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Endothelial cell dysfunction following prolonged activation of progesterone receptor

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Progestin-only contraceptives are associated with breakthrough bleeding in up to 50% of users. The causes of blood vessel rupture are not well understood. Here we report that both rmal and Norplant®-exposed endothelium express progesterone receptor. Experiments performed *in vitro* on endothelial cells isolated from human endometrium revealed that long-term progesterone exposure leads to suppression of endothelial cell proliferation, inhibition of migration and alteration in the profile of extracellular matrix proteins secreted by human endometrial endothelial cells. In addition, we detected increased levels of matrix metalloproteinase-9 in endothelial cultures treated with progesterone. The effect of progesterone on the cell cycle, along with the increased amounts of matrix-degrading enzymes, could account for breakdown of basement membrane components, vascular fragility and consequent vessel rupture leading to breakthrough endometrial bleeding.

Key words: angiogenesis/endothelial cell/migration/progesterone/proliferation

Introduction

During a woman's reproductive years, the endometrium undergoes cyclic changes of proliferation, differentiation, and cell death, in parallel ith the growth and maturation of ovarian ollicles and under the regulatory control of steroid hormones. Nonetheless, the participation of steroid hormones as direct mediators of endothelial physiology has been difficult to establish. We have recently demonstrated that progesterone receptor (PR) is functional in endometrial endothelial cells and that it regulates cell cycle progression in this cell type (Vázquez *et al.*, 1999).

Progesterone influences the expression of a variety of genes mediated by the progesterone receptor, a nuclear transcription factor (Horwitz *et al.*, 1985; Mauvais-Jarvis *et al.*, 1986; Savouret *et al.*, 1988; Press *et al.*, 1989; Kastner *et al.*, 1990). Ligand binding to this receptor unmasks a zinc finger DNA-binding domain that conveys target gene specificity and transcriptional activation via specific *cis*-acting sequences termed hormone response elements (Misrahi *et al.*, 1987, 1988; Theveney *et al.*, 1987). In the primate endometrium, the increase in circulatory progesterone causes a shift from the proliferative to the secretory phase by promoting metabolic changes and structural remodelling (Ferenczy and Bergeron, 1991). The levels of PR are increased in response to elevated oestradiol during the proliferative phase (Okulicz *et al.*, 1989). In the subsequent secretory phase, progesterone down-regulates the oestrogen receptor (Press *et al.*, 1986), the resulting effect redirects the hormonal response of the endometrium (Bhakoo and Katzenellenbogen, 1977; García *et al.*, 1988).

Exposure of the endometrium to progesterone under physiological conditions is cyclic and short. Interestingly, long-term use of progestins as a means of contraception can induce a significant

number of alterations in the endometrium. Vascular fragility and rupture is common, followed by endometrial atrophy. The reasons for breakthrough bleeding in the early stages of progestin contraceptives are not known. The bleeding problems generally occur in the first few months of therapy, subsequently the endometrium becomes atrophic and more women experience amenorrhoeic cycles (Fraser and Diczfalusy, 1980). Morphological studies have demonstrated that long-acting steroidal contraceptives can suppress the proliferative activity of the glands and stroma, cause underdevelopment of the arterioles, degenerative changes in the venules, and lesions in the vascular endothelium (Macqueo, 1980; Landgren *et al.*, 1982; Lan *et al.*, 1984). It is possible that under this regimen, long-term exposure to progestin interferes with the action of oestradiol by suppressing the synthesis of steroid receptors (Critchley *et al.*, 1993). This scenario has broad implications for the ability of a progestin-treated endometrium to respond to oestradiol (Fraser and Diczfalusy, 1980); however, further efforts are required to establish the mechanism of action of these progesterone-like drugs. At present, it appears that oestrogen and progesterone act together as inducers of bleeding and that a detailed study of steroid hormones on endometrial capillary endothelial cells might provide the basis for a more rational and effective clinical management of breakthrough bleeding disorders.

In this study, we investigated endometrial endothelial cell expression of PR *in vivo* and *in vitro*.

Materials and methods

Endometrial tissue

Norplant®-exposed, as well as unexposed ('normal'), endometrial specimens were obtained from women aged 18–40 years who underwent hysterectomy for benign conditions or by endometrial biopsy. The endometrial biopsies from normal women were performed on cycle days 13 and 21. The tissue was histologically normal, and the histological dating and serum oestrogen, progesterone, and LH drawn on the day of biopsy were consistent with stage of the cycle. Informed consent was obtained for the endometrial biopsies, and the protocol was approved by the Human Subjects Committee, Beth Israel Hospital (Boston, MA, USA). Norplant-exposed endometrium was obtained from women with 3–11 months of treatment.

Tissue culture and immunohistochemistry

Human endometrial endothelial cells (HEEC) as well as endothelial cells from other organs were isolated and cultured as described previously (Iruela-Arispe *et al.*, 1999).

For immunohistochemistry, tissue was frozen in OCT (Fisher Scientific, Pittsburgh, PA, USA) and sectioned on a Zeiss cryostat at 5 μm thickness. Sections were fixed for 15 min with vapours of 4% paraformaldehyde and washed in phosphate-buffered saline (PBS). Sections were then digested briefly with 0.25% trypsin (2.5 mg/ml) to unmask epitopes, incubated with 3% hydrogen peroxide in 70% methanol to minimize endogenous peroxidases and blocked with 10% normal go serum (Sigma, St Louis, MO, USA) for 2 Incubation with primary antibodies, PR (a generous gift from Dr Dean Edwards; Department of Pathology, University of Colorado Health Sciences Center, Denver, CO, USA), and platelet endothelial cell adhesion molecule (PECAM) (25 μg/ml; Pharmingen, San Diego, CA, USA), was performed for 2 h at room temperature. Specimens were then washed, incubated with biotinylated secondary antibodies and avidin–biotin–peroxidase for PR detection and alkaline phosphatase for PECAM visualization. Sections were counterstained with nuclear fast red.

For immunocytochemistry of cell cultures, endothelial cells were briefly fixed in 4% paraformaldehyde, washed in PBS and blocked with 1% goat serum. For detection of von Willebrand's factor (vWF), cells were incubated with polyclonal antibody to vWF (10 μg/ml. Dako, Carpinteria, CA, USA) followed by anti-rabbit biotinylated (3 μg/ml) and avidin–fluorescein isothiocyanate (Vector Laboratories, Burlingame, CA, USA). Endocytosis of acetylated low-density lipoprotein (Ac-LDL) was determined by incubation of cells in DMEM (Dulbecco's modified Eagle's medium) containing 10 μg/ml of 1,1'dioctdecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate acetylated low-density lipoprotein

(Dil-Ac-LDL) (Biochemical Technologies, Inc., Stoughton, MA, USA) at 37°C for 4 h. Cells were subsequently fixed for 30 min in 3% paraformaldehyde. Coverslips were then mounted in 50% glycerol in PBS and photographed on a Zeiss photomicroscope with Ektachrome 1600 ASA film.

For detection of PR on isolated cells, endothelial cells were cultured on coverslips and stimulated for 48 h with 20 nmol/l 17-β-oestradiol prior to fixation. PR antibodies were a generous gift from Dr Dean Edwards and were used as previously described (Vázquez *et al.*, 1999).

Isolation of total RNA and Northern blot analysis

Cultures of endothelial cells were treated for 2 days with 10 nmol/l 17-β-oestradiol to stimulate PR mRNA. Isolation of total RNA was performed following a single-step procedure (Chomczynski d Sacchi, 1987). Quality of RNA was determined electrophoresis on 1% agarose gels. For generation of Northern blots, 20 μg of total RNA was resolved on a 1% agarose gel and transferred to nylon membranes (Nytran; Schleicher & Schuell, Keene, NH, USA). RNA was then cross-linked by UV light and pre-hybridized at 42°C for 2–5 h in a solution containing: 50% formamide, 6×SSPE (1×SSPE = 150 mM NaCl; 10 mM NaH_2 PO_4; 1 mM EDTA), 1×Denhardt's solution, 0.1% SDS, and 100 μg/ml of heat-denatured salmon sperm DNA. Hybridization with [^{32}P]cDNA probes proceeded in the same solution at 42°C for 12–18 h. Probes were labelled by random priming using Multiprime kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and purified on Sephadex G-50 (Promega, Madison, WI, USA).

The progesterone receptor cDNA fragment was generated by reverse transcriptase and polymerase chain reaction utilizing the following primers that correspond to the human sequence of PR: (i) upstream, nucleotides 57–79; and (ii) downstream, nucleotides 584–605, considering the star codon as nucleotide number 1. The fragment was subcloned into pGEMT vector (Promega) and sequenced. Generation of the 28S rRNA cDNA gment has been described elsewhere (Iruela-Arispe *et al.*, 1991). For radiolabelled probes, plasmids were digested with *Eco*RI, fragments were resolved on a 2% agarose gel, and isolated by electroelution.

Proliferation assays

HEEC were made quiescent by incubation of confluent cultures for 2 days in the absence of serum and growth factors. Cultures were then seeded in 24-well plates in phenol red-free EBM (endothelial cell basal medium) (Clonetics, San Diego, CA, USA) supplemented with 1% fetal calf serum (FCS), basic fibroblast growth factor (FGF-2) (4 ng/ml), and vascular endothelial growth factor (VEGF) (50 ng/ml), in the presence of either progesterone (Sigma), the agonist R5020 (Promegestone; NEN Life Science Products, Boston, MA, USA), or vehicle control. Cells were washed and fixed in 10% TCA (trichloroacetic acid). Incorporation of [^{3}H]thymidine was determined by scintillation counting as previously described (Vázquez *et al.*, 1999). Experiments were done in quadruplicate and were performed with three independent isolates of cells from different specimens.

Migration assays

Migration assays were performed on modified Boyden chambers with an 8 μm pore polycarbonate membrane (Corning Costar Corporation, Cambridge, MA, USA) using 10 μg/ml of fibronectin as a haptotactic agent. The membrane was incubated with the fibronectin solution in phenol red-free DMEM for 2 h and was then blocked with 0.5% BSA for 1 h. Quiescent cells were harvested with trypsin (0.5 mg/ml). After inactivation of trypsin using DMEM containing 10% serum, cells were washed with Hanks' balanced salt solution, resuspended in phenol red-free EBM and added to the upper compartment of the chamber (1×10^5 cells/chamber). VEGF (50 ng/ml) was added to the lower chamber in the presence or absence of progesterone (0.1 μmol/l). After 3 h at 37°C and 5% CO_2, membranes were washed, fixed in 3% paraformaldehyde, stained with Toluidine blue and destained as previously described (Jasiulionis *et al.*, 1996).

Metabolic labelling

Cells were plated on 35 mm^2 dishes and incubated in the presence or absence of 0.1 μmol/l

progesterone for 1, 3 or 6 days. At the last 24 h of each time point, medium was substituted with fresh medium containing 50 μCi/ml of [^{3}H]proline. Conditioned media were spun to remove cell debris, and the following proteinase inhibitors were added: 6.3 mmol/l *N*-ethylmaleimide, 2 mmol/l phenylmethylsulphonyl fluoride, and 0.5 μg/ml of peptstatin A. Media were dialysed in 0.1 N acetic acid, lyophilized, and resolved on a 2–15% gradient sodium dodecyl sulphate–polyacrylamide electrophoretic gel. An equivalent number of counts (100 000 c.p.m.) was analysed for each experiment.

Zymography

Conditioned media were collected at the same time points as indicated in the metabolic labelling assays and clarified by centrifugation. Volumes of conditioned media representative of equalized cell number in each well were subjected to fractionation and zymographic analysis in gelatin substrate gels. Gels were incubated for 48 h at 37°C in a buffer containing: 50 mmol/l Tris, pH 8.0, 5 nmol/l $CaCl_2$, and 0.02% sodium azide. Gels were then fixed and stained with Coomassie blue. Clear bands were identified by electrophoretic motility. Gels were photographed by transillumination.

Results

To ascertain whether the pathological effects of Norplant could be directly related to prolonged activation of PR on endothelial cells, we initially examined the expression of PR on normal and Norplant-exposed endometrial specimens. Figure 1 shows a series of immunohistochemical analyses for simultaneous visualization of capillaries and PR expression. The receptor was detected in a subpopulation of endothelial cells in the vessels of normal secretory endometrium (Figure 1a). In addition, expression of PR was also seen in Norplant specimens (Figure 1b). Norplant biopsies were characterized by presence of erythrocytes throughout the stroma (Figure 1c). The presence of PR in endothelial cells was seen in all Norplant biopsies examined (seven in total). Although we did not perform rigorous quantification, it did not appear that the relative number of PR-positive endothelial cells changed upon use of Norplant. Nonetheless, we speculated that prolonged and constant exposure to progestins could lead to alterations in endothelial physiology. To evaluate the effect of long-term progestins, we isolated endometrial endothelial cells and initiated a series of experiments to test progesterone exposure to endothelial cell physiology.

Isolation of HEEC was performed as previously reported (Iruela-Arispe *et al.*, 1999). Primary cultures were purified to homogeneity and expressed typical endothelial markers. Confluent monolayers showed a cobblestone appearance (Figure 2a) and exhibited contact inhibition. Endometrial endothelial cultures expressed von Willebrand's factor and were able to endocytose low density lipoprotein (Figure 2b,c respectively).

More importantly, these cells retained expression of PR. PR transcripts were identified in early passage cultures of HEEC by Northern blot analysis (Figure 3A). The expression levels varied with the isolate and decreased significantly upon passage number. Endothelial cells isolated from other organs also expressed PR mRNA. Figure 3A sho transcript in human dermal endothelial cells, yet no expression was detected in umbilical vein endothelium or in lung-derived endothelium. We have previously demonstrated that PR is functional on dermal endothelial cells by direct binding analysis, transactivation assays, and competition with progesterone antagonists (Vázquez *et al.*, 1999). Presence of PR protein was further evaluated by immunocytochemistry on endothelial cell cultures. Variable degrees of protein levels were detected in the nucleus in most cells (Figure 3B).

The effects of progesterone on endothelial physiology appear to be suppressive in nature. We have previously shown that progesterone can inhibit proliferation of dermal endothelial cells (Vázquez *et al.*, 1999); here we demonstrate that HEEC proliferation induced by VEGF and FGF-2, can also be suppressed by the progestin analogue R5020 (Figure 4A). In addition, progesterone inhibits VEGF/FGF-2-driven endothelial cell migration, as we were able to show using a variable number of strains of HEEC primary cultures (Figure 4B). The specific mechanism that mediates this effect is unknown. Exposure to progesterc could affect attachment to the extracellular matrix, organization of the cytoskeleton, or signalling of

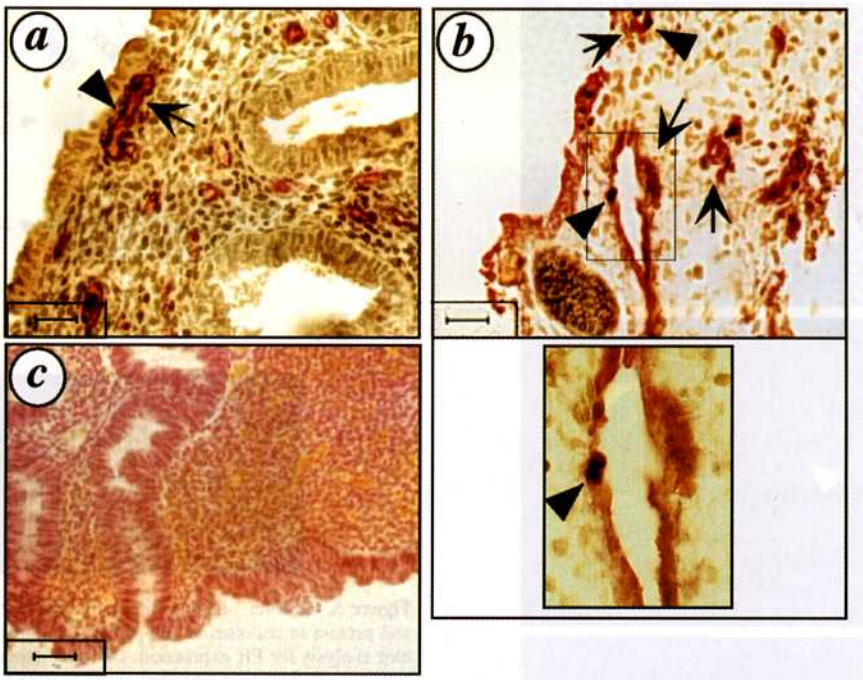

Figure 1. Progesterone receptor (PR) is expressed in a subset of endometrial endothelial cells. Endometrial biopsies from normal (**a**) and Norplant-exposed endometrium (**b–c**) were sectioned, fixed, and incubated with PR antibody to detect both PR A and B, as well as platelet endothelial cell adhesion molecule (PECAM) antibody to identify blood vessels (**a** and **b**). PR was detected by using a biotinylated secondary antibody and avidin–peroxidase method (in brown), while PECAM was identified by using an alkaline phosphatase method (in red). Arrows identify vessels, and arrowheads indicate positive nuclei. Negative control (**c**) included lack of both primary antibodies. Notice in **b** the presence of red blood cells (orange) diffused in the stroma and indicative of breakdown of blood vessels and haemorrhage. Bar indicates 50 μm.

small G proteins such as cdc42, Rho, and Rac, all of which could lead to suppression of migration.

We also evaluated whether progesterone could alter the profile of matrix proteins secreted by endothelial cells. Interestingly, exposure to physiological levels of progesterone for 3–6 days induced secretion of several extracellular matrix proteins in comparison with control cultures (Figure 5A). In addition, long-term exposure to the hormone also resulted in alteration of several secreted proteins. We do not know the identity of the proteins that were altered, yet it appears that progesterone can directly induce a large spectrum of modifications.

Finally, we have also examined the effect of progesterone on the secretion of matrix metalloproteinases by the endothelium. We found that exposure to progesterone for 3 days leads to increased levels of matrix metalloproteinase (MMP)-9 (Figure 5B). This increase appears to diminish after 6 days of progesterone treatment, yet concentrations do not return to baseline. This MMP can have a significant effect on the integrity of the basement membrane and perhaps lead to vascular instability.

Discussion

In the normal adult, the expansion of capillary networks is, for the most part, restricted to situations of tissue repair as response to injury. An exception is found in the human endometrium. The cyclic nature of endometrial growth requires recurrent growth of new capillaries (Rogers *et al.*, 1998). Unlike the capillary growth of tumours, vascular expansion and repair in the endometrium appears to be tightly regulated; indeed, this tissue constitutes one of the best examples of self-limiting angiogenesis within the normal adult. Nevertheless, major gaps exist in our current understanding of the neovascularization of this tissue, as well as the

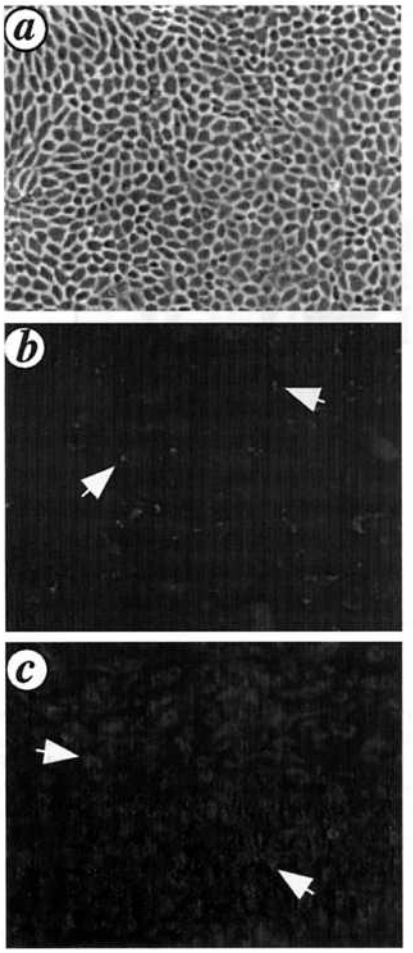

Figure 2. Isolation of human endometrial endothelial cells. Tissue specimens from human endometrium were used to purify endothelial cells to homogeneity (Iruela-Arispe *et al.*, 1999). Cultures formed a cobblestone monolayer (**a**) typical of endothelial cells. Expression of von Willebrand's factor (**b**) was verified by immunofluorescence. Endocytosis of acetylated low-density lipoprotein (**c**) further indicates the purity of the cultures. Arrows in **b** and **c** indicate positive cells.

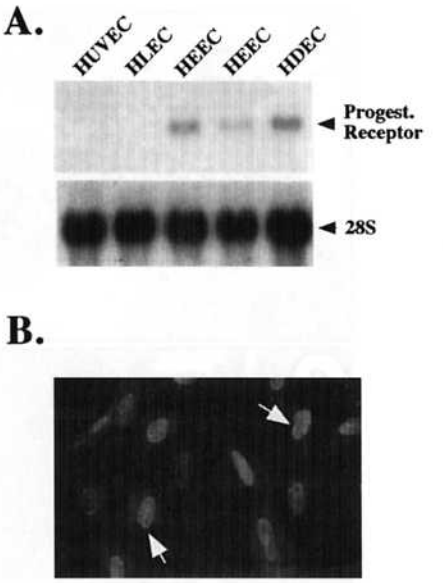

Figure 3. Presence of progesterone receptor (PR) transcript and protein in endothelial cell primary cultures. (**A**) Northern blot analysis for PR expression. Different lanes represent: human umbilical vein endothelial cells (HUVEC), human lung endothelial cells (HLEC), two strains of human endometrial endothelial cells (HEEC) and human dermal endothelial cells (HDEC). 28S probe was used for evaluation of loading and transfer efficiency. (**B**) After isolation and further purification, HEEC were briefly fixed with paraformaldehyde, blocked, and incubated with progesterone receptor antibodies that recognize PR A and B. A variable degree of PR in the nucleus can be seen in most cells.

mechanisms that lead to vascular repair upon menstruation. Even less is known about pathological states, such as breakthrough bleeding associated with use of long-term progestin-only contraceptives. In this study, we have provided evidence that progesterone directly affects several aspects of endometrial endothelial physiology, some of which could destabilize the vascular basement membrane resulting in capillary rupture and haemorrhage.

The hypothesis that endometrial endothelial cells could be directly affected by steroid hormones has been considered previously. Nonetheless, the lack of convincing data on expression of functional PR in these cells has discouraged further investigation. In fact, most of the available literature on expression of PR on endothelial cells is contradictory. Studies performed by Colburn and Buonassisi reported that endothelial cells express steroid receptors (Colburn and Buonassisi, 1978), while a later study stated that the expression of both progesterone and oestrogen receptor was only present in smooth muscle cells of spiral arteries (Perrot-Applanat *et al.*, 1988). Koji and Brenner defended the position that oestrogen receptors were not expressed by endothelial cells, vascular smooth muscle cells or even perivascular stromal cells (Koji and Brenner, 1993). The discrepanc between these studies might be a reflection of the immunocytochemical procedure followed. Vari-

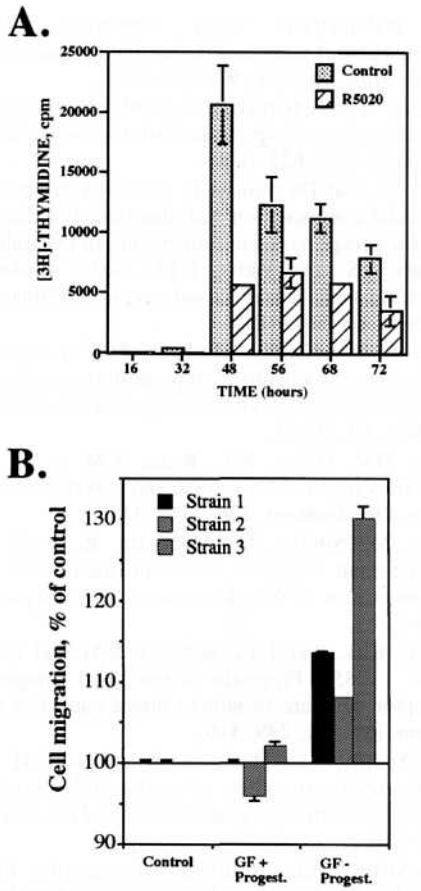

Figure 4. Effect of progesterone (Progest.) on endothelial cell proliferation and migration. (**A**) Quiescent human endometrial endothelial cells (HEEC) were cultured in 24-well plates in the presence of 0.1% fetal calf serum, vascular endothelial growth factor (VEGF) (50 ng/ml), basic fibroblast growth factor (FGF-2) (4 ng/ml) and in absence (control) or presence of the progesterone analogue R5020 (1 μmol/l). A pulse of [^{3}H]thymidine was given 8 h prior to harvesting the cell layer (time point indicated in the graph). (**B**) Quiescent HEEC were cultured in the presence of VEGF (50 ng/ml), FGF-2 (4 ng/ml), and in the presence or absence of progesterone (0.1 μmol/l) in phenol red-free Dulbecco's modified Eagle's medium for 3 h in a Boyden Chamber. Three independent strains of HEEC were used for each treatment. Graph represents the percentage of migrating cells versus the control (100%) in the absence of growth factors (GF) and progesterone. Each point was done in triplicate.

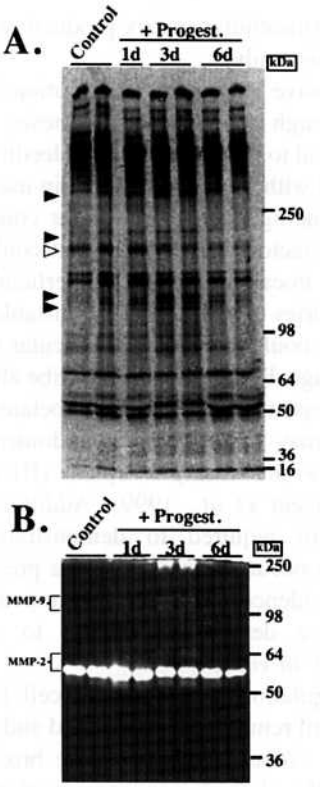

Figure 5. Effect of progesterone (Progest.) on extracellular secretion and gelatinase profile of human endometrial endothelial cells (HEEC). (**A**) HEEC were incubated in phenol red-free endothelial cell basal medium supplemented with growth factors and 0.1% charcoal filtered serum for 1–6 days in the presence or absence of 0.1 μmol/l progesterone. A pulse of [^{3}H]proline was given 24 h before each time point. Conditioned media was removed and precipitated by trichloroacetic acid. Equal counts (100 kc.p.m.s) were loaded on each lane and separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Molecular weight standards are indicated on the right. Arrowheads show a number of bands that appeared after 3 days of continuous progesterone treatment. (**B**) HEEC were incubated as described in **A**. Conditioned media were removed and subjected to gelatinase zymography. After migration was completed the gel was stained in Coomassie blue and destained to visualize gelatinases [matrix metalloproteinase (MMP)-2 and -9].

ability in the preservation of antigens, and sensitivity and specificity of antibodies have been a source of conflicting findings for other proteins. Furthermore, the identification of small capillaries in the endometrial stroma is complicated by the high cellularity of this tissue and difficulty in the identification of capillaries. We have recently performed a detailed study that demonstrates the presence of PR in a variety of endothelial cells (Vázquez *et al.*, 1999). Levels of PR were significantly lower than those displayed by stromal cells, yet the receptor was functional and activation resulted in endothelial cell cycle arrest (Vázquez *et al.*, 1999), suppression of migration, and altera-

tions in extracellular matrix production, including MMPs (this study).

Suppressive effects on proliferation and migration, although arresting angiogenesis, might not directly lead to the breakthrough bleeding episodes associated with prolonged progestin use. Nonetheless, depending upon extracellular concentrations of growth factors, cell cycle arrest could result in apoptosis. Focal cell death in superficial endometrial capillaries together with an unstable basement membrane could then result in vascular rupture and haemorrhage. Recent studies describe alterations in vascular basement membrane-associated components that may be involved in endometrial breakdown in women using Norplant (Hickey *et al.*, 1999; Vincent *et al.*, 1999). Additional investigations are required to demonstrate if these alterations occur *in vivo*. The data presented here provide evidence that prolonged activation of PR could have detrimental effects to endothelial physiology *in vitro*.

The regulation of endothelial cell function by steroids still remains an unexplored and potentially important avenue that could have broad implications for the clinical management of endometrial pathologies, in particular breakthrough bleeding with the use of long-term progestins.

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