Vascular endothelial growth factor production by circulating immune cells is elevated in ovarian hyperstimulation syndrome

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BACKGROUND: Ovarian hyperstimulation syndrome (OHSS) is an iatrogenic disease manifesting itself by ovarian enlargement and massive ascites with increased peritoneal capillary permeability. Although vascular endothelial growth factor (VEGF) is considered to play the main role in developing OHSS, its precise mechanism remains unclear. In this study, we examined possible roles of circulating immune cells in the pathogenesis of OHSS.

METHODS: Peripheral blood mononuclear cells (PBMC) and plasma were collected from healthy non-pregnant volunteers and from patients receiving ovulation induction for IVF. PBMC were cultured for 48 h. Plasma and/or medium concentrations of VEGF, estradiol and progesterone were measured using enzyme-linked immunosorbent assay and radioimmunoassay kits. RESULTS: VEGF production by cultured PBMC and plasma concentrations of VEGF taken from patients with early onset OHSS (n = 12) were significantly higher than those in non-pregnant volunteers and patients without OHSS whose oocyte retrieval rates were similar to that of OHSS patients. OHSS patients were further classified into a high plasma VEGF concentration group and a high culture medium VEGF group. There was no significant correlation among VEGF production by PBMC and plasma concentration of VEGF, estradiol or progesterone. CONCLUSION: Although mechanistic evidence has not been provided, our study does provide new evidence to suggest that circulating immune cells are involved in the pathogenesis of OHSS via VEGF production.

Keywords: HCG; OHSS; PBMC; VEGF

Introduction

Ovarian hyperstimulation syndrome (OHSS) is one of the most serious complications resulting from the use of gonadotrophins for ovulation induction (Elchalal and Schenker, 1997). OHSS is characterized by massive cystic enlargement of the ovaries associated with hemoconcentration, ascites and pleural effusion. The shift of fluid into the third space is induced by increased peritoneal capillary permeability (Rizk et al., 1997). High hemoconcentration occasionally causes venous thrombosis, leading to cerebral infarction (Elford et al., 2002). Although the pathophysiology of this syndrome has not yet been completely elucidated, it is speculated that vasoactive substances secreted by the ovaries under human chorionic gonadotrophin (HCG) stimulation play a key role in the increased capillary permeability observed in this syndrome (Elchalal and Schenker, 1997).

For vasoactive substances, the renin–angiotensin system and various cytokines including interleukins (IL) and tumor necrosis factor-α have been proposed (Chen et al., 2000; Teruel et al., 2002). Among these, vascular endothelial growth factor (VEGF), which promotes angiogenesis and vascular permeability (Clauss, 2000), is most likely involved in the pathogenesis of OHSS (McClure et al., 1994). VEGF was initially found in ascitic fluid samples from patients with OHSS. Later, VEGF was demonstrated to be secreted from progesterone-producing granulosa cells in the ovarian follicles, and the production of VEGF was stimulated by HCG (Neulen et al., 1995; Lee et al., 1997a). The following studies reported that serum VEGF was significantly higher in the group that developed severe OHSS compared with that in those who did not. Therefore, serum VEGF was proposed to be useful for predicting the risk of OHSS (Krasnow et al., 1996; Abramov et al., 1997; Lee et al., 1997b; Agrawal et al., 1998, 1999; Levin et al., 1998, Aboulghar et al., 1999). From these findings, it is speculated that VEGF is the principal mediator to increase capillary permeability in OHSS and this pathological condition is brought through HCG stimulation (Rizk et al., 1997). However, several studies reported that serum VEGF (D’Ambrogio et al., 1999; Chen et al., 2000) and follicular VEGF in the ovary (Geva et al., 1999) were not significantly related to OHSS.
Although recent work showed that there was a significant correlation between plasma and serum VEGF levels in patients undergoing controlled ovarian stimulation for IVF (Manau et al., 2006), it was widely recognized that plasma, rather than serum, should be used for analysis because VEGF released from platelets during the coagulation process interfered with the serum VEGF concentration (Verheul et al., 1997; Jelkmann, 2001). However, the results of plasma VEGF in OHSS patients were still controversial (Artini et al., 1998; Enskog et al., 2001; Pau et al., 2006). Thus, the pathological contribution of VEGF to OHSS remains unclear.

Several studies showed that follicular and serum levels of various cytokines including IL-8 were elevated in OHSS (Mathur et al., 1997; Rizk et al., 1997; Aboughar et al., 1999). Recently, we observed that HCG stimulates the production of IL-8 and chemoattractant factors by peripheral blood mononuclear cells (PBMC) in vitro (Egawa et al., 2002; Kosaka et al., 2002; Nakayama et al., 2002; Fujisawa, 2006). In addition, our preliminary experiments showed that HCG promoted VEGF production by PBMC (unpublished data). Accordingly, we hypothesized that immune cells produce VEGF and contribute to increased capillary permeability in OHSS. In this study, to examine this possibility, the basal production levels of VEGF by PBMC were examined in patients who either developed or did not develop OHSS during IVF treatment. The relationship between VEGF production by PBMC and plasma concentrations of VEGF as well as estradiol and progesterone was also analysed.

Materials and Methods

Study subjects
Ovarian stimulation and oocyte collection for IVF-embryo transfer treatment were performed as described previously (Fujisawa et al., 2002). In brief, administration of a GnRH agonist (buserelin acetate; Aventis Pharma Co., Tokyo, Japan) was initiated in the mid-luteal phase or in the early follicular phase. All patients subsequently received pure FSH (Serono Japan Co., Tokyo, Japan) or hMG (Organon Japan Co., Tokyo, Japan) from cycle day 3 until the dominant follicle reached a diameter of more than 18 mm. An injection of HCG (Mochida Pharmaceutical Co., Osaka, Japan) was followed 36 h later by oocyte retrieval.

PBMC were prepared as described previously (Hashii et al., 1998). Volunteers were recruited from healthy non-pregnant women (proliferative phase cycle days 7–12, n = 14; secretory phase, cycle days 16–24, n = 13) with a regular menstrual cycle.

PBMC were also collected from patients undergoing IVF treatment, who developed early onset OHSS according to published criteria (Golan et al., 1989), on the day of diagnosis [5–10 days after oocyte retrieval, (mean ± SD) 7.5 ± 1.5 days, n = 12]. Among these patients with early onset OHSS, two patients were recognized as being pregnant later in the same cycle. In addition, PBMC were prepared from the non-pregnant IVF-treated patients in the secretory phase (5–10 days after oocyte retrieval, 7.9 ± 1.7 days), who did not develop OHSS (n = 16). To adjust parameters such as FSH dosage, plasma progesterone and estradiol among OHSS and non-OHSS patients, non-OHSS patients whose number of oocytes retrieved had been matched with that of OHSS patients were randomly selected from patients receiving IVF-embryo transfer treatment. PBMC were also obtained from pregnant patients (12–22 days after oocyte retrieval) who developed late-onset OHSS (n = 7) and those who did not (n = 13).

Moderate OHSS was diagnosed by abdominal distension, enlarged ovaries (more than 6 cm in diameter) and ascites, and severe OHSS was manifested by additional breathing difficulties and/or hydrothorax, hemoconcentration (hematocrit >45%) and oliguria. Both moderate (n = 14) and severe OHSS (n = 2) were referred to OHSS in this study, but mild OHSS was excluded from the analysis.

There were no significant differences among groups undergoing IVF treatment with early and late-onset OHSS or without OHSS with regard to age, numbers of oocytes collected, total dose of FSH or blood sampling day (Table 1).

Informed consent for the use of PBMC in this study was obtained from all participants. Analysis of these samples was approved by the Ethical Committee of Kyoto University Hospital.

PBMC culture
PBMC were isolated by centrifugation with Ficoll-Hypaque from 20 ml of heparinized venous blood (Hashii et al., 1998). After centrifugation, PBMC were collected from the interphase layer and washed four times with RPMI 1640 (GIBCO, Grand Island, NY, USA). From the upper layer, plasma was also collected and stored at −20°C until assayed for VEGF, estradiol and progesterone. Then these cells were suspended in RPMI 1640 supplemented with 10% FCS (Dainippon Pharmaceutical Co., Osaka, Japan), 100 U/ml of penicillin and 100 μg/ml of streptomycin. PBMC (1 × 10⁶ cells/ml) were immediately subjected to culture in a 24-well tissue culture plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA).

Table 1: Profiles of subjects

<table>
<thead>
<tr>
<th></th>
<th>Age (years)</th>
<th>Number of oocytes</th>
<th>FSH dosage (IU)</th>
<th>Blood sampling (day)</th>
<th>Plasma estradiol (fmol/ml)</th>
<th>Plasma progesterone (pmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volunteers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative phase (n = 14)</td>
<td>29.2 ± 3.68</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>364 ± 9</td>
<td>&lt;0.95</td>
</tr>
<tr>
<td>Secretory phase (n = 13)</td>
<td>30.3 ± 4.09</td>
<td>–</td>
<td>–</td>
<td>9.5 ± 2.1</td>
<td>436 ± 132</td>
<td>35.6 ± 4.3</td>
</tr>
<tr>
<td>IVF patients</td>
<td></td>
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</tr>
<tr>
<td>Secretary without OHSS (n = 16)</td>
<td>32.7 ± 3.92</td>
<td>14.3 ± 3.9</td>
<td>1359 ± 260</td>
<td>7.5 ± 1.5</td>
<td>15320 ± 7183</td>
<td>781 ± 375</td>
</tr>
<tr>
<td>Early onset OHSS (n = 12)</td>
<td>32.3 ± 3.64</td>
<td>15.1 ± 4.1</td>
<td>1288 ± 186</td>
<td>7.9 ± 1.7</td>
<td>15730 ± 6313</td>
<td>806 ± 347</td>
</tr>
<tr>
<td>Pregnancy without OHSS (n = 13)</td>
<td>33.0 ± 3.22</td>
<td>14.8 ± 4.2</td>
<td>1338 ± 225</td>
<td>18.1 ± 3.1</td>
<td>17910 ± 7251</td>
<td>1075 ± 367</td>
</tr>
<tr>
<td>Late onset OHSS (n = 7)</td>
<td>29.2 ± 2.56</td>
<td>14.5 ± 4.7</td>
<td>1307 ± 224</td>
<td>17.5 ± 3.4</td>
<td>17580 ± 6721</td>
<td>1053 ± 352</td>
</tr>
</tbody>
</table>

Date are mean ± SD. There were no significant differences among groups undergoing IVF treatment with early and late-onset OHSS or without OHSS with regard to age, number of oocytes collected, total dosage of FSH and plasma estradiol and progesterone.
in triplicate at 37°C in a humidified atmosphere of 5% CO₂ in air for 48 h. After the culture, medium was collected and stored at −20°C until assayed for VEGF.

**Assay of VEGF, estradiol and progesterone**

The concentration of VEGF was measured by ELISA kit (R&D System Inc., Minneapolis, MN, USA). This kit detects the free form of VEGF 125/165 isoforms, which are dominant forms in the blood circulation (Jelkmann, 2001; Tissot van Pato et al., 2005). Using this ELISA kit, the minimal reliable value was 4 pg/ml after modifying the standard curve. The inter- and intra-assay coefficients of variation were 4.6% and 7.7%, respectively. By RIA kits (Immunotech, Marseille, France), blood concentrations of progesterone and estradiol were measured using plasma (Nahoul et al., 1993; Christin-Maitre et al., 1998; Couzin et al., 1999), which was isolated during PBMC preparation. Inter- and intra-assay coefficients of variation were 5.7% and 5.3% for the progesterone assay and 7.4% and 6.3% for the estradiol assay, respectively.

**Statistical analysis**

The data were expressed as mean ± SD. Differences in VEGF concentrations among the cultured PBMC derived from women in the proliferative and secretory phases and IVF-treated patients with or without OHSS were analyzed by Kruskal–Wallis analysis followed by Mann–Whitney analysis. The relationship between VEGF production and plasma concentrations of VEGF, estradiol and progesterone was analyzed by simple regression. Chi-square test was used for comparison of the positive rate of high-value groups between OHSS and non-OHSS groups. Differences were regarded as significant at the 5% level.

**Results**

**Plasma concentration of VEGF**

In the non-OHSS groups, about half of the plasma samples were under cut-off value (Fig. 1). Plasma concentration of VEGF derived from both patients with early and late-onset OHSS were significantly higher than those from non-pregnant patients without OHSS (5–10 days after oocyte retrieval), pregnant patients without OHSS (12–22 days after oocyte retrieval) and non-pregnant volunteers in proliferative and secretory phases (Table 2, middle column).

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma VEGF (pg/ml)</th>
<th>VEGF by PBMC (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volunteers (n = 16)</td>
<td>8.4 ± 8.2</td>
<td>3.5 ± 5.0</td>
</tr>
<tr>
<td>Early onset OHSS (n = 12)</td>
<td>25.1 ± 19.8</td>
<td>18.6 ± 18.9</td>
</tr>
<tr>
<td>Pregnancy without OHSS (n = 13)</td>
<td>5.1 ± 7.1</td>
<td>5.6 ± 8.0</td>
</tr>
<tr>
<td>Late onset OHSS (n = 7)</td>
<td>26.0 ± 18.0</td>
<td>27.6 ± 35.3</td>
</tr>
</tbody>
</table>

Plasma concentrations of VEGF in the OHSS group were significantly higher than those in non-OHSS and volunteer groups (P < 0.0001). VEGF production by cultured PBMC in the early onset OHSS was significantly higher than that of IVF-treated patients without OHSS in secretory phase, but it showed no difference from that of pregnant patients without OHSS (P = 0.143).

**High VEGF production by PBMC in OHSS**

![Diagram](https://example.com/diagram.png)

**Figure 1:** The relationship between VEGF concentration in plasma and VEGF production by cultured PBMC from OHSS and non-OHSS patients. Blood samples were obtained from volunteers and pregnant and non-pregnant IVF-treated patients with or without OHSS. Isolated PBMC were cultured for 48 h. Concentrations of VEGF in the plasma and the culture media were measured. Concentrations of VEGF in the plasma and PBMC culture media derived from patients with OHSS were significantly higher than those from other groups. However, there was no significant correlation between VEGF production by PBMC and plasma VEGF concentration in patients with or without OHSS and volunteers (arrow, R = 0.225, P = 0.0526). Dotted lines show the 95th percentile values of the samples in non-OHSS patients and volunteers in plasma VEGF (horizontal line) and PBMC production (vertical line). OHSS patients were divided into two groups, a group with high plasma VEGF (dotted ellipse) and a group with high VEGF production by PBMC (solid ellipse). All patients with OHSS except one belonged to either of the two high-value groups (P < 0.0001).

**Relationship between VEGF production by PBMC and plasma VEGF concentration**

Although not statistically significant, there appeared to be a weak positive correlation between VEGF production by cultured PBMC in early OHSS and plasma VEGF concentration.
PBMC and plasma VEGF concentration in patients with or without OHSS and volunteers (R = 0.225, P = 0.0526, Fig. 1). However, in patients with OHSS, there was no correlation between VEGF production by PBMC and plasma VEGF concentration (R = 0.181, P = 0.468).

Patients with OHSS were apparently further classified into a high plasma concentration group (dotted ellipse, Fig. 1) and a high PBMC production group (solid ellipse, Fig. 1). When values above the 95th percentile in the non-OHSS and volunteers groups were defined as high values both in VEGF production by PBMC and plasma VEGF concentration, all but one of the patients with OHSS belonged to either high value group (P < 0.0001, Fig. 1).

**Relationship between VEGF production by PBMC or plasma VEGF concentration and plasma steroid hormone levels in OHSS patients**

There was no significant correlation between VEGF production by PBMC and plasma estradiol (R = 0.130, P = 0.595) or progesterone (R = 0.018, P = 0.943) concentration in patients with OHSS. Similarly, plasma VEGF did not show any correlation with plasma estradiol (R = 0.048, P = 0.844) or progesterone (R = 0.113, P = 0.645) concentration.

**Discussion**

OHSS is a unique pathological model where peripheral capillary permeability in a wide area facing the peritoneal and/or pleural cavity is uncontrollably increased (Goldman et al., 1995). It is considered that HCG induces this event as a trigger and that the subsequent local production of biologically active molecules, including VEGF, plays important roles in promoting vascular permeability. In accordance with previous reports (Artini et al., 1998; Pau et al., 2006), this study showed that plasma VEGF concentration was higher in patients with OHSS.

Clinically, the shift of fluid into the third space in OHSS is usually limited to the peritoneal cavity, not throughout the whole body. Therefore, many investigators have speculated that luteinizing granulosa cells in the ovarian follicles are the main target cells responding to HCG by producing VEGF (Neulen et al., 1995; Lee et al., 1997a; Yamamoto et al., 1997; Otani et al., 1999; Sugino et al., 2000; Wulff et al., 2001; Wang et al., 2002) and the elevated local production of OHSS in the ovary induces increased peritoneal capillary permeability, resulting in a high concentration of circulating VEGF. However, Geva et al. (1999) did not find any differences in local follicular VEGF levels among the groups with and without OHSS, speculating that the increased capillary permeability found in OHSS is due to certain systemic effects. Pellicer et al. (1999) also reported that VEGF levels in the follicular fluid of patients who were at risk for OHSS were lower than those of patients who were not at risk. The present study showed that basal production of VEGF by circulating immune cells was significantly elevated in patients with early onset OHSS, providing a novel possibility that VEGF secretion by circulating immune cells contributes to the increased capillary permeability in OHSS. This also indicates that the functioning of immune cells is systemically affected in patients with OHSS. In general, immune cells migrate from blood circulation to stromal tissues around inflammatory sites, becoming activated (Rosales and Juliano, 1995; Miyasaka and Tanaka, 2004). It has been proposed that ovulation and subsequent corpus luteum formation mimic an inflammatory reaction and circulating immune cells are recruited in this tissue-remodeling site (Espey, 1994; Bukulmez and Arici, 2000). Accordingly, in patients with OHSS, the increase in local production of VEGF can be superimposed by migrating immune cells in the ovary.

Unexpectedly, there was no significant correlation between VEGF production by PBMC and plasma VEGF concentration in patients with OHSS, suggesting heterogeneity in the pathogenesis of OHSS. Although the precise reasons for such differences are unknown, it seems likely that circulating immune cells do not directly contribute to plasma VEGF concentration. Interestingly, patients with OHSS were composed of two distinct groups, a group with high plasma VEGF and a group with high VEGF production by PBMC. Most patients with OHSS belong to either high group, confirming the current concept that VEGF is an important factor for the pathogenesis of OHSS. Since standard approaches could not detect the presence of the latter group showing high VEGF production by PBMC, this may be one of the main reasons that the roles of VEGF in pathogenesis of OHSS remained unclear for more than a decade. Consequently, to further understand the pathophysiology and/or develop effective therapy for OHSS, the functional changes of PBMC in OHSS patients should be noted as a new factor. In this study, it remains unclear what populations of mononuclear cells mainly produce VEGF. The possible differences in PBMC populations should also be clarified among OHSS and non-OHSS patients.

In conclusion, this study demonstrated that basal production of VEGF by PBMC was significantly elevated in patients with OHSS. Although mechanistic evidence has not been provided, these findings suggest that circulating immune cells are involved in the pathogenesis of OHSS. Since OHSS is a unique pathological model for increased peritoneal vascular permeability, clinical data from this study will provide a new perspective on vascular pathophysiology.

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**References**


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