂ in air. The initial cell numbers were not determined because the admixture of unidentified free cells or tissue pieces or both varied considerably from sample to sample.

For the biopsies of endometriosis the connective tissue capsule was incised by scissors to facilitate cell migration out of the nodules and the entire nodule was subsequently incubated, as described above for the cell suspensions.

The curetted uterine mucosa was minced using two scalpel blades into 1- to 2-mm² pieces, washed with medium, and incubated in the same way.

The culture medium was changed twice a week. Confluent monolayers were dissociated with 0.25% solution of trypsin (Difco, Detroit, MI) and 0.025% EDTA (Merck, Darmstadt, FRG) in Ca²⁺- and Mg²⁺-free Tyrode's solution. Cultures from curettes material were occasionally treated with genetins (Sigma Chemical Co, St. Louis, MO) at a concentration of 100 µg/ml for 24 to 48 h to suppress the proliferation of fibroblasts (6). The cultures were observed daily through an inverted microscope (Diavert, Leitz, Wetzler, FRG) using phase contrast optics.

**Immunohistochemistry.** The cultures were fixed in cold methanol and acetone. After rehydration in phosphate buffered saline (PBS), the first antisera was applied and the cultured cells incubated at room temperature for 30 to 45 min in a moist chamber. After exhaustive washing with PBS (three times, 10 min) the fluorescein-labeled antibody (goat antirabbit or rabbit antimouse IgG conjugated with fluorescein isothiocyanate; diluted 1:20 and obtained from Nordic, Tilburg, The Netherlands) was added and the cells held at room temperature for another 30 to 45 min. After a second series of washes in PBS, cells were mounted in glycerol PBS or in gelvatol (Monsanto, St. Louis, MO) and viewed with a Leitz Dialux EB 20 microscope equipped with epifluorescent illumination using appropriate filters. Using the indirect immunofluorescence technique, cultured cells were tested with the following antisera: polyclonal antikeratin-antisemum pKer as well as polyclonal antivectin-antisemurum pVim (14,15), monoclonal anticytokeratin-antibody RGE 53 reacting only with cytokeratin 18 (16), monoclonal antibody against ovarian carcinoma OV-TL 3 (13) and antibody against Factor VIII-related protein (Central Laboratory of Bloodtransfusion, Amsterdam, The Netherlands).

**Electron microscopy.** Cell colonies in culture flasks were fixed for 1 to 1.5 h in 2% glutaraldehyde and 2% paraformaldehyde (Karnovsky) in 0.1 M phosphate buffer (pH 7.2; 420 mOsm) at 4°C. After rinsing in the same buffer the cultures were postfixed for 1 h in 1% osmic acid in 0.1 M phosphate buffer. The specimens were then dehydrated through graded ethanol solutions and transferred via a mixture of ethanol (100%) and epoxy resin (1:1) into pure Epon 812 as an embedding medium. Cultured colonies were cut out of the flasks and Beem capsules were stuck perpendicularly onto the substrate.

After polymerization, semi-thin sections were cut and stained with toluidine blue. Appropriate areas were selected under the light microscope and ultrathin sections cut with a diamond knife on a Reichert OM U3 ultramicrotome (Reichert, Vienna, Austria). The silver sections were picked up on copper grids, double contrasted with uranyl acetate and lead citrate, and examined in a Philips EM 300 electron microscope (Philips, Eindhoven, The Netherlands).

**RESULTS**

**Free cells and mesothelium.** Various amounts of free cells (red and white blood cells, macrophages) were present in cultures collected from the pelvic cavity. These cells were washed off during the first changes of medium.

Cells derived from the lining mesothelium of the pelvis were a constant component of cultures from uterine-tubal flushes and pelvic washings. Initially they formed epithelioid trabeculae. They proliferated intensively and in 1 to 2 wk epithelioid sheets and confluent monolayers

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**FIG. 1.** Peripheral part of a colony of endometrial epithelium: primary culture of uterine-tubal flush, 8 d. Phase contrast. X184.
developed. With prolonged cultivation, cells of fibroblastlike morphology appeared and often interacted with epithelial and stromal cells of the endometrium (see below). When primary cultures were not subcultured for several weeks, multilayered areas and local piling up could be observed in some cultures, resembling the structures described in cultures of pleural mesothelium by Mouriquand et al. (10).

Ultrastructurally cultured mesothelial cells were connected by moderate amounts of small fascia adherenslike junctions. Well-developed organelles and glycogen particles were clearly demonstrated, as well as electronlucent autophagic vacuoles of varying diameters. Microfilaments and intermediate-sized filaments were also present.

Generally most cells showed a preponderance of short, thin microvilli, but apart from some long, slender microvilli small irregular and blunt ones might be present. A regular phenomenon was the presence of a single intracytoplasmatic vacuole studded with fuzzy-coated microvilli. Among the cells collagen fibrils were detected. Glandular formation of mesothelial cells was not observed.

Both the polygonal and the elongated cell types contained intermediate filaments reacting intensively with antivimentin-antibodies and less strongly with antikeratin-antibodies (Table 1). As the latter reaction is characteristic for epithelial cells, we may assume that the elongated, fibroblastlike cells also originated from the mesothelial lining of the pelvic cavity.

Epithelium. In 65% of cultures from uterine-tubal flushes at laparoscopy a peculiar cell type occurred. Forming flat epithelioid colonies of bizarre, frequently elongated cells with sharp outlines (Fig. 1), they differed distinctly from the polygonal cells usually found in cultures of other epithelial tissues. They resembled the "tadpole"-shaped cells described by Varma et al. (23) in endometrium cultures. In larger colonies they curled around each other in a whorling pattern. Initially they proliferated intensively and formed colonies of various sizes, depending on the number of cells present in the explanted material. Incidentally, even areas of confluent monolayers were formed. However, this cell population had a finite life span. In some small colonies regressive changes could be observed during the 2nd wk. The cells flattened and moved outward. The structure of the epithelial mosaic loosened, and the colony did not grow further (Fig. 2). Some cells became spherical and detached from the substratum. Other colonies retained their morphologic appearance and remained in good condition for several weeks to about 2 mo. Attempts at dissociating with trypsin-EDTA led to considerable loss of cells. A few cells reattached to the culture vessel but did not increase in number. Cells floating in the medium disintegrated gradually.

In cultures of biopsies of endometriosis this cell type occurred in small colonies containing no more than 40 to 50 cells. They persisted for 3 to 4 wk. Thereafter they showed regressive changes, as described above.

The same cell type formed extensive areas in cultures of curedt endometrium. It was also present in cultures from jet washes and tubal washings, but never observed in cultures from pelvic washing before flushing the genital tract nor in those from malignant ascitic fluids. In one case of cultured tubal epithelium (derived from a washing of a ligated oviduct) ciliary movement could be observed during the first 2 wk in vitro.

Ultrastructural findings were comparable to those described earlier (2,3,9,23). The cells were large, tightly arranged, and of varying electron densities. The
clustered cells were connected by fascia adherens-like junctions. When the cells were sectioned in a favorable plane, the fascia adherens-like junctions seemed to be long and twisted (Fig. 3). Neighboring cells showed irregular protrusions or thin, short filopodia, sometimes interconnected by small junctions. The free surfaces exhibited thin bundles of subplasmalemmal microfilaments with protrusions of short, stiffened microvilli. Few cells seemed to be bi- or trimucleated; most were mononucleated. Nucleoli were well defined and nuclear bodies were common in most cases. The profiles of rough endoplasmic reticulum and long mitochondria were especially conspicuous. Polysomes and bundles of microfilaments as well as intermediate-sized filaments were found scattered throughout the cytoplasm. Glycogen particles were found either dispersed or distinctly clustered (Fig. 4). Microtubuli as well as electron-light vacuoles and electron-dense lysosomal structures were noted. Glandular organization of cells forming a true lumen was regularly present (Fig. 4). A few cells exhibited a single cytoplasmic vacuole with microvilli (Fig. 5), also observed in mesothelial cells.

The main differences between the tadpole-like epithelial cells and mesothelial cells were as follows: the former exhibited more and longer fascia adherens-like junctions; they were the only cells found frequently in glandular formations. In contrast to most mesothelial cells with their regular short and thin microvilli, tadpole-like cells showed irregular protrusions or thin and short filopodia. Collagen fibrils were never present among the latter.

By immunofluorescence (Table 1) fine networks of cytokeratin filaments (Fig. 6 A) could be detected throughout the cytoplasm, reacting intensely with the monoclonal RGE 53 antibody specific for glandular epithelium (16) (Fig. 6 B), clearly identifying them from the mesothelial cells that contained distinctly less cytokeratin. Filaments staining with antivimentin-antibodies were present in almost all cultured cells forming networks in the cytoplasm. The cultured epithelial cells stained very weakly with the monoclonal antibody OV-TL 3, as is also the case with frozen sections of the normal lining-glandular epithelium of the female genital tract. The antibody against Factor VIII-related protein did not react with the epithelial cells.

Stromal cells. A cell type growing in epithelial formations, but quite distinct from the true epithelial cells described above as well as from mesothelial cells, was regularly observed in most cultures from uterine-tubal flushes. These cells formed pavement-like colonies of polygonal cells with perinuclear cytoplasmic granulation (Fig. 7). They could be successfully subcultured with trypsin-EDTA and revealed a prolonged lifespan in comparison with the endometrial epithelium. However, in cultures from uterine-tubal flushes they were often overgrown by the more actively proliferating mesothelial cells.

This cell type also represented a regular component of cultures from nodules of endometriosis. Here they differed distinctly from fibroblasts occasionally present in this material as well as in cultures from curretted endometrium before treatment with 100 μg/ml genetin. This antibiotic selectively eliminates fibroblasts from mixed cultures (6). After a 24- to 48-h treatment stromal cells remained intact, whereas fibroblasts showed cytotoxic effects and gradually disintegrated. After treatment with genetin stromal cells were the most actively growing population in cultures of curretted endometrium. They could be subcultured up to 20 times.

Stromal cells were not present in cultures from tubal washings, from peritoneal washings before flushing, nor from ascitic fluid of various origin.

![Fig. 4. Lumen formed by two neighboring cells. Note small junctions (arrowheads), filaments (thin arrows), and glycogen (thick arrow). ×5460.](image)

![Fig. 5. A large cell with a multilobated nucleus and a single cytoplasmic vacuole provided with slender microvilli. ×1260.](image)
Ultrastructurally, in most instances stromal cells seemed to be large cells intermingled with a few small cells. Most of the cells were also arranged pavementlike, but no junctions were observed. At points of close contact between several cells, small, dense subplasmalemmal filamentous plaques were present. The free surfaces were smooth with occasionally a few short microvilli or short, irregular protrusions. Within the intercellular spaces bundles of collagenous fibrils in varying amounts were frequently observed (Fig. 9). The cells were mononucleated, the nuclei mostly bi- or multilobated, sometimes showing distinct nucleoli. With the exception of absence of glycogen and junctions, the presence and amount of organelles, electron-light vacuoles as well as the distribution of the cytoskeleton were very similar to those of the epithelial cells.

Stromal cells contained intermediate filaments that could be decorated with antivimentin-antibodies but not with the polyclonal or monoclonal anticytokeratin-antibodies, the monoclonal OV-TL 3, nor with the antibody against Factor VIII-related protein (Table 1).

Cellular interactions. Epithelial and stromal cells formed either pure or mixed colonies. In some cultures epithelial colonies persisted on the top of stromal monolayers for several weeks up to 2 mo. But, as described above, the epithelial cells degenerated and died earlier than the stromal cells. During the 3rd wk in vitro, very active growth of mesothelial cells occurred and these cells soon surrounded all other cell types in all cultures derived from cell suspensions from the pelvic cavity. In cultures from uterine and tubal flushes they often migrated into the loosened epithelial mosaic but did not invade the compact epithelial colonies. Rather they surrounded them, forming concentric layers around them (Fig. 9 A). At these sites the mesothelial proliferation was especially intensive, and often multilayered walls developed. In some cases mesothelial cells spread over the surface of the epithelium covering it by a more or less continuous cell layer (Fig. 9 B).

Using antikeratin antibodies, strongly fluorescent confluent epithelial sheets were detected at these sites lying under the less intensely reacting mesothelial cells.

**DISCUSSION**

In the majority (65%) of cultures originating from uterine-tubal flushes collected from the pelvic cavity at laparoscopy, proliferating epithelial and stromal cells of the mucous membrane could be found. They were indistinguishable from cultured uterine epithelium-glands or stroma from curetted material, from jet washes, or biopsies of endometriosis. In contrast they did not belong to the regular cell population of the pelvic cavity, as they were never observed in cultures of pelvic washings before flushing nor in cultures of ascitic fluid of varying origins. These findings favor the implantation theory of endometriosis (18).

Based on the study of histologic sections of cell material collected from the pelvic cavity after flushing, Beyth et al. (1) postulated the retrograde seeding of endometrial elements into the pelvic cavity, but the viability and proliferative capacity of the collected cells were not proven by these investigators.

Attempts to culture endometrial cells in monolayer have been reported (2-5,9,12,19,22,23). However, there

**Fig. 6.** Epithelial colony with positive fluorescence of a cytokeratin filamentous network using the polyclonal antiserum pKer (A) and the monoclonal antibody RGE 53 (B). Note less intensive staining in the surrounding mesothelial cells. Primary 11-d culture from an uterine-tubal flush. ×238.

**Fig. 7.** Stromal cells in a primary culture of uterine-tubal flush; 20 d. Phase contrast, ×184.
production were distinctly different from stromal cells lacking junctions and glycogen but with abundant collagen production.

Another possibility to classify various cell types as to their histologic origin is offered by the indirect immunofluorescence, identifying the tissue-specific intermediate filaments. Using antibodies to these proteins, we were able to detect cytokeratin filaments in the tadpole-shaped epithelial cells reacting intensely with the monoclonal RGE 53 antibody, specific for cytokeratin 18 occurring in glandular epithelium. Due to the specific reaction of cytokeratin filament-antibodies, this cell type could be reliably distinguished from stromal cells growing in epithelioid patterns. This is in accordance with the findings of Centola et al. (2) who reported staining of keratin by the PAP technique in endometrial epithelium in vitro.

Stromal cells are known to assume an epithelioid shape in vivo, e.g., as decidual cells during pregnancy. This property distinguishes them from the elongated fibroblasts in vitro. Both cell types contain intermediate filaments decorated by antivimentin-antibodies. The resistance of stromal cells to genetin has proven that they represent a peculiar cell type of mesenchymal origin, quite distinct from fibroblasts which are irreversibly damaged after application of this antibiotic.

None of our cultured cells reacted positively with the antibody against Factor VIII-related protein; the admixture of vascular endothelium can thus be excluded.

The weak reaction of the epithelial cells with the OV-TL 3 antibody corresponds to a similar reaction of the endocervical epithelial lining and tubal lining epithelium in frozen sections of these organs (13).

The limited life span of the epithelial cells in vitro, reported also by other authors (2,3,9,19,22,23) might be due to the lack of environmental or hormonal factors in vitro, or both.

 Cultures from tubal flushes (wash of tubes with isthmic tubal occlusion as well as with congenital aplasia of the uterus) did not contain stromal cells. No morphologic

![Fig. 8. Stromal cells with many polysomes, note intercellularly localized collagenous fibrils. X3700.](image)

has been some confusion concerning the identification of the cell types in vitro. Often no distinction was made between fibroblasts and stromal cells, and the epithelioid appearance of stromal cells in vitro has created difficulties in distinguishing them from the true epithelial elements. A reliable identification based on electron microscopic criteria was presented by Varma et al. (23) as well as by Dorman et al. (4), distinguishing among epithelium, stromal cells, and fibroblasts. This classification has been further confirmed by histochemical methods (20). Our ultrastructural studies are generally in agreement with the findings of the above-mentioned authors. The epithelial cells with true junctions, rigid microvilli, glycogen content, and lack of collagen

![Fig. 9. A, epithelial colony surrounded by a multilayered wall of mesothelial cells (23 d); B, mesothelial cells covering large parts of the same epithelial colony 14 d later. Primary culture of an uterine-tubal flush. Phase contrast, X86.](image)
differences were observed between the growth of the epithelium in these cultures and those from pure uterine mucous membrane (jet washed). Both contained the tadpole-like arrangement of cells, unusual in cultures of various other epithelia. This might be explained by the common origin of both organs from the millerian duct. The possibility of endometriosis originating from the tubal lining was postulated by Sampson (18). Moreover, cases of endometriosis in patients with congenital uterine aplasia have been observed in our clinic and by others (6,17).

Material collected from uterine tubal flushes at laparoscopy did contain viable, actively proliferating epithelial and stromal cells from the uterine or tubal mucous membrane or both. The pelvic cavity may provide a suitable environment for the attachment and further proliferation of these cells leading to the development of endometriotic foci.

The peculiar interaction of the compact epithelial colonies with mesothelial cells in our cultures points to a local stimulation of the latter by the adjacent endometrial epithelium. A similar reaction in vivo also postulated by Sampson (18) may be assumed during the development of the nodules of retroperitoneal endometriosis which are also covered by mesothelium (24).

REFERENCES


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