Background: $\alpha_2$-macroglobulin ($\alpha_2$M) is a multifactorial binding protein, found in follicular fluid, that is a naturally occurring inhibitor of vascular endothelial growth factor (VEGF). The aim of this study was to determine if there is a relationship between serum VEGF levels, $\alpha_2$M levels and the development of OHSS in hyperstimulated subjects undergoing IVF (those with 15 or more oocytes).

Methods: Venous blood was collected at the time of oocyte retrieval from subjects who yielded 15 or more oocytes. Serum samples were analysed for VEGF and $\alpha_2$M concentrations.

Results: There was no statistically significant difference in serum VEGF levels at the time of oocyte retrieval between hyperstimulated subjects who did and did not subsequently develop OHSS [3.95 (3.3–4.4) versus 3.85 (3.3–4.5); $P = 0.79$]. By contrast, the serum level of $\alpha_2$M was statistically significantly higher in the group of subjects who did not develop OHSS [2.27 (1.91–2.58) versus 1.67 (1.45–1.73)].

Conclusions: These results suggest that elevated $\alpha_2$M levels are associated with a decreased risk of developing OHSS. $\alpha_2$M may act by ‘removing and inactivating’ VEGF, with higher levels providing increased protection against the syndrome. $\alpha_2$M measurements may help to differentiate those for whom it is safe to proceed with embryo transfer from those for whom it is not, because of the risk of OHSS.

Key words: $\alpha_2$-macroglobulin/binding protein/OHSS/oocyte retrieval/VEGF

Introduction

Ovarian hyperstimulation syndrome (OHSS) is a serious and potentially life-threatening complication of ovarian stimulation. Mild cases occur in 8–23%, moderate forms in 0.005–7% and severe forms in 0.008–10% of treatment cycles (Schenker and Weinstein, 1978). The changes observed might be considered a magnification of processes that occur in a normal ovulatory cycle with an overriding or disruption of the usual homeostatic mechanisms. The major clinical components are marked ovarian enlargement and ascites. Severe cases are also associated with thromboembolic phenomena, respiratory distress and pre-renal failure.

The risk of developing OHSS is linked to the magnitude of ovarian response. Whilst follicle/oocyte number and serum oestradiol have both been used as predictive markers for the condition (Haning et al., 1983; Navot et al., 1992), only a relatively small percentage of cases are accurately predicted. This may imply that factors beyond the ovary play a key role in the pathogenesis of the condition.

The exact pathogenetic mechanism responsible for OHSS remains obscure but it is largely accepted that the basic event is an acute increase in capillary permeability both within the ovary and the peritoneal cavity (Goldsman et al., 1995). Over the past three decades, numerous substances have been investigated for the role of mediator but most have been discredited. However, recent evidence suggests an interaction between the immune and reproductive systems (Orvieto and Ben-Rafael, 1996), and at present, factors belonging to the immune system and exaggerated folliculogenesis are pathogenetic forerunners.

Vascular endothelial growth factor (VEGF) is a highly conserved multifunctional cytokine displaying a high specificity for endothelial cells (Ferrara and Davis-Smyth, 1997). VEGF expression and production within the ovary are critical for normal reproductive function (Geva and Jaffe, 2000). It is not only an important mediator of angiogenesis in the reproductive tract (Redmer and Reynolds, 1996; Smith, 1998), but also an important stimulator of vascular permeability (Ferrara et al., 1991). It is clear that the intraovarian angiogenesis that normally accompanies follicular development after the LH surge (Miyazaki et al., 1998) is abnormally exaggerated in hyperstimulated patients because of the increased number of follicles undergoing ovulatory changes. It is proposed that in patients who develop OHSS, the ovaries and the body’s normal homeostatic mechanisms are unable to combat the side-effects of this excessive response. The increased levels of VEGF, therefore, overwhelm the capillary bed in the ovaries and the peritoneal cavity, leading to increased permeability and to the
fluid shifts from the vascular to the extravascular space that typify this condition.

Studies on VEGF in OHSS patients have shown that it may be responsible for the acute increase in vascular permeability and development of ascites (McClure et al., 1994; Neulen et al., 1995). Among patients at risk for OHSS, serum VEGF levels have been reported to be significantly higher in patients who develop severe OHSS (Agrawal et al., 1999). The kinetics of VEGF in the plasma of seven patients who developed OHSS was closely correlated with the clinical course of the syndrome and with certain biological characteristics of OHSS and of capillary leakage such as leukocytosis and increased haematocrit (Abramov et al., 1997). It was found (McClure et al., 1994) that incubation of OHSS ascites with VEGF antibodies almost completely removed its capillary permeability inducing potential. The authors concluded that the major capillary permeability agent in OHSS ascitic fluid was VEGF. Furthermore, in a subsequent study, individual follicular fluid induced endothelial cell permeability, the extent of which was highly correlated with oocyte number. This effect was almost totally reversed by the addition of VEGF antibody. The authors concluded that VEGF is the factor responsible for the increased vascular permeability (Levin et al., 1998).

Whilst McClure et al. (1994) identified VEGF in OHSS ascites, others have endeavoured to measure VEGF levels in serum (Krasnow et al., 1996; Lee et al., 1997; Agrawal et al., 1997, 1998) and plasma (Artini et al., 1998). Some reports have suggested that levels are increased in subjects who ultimately develop OHSS (Krasnow et al., 1996; Agrawal et al., 1997; Lee et al., 1997) whilst others have not (Chen et al., 2000; Enskog et al., 2001). These studies have utilized a variety of assay techniques and usually have not specified if they are measuring total or free VEGF. Thus, the exact relationship is unclear.

It is well recognized that some patients with large numbers of oocytes do not develop OHSS whilst some with an apparently average degree of ovarian response do. If the syndrome is due to an increased level of VEGF secondary to an excessive degree of stimulation, it is possible that some women have a better intrinsic ability to neutralize the effects of the VEGF than others. This ability would, therefore, explain why comparable risks of OHSS.

α2-Macroglobulin (α2M) is a major serum protein associated with both the remodelling processes of ovulation and with the maintenance of the corpus luteum (Gaddy-Kurten et al., 1989). These processes may require the inactivation of specific luteolytic growth factors, such as VEGF. Recent work has shown that when VEGF binds to α2M, the receptor binding ability of VEGF is inactivated (Soker et al., 1993; Bhattacharjee et al., 2000). Therefore, α2M may act as a VEGF ‘removing and inactivating’ protein. Variations in endogenous levels of this naturally occurring inhibitor may explain why some women are less susceptible than others to developing OHSS despite similar degrees of ovarian stimulation.

The aims of this paper are 2-fold: (i) to measure the serum levels of α2M and VEGF in patients at the time of oocyte retrieval; and (ii) to determine if there is relationship between these levels and the development of OHSS.

Materials and methods

Subject selection

Patients undergoing ovarian stimulation for IVF between August 1998 and July 1999 were recruited to the study at the Regional Fertility Centre, Royal Maternity Hospital, Belfast. Ethical approval was obtained from the Queen’s University Belfast Research Ethical Committee. A total of 218 subjects were recruited of which 52 had more than 15 oocytes at oocyte retrieval. These were divided into two groups: group 1 ‘OHSS’ included 10 subjects who developed moderate/severe OHSS requiring hospital admission according to published criteria (Golan et al., 1989). Group 2 (‘no-OHSS’) included the remainder (n = 42), none of whom developed OHSS.

All subjects underwent a standard treatment protocol with: (i) pituitary down-regulation Synarel (Searle, High Wycombe, UK), a GnRH agonist which was commenced in the mid-luteal phase and administered nasally until the ovulatory HCG injection was given. Ovulation induction was with HMG (Menogon; Ferring, Langley, UK) or recombinant (r)FSH (Gonal F; Serono Pharmaceuticals, Feltham, UK; or Puregon; Organon Laboratories Ltd, Cambridge, UK).

Subjects were scanned on day 11 of stimulation to determine the degree of ovarian response. Those with fewer than four follicles >18 mm in diameter had their cycle abandoned (‘failed cycle’). The remainder received an HCG (Profasi; Serono) injection of 10 000 IU to induce final oocyte maturation. At 34–36 h after HCG administration, all ovarian follicles were aspirated transvaginally under ultrasound control. Subjects received Rapifen (Janssen–Cilag, High Wycombe, UK) intravenously as necessary for pain relief.

Immediately before commencing oocyte retrieval, a sample of peripheral blood was taken. The blood was allowed to clot, refrigerated and then centrifuged before the serum was withdrawn. The serum samples were stored at −70°C until required for analysis. Subjects thought to be at significant risk of OHSS (guideline: total oocyte number >15) were offered the option of embryo freezing rather than fresh embryo transfer.

All subjects who underwent embryo transfer received luteal phase support with daily vaginal progesterone pessaries (Cyclogest, 400 mg twice daily; Shire Pharmaceuticals Ltd, Hants, UK).

Hormone assays

VEGF

Circulating total serum VEGF concentrations were determined by an in-house radioimmunoassay, which was a modification of a published method (Anthony et al., 1997). Briefly, a double antibody method was used to separate bound from free [125I]recombinant human (rh)VEGF. rVEGF165 was used as standard (R&D Systems, Europe Ltd). Labelled VEGF ([125I]rhVEGF) was used as a tracer (Amersham Pharmacia Biotech, UK Ltd). Polyclonal goat anti-human VEGF antiserum (R&D Systems, Europe Ltd) at an initial dilution of 1:2000 was used as the primary antibody. The assay buffer was 0.2% bovine serum albumin in 0.04 mol/l phosphate buffer (pH 7.4) with 625 KIU Trasylol (Bayer AG, Germany) added per millilitre. Polyethylene glycol was added to assist in the precipitation of the VEGF–antibody complex.

Aliquots (100 µl) of standard or unknown serum (diluted in assay buffer with 0.14 mol/l NaCl) were incubated with 100 µl of diluted antibody. 100 µl [125I]rhVEGF (diluted 1:300 in assay buffer with 680 IU heparin/20 ml, i.e. 3.4 IU per tube) was added and tubes were incubated overnight at 4°C. A polyethylene glycol assisted double
The quantitative determination of α₂-polyethylene glycol added to each tube was used to separate antibody- and 100 ml donkey antigoat at 1:25 (IDS, UK Ltd) and 500 ml 4% antibody method [100 ml carrier normal goat serum at 1:2000 dilution (A2M) (g/l) levels in the ovarian hyperstimulation syndrome (‘OHSS’) and ‘no-OHSS’ groups. Serum samples were loaded onto racks of nine (eight in total) and placed in the Beckman Image Immunochemistry System. Prior to

antibody method [100 ml carrier normal goat serum at 1:2000 dilution and 100 ml donkey antigoat at 1:25 (IDS, UK Ltd) and 500 ml 4% polyethylene glycol added to each tube] was used to separate antibody-bound [125I]hVEGF from free [125I]hVEGF. The radioactivity in the bound fraction was then counted on a gamma counter (Wallac 1277 Gammamaster, automatic gamma counter). All samples were assayed in duplicate and at dilutions of 1/5 and 1/10 to allow more accurate readings on the most sensitive part of the standard curve.

Pooled sera samples were aliquoted and stored at −20 °C to run as controls in all assays. The intra- and inter-assay coefficients of variation (CV) were 5.8 and 10.4% respectively at 4 ng/ml. The antibody showed no cross-reactivity with other cytokines as specified in the R&D data sheet.

α₂-Macroglobulin
The quantitative determination of α₂M in serum was determined by rate nephelometry. The rate nephelometer measures the increase in the intensity of light scattered by particles suspended in solution as a result of complexes formed during an antigen–antibody reaction [α₂M (sample) + antibody → α₂M (sample) – antibody (aggregates)]. Serum samples were loaded onto racks of nine (eight in total) and placed in the Beckman Image Immunochemistry System. Prior to

analysis, reagents, buffers and diluents were checked. Each new reagent was calibrated. The Beckman α₂M test is standardized to the International Federation of Clinical Chemistry (IFCC) International Reference Preparation for Plasma Proteins, lot CRM 470, certified by the Bureau of Reference of the European Community (BCR).

The intra- and inter-assay coefficients of variation of the system were 2.8 and 3.3% respectively. The lower detection limit was 0.4 g/l (40 mg/dl) (95% confidence interval).

Statistical analysis
All data are presented as median (interquartile range). Due to the non-Gaussian distribution of the data, the non parametric Mann–Whitney $U$-test was used to assess differences between the ‘OHSS’ group and the ‘no-OHSS’ group within each parameter. In addition, differences in certain parameters between the two groups were also assessed by Student’s $t$-test for independent samples (TTIS) if it resembled a Gaussian distribution, i.e. the mean was in the middle of the range. All analyses were performed using the Statistica package (Statsoft Version 5.1, Hamburg, Germany). The level of significance was set at $P \leq 0.05$.

Results
OHSS requiring hospital admission occurred in 10 of the 218 (4.6%) subjects undergoing treatment cycles. The average number of days between oocyte retrieval and admission was 3 days (range 2–5). The syndrome was severe in five of 218 (2.3%) and moderate–severe in five of 218 (2.3%). Embryo transfer had taken place in five of the 10 OHSS patients and was successful in two subjects.

Demographics
There were no statistically significant differences between subjects with and without OHSS with respect to parity, duration of infertility, age, dose of gonadotrophins and oocyte number (Mann–Whitney $U$-test). The oocyte numbers were remarkably similar in the ‘OHSS’ and the ‘no-OHSS’ groups [19.5 (17–20) versus 20.0 (16–22); $P = 0.89$, not significant] (Table I).

VEGF levels
There was no statistically significant difference in total serum VEGF levels in the 10 subjects who developed moderate–severe OHSS and the 42 who did not [3.95 (3.3–4.5) versus 3.85 (3.3–4.5); $P = 0.79$, not significant] (Figure 1).

α₂-Macroglobulin levels
The levels of α₂M in the serum at the time of oocyte retrieval were statistically significantly higher in the 42 ‘no-OHSS’ subjects than in the 10 ‘OHSS’ subjects: [2.27 (1.91–2.58) versus 1.67 (1.45–1.73)].

Discussion
OHSS, which can be a life-threatening condition, is of significant concern because of the widespread and increasing demand for assisted conception. Despite the introduction of clinical measures to avoid the condition, its incidence remains relatively constant. Until now, the aetiological mechanisms have been based around aberrations in ovarian physiology.
Table 1. Comparison of subject characteristics and vascular endothelial growth factor (VEGF)/α2-Macroglubulin (α2M) serum levels at the time of oocyte retrieval in patients who did and did not develop ovarian hyperstimulation syndrome (OHSS)

<table>
<thead>
<tr>
<th></th>
<th>OHSS (n = 10)</th>
<th>No OHSS (n = 42)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parity</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of infertility (years)</td>
<td>5 (4–7)</td>
<td>4 (3–6)</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years)</td>
<td>32 (30–40)</td>
<td>31 (29–33)</td>
<td>NS</td>
</tr>
<tr>
<td>Oocyte number</td>
<td>1700 (1700–1700)</td>
<td>1700 (1700–1900)</td>
<td>NS</td>
</tr>
<tr>
<td>Serum VEGF (ng/ml) at oocyte retrieval</td>
<td>19.5 (17–20)</td>
<td>20 (16–22)</td>
<td>NS</td>
</tr>
<tr>
<td>Serum α2M (g/l) at oocyte retrieval</td>
<td>3.95 (3.3–4.4)</td>
<td>3.85 (3.3–4.5)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>1.67 (1.45–1.73)</td>
<td>2.27 (1.91–2.58)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Values are medians (interquartile range).

* Mann-Whitney U-test; NS = not significant.

This is the first paper to demonstrate that lower α2M levels are associated with an increased incidence of OHSS. Further, we have shown that whilst total serum VEGF levels are elevated in line with the degree of ovarian stimulation, the levels are not additionally elevated in subjects who subsequently develop OHSS.

In the reproductive system, VEGF is one of the most important inducers of angiogenesis (Ratcliffe et al., 1999). VEGF mRNA expression was significantly higher in granulosa cells obtained from patients with an elevated number of oocytes and a high fertilization rate (Doldi et al., 1997), confirming the role this factor plays in the regulation of vascular development during follicular growth and luteal differentiation (Ratcliffe et al., 1999). In addition to producing proliferation of endothelial cells, VEGF induces vascular permeability (Dvorak et al., 1995). Therefore, it is reasonable to assume that during ovarian stimulation, an excessive response leads to an excess of VEGF, which overspills the ovarian capillary bed.

Some workers acknowledge the biological relationship between VEGF and OHSS but are uncertain as to whether it is a causal or casual finding, although the initial work by McClure would suggest a causal relationship (McClure et al., 1994). A study recent study (Geva et al., 1999) found no differences between follicular fluid VEGF levels among an OHSS group, a hyperstimulated group that did not develop OHSS and a low responder group. Others workers have reported similar findings (Chen et al., 2000; Enskog et al., 2001); there were no statistically significant differences between ‘OHSS’ and control patients in VEGF levels up to and including the day of embryo transfer.

In another study, the serum from ten patients who had developed severe OHSS was analysed. The authors concluded that VEGF is not an important clinical marker for the course OHSS (Ludwig et al., 1998). The following year, however, this same group addressed the issue of ‘total’ and ‘free’ VEGF; free VEGF being the biologically active component. They reported that the concentration of free VEGF was significantly higher in patients who developed OHSS compared with the control group. No such difference existed with respect to total circulating VEGF. The authors concluded that the course of severe OHSS cannot be predicted by the overall pattern of circulating free VEGF and that other factors must be involved in the pathogenesis (Ludwig et al., 1999). All of these findings provide circumstantial support for the theory proposed in this study although the authors of these various reports did not realize this at the time of publication.

Ovulation, with its characteristic vascular changes and proteolysis, is typical of an inflammatory response (Buscher et al., 1999; Tsafirri and Reich, 1999). One of the most prominent features of an inflammatory response is the increased capillary permeability caused by inflammatory mediators. In addition to VEGF, other agents implicated in the process of ovulation are known to increase vascular permeability (Gao et al., 1992; Tilton et al., 1999; Tsafirri and Reich, 1999). Therefore, the development of OHSS may depend on a cascade of events rather than one individual factor. VEGF and inflammatory mediators such as the kallikrein–kinin system may act synergistically to increase capillary permeability, the single most important feature in the development of OHSS.

α2M is a major serum protein that is present in follicular fluid in high concentration (Curry et al., 1990). Studies have shown that α2M mRNA and protein are low in developing follicles, increase at the time of ovulation and are maximally expressed in the corpus luteum (Gaddy-Kurten et al., 1989). Tissue-specific increases in α2M mRNA occur in response to the LH surge/HCG injection and to prolactin in periovulatory follicles and early luteinization. Decreases in α2M protein content of the corpus luteum coincide with termination of the functional lifespan of this structure. Furthermore, changes in the amount of α2M protein in follicles during development and ovulation may be associated not only with changes in the synthesis of α2M by follicular cells, but also with changes in vascular permeability controlling access of serum-borne α2M to the follicles. Although the cyclical expression of α2M in the ovary is very similar to that of VEGF, there was no statistically significant correlation between serum VEGF and α2M levels at the time of oocyte retrieval in the two groups of subjects.

The outstanding characteristic of α2M is its ability to bind to a wide range of physiologically important molecules (James, 1990). The temporal expression of α2M protein in the ovary suggests that this inhibitor is necessary to maintain proteolytic homeostasis during the periovulatory period allowing regulation of corpus luteum formation (Gaddy-Kurten et al., 1989).
Once $\alpha_2$M binds a molecule, a conformational change results in its rapid clearance from the circulation by the reticuloendothelial system (James, 1990).

$\alpha_2$M has been shown to bind VEGF. The binding of VEGF to $\alpha_2$M leads to inactivation of VEGF since complexed VEGF can no longer bind to the VEGF receptors of vascular endothelial cells (Soker et al., 1993; Bhattacharjee et al., 2000). Therefore, the level of $\alpha_2$M at the time of oocyte retrieval may influence whether or not a patient develops the syndrome since it is the concentration of free hormone that determines its acute effects on target cells. The concept of an interplay between oocyte number and hence VEGF level and the level of $\alpha_2$M at the time of oocyte retrieval might explain the inter- and intra-individual variation in development of the syndrome.

Although the concept of a naturally occurring inhibitor of VEGF is novel and exciting, certain limitations of this study must be addressed. Firstly, in OHSS the usual homeostatic mechanisms are overridden or disrupted. Therefore, from ‘no-OHSS’ to ‘severe OHSS’ is probably a continuum of the one clinical entity. It is quite likely that some patients with at least moderate OHSS fail to report or recognize their symptoms. Furthermore, the incidence of severe OHSS is very low, so, despite recruiting a considerable number of patients into the study, few actually developed severe OHSS. However, even though the number of study patients was small, there was a clear-cut difference in the mean serum $\alpha_2$M levels and the biological data with no overlap in >75% of cases. Our results suggest that those patients with higher levels of $\alpha_2$M are less at risk of OHSS and, by extrapolation, possibly because more of the excess VEGF is bound. Development of the syndrome would, therefore, depend on the interplay between oocyte number and hence VEGF level and the level of $\alpha_2$M at the time of oocyte retrieval. Since $\alpha_2$M is rapidly cleared by the reticuloendothelial system once it binds to a molecule, the level in the circulation is not static. This concept might explain why patients develop the syndrome in some cycles and not others despite lower levels of ovarian stimulation. Analysis of $\alpha_2$M levels at time of hospital admission and at resolution of symptoms would be needed to explore this concept further.

Although numerous strategies have been proposed to reduce the incidence of OHSS, none is completely effective. However, many attempts have been made to modify the treatment to avoid cancellation of a cycle. An effective and widely used option is prolonged ‘coasting’ to allow estradiol concentrations to drop (Dhont et al., 1998; Waldenstrom et al., 1999; Fluker et al., 1999). However, if $\alpha_2$M is the determining factor for the development of OHSS, the incorporation of a routine assay of $\alpha_2$M at the time of oocyte retrieval may increase the likelihood of identifying those patients at risk. The level of $\alpha_2$M could be considered in conjunction with the oocyte number and signs of an increased inflammatory response, such as increased peritoneal fluid at the time of oocyte retrieval. This would assist in differentiating those patients for whom it is safe to proceed with embryo transfer from those for whom it is not, because of the risk of OHSS.

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