OVARian HYPERSTIMULATION syndrome (OHSS) is an iatrogenic complication of ovulation induction for the treatment of infertility. The syndrome ranges in severity from mild to severe. Its incidence is 0.1–4% of ovulation induction treatments (1), but the low incidence is increasing worldwide through the expansion of infertility treatments. The clinical characteristics of the syndrome stem from an intense inflammatory reaction that results in massive accumulation of extracellular protein-rich fluid, which manifests as massive ascites and pleural and pericardial effusion, combined with profound intravascular volume depletion and hemoconcentration (2). Other severe clinical manifestations that may accompany the syndrome include a state of hypercoagulability, thromboembolic phenomena, adult respiratory distress syndrome, and death.

Vascular Endothelial Cadherin Regulates Vascular Permeability: Implications for Ovarian Hyperstimulation Syndrome

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Context: Ovarian hyperstimulation syndrome (OHSS) is an iatrogenic complication of treatment with fertility drugs. It is characterized by increased vascular permeability and simultaneous overexpression of vascular endothelial growth factor (VEGF) in ovarian cells.

Objective: We tested the hypothesis that the endothelium and endothelial cell-to-cell junctions are downstream targets of VEGF during OHSS pathogenesis. We investigated the potential involvement of vascular endothelial (VE)-cadherin, an interendothelial adhesion molecule, in the capillary hyperpermeability in OHSS.

Design: Human endothelial cells from umbilical veins (HUVEC) were used as an in vitro model of OHSS.

Intervention: Cell cultures were treated with varying doses of estradiol (E2), human chorionic gonadotropin (hCG), VEGF, and anti-human VEGF antibodies, either alone or in combination, and the effect on VE-cadherin release was evaluated at different time points.

Results: Culturing of HUVEC with high doses of E2 produced no significant changes in VE-cadherin concentration, but hCG and VEGF produced a significant increase in VE-cadherin release. Time-course experiments showed that VE-cadherin was secreted 12 h after VEGF addition. Antihuman VEGF antibodies prevented these changes. Permeability assays demonstrated that, although E2 did not alter the arrangement of HUVEC in vitro, hCG and VEGF caused changes in the actin fibers indicative of increased capillary permeability. VEGF also induced an increase in paracellular permeability of HUVEC at the same doses used in the previous experiments.

Conclusions: Adhesion molecules like VE-cadherin may play a role in the development and progression of increased capillary permeability in severe OHSS. (J Clin Endocrinol Metab 92: 314–321, 2007)

However, the pathophysiology of the syndrome is not completely understood, and no specific therapy or prevention is available.

It is thought that certain systemic and ovarian biosynthetic cytokines and vasoactive and angiogenic factors that are produced in excess during induction of ovulation initiate the cascade of events that leads to OHSS. Because increased capillary permeability is the primary initial change leading to the full appearance and maintenance of OHSS (3), recent investigations have focused on vasoactive substances. Many cytokines and vasoactive and angiogenic factors, such as vascular endothelial growth factor (VEGF), have been implicated as major mediators of the pathogenesis of capillary leakage and endothelial damage in OHSS (4–7). Gómez et al. (5) showed that vascular permeability could be reduced by blocking VEGF receptor-2 and suggested that this approach could be used to prevent OHSS in humans. However, there is no specific prognostic marker for early prediction of the resolution course of OHSS.

Endothelial cell-to-cell junctions are complex structures formed by different adhesive molecules (8, 9). Endothelial cells have tight and adherens junctions that have a general organization similar to that described for epithelial cells (10, 11). Adherens junctions are ubiquitous along the vascular tree and are formed by transmembrane proteins belonging to...
the cadherin superfamily (12). The cadherins are single-chain transmembrane polypeptides that mediate homophilic, calcium-dependent adhesion. They are specifically associated with the adherens junction region, where they form multimeric complexes with cytoplasmic catenin proteins. Endothelial cells express a cell-specific cadherin called vascular endothelial (VE)-cadherin, also known as CD144/cadherin-5 (8). This specific cadherin is structurally related to other classical cadherins and is a 125-kDa single-pass transmembrane glyoprotein. It associates as cis dimers via extracellular domains of the cell to promote intercellular homophilic adherens junctions (10). VE-cadherin has five extracellular calcium-binding domains and links to the actin filament network through proteins of the armadillo family, the catenins (13). It has multiple possible cytoplasmic binding partners; it binds to β-catenin and thus allows a link to α-catenin, plakoglobin, p120, and the actin cytoskeleton. Although the extracellular domain of VE-cadherin is required for homophilic adhesion and clustering, the intracellular association to catenins and the cytoskeleton are needed for stabilization of the junctional complex and for full control of paracellular permeability (14). In addition, catenins, when released into the cytoplasm, may translocate to the nucleus and modulate cell transcription (15). This suggests that the cadherin-catenin complex may play a role in intracellular signal transduction after homotypic cell-to-cell adhesion.

VE-cadherin plays a morphogenic role in vascular development. Its expression is required for the normal organization of the vascular tree in the embryo. A null mutation in the VE-cadherin gene results in embryonic lethality by 9.5–10 d gestation with normally differentiated endothelial cells unable to form vascular structures (16). VE-cadherin also plays a key role in regulating endothelial cell permeability and migration and assembly of new blood vessels (14, 17). Lam-pugnani et al. (18) showed that tyrosine phosphorylation of the adherens junction component of VE-cadherin, p120, and β-catenin induced loss of confluence. Increased tyrosine phosphorylation at the sites of cell-cell and cell-matrix contacts has been correlated with biological processes such as cell migration, cell motility, and metastatic spread of tumor cells (19–22). Interestingly, endothelial adherens junctions are known to be a downstream target for VEGF signaling. Thus, it has been suggested that tyrosine phosphorylation may be involved in the loosening of cell-cell contacts in established vessels that modulates transendothelial permeability and allows sprouting and cell migration during angiogenesis (23).

VEGF is a potential mediator in the development of OHSS because it has vasoactive properties (4–6) and is thought to mediate redistribution of adherens junction proteins and the loss of the endothelial cell barrier architecture. In women who develop OHSS, VEGF is expressed and produced by granulosa-lutein cells (24) and is released into the follicular fluid (25) in response to human chorionic gonadotropin (hCG), increasing capillary permeability. Similarly, Albert et al. (25) have demonstrated that hCG stimulates the release of VEGF in human endothelial cells, which, in turn, acts in an autocrine manner to increase vascular permeability. In addition, plasma VEGF levels correlate with the clinical picture of OHSS, and changes in VEGF in ascites have been corre-
Briefly, this assay uses a quantitative “sandwich” enzyme immunoassay technique by which a polystyrene microtiter plate is precoated with a monoclonal antibody specific for the soluble VE-cadherin molecule. Standards and samples are introduced into the wells, and soluble VE-cadherin is bound by the immobilized antibody. After unbound proteins are washed away, the second enzyme-linked monoclonal antibody specific for the soluble VE-cadherin is added to the wells to sandwich the soluble VE-cadherin immobilized during the first incubation. After a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells, and color develops in proportion to the amount of soluble VE-cadherin bound in the initial step. Color development is then stopped, and the intensity of the color is quantified spectrophotometrically. A curve is prepared by plotting OD against concentration of soluble VE-cadherin in the standard wells. The concentration of soluble VE-cadherin in unknown samples is then determined by comparing the OD of the samples to this standard curve. Interassay and interassay coefficients of variation were 4.1% and 7.2%, respectively. No cross-reactivity between soluble VE-cadherin and other adhesion molecules has been found using this assay. Experiments were performed in triplicate.

We designed a series of time-course experiments to characterize the initiation and duration of effect of VEGF on HUVEC. Maintaining a fixed dose of 100 pg/ml VEGF, the conditioned media were collected at 0-, 3-, 6-, 12-, 24-, and 48-h intervals and stored at −80°C until assay. Experiments were performed in triplicate.

**Blocking experiments**

Blocking experiments were performed using antihuman VEGF. Monolayers stimulated with a fixed dose of 10 pg/ml VEGF were treated with increasing doses of anti-VEGF (0, 75, 150, and 300 pg/ml). Experiments were performed in triplicate.

**Cell proliferation experiments**

Cellular proliferation, viability, and activation were tested with a colorimetric assay, Cell Proliferation kit I [3-(4,5-dimethyl-2-y)-2,5-difeniltetrazolium bromide (MTT); Roche Diagnostics Co., Indianapolis, IN] for nonradioactive quantification. This assay was first described by Mosmann (27) and is designed for spectrophotometric quantification of cell growth and viability without the use of radioactive isotopes. It is used to measure cell proliferation in response to growth factors, cytokines, and nutrients and also for measuring cytotoxicity. It is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. This cellular reduction involves the pyridine nucleotide cofactors NADH and NADPH. The formazan crystals solubilize, and the resulting colored solution is quantified using a scanning multimode spectrophotometer.

After the previously described experiments were performed, 50 μl of MTT labeling reagent (final concentration 0.5 mg/ml) was added to each well. The microtiter plate was incubated for 4 h in a humidified atmosphere (37°C, 5% CO₂). Then, 500 μl of the solubilization solution was added to each well, and the plate was left to stand overnight in the incubator in a humidified atmosphere. After complete solubilization of the purple formazan crystals was verified and the spectrophotometric absorbance of the samples was measured using a microtiter plate (ELISA) reader. Absorbance of the formazan product was measured at 570 nm, according to the filters available for the ELISA reader.

**Permeability assays**

HUVEC were seeded on 12-well plates, grown to confluence, and incubated with E₂ (10⁻⁵ mol/liter), hCG (100 IU/ml), VEGF (10 and 100 pg/ml), hCG (100 IU/ml) + VEGF (100 pg/ml), and VEGF (10 pg/ml) + anti-VEGF (150 pg/ml) for 24 h (n = 3). Cell monolayers were then incubated for 8 min at 4°C with Triton X-100 solution (Roche Molecular Biochemicals, Mannheim, Germany). Finally, 40 μl of TRITC-phalloidin (final concentration 20 mg/ml) was added for 45 min at 4°C. Fluorescent microscopy was used (Nikon Eclipse E400) to observe rearrangement of the actin filaments after each treatment. Tissue images were captured with a digital camera (Olympus Inc., Tokyo, Japan).

HUVEC cells were seeded on Transwell filters (5-μm pore size; Costar, Cambridge, MA) in 24-well dishes and cultured with 400 μl of the culture medium described before in the upper chamber and 1200 μl of the same growth medium in the lower chamber. Cells were grown for three days without changing the medium until they reached confluence. For the assay, 5 μl of FITC-albumin (final concentration 2 mg/ml) was added to the upper chamber. Immediately after, 5 μl of culture medium alone or with VEGF (10 and 100 pg/ml), VEGF (10 pg/ml) + anti-VEGF (150 pg/ml), and VEGF (100 pg/ml) + anti-VEGF (150 pg/ml) was added. At the indicated time points (2, 4, 6, 12, and 24 h), a 50-μl sample was removed from the lower compartment and replaced with the same volume of growth medium to maintain hydrostatic equilibrium. Samples were stored at −80°C. The fluorescent content was measured at 484 nm absorption/emission wavelengths for FITC-albumin (SPEC-TRAFluor Plus; 94930; TECAN, Männedorf, Switzerland).

**Statistical analysis**

Data are expressed as the mean ± SEM. ANOVA was used to compare groups. Bonferroni’s and Scheffé’s tests were applied when ANOVA showed statistical differences. Soluble VE-cadherin concentrations were compared using the Student’s t test and analysis of covariance.

A P value < 0.05 was considered statistically significant for all comparisons. Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL).

**Results**

**Release of VE-cadherin by HUVEC**

We first tested the effect of E₂ on the release of VE-cadherin by HUVEC. Figure 1A shows the effect of increasing doses of E₂ on this release. There was a significant increase in the release of VE-cadherin only when 10⁻³ mol/liter E₂ was added to the culture medium. However, the morphological appearance of cells suggested a toxic effect rather than a physiological action. E₂ at concentrations less than 10⁻³ mol/liter did not significantly affect the release of VE-cadherin.

The addition of increasing doses of hCG to HUVEC produced a significant increase (P < 0.05) in the concentration of VE-cadherin (Fig. 1B). Addition of increasing doses of VEGF also induced a significant increase (P < 0.05) in VE-cadherin in HUVEC (Fig. 1C). The morphological appearance of cells suggested a toxic effect of VEGF at high concentrations (≥ 1000 pg/ml).

We also investigated the effect of VEGF with a fixed dose of hCG by adding increasing concentrations of VEGF together with 1000 IU of hCG and measuring VE-cadherin after 24 h. A 100-pg/ml dose of VEGF produced a significant increase (P < 0.05) in the release of VE-cadherin by HUVEC (Fig. 1D).

We conducted further experiments to determine whether the effect of VEGF could be blocked by increasing doses of anti-VEGF. Dose-response experiments showed that 75 and 150 pg/ml anti-VEGF significantly reduced (P < 0.05) the release of VE-cadherin (Fig. 2). The addition of 300 pg/ml anti-VEGF produced a toxic effect, providing an explanation for the increase in VE-cadherin due to cell death.

Time-course experiments are shown in Fig. 3. VEGF induced an increase in VE-cadherin concentration within 3 h. However, a significant increase (P < 0.05) was only observed after 12 h.

**Quantification of cell proliferation**

MTT was used to quantify cellular proliferation and activation in response to E₂, hCG, VEGF, and anti-VEGF. There was no increase in the number of living cells that resulted in
an increase in the total metabolic activity of the samples. At doses of E2, VEGF, or anti-VEGF producing a toxic effect in the HUVEC monolayer, MTT concentration decreased, showing that the increase in VE-cadherin is related to a cytotoxic effect of these molecules at high concentrations.

Permeability assays

Cytoskeletal rearrangements. We subsequently investigated the morphological changes induced in cultured HUVEC that could be responsible for augmented vascular permeability in women with OHSS. Using fluorescent microscopy we examined actin filament organization after adding TRITC-phalloidin to cells cultured in basal conditions and treated with 10^{-3} mol/liter E2, 1000 IU hCG/ml, 100 pg/ml VEGF, and 100 pg/ml VEGF + 1000 IU hCG. Figure 4A shows the normal disposition of cells in basal conditions, and Fig. 4B shows the lack of effect of E2 added to the culture medium. In contrast, the addition of hCG induced contraction of the endothelial membrane (Fig. 4C). Addition of VEGF and VEGF + hCG induced a considerable change in cellular shape due to a rearrangement of actin filaments being irregularly aligned within the cells (Fig. 4, D and E).

The morphological effect of VEGF was observed within 10 min (Fig. 5A). However, the maximal effect was observed at 12 and 24 h (Fig. 5, D and E). After 48 h, the VEGF effect on endothelial cells was toxic rather than physiological (Fig. 5F).

To confirm the specificity of VEGF action, experiments were performed using VEGF antibody. Addition of antihuman VEGF antibody (150 pg/ml) to the culture medium inhibited the morphological changes induced by VEGF (10 pg/ml) in endothelial cells.

Albumin permeability. We investigated the effects of VEGF on the integrity of intercellular junctions by measuring the permeability of a confluent endothelial monolayer to labeled albumin. Confluent monolayers of human endothelial cells cultivated on Transwell filter inserts were treated with VEGF (10 and 100 pg/ml). The permeability of the monolayer to FITC-albumin was determined at several time points by mea-
suring the fluorescence intensity of the medium in the lower compartment. In experiments using basal conditions, the level of FITC-albumin in the lower compartment increased slowly over time. When the monolayer was cultured in the presence of VEGF, there was a significant time-dependent increase in FITC-albumin permeability compared with the control monolayer; the increase was significant with 100 pg/ml VEGF within the interval of 6–12 and 24 h (Fig. 6). Addition of antihuman VEGF antibody (150 pg/ml) to the culture medium inhibited the effect of 10 pg/ml VEGF.

Discussion

This study sought to improve the understanding of the biochemical and molecular mechanisms for increased cell permeability in OHSS by using an in vitro model of HUVEC. Specifically, we set out to investigate the possible involvement of an endothelial cell adhesion molecule, VE-cadherin, in the systemic acute-phase response of OHSS.

The first question addressed was whether the conditions established in vivo affect the endothelium in terms of release of adhesion molecules such as VE-cadherin. We have previously observed in vivo in women at risk of developing OHSS that ovarian stimulation with gonadotropins elevates serum E2; however, only after hCG administration was there a sudden and significant increase in serum VEGF, the main vasoactive mediator (6). The data obtained in the current study are consistent with these earlier findings. First, we observed that E2 alone did not increase the release of VE-cadherin unless a toxic concentration was used (10−3 mol/liter E2). It is known that hypoxic cells secrete VEGF (28); thus, it is possible to thereby account for the effect of E2 on VEGF release. Second, addition of hCG to endothelial cells induced a significant release of VE-cadherin by HUVEC. It was previously shown that endothelial cells release VEGF within minutes of adding hCG (25). Third, we observed that a combination of VEGF and hCG increased the release of VE-cadherin, suggesting that VEGF may have an autocrine action on adhesion molecules. In fact, it has been shown that the shedding of VE-cadherin, which occurs during endothelial cell apoptosis, can be blocked by an inhibitor of metalloproteinases (29). This may reflect reversible endothelial cell damage, taking into account that OHSS is a self-limited event.

The finding that E2 alone was unable to modify the release of VE-cadherin suggests that it is irrelevant to OHSS pathogenesis. These data confirm accumulated in vivo evidence. It is known that, despite high serum E2 levels, OHSS rarely develops if hCG is withheld (3, 30). We have also determined, by studying women with enzymatic deficiencies of the ovaries, that OHSS can occur with very low serum E2 levels (31). Thus, our in vitro findings are consistent with clinical observations and also validate the in vitro model. The use of extreme supraphysiological concentrations of E2 in our model had no influence in the results obtained, because 10−6–10−4
mol/liter E₂ was not toxic to cells, and had the same lack of effect on VE-cadherin concentration (Fig. 1A) and cell shape (Fig. 4B).

One of the main actions of VEGF is to increase vascular permeability (5, 6). Thus, on the basis of our findings, we postulate that the endothelial adherens junction, particularly VE-cadherin, is a downstream target of VEGF in endothelial cells, generating an acute response that manifests as changes in vascular permeability. The fluorescent microscopy experiments were designed to investigate this hypothesis.

Integrity of the endothelial cytoskeleton is important to the functional competence of an endothelial barrier. Increased endothelial permeability to solutes and water is dependent first on the shape and configuration of endothelial cells, determined by alterations in cytoskeletal elements such as actin filaments (32), and second on the appearance of interendothelial gaps and disorganization of endothelial junctional proteins (33). We used fluorescent microscopy to investigate the HUVEC monolayers. In HUVEC treated with similar doses of hCG and VEGF that increased the release of VE-cadherin, we found irregular alignment and rearrangement of the actin cytoskeleton and considerable changes in cell shape and gap formation between adjacent cells. These effects were not observed in endothelial cells that had been treated with only E₂. Moreover, this effect was reversed by anti-VEGF. These experiments show that: 1) E₂ alone does not increase vascular permeability, 2) hCG acts through VEGF, and 3) blocking VEGF action is a valid option for preventing or reverting the changes induced in VE-cadherin by both hCG and VEGF.

We demonstrated that VEGF increases FITC-albumin permeability across endothelial monolayers and suggest that this is due to rearrangement of endothelial junctional proteins like VE-cadherin, as previously described (34). VEGF-

![Fig. 5. Effect of a fixed dose of VEGF (100 pg/ml) in the organization of actin filaments and endothelial membrane in a time-course manner. Control (A), 10 min (B), 3 h (C), 6 h (D), 12 h (E), 24 h (F), and 48 h (G). TRITC-phalloidin, final concentration 20 mg/ml.](image-url)
induced modulation of adhesion molecules might change the lateral clustering of the extracellular domains of junctional proteins such as VE-cadherin and, consequently, alter cadherin adhesive strength. It remains to be examined whether the redistribution of VE-cadherin at cell-cell contacts in a zigzag pattern, which has previously been correlated with destabilization of endothelial junctions (35), reflects this kind of action.

Endothelial-cell-leukocyte adhesion molecules have been implicated as mediators in many disorders, including neutrophil-mediated lung injury in rats and autoimmune encephalomyelitis (36). OHSS may be another disorder in which these adhesion molecules are involved in exaggerated leukocyte recruitment and transendothelial migration, causing tissue damage and capillary hyperpermeability. Our results are consistent with several findings that suggest the involvement of cell adhesion molecules in ovarian physiology (37). It appears that cell adhesion molecules may play a role in ovarian physiological processes such as folliculogenesis, ovulation, corpus luteum formation, and luteolysis, and as suggested by our results, in the pathophysiology of OHSS that results from the exaggeration of these processes.

The participation of cell adhesion molecules in OHSS that are major mediators of inflammation, such as VE-cadherin, is clear, because inflammation may be a major physiologic phenomenon in the reproductive processes of the ovary. Elevated serum levels of VE-cadherin may contribute to the evolution and progression of OHSS as a result of its inflammatory role. VE-cadherin participates in the adhesion, extravasation, recruitment, and activation of leukocytes and endothelial cells at sites of inflammation. Thus, VE-cadherin may help initiate and propagate intense local and systemic inflammatory reactions that result in increased capillary permeability.

The significance of our findings may be limited by the fact that the cells we used are not apparently involved in the pathogenesis of OHSS. The ideal model would be endothelial ovarian cells from in vitro fertilization patients (38). However, the cells that we used have receptors for E2, hCG, and VEGF, and our main hypothesis was that the entire endotHELium of the human body might be involved in the pathogenesis of OHSS. Thus, cells obtained from the umbilical vein are a valid model for testing our hypothesis. Even more, these findings might be clinically relevant as preliminary observations in women undergoing controlled ovarian hyperstimulation who develop OHSS showed a 4-fold increase in VE-cadherin levels after hCG administration, that persisted elevated until OHSS resolved (39).

Taking all the experiments and accumulated clinical evidence together, we suggest that endothelial junctional proteins, along with the ovary, are a primary target of hCG. As a result, VEGF is stimulated, leading to an acute biological response in the capillaries that causes increased permeability. During OHSS, VEGF mediates increased vascular permeability and endothelial migration at least partly through modulation of VE-cadherin function. Blocking this action prevents the changes induced in VE-cadherin and the resultant loss of endothelial barrier architecture. This provides a rationale for new therapeutic approaches to prevent and/or treat OHSS and other clinical conditions with ascites such as malignant ovarian disease.  

Acknowledgments

We thank Raúl Gómez for his cooperation and critical discussion in the design of this project.

Received June 8, 2006. Accepted October 2, 2006.

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This work was supported by FISs PI03/0108 and SAF 2004–06028 research grants.

Disclosure Statement: The authors have nothing to disclose.

References

5. Gómez R, Simón C, Remohi J, Pellicer A 2002 Vascular endothelial growth factor receptor-2 activation induces vascular permeability in hyperstimulated rats, and this effect is prevented by receptor blockade. Endocrinology 143: 4339–4348
6. Gómez R, Simón C, Remohi J, Pellicer A 2003 Administration of moderate and high doses of gonadotropins to female rats increases ovarian vascular endothelial growth factor (VEGF) and VEGF receptor-2 expression that is associated to vascular hyperpermeability. Biol Reprod 68:2164–2171