The role of endothelial cells in the pathogenesis of ovarian hyperstimulation syndrome

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Ovarian hyperstimulation syndrome (OHSS) is an iatrogenic complication of treatment with fertility drugs. Using human lung microvascular endothelial cells (HUMEC-L) as an in-vitro model of OHSS, we have tested the hypothesis that the endothelium is a target of HCG in the pathogenesis of OHSS. Since OHSS is characterized by increased capillary permeability, we have investigated the production and action of vasoactive agents. When HUMEC-L were cultured with high doses of estradiol (E2), no significant changes were observed in the secretion of vascular endothelial growth factor (VEGF), interleukin (IL)-6 or IL-1β. However, the addition of HCG resulted in a significant increase in the secretion of VEGF and IL-6. Time–course experiments showed that VEGF was secreted within minutes of HCG addition, whereas IL-6 was significantly increased only after 48 h in culture. The secretion of IL-1β was unchanged by these hormonal conditions. The presence of HCG receptors was demonstrated in HUMEC-L in basal conditions as well as after the addition of E2. The expression of VEGF receptors was also investigated. High doses of E2 were unable to increase the expression of KDR, flt-1 and sfl-t, but the addition of HCG significantly upregulated the KDR concentration in endothelial cells, while no change was observed for flt. Permeability assays demonstrated that while E2 alone did not change the arrangement of HUMEC-L in vitro, the presence of HCG caused changes in the actin fibres corresponding to increased capillary permeability. Anti-human VEGF antibodies were able to overcome these changes. In conclusion, these experiments show that the endothelium may be a primary target of HCG, causing an acute release of VEGF and a significant increase in IL-6 and resulting in an autocrine–paracrine action that may increase vascular permeability.

Key words: capillary permeability/endothelium/IL-6/ovarian hyperstimulation syndrome/VEGF

Introduction

Ovarian hyperstimulation syndrome (OHSS) is an iatrogenic and potentially life-threatening complication of treating women with fertility drugs. It is assumed that certain ovarian biosynthetic components produced in excess during ovulation induction initiate the cascade of events resulting in OHSS. Since enhanced capillary permeability is the major initial change leading to the full appearance and maintenance of OHSS (Navot et al., 1995), recent investigations have focused on vasoactive substances.

Vascular endothelial growth factor (VEGF), also named vascular permeability factor, and the cytokine interleukin (IL)-6 are potential mediators in the development of OHSS for three important reasons: (i) they have vasoactive properties (Senger et al., 1983; Motro et al., 1990); (ii) they have been identified in follicular fluid and their mRNA transcripts and proteins have been detected in granulosa–luteal cells (Yan et al., 1993; Neulen et al., 1995; Gordon et al., 1996; Loret de Mola et al., 1996); and (iii) they are increased in serum, peritoneal fluid and/or follicular fluid of women who develop OHSS as compared with controls (Loret de Mola et al., 1996; Revel et al., 1996; Abramov et al., 1997; Agrawal et al., 1998, 1999; Artini et al., 1998; Ludwing et al., 1999).

We have investigated the roles of VEGF, IL-6 and IL-1β as mediators of HCG action in the development of OHSS. In a prospective-controlled clinical trial (Pellicer et al., 1999), we showed that both VEGF and IL-6 increase in the blood of women at risk of developing OHSS. While the levels of IL-6 are elevated before HCG administration, increases in VEGF seem to be the result of HCG action on its target cells (Pellicer et al., 1999), suggesting that VEGF may be the primary molecule involved in the pathogenesis of OHSS.

Another point of interest observed in our studies was the high level of VEGF in serum, whereas follicular fluid VEGF values were lower in women at risk of developing OHSS. This finding suggested that cells other than the follicular components

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of the ovary could be a potential cellular source and target of VEGF (Pelllicer et al., 1999). Therefore, we also have to consider the endothelium as a source of these molecules. In fact, there is convincing evidence that the human endothelium is a source of both VEGF (Banerjee et al., 1997) and IL-6 (Van der Meeren et al., 1991).

Once VEGF and IL-6 are released, they may act at paracrine and autocrine levels inducing the vascular changes leading to OHSS. Receptors for both VEGF (Shweiki et al., 1993) and IL-6 (Mantovani et al., 1997) have been described in endothelial cells, and both have been shown to increase capillary permeability in human (Goldsman et al., 1995) and animal (Rizk et al., 1997; Schenker, 1999) models. Increased capillary permeability is characteristic of OHSS (Navot et al., 1995; Schenker, 1999) and is responsible for the leakage of fluid to the third space in these patients.

Thus, our hypothesis is that the endothelium is a source and target of the vasoactive substances released in response to the conditions clinically induced in women who develop OHSS. To test this possibility, we have developed an in-vitro model using high estradiol (E2) and HCG concentrations in human microvascular endothelial cells, and have investigated their ability to express, produce and secrete the vascular mediators potentially involved in the pathogenesis of OHSS. The aim was to gain knowledge about the cascade of molecular events acting in OHSS as an aid to help prevent and successfully treat the syndrome.

Materials and methods

Chemicals

17-β estradiol was purchased from Sigma Chemicals Co. (St Louis, MO, USA) and HCG (Profasi) was obtained from Serono Laboratories (Madrid, Spain). Recombinant human VEGF and anti-human VEGF were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Anti-human flt (fms-like tyrosine kinase receptor) and anti-human KDR (tyrosine kinase receptor) were purchased from Santa Cruz (Los Angeles, CA, USA). Anti-rabbit IgG fluorescein isothiocyanate (FITC) for the flt receptor and anti-mouse IgG FITC for the KDR receptor were obtained from Sigma. Phosphate-buffered saline (PBS), bovine serum albumin (BSA), Tween-20 and Bouin’s solution were also purchased from Sigma.

Cell preparation and culture

Human lung microvascular endothelial cells (HUMEC-L) from three women aged <35 years were obtained from Clonetics® (Bio-Whittaker, Inc., Walkersville, MD, USA) to establish monolayers. Estrogen receptor (ER)-β has been localized to the cell nuclei of normal human lung (Taylor and Al-Azzawi, 2000). Cryopreserved cells were thawed in a 37°C water bath for 1–2 min. The cells (1 × 10^5) were resuspended in 1 ml of endothelial growth medium (Promocell®, Heidelberg, Germany) supplemented with 25% fetal bovine serum, 0.4% endothelial cell growth supplement, 0.1 ng/ml epidermal growth factor, 1 µg/ml hydrocortisone, 1 ng/ml basic fibroblast factor, 50 ng/ml amphotericin B and 50 ng/ml gentamycin as they became more confluent. The cells were grown until they reached 70–90% confluence and placed in 24-well culture plates (Becton-Dickinson, NJ, USA) for the experiments. The endothelial cells were morphologically checked every day under a phase contrast microscope (Nikon, ELWD 0.3, Tokyo, Japan).

Dose–response and time–course experiments

First, dose–response experiments (n = 3) were performed by adding E2 at different concentrations to the endothelial cell monolayers (0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} mol/l E2) for 24 h. The conditioned medium was collected and stored at −70°C for subsequent IL-1β, IL-6 and VEGF determinations employing commercially available ELISA kits (Quantikine; R&D Systems). Intra- and inter-assay coefficients of variation were respectively 4.1% and 8.5% for IL-1β, 2.7 and 4.5% for IL-6, and 5.4 and 7.3% for VEGF. A total of three experiments was performed.

The second point tested was the effect of adding increasing doses of HCG for 24 h. A range from 0–1000 IU (0, 1, 10, 100, 1000 IU) was added for 24 h, considering that 100–200 IU is the maximal dose found in vivo after 24 h (De los Santos et al., 1993). Again the medium was collected and IL-1β, IL-6 and VEGF were measured by ELISA. A total of three experiments was performed.

Subsequently, we planned a series of time–course experiments to define the initiation and duration of the effect of E2 and HCG on HUMEC-L. Maintaining a fixed dose of 10^{-4} mol/l E2 and 1000 IU HCG, the conditioned media were collected at 0, 3, 6, 12, 24 and 48 h intervals and stored. A total of six experiments was performed.

In the next series of experiments, we tested whether VEGF was able to increase the release of IL-6 by HUMEC-L, as we know that there is a correlation between the immune system and angiogenesis (Uno et al., 2000). Increasing concentrations of VEGF (0, 10, 100, 1000 and 10 000 pg/ml) were employed in three experiments. Subsequently, in three more experiments, we developed time–course experiments in which VEGF (10 pg/ml) was added to the endothelial cell monolayer and the conditioned medium was collected after 0, 3, 6, 12, 24 and 48 h in order to find the beginning and duration of such an effect.

Immunohistochemistry

Endothelial cell monolayers were cultured in 2-well culture plates (Nunc®, Lab-Tek, Napierville, IL, USA). After the experiments were completed, cells were fixed/permeabilized for 30 min with Bouin’s solution. The avidin–biotin immunoperoxidase method and polyclonal LH/HCG receptor antibody raised against a synthetic N-terminus 15–38 amino acid sequence (from Dr Patrick Roche at the Mayo Clinic, Rochester, MN, USA) were used for immunostaining the cells for receptors (Toth et al., 1994; Zhou et al., 1999). Briefly, the slides were incubated overnight at 4°C with 1:300 dilution of receptor antibody. After rinsing with PBS, the slides were incubated for 90 min at room temperature with 1:100 dilution of secondary antibody. Antibody preabsorbed with excess receptor peptide was used for the procedural controls. The degree and pattern of immunostaining were visually determined under an Olympus microscope (Olympus Corp., New Hyde Park, NY, USA). Representative photographs were taken and processed using computerized equipment. A total of three experiments was performed.

Competitive (c)RT–PCR

We tested the presence of the VEGFR receptors KDR, flt-1 and sflt-1 at the mRNA level in HUMEC-L and the effects of E2 (10^{-4} mol/l) on these receptors after 24 h in culture (n = 9). RNA was extracted using Trizol (Gibco BRL, Life Technologies, Grand Island, NJ, USA) (Chomczynsky and Sacchi, 1987). RT was carried out from 0.5 µg RNA using the Advantage RT-for-PCR KIT (Clontech, Palo Alto, CA, USA) according to manufacturer’s instructions and the product was diluted to a final volume of 100 µl with diethylpyrocarbonate-treated water and stored at −20°C until the PCR.

In order to construct competitive fragments, floating primers for VEGF receptors and β-actin were employed. They were designed from the sequence of target cDNA between the 3' and 5' binding
Figure 1. In-vitro secretion of IL-1β, IL-6 and VEGF by HUMEC-L after 24 h of treatment with increasing concentrations of E2 (A) or increasing doses of HCG with a fixed dose 10^-4 mol/l of E2 (B). Control: HUMEC-L in basal conditions, n = 3. *P < 0.05.

Figure 2. Time-course experiments of the release of VEGF (A) and IL-6 (B) by HUMEC-L under hormonal stimulation with 10^-4 mol/l E2 and 1000 IU HCG (n = 6). Time 0 represents the secretion of both cytokines 5 min after the addition of E2 and HCG. *P < 0.05.

sites followed by the reverse complementary 3' binding site (Krussel et al., 1998).

RT products from isolated HUMEC-L were subjected to 35 cycles of PCR in an Eppendorf Mastercycler personal (Eppendorf, Hamburg, Germany). The products were then resolved by 1% agarose gel electrophoresis, stained with ethidium bromide, and the band was extracted from the gel with the Sephaglass™ Band Prep Kit (Pharmacia Biotech, Cambridge, UK), and the concentration of the resulting DNA was quantified by spectrophotometry.

The resulting products had a deletion between 3' and 5' primer binding sites compared with the target cDNA to be detected. PCR products were confirmed by independent sequence analysis. An equal amount of competitive fragment was added to each reaction as an internal standard.

Flow cytometry

Flow cytometry was employed to quantify the effect of the different hormone treatments on VEGF receptor contents at the protein level (n = 3). Controls and treated cells were detached from plastic wells after incubation employing trypsin/EDTA (Promocell, 5 min at room temperature) by pipetting. The cells were collected by centrifugation and then washed with PBS and resuspended in 300 µl of PBS–BSA 1% (blocking buffer) for 1 h at 4°C. Subsequently, cells were centrifuged and washed with PBS–Tween 20 (PBS–T). The cells were then coated, following manufacturer instructions, with 200 µl antibody against human KDR (2 µg/ml) or flt-1 (2 µg/ml) receptors for 1 h at room temperature. The cells were washed again with PBS–T, and afterwards coated with 200 µl anti-rabbit IgG FITC (1:160) for the flt receptor or anti-mouse IgG FITC (1:64) for the KDR receptor for 1 h at room temperature. Cells were then centrifuged, resuspended, washed with PBS–T, and finally resuspended in 500 µl of PBS and analysed in the flow cytometer.

All flow cytometry determinations were performed in an Epics XL flow cytometer (Beckmann-Coulter) using an argon-ion laser tuned at 488 nm and 15 mW. FITC fluorescence was collected by 575 DL/525BP and propidium iodide was collected by 600DL/575BP filters. Data were collected in 4-decade logarithmic amplification. Debris was excluded by analysis of scatter properties. At least 10 000 events per sample were stored in list-mode files. Data were expressed as the percentage of stained cells and fluorescence relative units.

Blocking experiments

Blocking experiments were performed using anti-human VEGF. Initially, dose–response experiments (n = 3) were planned at the ED50 suggested by the manufacturer. A dose of 300 pg/ml was then employed to test this effect over time (0, 3, 6, 12, 24 and 48 h).

Permeability assays

HUMEC-L were seeded on 24-well plates, grown to confluence, and then incubated with E2 (10^-4 mol/l), E2 + HCG (1000 IU), or E2 +
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Figure 3. The release of IL-6 by HUMEC-L in response to VEGF. (A) Dose–response experiments with increasing concentrations of VEGF and a fixed dose of E2 (10^{-4} mol/l) and HCG (1000 IU) for 24 h. (B) Time–course experiments with a fixed dose of VEGF (10 pg/ml), E2 (10^{-4} mol/l) and HCG (1000 IU). Control: HUMEC-L in basal conditions. *P < 0.05.

HCG + anti-human VEGF (300 pg/ml) for 24 h (n = 3). After these treatments, the cell monolayers were rinsed twice with PBS and fixed with 4% paraformaldehyde in PBS at 4°C. TRITC–phalloidin (20 mg/ml) was then added to the cell layers for 30 min at 4°C. Confocal analysis was performed with an NRC 1024 instrument (Bio-Rad, Hemstead, UK). The excitation line used was 488 (TRITC). The filter used was HQ515/10 (TRITC). Transmitted light images were acquired for every field.

Statistical analysis
Data were expressed as mean ± SEM. Analysis of variance (ANOVA) was employed to compare among groups. Bonferroni’s and Scheffé’s tests were applied when ANOVA showed statistical differences. Pearson’s coefficient of correlation was also employed. Significance was defined as P < 0.05. The statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA).

Results
Secretion of VEGF, IL-6 and IL-1β by HUMEC-L
We first tested the effect of E2 on the release of IL-1β, IL-6 and VEGF by human endothelial cells. Figure 1A shows the effect of increasing doses of E2 on HUMEC-L. There was a significant increase in the release of VEGF when 10^{-3} mol/l E2 was added to the culture medium. However, the morphological appearance of cells suggested a toxic effect rather than a physiological action, and therefore this dose was not used (data not shown). Apart from this result, E2 did not cause any significant differences in the release of IL-1β, IL-6 or VEGF.

When HCG was added in increasing doses to HUMEC-L cultured with a fixed dose of 10^{-4} mol/l E2, a significant increase (P < 0.05) in the secretion of VEGF was observed when 1000 IU HCG was added (Figure 1B). No change was detected for IL-1β accumulation in the conditioned medium.

The time–course experiments are shown in Figure 2. E2 and HCG induced a dramatic increase in VEGF secretion within 5 min (Figure 2A). However, a significant increase (P < 0.05) in IL-6 release was only observed after 48 h of culture with both E2 and HCG (Figure 2B).

Experiments were also performed to test whether VEGF has an autocrine role in the endothelium. To this end, we added increasing concentrations of VEGF with a fixed dose of E2 and HCG and measured the secretion of IL-6 after 24 h. A dose of 10 pg/ml VEGF resulted in a significant increase (P < 0.05) in the secretion of IL-6 by HUMEC-L in culture medium (Figure 3A). This dose of VEGF required 48 h in the time–course experiments to significantly increase (P < 0.05) the release of IL-6 in vitro (Figure 3B).

Blocking experiments were designed to further investigate
a trigger effect of VEGF on IL-6 secretion in the presence of E2 and HCG. Dose–response experiments showed that 300 pg/ml of anti-VEGF was able to significantly reduce (P < 0.05) the secretion of IL-6 (Figure 4A). Employing this dose of anti-human VEGF, the release of IL-6 by HUMEC-L was blocked during 48 h (Figure 4B).

Presence and regulation of HCG and VEGF receptors in HUMEC-L

We tested the presence of HCG receptor protein in HUMEC-L, and the effect of E2 on its expression. Immunohistochemistry showed the presence of intracytoplasmic receptors for LH/HCG (Figure 5). A receptor antibody preabsorption control showed no immunostaining (not shown). A dose of 10^{-4} mol/l E2 had no effect on the expression of HCG receptors (Figure 5).

A second series of experiments was designed to show the presence of mRNA transcripts for the VEGF receptors KDR, flt-1 and s-flt. HUMEC-L expressed mRNA for all three receptors and increasing concentrations of E2 in the absence of HCG had no effect on the expression of these transcripts (Figure 6).

In addition, experiments were designed to determine the presence and regulation of KDR and flt-1 by employing flow cytometry. The percentage of HUMEC-L stained for the flt-1 receptor did not change between the controls and the presence of E2 alone or E2 and HCG together. However, the cells treated with E2 and HCG showed a significantly increased (P < 0.05) KDR production in HUMEC-L, as determined by the percentage of positively stained cells (Figure 7A). There was no difference among the cells in the different culture conditions in terms of fluorescence relative units, i.e. the fluorescence intensity in each positive cell (Figure 7B).
Figure 7. Effect of E2 (10^{-4} \text{ mol/l}) alone or E2 (10^{-4} \text{ mol/l}) and HCG (1000 IU) on VEGF receptor expression at the protein level as assessed by flow cytometry of HUMEC-L cells (n = 3). (A) Percentage of stained cells. Difference between * and **, P < 0.05; (B) fluorescence relative units.

Permeability assays

Finally, we analysed HUMEC-L for morphological changes that could be responsible for an augmented vascular permeability observed in women with OHSS. To this end, the organization of the actin filaments was tested using confocal microscopy after adding TRITC-phalloidin to cells cultured in basal conditions, after E2 and E2 plus HCG treatment. Figure 8A shows the normal disposition of the cells in basal conditions and this was not affected by the addition of E2 to the culture medium (Figure 8B). However, the addition of HCG induced contraction of the endothelial cells manifestly by considerable morphological changes in cell shape due to a rearrangement of actin filaments being irregularly aligned within the cells (Figure 8C), and this has been correlated with endothelial permeability. The reversal of this effect was tested with VEGF antibody. Anti-human VEGF antibody added to the culture medium was able to inhibit the effect of HCG on the morphological changes in endothelial cells (Figure 8D).

Discussion

This study was designed to understand the biochemical and molecular mechanisms of OHSS by employing an in-vitro model of human endothelial cells from lung microvasculature. The significance of our findings may be limited by the facts that the study was conducted in vitro, the actual cells employed are not apparently involved in the pathogenesis of OHSS, and E2 and HCG were added at supraphysiological concentrations.

With regard to the cells, it should be understood that the ideal model would be the use of endothelial ovarian cells from IVF patients (Ratcliffe et al., 1999), but we were unable to reproduce these cultures. The cells employed were obtained from capillaries, had receptors for both E2 and HCG, and our main hypothesis was that the entire endothelium of the human body may be involved in the pathogenesis of OHSS. Thus, cells obtained from the lung could be employed to test our hypothesis.

The second main criticism of our model is the use of supraphysiological concentrations of E2 and HCG. This is true for E2, but not completely true for HCG, since we have described maximal HCG concentrations in vivo after 24 h of HCG administration, being in the range of 100–200 IU (De los Santos et al., 1993). While the addition of HCG did produce changes in the monolayers, E2 had no significant effect at any concentration tested. We feel that the use of extreme supraphysiological concentrations of E2 in our model has no influence in the results obtained, since 10^{-4} \text{ mol/l} E2 was not toxic to the cells and had the same lack of effect as the lower doses in terms of VEGF synthesis, KDR receptor up-regulation and changes in actin filaments as physiological doses such as 10^{-8} \text{ mol/l}.

With these limitations in mind, the first question addressed was whether or not the conditions established in vivo affect the endothelium in terms of release of vasoactive substances. We have previously observed in vivo in women at risk of developing OHSS that ovarian stimulation with gonadotrophins, and the subsequent increase in serum E2, is able to elevate serum IL-6. However, only after HCG administration there is a sudden and significant increase in serum VEGF, the origin of which may not be the cellular components of the ovarian follicle, since VEGF levels in follicular fluid are even decreased in women at risk of developing OHSS. In contrast, IL-1β is basically unaltered in serum by hormonal stimulation (Pellicer et al., 1999).

The data obtained in the current study mimic these earlier findings. First, we observed that E2 alone was unable to increase the release of VEGF, IL-6 or IL-1β, unless a toxic concentration was employed (10^{-3} \text{ mol/l} E2). It is well known that hypoxic cells react with the secretion of VEGF (Tuder et al., 1995); thus, it is easy to explain the toxic effect of E2. Secondly, the addition of HCG induced a significant release of VEGF by HUMEC-L. This action was acute in the sense that we were able to detect high VEGF concentrations within minutes of adding HCG to the endothelial cells in the time-course experiments. HCG was also able to induce the release of IL-6. This was not observed in the first dose–response experiments conducted for 24 h. However, experiments conducted for 48 h showed an enhancement of IL-6 release by HUMEC-L. Thus, it seems that HCG has an acute and a chronic effect, the former seen with VEGF and the latter with IL-6.

Moreover, VEGF was also able to enhance the release of IL-6 after 48 h, an effect that was blocked by anti-human VEGF. These experiments initially showed that VEGF may have an autocrine action on endothelial cells and provided a clue for the development of new strategies to prevent and/or
Figure 8. Capillary permeability of HUMEC-L cells employing confocal microscopy to analyse actin filaments. (A) Control; (B) cells treated with $10^{-4}$ mol/l E2; (C) cells treated with $10^{-4}$ mol/l E2 and 1000 IU HCG; (D) cells treated with $10^{-4}$ mol/l E2 and 1000 IU HCG plus anti-VEGF (300pg/ml); n = 3.

Figure 9. Proposed model for the pathogenesis of OHSS.
treat OHSS. A novel strategy is necessary because the action of HCG upon the endothelium cannot be overcome with HCG-blocking antibodies, since this gonadotrophin is necessary for oocyte and follicular maturation. Thus, a possible way of preventing the cascade of events leading to OHSS may be by neutralizing VEGF and/or blocking its receptors.

The irrelevant role of E₂ in the pathogenesis of OHSS was further tested when the presence and regulation of HCG and VEGF receptors were investigated. E₂ alone was unable to up-regulate either type of receptor at the mRNA or protein levels. Again, it was the addition of HCG that induced a cascade of events resulting in a significant increase of the KDR receptor.

These data are confirmatory of the accumulated evidence in vivo. We know that OHSS rarely develops if HCG is withheld despite high serum E₂ levels (Navot et al., 1995), and we have also learned from women with enzymatic deficiencies yielding very low levels of E₂ after aggressive stimulation of the ovaries, that OHSS can occur with very low serum E₂ levels (Pellicer et al., 1991). Thus, our findings in vitro are consistent with the clinical observations (Pellicer et al., 1991, 1999) and also act as validation of the in-vitro model.

It is worth noting that HCG induced an up-regulation of the VEGF receptor KDR in human endothelial cells. KDR has been shown to be the most functional receptor for human VEGF. This receptor transduces signals for mitogenicity, chemotaxis, angiogenesis and cytoskeletal reorganization (Waltenberger et al., 1994) and the fact that KDR receptors are significantly increased after HCG indicates that the endothelium is sensitive to this hormone, and its alteration could result in endothelial dysfunction (Waltenberger et al., 1994). One of the actions of VEGF is to increase vascular permeability (McClure et al., 1994). Thus, on the basis of these findings, we postulate that HCG induces the secretion and reception of VEGF in the endothelial cells, generating an acute response manifested by changes in vascular permeability. The confocal microscopy experiments were designed in keeping with this concept.

The integrity of the endothelial cytoskeleton is important for the functional competence of an endothelial barrier (Suttorp et al., 1991). Increased endothelial permeability to solutes and water is dependent firstly on the shape and configuration of endothelial cells, as determined by alterations in cytoskeletal elements such as actin filaments (Malik et al., 1989), and secondly on the appearance of interendothelial gaps and disorganization of endothelial junctional proteins (Maruo et al., 1992). VEGF can acutely impair endothelial cell integrity in vivo (McClure et al., 1994) and in vitro (Maruo et al., 1992; Titon et al., 1995; Hippenstiel et al., 1998; Kevil et al., 1998). The capillary permeability in vitro has been studied by analysing the transendothelial albumin flux (Goldblum et al., 1990; Downing et al., 1992; Maruo et al., 1992; Biffi et al., 1995; Lee et al., 1998), tight junctional resistance (Deli et al., 1995) and the organization of actin filaments (Maruo et al., 1992; Lee et al., 1998). Kevil et al. demonstrated that VEGF increases albumin permeability across endothelial monolayers and suggested that this is due to rearrangement of endothelial junctional proteins (Kevil et al., 1998) as described by other authors (Maruo et al., 1992; Liu et al., 1995; Dobrogowska et al., 1998; Bates et al., 1999). Maruo et al. analysed the capillary permeability induced by IL-6, and showed that in some cells the actin filaments appeared to be aligned irregularly within cells (Maruo et al., 1992). Finally, associated with this increase in permeability, a considerable morphological change in cell shape, gap formation between adjacent cells and rearrangement of actin filaments have also been observed in IL-6 treated cells (Maruo et al., 1992). However, changes in cell shape can be also related to other functions than permeability, and this should be kept in mind.

We employed confocal microscopy to analyse the monolayers. An irregular alignment and rearrangement of the actin filaments and considerable morphological changes in cell shape and gap formation between adjacent cells were observed in HUMEC-L treated with E₂ + HCG, but not in the endothelial cells treated only with E₂. Moreover, this effect was reversed by anti-VEGF. These experiments convey several messages: (i) E₂ alone does not increase vascular permeability; (ii) HCG acts through VEGF; and (iii) blocking VEGF action is a valid alternative to overcome the changes induced in the endothelium by HCG.

Changes in IL-1β in response to E₂ and HCG were also tested, but we found none, consistent with our in-vivo studies (Pellicer et al., 1999). Although a role for IL-1β cannot be definitely ruled out because the entire system was not explored, we feel quite confident that IL-1β is probably not involved in the pathogenesis of OHSS.

Taking all the experiments and the accumulated clinical experience together, we suggest that the endothelium, along with the ovary, is a primary target of HCG (Figure 9). As a result VEGF and its KDR receptor are stimulated, resulting in an acute biological response in the capillaries causing increased permeability. Blocking VEGF action with specific antibodies prevents the changes induced by HCG, providing the rationale for new therapeutic approaches to prevent and/or treat OHSS. There is no evidence to suggest a critical role for IL-6 or IL-1β in the pathogenesis of OHSS, although the former is released by the endothelial cells under hormonal stimulation and might be involved in the long-term maintenance of the observed vascular changes.

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