Morphology and functional characteristics of human ovarian microvascular endothelium

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Corpus luteum formation is characterized by a period of extensive vascularization, as capillaries in the thecal layer of the collapsed follicle following ovulation invade the previously avascular granulosa layer. In order to study these processes in vitro we have developed an endothelial cell preparation from the specific microvasculature of the ovarian follicle. Follicular aspirates, obtained at oocyte collection for in-vitro fertilization (IVF), were filtered to obtain fragments of follicle wall. These were set in Matrigel and then cultured allowing the growth of capillary-like structures through the matrix. Upon emergence from the Matrigel the growing cells formed monolayers with the characteristic cobble-stone morphology of endothelial cells. Immunocytochemistry demonstrated the presence of a range of endothelial-specific markers including von Willebrand factor (vWF), Ulex europeus agglutinin (UEA)-1, CD31 and E-selectin, as well as VCAM-1, which is normally associated with stimulated endothelial cells. RT-PCR analysis showed the expression of two receptors for vascular endothelial growth factor (flt-1 and KDR), and the endothelial nitric oxide synthase, adding further evidence of their identity as human ovarian microvascular endothelial cells (HOMEC). Thus, the novel preparative procedure described now allows the generation of HOMEC cultures from readily available material resulting from IVF procedures.

Key words: angiogenesis/cell culture/endothelial cells/immunocytochemistry/ovary

Introduction

Angiogenesis is critical for many physiological processes including organ development and tissue repair. Tissues of the female reproductive tract uniquely manifest physiological growth and regression of microvasculature (Scott and Bicknell, 1993; Ferrara, 1996; Ferrara et al., 1992; Nelson et al., 1992). Physiological angiogenesis is an especially important feature of corpus luteum development where microvascular endothelial cells located in the thecal layer of the follicle invade the avascular granulosa layer to form an extensive capillary network. This has recently been examined in an elegant study using quantitative estimations of vascularity throughout the ovarian cycle (Suzuki et al., 1998) which confirmed the development of capillaries in the luteinized granulosa layer of the human corpus luteum. This neovascularization is essential for the blood-borne delivery of substrates for steroidogenic cells within the ovary (Augustin et al., 1995; Christenson and Stouffer, 1996a; Ferrara et al., 1998) and enables oestradiol and progesterone to be released into the circulation.

This process of neovascularization is under the control of heparin-binding growth factors, particularly vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), produced by the granulosa-derived cells of the corpus luteum (Anthony et al., 1994; Asaki and Tamura, 1993; Kamat et al., 1995; Shibuya, 1995; Redmer et al., 1996). Studies have shown that in the developing corpus luteum up to 98% of the proliferating cells are of endothelial origin. Investigations into the rate of proliferation showed that endothelial proliferation is greatest during the luteal phase (Rodger et al., 1997). The resulting mature corpus luteum thus contains a considerable percentage of endothelial cells (Bagavandoss and Wilks, 1991; Redmer and Reynolds, 1996; Jablonka-Shariff et al., 1993).

Studies on the control of angiogenesis involving microvascular endothelium would be facilitated by the development of representative, in-vitro physiological models. The use of transformed endothelial cell lines (Hughes, 1996) for studies on angiogenesis is limited by their reduced responsiveness to growth factors. Endothelial cell preparations from large vessels such as the umbilical vein have the advantage of adequate responsiveness to growth factors and have been widely used (Gimbrone et al., 1974; Cockerill et al., 1994). However, there are important differences between these preparations and those from microvascular endothelium (Craig et al., 1998; Fajardo, 1989). In particular, the morphology and responsiveness of microvascular endothelium varies according to the tissue of origin (Richard et al., 1998). Ideally, therefore, a representative model for angiogenesis in the corpus luteum would involve the use of endothelial cells from this tissue. In the present study we aimed to: (i) develop a reproducible and convenient method for the isolation and culture of human ovarian microvascular endothelial cells, and (ii) undertake their morphological and functional characterization.

Materials and methods

Cell cultures

Ethical approval for the use of human tissue in these studies was obtained. All cultures were maintained in M199 supplemented with 10% (v/v) fetal calf serum (heat inactivated), penicillin

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(100 units/ml), streptomycin (100 µg/ml), amphotericin B (2.5 µg/ml) and L-glutamine (2 mM/ml), all from Life Technologies, Paisley, UK.

Human umbilical vein endothelial cells (HUVEC) were harvested from fresh umbilical cords obtained at Caesarean section using the method of Jaffe et al. (1973). Cultures were maintained in supplemented M199 with additional endothelial cell growth supplement (ECGS; 150 µg/ml; Sigma, Poole, UK) using 5% CO₂ in air at 37°C. HUVEC cultures were grown to confluence on 1% (w/v) gelatin-coated 50 cm² flasks. Culture medium was changed every 3–4 days and cells were used up to and including passage 4.

The ECV304 cell line, a spontaneously transformed HUVEC cell line, was obtained from the European Collection of Animal Cell Cultures (CAMR, Salisbury, UK). Cells were maintained in the supplemented M199 with no additional growth factors in 5% CO₂ in air at 37°C. Cultures were grown to confluence on uncoated flasks. Medium was changed every 3–4 days.

Human foreskin fibroblasts for control studies were kindly supplied by the Department of Medical Oncology, Southampton General Hospital and were maintained in supplemented M199 with no additional growth supplement, in 5% CO₂ in air at 37°C. Culture medium was changed every 3–4 days. Cells were used up to passage 10.

In order to isolate human ovarian microvascular endothelial cells (HOMECL), follicular aspirates were obtained at the time of oocyte recovery for in-vitro fertilization. The treatment protocol, described in detail by Jenkins et al. (1991), involved down-regulation of pituitary function with gonadotrophin releasing hormone analogue and stimulation of multifollicular development with human menopausal gonadotrophin. Follicular aspirates were filtered through 70 µm cell strainers (Becton Dickson, Cowley, UK) and the tissue pieces retained were resuspended in M199 by backwashing. Centrifugation (100 g, 10 min) yielded a small pellet which was cooled before resuspension in 0.5 ml of Matrigel (Becton Dickson) at 4°C. Aliquots (50–100 µl) of this mixture were dispensed onto the surface of uncoated 25 cm² flasks and the Matrigel containing the tissue fragments was allowed to set. Cultures were maintained in supplemented M199 with ECGS and VEGF in 5% CO₂ in air at 37°C. Cultures were grown to confluence on uncoated flasks. Medium was changed every 3–4 days.

Histochemistry

In order to confirm the ovarian origin of the fragments obtained by follicle aspiration, histochemical staining for the steroidogenic marker 3β-hydroxysteroid dehydrogenase (3βHSD) was carried out. HOMECL cultures exhibiting cell morphology consistent with endothelial cobblestone growth and the original tissue fragment in Matrigel from which the cells had originally grown were stained according to the method of Aldred and Cooke (1983). Briefly, cultures were washed with HBSS and incubated at 37°C for 2 h with a mixture containing 100 µl 5α-androstane-3β-ol-17-one (2 mg/ml) in dimethyl formamide, 1 ml nitroblue tetrazolium (1.6 mg/ml), 800 µl NAD*-free salt (3 mg/ml) and 4 ml of phosphate-buffered saline (all additions from Sigma). Cultures were then frozen for 1 h at –80°C and thawed at room temperature. The presence of staining for 3βHSD was observed by light microscopy.

Immunocytochemistry

Cells were characterized by fluorescence immunostaining using a panel of endothelial cell-specific antibodies, listed with dilutions in Table I. Confluent and subconfluent cells were plated on 4-well chamber slides and allowed to settle overnight. Plated cells and capillary-like structures in association with Matrigel were fixed in ice cold methanol for 30 min and washed with PBS containing 0.1% Triton. Slides with primary antibodies were incubated overnight at room temperature. After washing in PBS, biotinylated secondary antibodies (10 µg/ml; Vector, Peterborough, UK) were added and slides incubated for 1.5 h at room temperature. After washing and incubation with fluorescein isothiocyanate-conjugated streptavidin (20 µg/ml; Vector) for 1 h at room temperature and nuclear counterstaining with propidium iodide (1 µg/ml; Sigma) specimens were mounted in Mowiol (Harlow Chemicals, Harlow, UK). Slides were viewed under epifluorescence using a Leica TSC 4D confocal microscope.

Polymerase chain reaction

RNA was extracted from cultured cells using RNezol-B (Biogenesis Ltd, Poole, UK) following standard conditions and reverse transcription (RT) carried out using the Stratagene First Strand Kit (Stratagene Ltd., Cambridge, UK). The polymerase chain reaction (PCR) was carried out for 40 cycles using 2 µl RT solution containing the complementary DNA (cDNA) and 2.5u Taq polymerase (Promega, Southampton, UK) at 72°C, 91°C and 54°C. Primers used for generating the specific PCR product from cDNA for the VEGF receptor flt-1 were (5’ to 3’): sense: GTCA GAA GAG GAT GAA GGT GTC TA and antisense: CAC AGT CCG GCA CGT AGG TGA TT. These primers were designed to generate a 414 base pair PCR product (de Vries et al., 1992). For amplifying cDNA encoding the other VEGF receptor KDR (flk-1) the primers sense: ACG CTG ACA TGT ACG TGC TAT and antisense: GCC AAG CTT GTA CCA TGT GAG were used with the same cycle number and temperature for flt-1 to generate a PCR product of 421 base pairs (Charnock-Jones et al., 1994). For amplifying cDNA encoding endothelial derived nitric oxide synthase (eNOS) the primers sense: GTG ATG GCG AAG CGA GTG AA and antisense: ACA TCT CCA TCA GGG CAG CT were used (Janssens et al., 1992) with the same cycle number and temperature for flt-1 and KDR to generate a 244 base pair product. PCR products were subjected to gel electrophoresis, stained with ethidium bromide and photographed under ultraviolet light. Authenticity of products was confirmed by Taq cycle sequencing with primers, free nucleotides and fluorescent dideoxy terminators. Polyacrylamide gels were scanned in an Applied Biosystems Model 373A computer linked automatic sequencing system.

Results

Illustrations of the outgrowth of endothelial cells from the tissue fragments set in Matrigel onto the surrounding plate are shown in Figure 1. After approximately six days in culture, cells were seen to invade the matrix, forming capillary-like structures before moving out on to the surface of the plastic culture flask (Figure 1A). Initially, the cells on the plastic surface had an elongated appearance which became polygonal as the cells became confluent. This cobblestone appearance, typical of endothelial cells, is shown in an area of confluent cells in Figure 1B. Areas of cobblestone cells removed from the primary cultures appeared to be of uniform morphology and were uniformly stained by the various endothelial cell markers investigated (see below). When replated on Matrigel the cells formed complex capillary-like networks, whereas on areas of the culture flask that were not covered by matrix,
monolayers reformed (Figure 1C). Our results therefore suggest that the cultures were not substantially contaminated with other cell types. The cellular morphology and characteristics of the replated cells appeared to be similar to that of the endothelial cells from the original outgrowths.

Table II shows the results of immunocytochemical studies on the cells described above in comparison with HUVEC and ECV304 cells. Fibroblasts were used as a negative control. Specific immunofluorescence for the endothelial cell markers CD31, vWF and UEA-1 was seen in monolayers of cells from follicular aspirates with an intensity similar to positive HUVEC cultures. The ECV304 cell line was negative for CD31 and vWF but showed positive staining for UEA-1. HUVEC and monolayers of cells from follicular aspirates both showed positive immunofluorescence for the adhesion molecule E-selectin but only the latter expressed VCAM-1 though at a low level. The ECV304 cells were negative for these adhesion molecules. The fibroblast preparation was negative throughout as expected. Immunofluorescence for endothelial markers was maintained following capillary formation, as illustrated for CD31 in Figure 2.

Histochemical staining for 3β-HSD was seen within some of the tissue fragments filtered from follicular aspirates and in some specimens endothelial cells were seen growing out from positively stained fragments indicating an origin in a tissue layer containing steroidogenic cells (data not shown).

RT-PCR analysis showed the presence of the correct size double stranded PCR products for the VEGF receptors KDR

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**Table I. Summary of antibodies and dilutions used for immunocytochemistry**

<table>
<thead>
<tr>
<th>Antibody/ Lectin</th>
<th>Antibody source</th>
<th>Type of antibody</th>
<th>Stock concentration (mg/ml)</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Von Willebrand Factor (vWF)</td>
<td>Sigma (F-3520)</td>
<td>Rabbit IgG</td>
<td>12</td>
<td>1:2500</td>
</tr>
<tr>
<td><em>Ulex europeus</em> agglutinin-1 (UEA-1 lectin)</td>
<td>Sigma (L 4889)</td>
<td>—</td>
<td>1</td>
<td>1:10</td>
</tr>
<tr>
<td>E-selectin (CD62E)</td>
<td>R &amp; D Systems (BBA 16)</td>
<td>Mouse IgG1</td>
<td>1</td>
<td>1:100</td>
</tr>
<tr>
<td>PECAM-1 (CD31)</td>
<td>Dako, Bucks, UK (M 0823)</td>
<td>Mouse IgG</td>
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<td>1:40</td>
</tr>
<tr>
<td>VCAM-1 (CD106)</td>
<td>Serotec, Oxford, UK (MCA1131)</td>
<td>Mouse IgG1</td>
<td>0.02</td>
<td>1:10</td>
</tr>
</tbody>
</table>

PECAM-1 = platelet endothelial cell adhesion molecule-1.
Table II. Immunocytochemical characterization of HOMEC, HUVEC, ECV304 and fibroblast cells

<table>
<thead>
<tr>
<th>Antibody/lectin</th>
<th>HOMEC</th>
<th>HUVEC</th>
<th>Fibroblast</th>
<th>ECV304</th>
</tr>
</thead>
<tbody>
<tr>
<td>Von Willebrand factor</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>UEA-1</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>E-selectin (CD62E)</td>
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<td>+++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PECAM-1 (CD31)</td>
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<td>+++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>VCAM-1 (CD106)</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Immunofluorescence: + = weak; ++ = moderate; +++ = intense; – = none.

Figure 2. 3D combined confocal image of specific immunofluorescence for CD31 in a HOMEC capillary-like structure in association with Matrigel. Nuclear counterstaining with propidium iodide.

and flt-1, in both HOMEC and HUVEC, but not in the transformed ECV304 cells (Figure 3A). Expression of eNOS was also seen in HOMEC and HUVEC as demonstrated by RT-PCR, but an equivalent PCR product was not detectable in ECV304 cells (Figure 3B).

Discussion

Human ovarian microvascular endothelial cells cultured from follicular aspirates in the present study exhibited features characteristic of endothelial cells. Firstly, the cobblestone morphology seen when cells formed a contact inhibited monolayer was similar to that observed in HUVEC cultures. As with HUVEC and ECV304 cells, formation of capillary-like architecture by HOMEC was seen on the artificial matrix, Matrigel, which closely mimics the physical properties and functional characteristics of basement membrane. This capillary-like formation appears to be a common characteristic of endothelial cells (Koolwijk et al., 1996; Kubota et al., 1996) and has been demonstrated in ovarian endothelial cells derived from the porcine fetus (Plendl et al., 1996).

Secondly, immunocytochemical analysis showed that HOMEC possess the endothelial cell specific markers CD31, vWF, E-selectin and UEA-1 which were evident with the same fluorescence intensity as seen in HUVEC cultures. However, in contrast to HUVEC, HOMEC constitutively expressed the adhesion molecule VCAM-1 whose expression is more typically seen in endothelial cells stimulated by cytokines (Haraldsen et al., 1996). Its expression in unstimulated HOMEC in the present study may reflect the special role of these cells in interacting with luteal cells and the tissue matrix during the rapid formation of the corpus luteum in vivo.

Currently available models for the study of ovarian endothelial cells have been based on the isolation of cells directly from the corpus luteum. Some studies have involved the purification of dispersed cells from the primate corpus luteum using immunomagnetic separation (Christenson and Stouffer, 1996b). In our experience, this method proved unsatisfactory for the isolation of endothelial cells from the human corpus luteum because problems associated with the incomplete dispersion of tissue did not allow effective separation of endothelial cells from luteal cells (unpublished data). Ethical difficulty of obtaining human tissue from surgical specimens is also an important limitation of this approach. The method described in the present study allows readily available human tissue obtained at egg collections for IVF to be utilized in a simple technique for the culture of HOMEC. This model also has the advantage of using endothelial outgrowth from the theca layer.
which would mimic to some extent the processes involved in corpus luteum development in vivo. Cells isolated using this method are at a stage when they might be expected to have the ability to generate the new capillary growth associated with corpus luteum formation after ovulation. Cells isolated from the mature corpus luteum, however, would be at a stage where proliferation has been completed. Further studies will be required to establish the functional properties of isolated endothelial cells using our method, and also to determine whether the hormonal treatment used in the IVF cycle has an influence on their functional activity. Evidence from bovine models suggests that sub-populations of endothelial cells in the corpus luteum may exist, showing differences in cytoskeleton and responsiveness to growth factors (Spanel-Borowski and van der Bosch, 1990; Fenyves et al., 1993). Establishing the extent of heterogeneity in the HOMEC cultures derived in the present study would require further work.

VEGF plays an important role in corpus luteum formation in a number of species and the present study shows that its effects could be mediated by either or both of the main VEGF receptors, flt-1 and KDR, which are being expressed by HOMEC. The activation of flt-1 has been shown to mobilize calcium ions which are important for eNOS activity (Ahmed et al., 1997). As eNOS was also shown to be expressed in HOMEC, the constituents for important endothelial mechanisms in relation to VEGF action are in place in these cells. The present observations are qualitative in nature and further studies are required to evaluate the role of these mechanisms, and to establish whether they had been influenced in our study by prior hormonal treatment used in the IVF cycle. Survival of microvascular endothelium under serum-free conditions has been demonstrated and it has been postulated that VEGF may act as a survival factor for microvascular endothelial cells under these conditions (Gupta et al., 1997). The proliferative activity of microvascular luteal endothelial cells isolated from the primate corpus luteum has been shown to be stimulated by VEGF (Christenson and Stouffer, 1996b). Taken together, the available evidence thus suggests that VEGF has an important role in controlling the function of microvascular endothelium in the ovary.

A special feature of the ovary is the potential for interaction between steroidogenic and vascular tissues. In a bovine model microvascular endothelial cells were seen to enhance the growth of granulosa cells (Spanel-Borowski et al., 1994). Future studies of HOMEC, luteal cells and matrix interactions will be of great interest, and the present study has shown the feasibility of utilizing an available source of normal human material for such studies.

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References


Characteristics of human ovarian endothelium


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