Menstrual effluent induces epithelial–mesenchymal transitions in mesothelial cells

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BACKGROUND: Menstrual effluent affects mesothelial cell (MC) morphology. We evaluated whether these changes were consistent with epithelial–mesenchymal transitions (EMT). METHODS: Monolayer cultures of MC were incubated overnight in conditioned media, prepared from cells isolated from menstrual effluent, with or without kinase and ATP inhibitors. Changes in cell morphology were monitored using time-lapse video microscopy and immunohistochemistry. Effects on the expression of EMT-associated molecules were evaluated using real-time RT–PCR and/or Western blot analysis. RESULTS: Incubation in conditioned media disrupted cell–cell contacts, and increased MC motility. The changes were reversible. During the changes the distribution of cytokeratins, fibrillar actin and α-tubulin changed. Sodium azide, an inhibitor of ATP production, and Genistein, a general tyrosine kinase inhibitor, antagonized these effects. Wortmannin, a phosphatidylinositol 3-kinase inhibitor, and SU6656, an Src tyrosine kinase inhibitor, only partially antagonized the effect. The expression of Snail and vimentin was markedly up-regulated, whereas the expression of E-cadherin was decreased and cytokeratins were altered. CONCLUSIONS: In MC, menstrual effluent initiates a reversible, energy-dependent transition process from an epithelial to a mesenchymal phenotype. Involvement of the (Src) tyrosine kinase signalling pathway and the changes in the expression of cytokeratins, Snail, vimentin and E-cadherin demonstrate that the morphological changes are EMT.

Key words: E-cadherin/endometriosis/mesothelial cells/Snail/vimentin

Introduction

Endometriosis is characterized by lesions outside the uterine cavity, which consist of functional endometrial glands and stroma. This benign disease occurs during the reproductive years, and affected women suffer from abdominal pain, dysmenorrhea, dyspareunia and infertility. Although the ideas about the pathogenesis of endometriosis remain controversial, most reports support Sampson’s theory that endometriosis results from the adherence of retrogradely shed endometrium to the peritoneal lining, followed by invasion and growth.

The peritoneal lining consists of mesothelial cells which form a monolayer of epithelium-like cells covering the surface of the abdominal cavity. These cells are strongly connected by well developed cell–cell junctional complexes, including tight junctions, adherens junctions, gap junctions and desmosomes (Mutsaers, 2002). An undamaged mesothelial architecture provides an important protective barrier against invading microorganisms and dissemination of ectopic cells (Mutsaers, 2002).

A traumatized or injured peritoneal surface has been shown to result in enhanced peritoneal tumour dissemination and tumour growth (Bouvy et al., 1996; Mathew et al., 1997; Reymond et al., 1998; Gutt et al., 2001). Such damage may be caused by tissue handling and drying out during laparotomy, increased intra-abdominal pressure during CO2 laparoscopy, or port site entry of trochars (Bouvy et al., 1996; Mathew et al., 1997; Reymond et al., 1998; Gutt et al., 2001). It has also been found that continuous ambulatory peritoneal dialysis is associated with recurrent episodes of peritonitis, which results in mesothelial detachment and in persistent peritoneal denudation (Andreoli et al., 1994; Yanez-Mo et al., 2003). This leads to increased intra-abdominal dissemination of cancer cells in these patients compared with non-dialysis patients (Bargman, 2000).

The early pathogenesis of endometriosis remains to be elucidated with regard to the initial interactions between menstrual endometrium and mesothelial cells. In our previous studies (Demir Weusten et al., 2000; Koks et al., 2000), shed menstrual effluent has been shown to induce morphological...
changes in mesothelial cells, which include disruption of intercellular junctions, retraction and exfoliation, and subsequent exposure of underlying extracellular matrix. These morphological changes appeared not to be due to cell death and were likely the result of cellular remodelling (Demir Weusten et al., 2000). We hypothesize that retrogradely shed menstrual effluent interrupts the mesothelial lining by inducing cellular remodelling. This leads to the exposure of submesothelial extracellular matrix and may facilitate the adherence of endometrium fragments on peritoneum.

Similar phenotypical changes have been described by other investigators and were referred to as epithelial–mesenchymal transitions (EMT) (Boyer et al., 2000). This cellular process is a manifestation of epithelial plasticity during embryo and organ morphogenesis, wound healing and tumour progression. During EMT, epithelial cells shift from an epithelial to a mesenchymal phenotype by reorganizing their cytoskeleton. The cellular features of epithelia prior to transformation are loss of polygonal morphology, adhesive cell contacts and cell polarity, development of a fibroblast-like shape with basal cytoplasmic projections, and increased cell motility (Hay, 1995). At the molecular level the zinc finger transcription factor, Snail, has been recently implicated in the switching mechanism for EMT (Nieto, 2002). Snail binds to E-boxes in the E-cadherin promoter and directly represses E-cadherin expression (Cano et al., 2000), resulting in the dissociation of intercellular junctions and an increase in the pool of cytoplasmic β-catenin, a key component of the Wnt signalling pathway. Many transforming epithelia also change their intermediate filaments from cytokeratin to vimentin, a cytoskeletal shift that seems to be mandatory for the start of the transformation process (Perez-Pomares and Munoz-Chapuli, 2002).

In several cell culture models, EMT are induced by a number of tyrosine kinase receptor binding growth factors, such as epithelial growth factor (EGF), insulin-like growth factors (IGF), fibroblast growth factor (FGF), hepatocyte growth factor/scatter factor (HGF/SF) or transforming growth factor-β (TGF-β) (Gavrilovic et al., 1990; Valles et al., 1990; Piek et al., 1999; Morali et al., 2001; Strutz et al., 2002). In these cases, several kinase signalling pathways have been implicated, which involve oncogenic Src, Ras, Raf, phosphatidylinositol-3 kinase (PI3K), Akt kinase, extracellular response kinase (ERK) and mitogen-activated protein (MAP) kinase as well as small G proteins (Rho and Rac) (Chan et al., 2002).

To date, the mechanisms which underlie the effects of menstrual effluent on mesothelial cells are not known. Therefore, we investigated whether the morphological changes in mesothelial cells induced by shed menstrual effluent can be characterized as EMT. To this end, it was evaluated whether the morphological changes are reversible, energy dependent, result from kinase-dependent remodelling of the cytoskeleton (Kellie et al., 1991; Boyer et al., 2000; Timpson et al., 2001; Frame et al., 2002), and involve changes in the expression of Snail, E-cadherin, vimentin and cytokeratin.

Materials and methods

Tissue

The use of human tissue in this study was approved by the Medical Ethical Committee of the Academic Hospital Maastricht, and all women signed a written informed consent. Human omentum (n = 8) was obtained from female patients undergoing abdominal surgery for benign indications. Anterogradely shed menstrual effluent (n = 60) was collected by healthy volunteers (n = 11) who had no history of endometriosis and had a regular ovulatory cycle. The donors collected menstrual effluent in a menstrual cup (Keeper, The Hague, The Netherlands) for 3 h (Koks et al., 1997). Peripheral blood sera were collected from the same individuals.

Mesothelial cell isolation and culture

Isolation of human omental mesothelial cells (HOMEC) and preparation of conditioned media were performed as described in a previous report (Demir Weusten et al., 2000). Briefly, the omental tissue was minced and incubated with collagenase (2 mg/ml, ICN Biochemicals B.V., The Netherlands) in routine medium [Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 supplemented with 10% fetal calf serum (FCS), penicillin 100 IU/ml, streptomycin 100 μg/ml and l-glutamine 2 mM/l, amphotericin 0.25 μg/ml, all from Gibco Life Technologies, The Netherlands] for 20 min at 37°C. The non-digested tissue was removed by a 400 μm stainless sieve (Sigma–Aldrich Chemie B.V., The Netherlands). Subsequently, the cell suspension was sieved through a 100 μm nylon mesh filter (Micronic, The Netherlands) and a 10 μm polyamide filter (Stokvis & Smits, The Netherlands). The cells retained on both of these filters were resuspended in culture medium [minimum essential medium (MEM)/fetal bovine serum supplemented with 10% FCS, 1% ITS (insulin, transferrin and selenium), non-essential amino acids, l-glutamine 2 mM/l, penicillin 100 IU/ml, streptomycin 100 μg/ml and amphotericin 0.25 μg/ml]. Culture medium was from Sigma–Aldrich Chemie B.V. Remaining contaminating stromal cells were separated from mesothelial cells by differential plating. After 30 min of incubation at 37°C, the non-adhering cells were collected and placed in a new flask.

The mesothelial cells are cultured in MEM/fetal bovine serum, which suppresses the growth of fibroblasts. The cells in the cultures are all positive for both cytokeratin and vimentin and negative for the endothelial marker CD34, as was shown previously (Demir Weusten et al., 2000). Confluent HOMEC cultures from passage two were used in the experiments.

Preparation of menstrual serum, conditioned media

After collection, the menstrual effluent was centrifuged at 1200 g for 10 min. The serum was stored and referred to as menstrual serum. The remaining tissue was immediately resuspended in routine medium (1:7 v/v), layered on a Ficoll-Paque gradient (Sigma–Aldrich Chemie B.V.) and centrifuged at 1200 g for 30 min. Endometrial and inflammatory cells were collected from the interphase, washed and cultured in routine medium for 24 h at 37°C and 5% CO2. After culture, the medium was removed and centrifuged at 1500 g for 10 min. This supernatant was referred to as conditioned medium. Sera and conditioned media prepared from different individuals were pooled (n = 10 and n = 12 respectively), filter-sterilized and stored at −80°C until use. Unless it is indicated, pooled sera or pooled conditioned media were used in all experiments.

Induction of morphological transitions in HOMEC

Mesothelial cells were grown in 24-well plates until confluence and subsequently cultured overnight in one of the above-mentioned sera.
and media preparations. Mesothelial cells cultured in peripheral blood sera and routine medium served as controls for the changes in cell morphology. Prior to use in the experiments, sera were diluted with routine medium (1:1 v/v). To test whether the morphological changes were reversible, sera and media were replaced with fresh routine medium and the cultures were continued for 4 days. These experiments were repeated 12 times.

**Visualization of morphological transition with time-lapse video imaging**

Confluent HOMEC monolayers were prepared in 6-well plates which were placed between temperature control plates on a translucent thermo-stage and viewed with a Leica MZFLIII stereomicroscope, equipped with a Donpisha 3-CCD camera. The dish was obliquely transilluminated. Cells were cultured in routine medium or conditioned media. HEPES (45 mmol/l, Gibco Life Technologies) was added to the cultures to compensate for any pH changes in the media. Digital images were prepared every 2 min for a period of 20 h and simulated into a video film using Fast Movie Processor 1.44 software.

**MTT assay**

Effects of conditioned media on the viability of mesothelial cells were determined using the MTT assay. Viable cells convert the soluble substrate MTT to an insoluble, coloured formazan salt. After culturing for 4 days, the viable cells were determined using the MTT assay. Viable cells convert the soluble substrate MTT to an insoluble, coloured formazan salt. After culturing for 4 days, the viable cells were determined using the MTT assay.

**Immunochemistry**

Mesothelial cell monolayers were incubated overnight in routine medium or conditioned media. The morphological changes in cells were characterized by conventional light and immunofluorescence microscopy. A fluorescent isothiocyanate-conjugated secondary antibody (goat anti-mouse IgG, diluted 1:20; Dako, A/S, Denmark) was used to visualise the primary antibodies, which were directed to cytoskeletal proteins to detect the changes in cell architecture. These antibodies were a pan-cytokeratin antibody against cytokeratins 5, 6, 8, 17, 19 (diluted 1:100, MNF116; Dako A/S), an α-tubulin antibody (1:400; Sigma–Aldrich Chemie B.V.). A direct rhodamine-conjugated phalloidin method was used for fibrillar actin (Friedman et al., 1984). In the negative controls the primary antibodies were omitted from the incubation solution.

**Blocking of energy supply and signalling pathways**

To test whether the energy supply of mesothelial cells is related to the morphological changes, sodium azide was added to the routine and conditioned media. Sodium azide inhibits haem-containing proteins, including the cytochromes in mitochondria which are responsible for ATP production, thus blocking the cellular energy supply (Bershadsky and Gelfand, 1981). During the overnight incubation, the routine and conditioned media were supplemented with 0.1% sodium azide.

To evaluate the involvement of kinases in the morphological remodelling of mesothelial cells, HOMEC were incubated overnight in routine or conditioned media in 24-well plates, in the presence or absence of kinase inhibitors. The inhibitors used and their concentrations are listed in Table I. Prior to overnight incubation, HOMEC were preincubated for 30 min in routine medium with vehicle or the inhibitor of interest.

**Kinase inhibitors used in this study**

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<th>Name</th>
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<td>src family kinases (src, fyn, yes, lyn)</td>
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**RNA isolation and complementary DNA synthesis**

HOMEC were cultured overnight either in routine medium or in conditioned media (n = 7). After removal of the medium, total RNA was extracted and purified using Trizol reagent, according to the instructions of the manufacturer (Gibco Life Technologies). Total RNA (1 μg) was incubated with random hexamers (1 μg/μl; Promega, USA) at 70°C for 10 min. The samples were chilled on ice for 5 min. To this, a reverse transcriptase mix consisting of 5×RT–buffer (4 μl), 10 nmol/l dNTP mix (1 μl) (Pharmacia, Sweden), 0.1 mol/l dithiothrietol (2 μl) and superscript II reverse transcriptase (200 IU/μl) was added and the samples were incubated at 42°C for 1 h. The reverse transcriptase was inactivated by heating the samples at 95°C for 5 min. The cDNA was stored at −20°C until further use. In each real-time RT–PCR reaction, 50 ng of cDNA template was used.

**Real-time RT–PCR for Snail, E-cadherin and vimentin expression**

Primers and probes for human Snail, E-cadherin, vimentin and cyclophilin A were purchased from the manufacturer as pre-developed assays (Perkin–Elmer Applied Biosystems, USA). Human cyclophilin A was selected as an endogenous RNA control to normalize for the differences in the amount of total RNA added to each reaction. Human endometrial tissue was used as a positive control in real-time RT–PCR amplifications.

All RT–PCR reactions were performed using an ABI Prism 7700 sequence detection system (Perkin–Elmer Applied Biosystems). The thermal cycling conditions comprised an initial decontamination step at 50°C for 2 min, a denaturation step at 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 1 min. Experiments were performed in duplicate for each sample. Quantitative values were obtained from the threshold cycle number (Ct) at which the increase in the signal associated with exponential growth of RT–PCR products is first detected with the ABI Prism 7700 sequence detector software (Perkin–Elmer Applied Biosystems). The fold-change in expression was calculated using the ΔΔCt method with the cyclophilin A mRNA as an internal control (for details, see user bulletin #2 for the ABI PRISM 7700 Sequence Detection System, available at http://www.uk1.uni-freiburg.de/core-facility/tagman/user_bulletin_2.pdf).

**Electrophoresis and Western blot analysis of E-cadherin, vimentin and cytokeratin**

Confluent monolayers of HOMEC were cultured overnight in routine medium or conditioned media in 24-well plates, in the presence or absence of kinase inhibitors. The inhibitors used and their concentrations are listed in Table I. Prior to overnight incubation, HOMEC were preincubated for 30 min in routine medium with vehicle or the inhibitor of interest.
20 mmol/l Tris–HCl, pH 8, 137 mmol/l NaCl, 1 mmol/l MgCl₂, 1 mmol/l CaCl₂, 10% glycerol, 1% Triton X-100, 1 mmol/l phenylmethylsulphonyl fluoride and protease inhibitor cocktail (Boehringer, The Netherlands). Unsolubilized cell rests were pelleted and the supernatants were stored at –80°C for later use. Protein concentrations were determined with the bicinchoninic acid assay (BCA assay; Sigma–Aldrich).

After the proteins (15 μg/lane) had been electrophoretically separated on a 10% sodium dodecyl sulphate–polyacrylamide gel, they were transferred to a nitrocellulose membrane (Schleicher and Schuell, The Netherlands). The membranes were stained with Ponceau S to evaluate whether the proteins were equally loaded. Non-specific binding sites were blocked with 5% non-fat dry milk in phosphate-buffered saline containing 0.05% Tween 20 (PBST), overnight at 4°C. After washing three times for 5 min with PBST, the blot membrane was incubated with anti-E-cadherin (diluted 1:500; Santa Cruz Biotechnology, USA), anti-vimentin (diluted 1:2000; Cappel, Organon Teknika, USA), and anti-pan-cytokeratin, against cytokeratins 5, 6, 8, 17, 19 (diluted 1:2000, MNF116; Dako A/S), for 1 h at room temperature. At the end of this period the blot membrane was washed three times for 5 min in PBST and then incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (diluted 1:1000, Dako A/S) for 1 h at room temperature. The antibodies were detected by enhanced chemiluminescene using SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA). The bands were analysed using Kodak X-OMAT film.

Results

Culturing mesothelial cells with menstrual sera as well as with media conditioned by shed menstrual cells resulted in morphological changes of these mesothelial cells from an epithelial-like to a fibroblast-like shape (Figure 1a–d). When menstrual sera and conditioned media were removed and the cultures were continued in routine medium for another 4 days, the typical mesothelial morphology was restored (Figure 2a–c). Cell viability, as measured with the MTT assay, was not different for cells cultured in routine medium and in conditioned media.

Time-lapse video microscopy of confluent HOMEC monolayers showed that culture in the presence of conditioned media caused disruption of cell–cell contacts between the mesothelial cells, and led to increased occurrence of membrane ruffling, which gave rise to formation of lamellipodia and filapodia. At the end of the experiment, i.e. after 20 h of culturing, the cells had lost their well-spread morphology and became refractile elongated cells, with stellate, spindle or fibroblast-like appearances, and demonstrated increased motility. These changes were not observed in control routine medium. Time-lapse videos are presented at the website: http://www.grow.unimaas.nl/biology_merg_movies.htm

Immunostainings with antibodies to pan-cytokeratin, fibrillar actin and α-tubulin showed a change in the localization of these cytoskeletal proteins as an effect of conditioned media (Figure 3). In control cultures, cytokeratin filaments were found condensed around the nucleus of mesothelial cells, forming ‘bird nests’ (Figure 3a), whereas after culture in conditioned media the cytokeratin filaments were concentrated in the spindles and extensions (Figure 3b). Fibrillar actin staining was concentrated at the periphery of the cells in control cultures (Figure 3c). After culturing the cells in conditioned media, fibrillar actin was distributed throughout the cytoplasm (Figure 3d). α-Tubulin staining highlighted the presence of a normal microtubule network that extended throughout the cytoplasm of mesothelial cells which were cultured in routine medium (Figure 3e). After incubation in conditioned media, the microtubule network depolymerized as shown by the homogeneous, non-fibrous nature of α-tubulin staining in morphologically changed cells (Figure 3f).

Addition of sodium azide to the conditioned media prevented the morphological alterations in mesothelial cells which were induced by conditioned media from menstrual effluent (Figure 4).

Figure 1. Epithelial–mesenchymal transitions of cultured mesothelial cells induced by menstrual sera and conditioned media. (a) Routine medium, (b) peripheral blood sera, (c) menstrual sera, (d) conditioned media prepared from cells isolated from shed menstrual effluent. Scale bars = 100 μm.

Figure 2. Restoration of the morphological transitions in mesothelial cells. Mesothelial cell morphology after overnight culture in (a) routine medium and (b) conditioned media, and (c) 4 days after replacement of the conditioned media with routine medium. Scale bars = 100 μm.
Genistein was toxic to mesothelial cells at the higher concentrations used (500 μmol/l and 1 mmol/l), whereas SU 6656 did not show any toxicity at all. Genistein was effective in preventing the morphological alterations induced by conditioned medium at 125 μmol/l and 250 μmol/l concentrations (Figure 5). At the highest concentrations used, SU 6656 was also a potent inhibitor of the effects of conditioned medium; however, the reversal was not as complete as observed for Genistein (Figure 5). Wortmannin was toxic at the highest dose (4 μmol/l). At lower dosages, Wortmannin prevented the morphological changes induced by conditioned media, but not completely (Figure 5).

All conditioned media tested induced mesothelial cell remodelling and concomitantly mRNA expression of Snail and vimentin in mesothelial cells, whereas E-cadherin mRNA levels were reduced (Figure 6). Furthermore, the expression of vimentin protein was increased and the expression of E-cadherin protein was reduced after culture in conditioned media compared with culture in routine medium (Figure 7). Western blot analysis with pan-cytokeratin antibody revealed alterations in the expression patterns of acidic type cytokeratins (<55 kDa) as well as heterodimers of acidic and basic type cytokeratins in mesothelial cells after culture in conditioned media (Figure 7).

Discussion
Conditioned media, prepared from cells isolated from menstrual effluent, induced morphological alterations in mesothelial cells in vitro. The typical epithelial-like polygonal

![Figure 3](image)

**Figure 3.** Cytoskeletal rearrangements in mesothelial cells after overnight culture in conditioned medium. (a, c, e) Cells cultured in routine medium. (b, d, f) cells cultured in conditioned medium. The cells are stained for cytokeratin filaments (a, b), actin filaments (c, d), and α-tubulin microtubules (e, f). Scale bars = 40 μm.
morphology of the mesothelial cells changed into a fibroblast-like morphology. Cells dissociated from each other and cell movements increased, as was revealed by time-lapse video microscopy. The effects were fully reversed when the conditioned media were replaced with routine medium and culture was continued for another 4 days.

Similarly, reversible changes in cell morphology have been described as a process called cell scattering, associated with EMT (Boyer et al., 1989, 1996; Hay, 1995). The loss of cell–cell cohesion, presumably due to damaged intercellular junctions and increased cell motility, strongly suggests concomitant changes in the cytoskeleton and this was indeed observed. The changes in immunofluorescence staining of cytokeratin, actin filaments and \( \alpha \)-tubulin demonstrated marked cytoskeletal rearrangements, whereas the MTT assay showed no change in cell viability. Also, the process was found to be reversible. These observations are consistent with our earlier findings (Demir Weusten et al., 2000), and provide evidence that the morphological alterations in mesothelial cells are not the result of an increase in the number of dying cells, but of cytoskeletal remodelling.

Sodium azide antagonized the EMT-inducing effects of conditioned medium. This compound inhibits haem-containing proteins, such as cytochromes in mitochondria which are responsible for ATP production, thus blocking the cellular energy supply (Vanzetti, 1966; Bershadsky and Gelfand, 1981). In other studies, sodium azide has also been shown to prevent the induced disassembly of microfilaments and microtubules (Ma et al., 1995). Apparently, depletion of the ATP pool interferes with ATP-dependent processes such as the phosphorylation of proteins (Wang, 1991), which will prevent the cytoskeletal reorganization. The characteristics of this process, including the reversibility, are consistent with EMT (Boyer et al., 1989; Mutsaers, 2002).

The EMT are a consequence of the activation of signalling cascades through membrane-associated proteins (Boyer et al., 2000). Extracellular matrix components such as collagens, as well as soluble factors, including EGF, HGF/SF, members of the FGF and TGF-\( \beta \) families, have been shown to be directly involved in the induction of EMT (Savagner, 2001). So far, few studies have been performed on EMT in mesothelial cells, and TGF-\( \beta \) (Yang et al., 2003), tumour necrosis factor-\( \alpha \) (TNF-\( \alpha \)) (Zhu et al., 2002) and HGF/SF (Rampino et al., 2001) have been implicated in EMT. Yang et al. (2003) reported similar cytoarchitectural changes of mesothelial cells as a result of continuous incubation for 7 days with TGF-\( \beta \). The mesothelial cells maintained their fibroblastic phenotype despite the withdrawal of TGF-\( \beta \). In the current study the changes were reversible and occurred within 18 h. It is therefore not likely that these changes are mediated by TGF-\( \beta \). TNF-\( \alpha \) and HGF/SF are likely candidates and their role in this process is currently under investigation.

The induction of EMT involves the Src tyrosine kinase and Ras signalling pathways. Ras functions by activating MAP kinase and PI\( _3 \)K signalling pathways (Boyer et al., 1997; Potempa and Ridley, 1998), whereas Src tyrosine kinases phosphorylate adhesion kinase in cellular focal adhesion points, which results in focal adhesion loss during transformation (Fincham and Frame, 1998; Chaudhary et al., 2002).

Genistein, a general protein tyrosine kinase inhibitor, fully antagonized the EMT-inducing effects of conditioned media. This indicates that tyrosine kinases are essential mediators in
EMT induction. However, the Src family protein tyrosine kinase inhibitor, SU 6656, was not able to fully antagonize the EMT-inducing properties of conditioned medium, indicating that other mechanisms may be involved. This is supported by the fact that Wortmannin, a PI3K inhibitor, was able to partially prevent EMT. These findings indicate that Ras and Src tyrosine kinases signaling pathways are involved in EMT induction in mesothelial cells by menstrual effluent. This is consistent with studies on the characterization of EMT in other cell types (Kellie et al., 1991; Hay and Zuk, 1995; Thomas et al., 1995; Gelman et al., 1998; Timpson et al., 2001; Frame, 2002; Frame et al., 2002).

The transcription factor Snail plays a key role in triggering EMT in Drosophila and cultured epithelia (Alberga et al., 1991; Batlle et al., 2000; Cano et al., 2000). Parallel to these reports, the present study provides evidence that the up-

Figure 5. Effects of kinase inhibitors on conditioned medium-induced epithelial–mesenchymal transitions in mesothelial cells. (a–d) Routine medium (a), supplemented with (b) Wortmannin, (c) Genistein, (d) SU 6656. (e–h) Conditioned medium (e), supplemented with (f) Wortmannin, (g) Genistein, (h) SU 6656. Scale bars = 100 μm.
regulation of Snail in mesothelial cells that are cultured in conditioned media is associated with the acquisition of a fibroblastoid phenotype. One of the direct targets of Snail is the promoter of E-cadherin. Snail directly represses E-cadherin promoter activity and E-cadherin expression (Batlle et al., 2000; Cano et al., 2000). When the mesothelial cells were cultured in conditioned media, E-cadherin mRNA expression was down-regulated and E-cadherin protein could no longer be detected. This will result in a disturbance of epithelial cell organization, probably due to a loss of cell–cell contacts. In addition, during this process vimentin expression was strongly increased in the mesothelial cells. This appears to be mandatory to start EMT in mesothelial cells, since it is well known that many transforming epithelia change their intermediate filaments from cytokeratin to vimentin (Greenburg and Hay, 1988). In the mesothelial cells, cytokeratin expression was also markedly altered.

Recently, transition of peritoneal mesothelial cells from an epithelial to mesenchymal phenotype was shown to be induced in vivo and ex vivo when these cells were subjected to continuous peritoneal dialysis (Yanez-Mo et al., 2003). The authors suggested that long-term exposure of the mesothelial cells to the irritating dialysis solutions may lead to complete transition of the mesothelial cells, which could be responsible for tissue fibrosis and failure in ultrafiltration. Patients undergoing continuous ambulatory peritoneal dialysis have an increased risk of the intra-abdominal spread of tumour cells compared with non-dialysis patients (Bargman, 2000). This indicates that the mesothelium serves as an effective barrier against the adhesion of cells. Our earlier findings that endometrial fragments preferably adhere to damaged areas with exposed submesothelial structures (Groothuis et al., 1998; Koks et al., 1999) confirm this.

Based on the evidence provided in this study, we conclude that the morphological alterations induced by factors released from shed menstrual effluent induce EMT. The implication for the clinic is that larger amounts of retrogradely shed menstrual effluent, and a longer exposure to this effluent, will likely lead to a greater insult for the mesothelium and an increased adhesion of retrogradely shed menstrual endometrial tissue. This is supported by the increasing risk for endometriosis when (retrograde) blood flow is heavier and the menstrual periods are longer (Sanfilippo et al., 1986; Darrow et al., 1993; Eskenazi and Warner, 1997).

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References
Reversible transition towards a fibroblastic phenotype in a rat carcinoma cell line. Int J Cancer 4(Suppl), 69–75.


Fincham VJ and Frame MC (1998) The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility. EMBO J 17, 81–92.


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