Vascular endothelial growth factor levels in serum and plasma from patients undergoing controlled ovarian hyperstimulation for IVF

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BACKGROUND: Vascular endothelial growth factor (VEGF) has been investigated as a marker of ovarian response to controlled ovarian hyperstimulation and as a predictor of ovarian hyperstimulation syndrome (OHSS) in IVF cycles. In most studies, serum has been used for circulating VEGF concentration measurement, but it has been suggested that plasma is the preferred medium to measure VEGF levels because of the potential contribution of VEGF released from platelets during blood clotting. This study investigated VEGF concentrations in paired serum and plasma samples from patients undergoing controlled ovarian hyperstimulation for IVF.

METHODS: Serum and plasma VEGF levels, as well as the number of platelets, were measured in 30 IVF patients who comprised three study groups delineated according to the estradiol (E2) serum concentration reached on the day of HCG administration: 10 patients having low E2 serum levels (<1500 pg/ml, group L), 10 patients having intermediate E2 serum levels (1500–3000 pg/ml, group I) and 10 patients having high E2 serum levels (>3000 pg/ml, group H). RESULTS: There was a statistically significant correlation between plasma and serum VEGF levels (r = 0.61; P < 0.005) for the entire population studied, although serum values were higher by a factor of ~6-fold. No significant correlation was found between peripheral blood VEGF concentrations and serum E2 or follicle number on HCG day or the number of oocytes collected. Similarly, paired serum and plasma VEGF measurements did not correlate with platelet count.

CONCLUSIONS: Serum and plasma VEGF concentrations are strongly correlated in paired samples from infertile patients undergoing controlled ovarian hyperstimulation. However, neither serum nor plasma VEGF levels were correlated with parameters associated with ovarian follicular activity. Peripheral blood VEGF levels were not correlated with platelet count.

Key words: controlled ovarian hyperstimulation/IVF/plasma VEGF/serum VEGF

Introduction

Vascular endothelial growth factor (VEGF) is a cytokine showing potent angiogenic and endothelial cell mitogenic properties, which are considered to be key factors associated with rapid vascular growth in a variety of physiological and pathological conditions (Ferrara and Davis-Smyth, 1997; Lebovic et al., 1999; Ferrara, 2004). In the ovary, VEGF is produced by both theca and granulosa cells; it is expressed and secreted in the premenopausal human ovary in a cyclic manner and regulated by gonadotrophin secretion during the menstrual cycle (Geva and Jaffe, 2000).

A higher ovarian production of VEGF is observed in response to FSH, LH and HCG, and the local production of VEGF in response to the surge in LH/HCG mediates the conversion of the avascular pre-ovulatory follicle into a highly vascular corpus luteum (Christenson and Stouffer, 1997; Neulen et al., 1998; Lebovic et al., 1999). Marked changes in the vascular system accompany follicular development, and thus, the pre-ovulatory follicle provides a unique physiological example of rapid growth associated with significant changes in follicular vascularity including greater microvascular perfusion (Tanaka et al., 1989; Zackrisson et al., 2000), increased capillary density (Cavender and Murdoch, 1988) and enhanced capillary permeability (Okuda et al., 1983; Neeman et al., 1997). These processes are magnified in IVF cycles where ovarian stimulation with exogenous gonadotrophins is used to induce multiple follicular growth and maturation.
On the contrary, because VEGF stimulates vascular permeability, this cytokine has been proposed as a promising molecule in the understanding of ovarian hyperstimulation syndrome (OHSS), a complication of controlled ovarian hyperstimulation for IVF (García-Velasco and Pellicer, 2003). Thus, VEGF is critical for normal ovarian function, but increased production of VEGF may be associated with vigorous gonadotrophin ovarian stimulation and OHSS. A good number of studies have focused on the use of peripheral blood VEGF concentration as a marker of ovarian response to controlled ovarian hyperstimulation (Lee et al., 1997; Moncayo et al., 1998; Artini et al., 1998a; Pellicer et al., 1998, 1999a; Battaglia et al., 2000; Manau et al., 2000; Unkila-Kallio et al., 2000; Agrawal et al., 2002; Licht et al., 2002; Manau et al., 2002; Tokuyama et al., 2002; Dorn et al., 2003; Molskness et al., 2004; Ocal et al., 2004; Asimakopoulos et al., 2005; Oliveira et al., 2005; Babayof et al., 2006) or as a predictor of OHSS (Krasnow et al., 1996; Abramov et al., 1997; Agrawal et al., 1998; Artini et al., 1998b; Ludwig et al., 1998; Aboulghar et al., 1999; Agrawal et al., 1999; D’Ambrogio et al., 1999; Geva et al., 1999; Ludwig et al., 1999; Pellicer et al., 1999b; Chen et al., 2000a,b; Enskog et al., 2001; Ogawa et al., 2001; Artini et al., 2002; Mathur et al., 2002; Wang et al., 2002; McElhinney et al., 2002a,b; García-Velasco et al., 2004; Tozer et al., 2004; Griesinger et al., 2006; Pau et al., 2006) in IVF cycles. These relevant English language articles were identified in the MEDLINE database using ‘VEGF and IVF’ and ‘VEGF and OHSS’ as subject heading terms.

In 31 (74%) of those studies, serum was used for circulating VEGF concentration measurement, whereas VEGF was measured in plasma in the remaining 11 (26%) studies. This is noteworthy, considering that previous work has suggested that plasma is the preferred medium to measure VEGF levels, because a significant and highly variable platelet-mediated secretion of VEGF during the clotting process would invalidate the use of serum as an indicator of circulating VEGF levels in both healthy subjects (Webb et al., 1998; Larsson et al., 2002; McIlhenny et al., 2002; Kusumanto et al., 2003) and disease states (Banks et al., 1998; Gunsilius and Gastl, 1999; Kusumanto et al., 2003; Ohta et al., 2003; Ferrero, 2004). When VEGF is measured, the difference between plasma and serum is relevant, and this may make the interpretation of data in the literature very difficult.

Therefore, on the above evidence, this prospective study was undertaken to measure both serum and plasma concentrations of VEGF in patients undergoing controlled ovarian hyperstimulation for IVF, which, to our knowledge, has not been reported previously. Also, because it has been suggested that non-homogeneous serum concentrations of estrogen may explain marked differences in circulating VEGF levels in women undergoing controlled ovarian hyperstimulation (Bussen and Dietl, 2003), this parameter was also considered in this study.

**Materials and methods**

**Patients**

This study enrolled 30 women with primary infertility undergoing IVF who consented to the study, which was approved by the Investigation and Ethics Committee of our hospital. All patients had both ovaries and had regular menstrual cycles every 27–32 days and normal ovulatory function as shown by midluteal serum progesterone and prolactin determinations, and ultrasonographic scanning. The women were 32–39 years old, the mean (±SE) age being 35.3 ± 0.5 years. All had normal blood pressure and body mass index (range 19–26 kg/m²), were non-smokers and were not taking any medication or involved in intensive exercise. Patients underwent IVF because of male factor or unexplained infertility. Patients with endometriosis were not included in this study, because an increase in circulating VEGF levels in diseased females has been debated in the literature (Pellicer et al., 1998; Gagné et al., 2003; Matalliotakis et al., 2003). The 30 patients comprised three study groups delineated according to the estradiol (E₂) serum concentration reached on the day of HCG administration: 10 consecutive patients having low E₂ serum levels (<1500 pg/ml, group L); 10 consecutive patients having intermediate E₂ serum levels (1500–3000 pg/ml, group M) and 10 consecutive patients having high E₂ serum levels (>3000 pg/ml, group H). This was performed in an attempt to investigate potential differences in circulating VEGF concentration based on E₂ serum levels.

**Ovarian stimulation regimen**

Controlled ovarian hyperstimulation for IVF was performed according to a protocol previously reported, including ovarian stimulation under pituitary suppression with a GnRH agonist (Manau et al., 2002). Briefly, daily s.c. leuprolide acetate was started in the midluteal phase of the previous cycle, and gonadotrophin stimulation of the ovaries was started 13–14 days later when the serum E₂ concentration declined to <50 pg/ml and a vaginal ultrasonographic scan showed an absence of follicles of ≥10 mm diameter. On days 1 and 2 of ovarian stimulation, 450 and 300 IU/day of recombinant FSH (Gonal-F, Serono S.A., Madrid, Spain), respectively, was administered s.c. On days 3 and 4 of ovarian stimulation, 150 IU/day of FSH was administered to each patient. From day 5 onwards, FSH was administered on an individual basis according to the ovarian response as assessed by sequential transvaginal ultrasonography and serum E₂ measurements. HCG (5000 IU) (Profasi; Serono S.A.) was administered i.m. when a consistent rise in serum E₂ concentration was associated with the presence of two or more follicles of ≥18 mm diameter. Oocyte aspiration was performed by vaginal ultrasonography 35–36 h after HCG injection.

**Blood sample collection and processing**

Blood samples for E₂ and VEGF measurement were taken from all patients on the day of HCG administration. Samples were collected at room temperature in three different tubes for analysis of serum E₂ levels and VEGF concentrations in serum and in plasma. Serum samples were collected in vacutainer tubes without additive (366468, Becton Dickinson, Rutherford, NJ, USA). Plasma samples were collected in vacutainer tubes containing EDTA as an anticoagulant (368861, Becton Dickinson). As previously recommended (Hombrey et al., 2002; Ferrero et al., 2004), samples were processed between 30 and 60 min after collection. The samples were centrifuged at 3000 g for 10 min, and the serum or plasma was carefully transferred to new tubes. The serum and plasma closest to the cells were not used. For this study, two serum aliquots were obtained. E₂ was measured in one of the serum aliquots for clinical monitoring, and the second aliquot and the corresponding matched plasma sample were stored at −70°C from collection until tested. Frozen serum and plasma samples from each patient for VEGF measurement were examined in one run within 3 months of collection. Platelet counts were also recorded on the day of HCG injection in all patients.
Hormone analyses and ultrasonography

E2 in serum and VEGF in serum and plasma were measured using commercially available kits as reported previously (Manau et al., 2002; Balasch et al., 2004). E2 concentrations in serum were estimated by a competitive immunoenzymatic assay (Immuno 1, Technicon; Bayer, Tarrytown, NY, USA). The sensitivity was 10 pg/ml, and the inter-assay coefficient of variation (CV) was 5%. Serum and plasma VEGF concentrations were quantified using enzyme-linked immunosorbent assay (ELISA) (Quantikine Human VEGF Immunoassay, R&D Systems Inc., Minneapolis, MN, USA) that recognizes the soluble isoforms VEGF121 and VEGF165, which are the most frequently expressed spliced variants of the VEGF A gene. The sensitivity of the assay was 7 pg/ml. Intra- and inter-assay CV in serum samples were 4.5 and 7%, respectively. The corresponding figures in plasma samples were 6.7 and 8.8%. This assay for the measurement of VEGF is the most widely used (Hormbrey et al., 2002). Thus, the ELISA method for VEGF measurement was used in most (95%) of studies identified in our literature search and quoted in the Introduction section; in fact, in most (80%) of cases, the ELISA was obtained from R&D Systems.

Ultrasonic scans were performed using a Toshiba Eccocee SAA-340A EF unit (Toshiba Co., Tokyo, Japan) equipped with a 5–7 MHz endovaginal probe (PVF-641VT).

Statistical analysis

Data were analysed by Statistics Package for Social Sciences (SPSS) statistical software using the non-parametric Mann–Whitney U-test, the Kruskal–Wallis test and Spearman’s correlation coefficient when appropriate. Results are expressed as mean ± SE and were considered significant at a P-value of <0.05.

Results

The mean ages of patients were similar in the three groups studied (group L: 36.8 ± 0.7 years; group I: 36.4 ± 0.6 years and group H: 35.7 ± 0.6 years). Infertility aetiologies were also similar in groups L (male factor, n = 6; unexplained infertility, n = 4), I (male factor, n = 6; unexplained infertility, n = 4) and H (male factor, n = 5; unexplained infertility, n = 5). Similarly, there were no differences with respect to the mean body mass index in groups L (22.1 ± 0.78 kg/m²), I (23.5 ± 0.66 kg/m²) and H (22.9 ± 0.9 kg/m²). All patients had multiple follicular development (13.8 ± 1.76 follicles of >10 mm on the day of HCG) and successful oocyte retrieval (10.5 ± 1.3 oocytes). As expected, statistically significant differences were observed regarding the peak E2 levels reached during ovarian stimulation in groups L (1320 ± 70 pg/ml), I (2367 ± 155 pg/ml) and H (3675 ± 208 pg/ml) (P < 0.005). However, both serum and plasma VEGF levels were similar in groups L, I and H (Figure 1). No patient developed OHSS.

There was a statistically significant correlation between plasma and serum VEGF levels (r = 0.61; P < 0.005) for the entire population studied (Figure 2), although serum values were higher by a factor of ~6-fold (296.8 ± 15.4 versus 47.9 ± 6.8 pg/ml; P < 0.001). Data regarding correlations between serum and plasma VEGF concentrations and the main parameters associated with ovarian follicular activity are presented in Table I. No significant correlation was found between peripheral blood VEGF concentrations and serum E2 or follicle number on HCG day or number of oocytes collected. Similarly, paired serum and plasma VEGF measurements did not correlate with platelet count (Table I).

Discussion

The development of the ovarian follicle from a small pre-antral follicle to the large pre-ovulatory follicle and the subsequent formation of a corpus luteum after ovulation are essential processes in mammalian reproduction. The pre-ovulatory follicle provides a unique physiological example of rapid growth accompanied by neovascularization, two processes that are characteristic of malignancy (Neeman et al., 1997). Several factors have been identified that have angiogenic activity, the most potent and specific of which is VEGF (Ferrara and Davis-Smyth, 1997; Ferrara, 2004). In fact, many striking morphological, biophysical and biochemical similarities between the pre-ovulatory follicle and solid tumours have been reported, with both systems showing a similar pattern of expression of the angiogenic factor, VEGF (Neeman et al., 1997). However, whereas the significance of circulating levels of VEGF in cancer patients is now
Correlation of serum and plasma VEGF concentrations, with...d and the prognostic importance of microvessel...demic angiogenesis after transfection experiments, inhibi...ondary angiogenic stimulators and inhibitors from the tumour vasculature (Ferrero, 2004). On this evidence, it has been suggested that, although the serum VEGF levels are affected by blood platelets, platelet-derived VEGF levels also reflect biology of cancer cells and that serum would be the more useful specimen for measurement of circulating VEGF in cancer patients for prognosis (George et al., 2000; Lee et al., 2000; Salgado et al., 2001). Overall, it has been stressed that the fact that serum VEGF levels appear more strongly prognostic than plasma may be because platelets deliver VEGF to tumours or endocytose the increased VEGF load released by cancer cells or may be because the estimation of in vivo plasma VEGF has not been accurate (Hormbrey et al., 2002).

Most of the early studies showing prognostic information of VEGF in cancer patients were performed using serum samples. Careful reexamination using plasma samples has confirmed the concept that the concentration of circulating free VEGF is increased in malignant disease (Jelkmann, 2001; Hormbrey et al., 2002). In addition, paired serum and plasma VEGF measurements have been reported to be strongly correlated both in cancer patients and in control subjects, with correlation coefficients ranging from 0.56 to 0.68 for malignancies (Adams et al., 2000; George et al., 2000) and from 0.68 to 0.73 in healthy subjects (George et al., 2000; Larsson et al., 2002). This is in agreement with results in the current investigation where a strong correlation was found between VEGF values in serum and plasma in patients undergoing controlled ovarian hyperstimulation for IVF. Also, in keeping with previous work (Webb et al., 1998), serum values in our study were higher than matched plasma values by a factor of ∼6-fold. Because we were measuring paired plasma and serum samples, we were extremely vigilant in our sample collection. Thus, all samples were collected by identical means at room temperature, were processed at a standardized time between 30 and 60 min after collection and were then immediately stored until immunoassay as previously recommended (Hormbrey et al., 2002; Ferrero et al., 2004). This ensured thorough platelet activation before centrifugation and freezing serum samples.

In contrast with previous studies in cancer patients, serum VEGF showed no more biological significance than plasma VEGF in IVF patients who underwent controlled ovarian hyperstimulation uneventfully. In fact, neither serum nor plasma VEGF concentrations on the day of HCG administration were correlated with the total number of follicles, the total number of oocytes, the number of oocytes or peak serum $E_2$ levels, all of which are considered as traditional parameters of ovarian follicle activity. Furthermore, both serum and plasma VEGF levels were similar in the three groups of patients.

being elucidated, the significance of peripheral blood VEGF concentrations in healthy individuals is unclear (Jelkmann, 2001; McIlhenny et al., 2002).

Many tumour cell lines secrete VEGF in vitro, suggesting the possibility that this diffusible molecule may be a mediator of tumour angiogenesis. In situ hybridization studies have demonstrated that the VEGF mRNA is expressed in the vast majority of human tumours so far examined, including carcinoma of the ovary among many others (Ferrara, 2004). The key role for VEGF in tumour biology is supported by observations of enhanced angiogenesis after transfection experiments, inhibition of tumour growth by anti-VEGF-blocking monoclonal antibodies and the prognostic importance of microvessel density in human tumours (Hormbrey et al., 2002; Ferrara, 2004).

Correlations have been demonstrated between the degree of tumour vascularization, VEGF expression and prognosis in several cancers; this has stimulated evaluation of circulating concentrations of VEGF as a potential objective prognostic indicator in malignancies (Hormbrey et al., 2002; Ferrara, 2004). Interestingly, however, controversy exists whether serum or plasma VEGF levels would provide the best prognostic information or best reflect what is happening at the tumour site, i.e. which would be the more sensitive marker of the status of cancer. Plasma levels represent free VEGF circulating levels,
included in this study, thus providing evidence against the hypothesis that non-homogeneous serum concentrations of estrogen may explain why the higher VEGF levels are observed in some supposedly high-risk IVF women who subsequently developed OHSS (Bussen and Dietl, 2003). It could be argued that peripheral blood VEGF concentration was measured only on the day of HCG injection in this study, whereas VEGF expression in the human ovary is an LH/HCG-dependent process during ovulation and luteal phase (Geva and Jaffe, 2000). However, we previously reported no significant changes in plasma VEGF levels throughout the luteal phase (including the day when the HCG ovulatory injection was administered) in IVF patients not developing OHSS (Manau et al., 2002). Our study results are also in keeping with recent work showing no correlation between plasma or serum levels of VEGF and E2 levels throughout the menstrual cycle in healthy female volunteers (McIlhenny et al., 2002).

While many studies have reported a high (Gunsilius and Gastl, 1999; George et al., 2000; Lee et al., 2000) or significant but weak (Adams et al., 2000; Spence et al., 2002) correlation between serum VEGF levels and platelet counts in cancer patients, the number of platelets was not correlated with peripheral blood VEGF concentrations in healthy controls (Salven et al., 1999; George et al., 2000). This is in agreement with the present study where infertile but otherwise healthy women were included. The correlation of serum VEGF with platelet counts in patients with malignancies but not in control subjects may be attributable to the scavenging of VEGF from the tumour source by platelets (George et al., 2000).

On the above-discussed data, we believe that plasma should be the preferred medium to measure VEGF levels in healthy patients such as women undergoing controlled ovarian hyperstimulation, because serum VEGF measurement shows no more biological significance than plasma VEGF in such patients, and also to avoid platelet-mediated secretion of VEGF during the clotting process. The latter effect may explain the very high variability in serum VEGF values observed in our study mainly in samples where plasma VEGF concentration was around the detection limit (see Figure 2). Although, as discussed above, platelet number failed to reach a statistically significant correlation with serum VEGF concentration in this study and previous reports, this may be explained by a wide interindividual variability in platelet VEGF content and production (Banks et al., 1998).

In conclusion, this study shows that serum VEGF concentration correlates strongly with plasma VEGF concentration in paired samples from infertile patients undergoing controlled ovarian hyperstimulation for IVF. However, neither serum nor plasma VEGF levels were correlated with a number of traditional parameters associated with ovarian follicular activity such as serum E2, follicle number or number of oocytes collected. Finally, peripheral blood VEGF levels were not correlated with platelet count.

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References


Serum and plasma VEGF levels in IVF cycles


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