Poor responder–high responder: the importance of soluble vascular endothelial growth factor receptor 1 in ovarian stimulation protocols

Joseph Neulen1,5, Daniela Wenzel2, Carsten Hornig3, Edda Wünsch1, Klaus Grunwald1, Reinhard Büttner4 and Herbert Weich2

1Department of Gynecological Endocrinology and Reproductive Medicine, University Clinic, RWTH Aachen, 2Ges. Biotechnologische Forschung, 3Receptor Ligand Technologies GmbH (RELIATech), Braunschweig and 4Department of Pathology, University Clinic, RWTH Aachen, Germany

5To whom correspondence should be addressed at: Department of Gynecological Endocrinology and Reproductive Medicine, RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany. E-mail: jneulen@post.klinikum.rwth-aachen.de

This study was designed to detect vascular endothelial growth factor (VEGF) and its soluble receptor (sVEGFR-1) in follicular fluid specimens and to evaluate the importance of sVEGFR-1 with respect to ovarian response to gonadotrophin stimulation. A total of 69 patients was treated for IVF with recombinant human follicle stimulating hormone (FSH). Concentrations of VEGF and sVEGFR-1 were quantified in follicular fluids from oocyte retrievals. Patients were designated to three groups with respect to the number of harvested oocytes: group A, 1–5 oocytes; group B, 6–10 oocytes; group C, >10 oocytes. In group A, 1133 ± 870 pg VEGF/ml follicular fluid per oocyte were quantified, in group B 426 ± 262 pg VEGF/ml per oocyte, and in group C 274 ± 179 pg VEGF/ml per oocyte. Soluble VEGFR-1 concentrations resulted in 1200 ± 523 pg/ml follicular fluid per oocyte in group A, 255 ± 193 pg/ml per oocyte in group B, and 79 ± 69 pg/ml per oocyte in group C. No free sVEGFR-1 could be detected in any follicular fluid. An index to estimate the biological activity of VEGF by dividing VEGF/sVEGFR-1 revealed an increasing availability of VEGF with higher ovarian response to gonadotrophin therapy. In group A this index was 1.03, in group B 1.71, and in group C 3.21. A delicate balance between VEGF and sVEGFR-1 is necessary to allow an adequate ovarian reaction to gonadotrophin therapy. Excess of bio-active VEGF increases the risk for ovarian hyperstimulation syndrome. Excess of sVEGFR-1 results in poor response and goes in parallel with reduced chances for conception.

Key words: gonadotrophin therapy/ovarian function/sVEGFR-1/VEGF

Introduction

Vascular endothelial growth factor (VEGF) is produced by theca interna and granulosa cells in the human ovary (Yamamoto et al., 1997). All types of VEGF are produced by granulosas cells, predominantly VEGF-A121 and VEGF-A165 (Yan et al., 1993; Neulen et al., 1996; Laitinen et al., 1997; Otani et al., 1999). Most intense production can be observed during the peri-ovulatory phase (Kamat et al., 1995). Recent results indicate that gonadotrophins influence VEGF mRNA expression and protein production with luteinizing hormone/ human chorionic gonadotrophin (LH/HCG) having a prominent effect in granulosa cells (Neulen et al., 1998). Clinically, VEGF production is linked to the pathophysiology of ovarian hyperstimulation syndrome (OHSS) (Neulen et al., 1995). Several reports show high VEGF concentrations in serum (Krasnow et al., 1996) or urine (Robertson et al., 1995) to be correlated with an increased risk of this severe iatrogenic condition. Measurements of VEGF in follicular fluids give ambiguous results regarding the indicative value of high VEGF concentrations and increased risk of OHSS (Pellicer et al., 1999). It can be assumed that differences occur due to the measurement of free or bound VEGF. One widely used commercial VEGF quantification kit obviously detects only free VEGF (Banks et al., 1998). Other reagents are not characterized.

VEGF mediates its effects through two specific receptors, fms-like tyrosine receptor-1 (flt-1 = VEGFR-1) (Shibuya et al., 1990) or kinase inserted tyrosine domain receptor (KDR = VEGFR-2) (Termar et al., 1992). VEGFR-1 is also produced as a soluble receptor (sVEGFR-1) by alternative splicing of the precursor mRNA (Kendall et al., 1996). It is not yet clear whether proteolytic shedding produces additionally sVEGFR-1. Obviously, sVEGFR-1 acts as a negative modulator for the bioactivity of VEGF (Hornig and Weich, 1999). Both receptors are produced by endothelial cells. Endothelial cells can be accepted as a prominent source
of sVEGFR-1 (Hornig et al., 2000). How the production of membrane bound or soluble VEGFR-1 is regulated in endothelial cells remains to be determined.

This study was designed to detect sVEGFR-1 in follicular fluids and to evaluate the importance of sVEGFR-1 with respect to ovarian response to gonadotrophin stimulation.

Material and methods

Pooled follicular fluids were obtained from 69 individual women undergoing oocyte retrieval for IVF. Reasons for infertility were tubal occlusion (36 couples), male infertility (24 couples), and unexplained infertility (nine couples). Written consent was obtained and the experimental design was approved by the local ethical committee.

In these patients, multiple follicular development was achieved with recombinant human FSH (rhFSH 150–225 IU per day, Gonal-F®, Serono, Munich, Germany) until follicular maturity. Gonadotrophin therapy was preceded by complete desensitization of the pituitary gland with 0.1 mg per day of tryptojen (Decapeptyl®, Ferring, Kiel, Germany). For ovulation induction 5000 or 10 000 IU HCG (Pregniesin®, Serono) were injected 36 h prior to ultrasound-guided transvaginal follicle aspiration when at least two follicles reached 18 mm in diameter.

Patients were designated to three groups with respect to the number of harvested oocytes: group A (low responder; 22 patients; mean age: 34.8 ± 2.6 years); 1–5 oocytes; group B (average responder; 21 patients; mean age: 33.2 ± 4.5 years); 6–10 oocytes; group C (high responder; 26 patients; mean age: 31.6 ± 3.5 years); >10 oocytes. Mean total rhFSH dose in group A was 2270 IU/cycle, in group B 1865 IU/cycle, and in group C 1625 IU/cycle. To induce ovulation mean total dose of HCG in group A was 9545 IU, in group B 8095 IU, and in group C 8653 IU. Only pregnancies in the eighth week were counted.

After oocyte collection follicular fluids were centrifuged at 500 g to remove blood cell contaminations. Aliquots of the individually pooled follicular fluids were stored at −20°C until VEGF and sVEGFR-1 quantification.

Serum oestradiol at the day of HCG injection was quantified with an automated technique (Bayer/Chiron Diagnostics, Neuss, Germany) according to the manufacturer’s recommendations.

VEGF (also described as VEGF-A) in follicular fluids was quantified with a commercially available sandwich enzyme-linked immunosorbent assay (ELISA; R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer’s recommendations.

Total (free and VEGF complexed) sVEGFR-1 was quantified with sandwich ELISA technique as described previously (Hornig et al., 1999) and applied according to the manufacturer’s (RELIAtech, Braunschweig, Germany) specifications. The determination of free, uncomplexed sVEGFR-1 was performed as described recently (Hornig et al., 2000) by a modified ELISA (RELIAtech). Undiluted follicular fluid samples were processed in duplicates.

For immunoblotting of sVEGFR-1 pooled follicular fluids with highest sVEGFR-1 concentrations were used (Hornig et al., 2000). In short, VEGFR-1 from 5 ml aliquots of follicular fluid which were adjusted to 0.5 mmol/l NaCl were concentrated by a Hitrap heparin-agarose column (Pharmacia Biotech, Freiburg, Germany), eluted with 1.5 mmol/l NaCl, concentrated by trichloroacetic acid (TCA) precipitation, and separated on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). After blotting, mouse monoclonal Flt-11 antibody (Sigma, Taufkirchen, Germany) was used for detection of sVEGFR-1.

Immunohistochemical stainings were performed on 5 μm cryostat sections of snap-frozen ovary tissue specimens from a premenopausal patient undergoing surgery for diagnosis of endometriosis during the follicular phase of cycle. After acetone fixation at −20°C for 10 min, sections were blocked for 15 min with 5% horse serum and incubated at 4°C with 1:200 dilution of the mouse monoclonal antibody anti-VEGF-1 (FLT-19) (Hornig et al., 1999). After washing 3×5 min with 2% non-fat dry milk dissolved in phosphate buffered saline (PBS), the sections were incubated for 30 min with 1:500 dilution of goat anti-mouse IgG-biotin labelled antiserum (DAKO, Copenhagen, Denmark) at room temperature, washed again three times and incubated for 30 min with the ABC complex (VectorLab, Burlingame, CA, USA) according to the manufacturer’s instructions. To visualize immuno-complexes, the slides were finally incubated in 50 ml diaminobenzidine solution (30 mg DAB, 0.3 g Tris-Cl pH 7.6) for 2 min at room temperature and counterstained with hemalaun (Merck, Darmstadt, Germany) for 2 min (Partanen et al., 1999).

Values are indicated as mean ± SD. To determine whether there were differences at all between the three patient groups an analysis of variance (ANOVA) test was performed for oestradiol, VEGF and sVEGFR-1 results. For further statistical analysis Student’s t-test was applied.

Results

At the day of HCG injection, in group A oestradiol concentrations were: 2408 ± 1513 pmol/l. An average of 3.6 oocytes per patient could be harvested. Four patients became pregnant. In group B oestradiol concentrations were: 3761 ± 2573 pmol/l. An average of 8.4 oocytes per patient could be harvested. Four patients became pregnant. In group C oestradiol concentrations were: 5767 ± 3160 pmol/l (oestradiol group A versus group B: P = 0.0218; group B versus group C: P = 0.0081; group A versus group C: P < 0.0001). An average of 14.5 oocytes per patient could be harvested. Five patients became pregnant.

VEGFR-1 antibodies detected specific antigen in blood vessels throughout the ovary. Intense staining in the vicinity of follicles indicated a higher capillary network around antral follicles. No staining could be detected in theca interna or granulosa cells (Figure 1).

Recombinant sVEGFR-1 (domain 1–5) rendered in Western blots a single band by immunoblotting at about 75 kDa. Recombinant sVEGFR-1 (domain 1–6) yielded a molecular weight of 90 kDa. Follicular fluid revealed a specific staining with two distinct bands for sVEGFR-1 at a molecular weight of about 110 kDa (Figure 2). This indicates that native human sVEGFR-1 is more intensely glycosylated than recombinant sVEGFR-1. The second band may be due to heparin leaking from the separation column (Hornig et al., 2000). The molecular weight of the naturally occurring sVEGFR-1 of 110 kDa was demonstrated by several groups, independently. This molecule was isolated from human amniotic fluid and sequenced previously (Banks et al., 1998; He et al., 1999). In the Western blot experiments of the current study, this naturally occurring sVEGFR-1 was compared with two forms of recombinant sVEGFR-1 produced in insect cells. The recombinant forms are smaller than the natural form due to less glycosylation by insect cells. Deglycosylation of natural human sVEGFR-1 and sVEGFR-1 produced by insect cells renders soluble receptors of equal size (Hornig et al., 2000).
Role of soluble VEGF receptor 1 in ovarian stimulation

Figure 1. Immunohistochemical visualization of vascular endothelial growth factor receptor 1 (VEGFR-1) in ovarian blood vessels. Intense staining in the vicinity of follicles indicated a higher capillary network around antral follicles. No staining was detected in theca interna or granulosa cells.

Figure 2. Western blot showed recombinant soluble VEGFR-1 (domain 1–5) with a molecular weight of 75 kDa and recombinant sVEGFR-1 (domain 1–6) with a molecular weight of 90 kDa respectively. Soluble VEGFR-1 from human follicular fluids rendered a molecular weight of 110 kDa due to more intense glycosylation. The second band probably arose from heparin leaking after concentration of sVEGFR-1 through a heparin–sepharose column.

The mean values ± SD were quantified in group A, 4226 ± 2862 pg VEGF/ml follicular fluid, in group B 3559 ± 2417 pg/ml, and in group C 3727 ± 2076 pg/ml (differences not statistically significant). In group A this resulted in 1133 ± 870 pg VEGF/ml per oocyte, in group B in 426 ± 262 pg VEGF/ml per oocyte, and in group C in 274 ± 179 pg VEGF/ml per oocyte (Figure 3) (differences group A versus group B: $P < 0.0001$; group B versus group C: $P = 0.0085$; group A versus group C: $P < 0.0001$).

Soluble VEGFR-1 concentrations revealed a significant negative correlation with the number of harvested oocytes: group A 4110 ± 1060 pg sVEGFR-1/ml follicular fluid, group B 2080 ± 1520 pg/ml, and group C: 1160 ± 810 pg/ml (group A versus group B: $P < 0.0001$; group B versus group C: $P = 0.0217$; group A versus group C: $P < 0.0001$). Soluble VEGFR-1 concentrations in serum are below the detection limit of the applied assay. These values resulted in 1200 ± 523 pg sVEGFR-1/ml per oocyte in group A, 255 ± 193 pg sVEGFR-1/ml per oocyte in group B, and 79 ± 69 pg sVEGFR-1/ml per oocyte in group C (all $P$ values < 0.0001).

No free sVEGFR-1 could be detected in any follicular fluid (Figure 4).
are women with elevated FSH concentrations re-
fl
mainly observed in the perimenopausal phase (Muasher, 1992).
reduced inhibin production by granulosa cells. This condition is
demonstrated either by immunohistochemistry or by Northern
blot technique (Yan
et al., 1998). Obviously different from these patients
that diminished ovarian reserve might result in low response
from patients undergoing ovarian stimulation adapted by low,
response to gonadotrophin therapy. There is evidence
that the intrafollicular amount re-
fl
be assumed with the amount of sVEGFR-1 in follicular
uids suggesting that elevated VEGF concentrations are a warning sign for
insufficient oxygen supply (Barroso
et al., 1999).
Elevated serum concentrations of free VEGF have been
correlated with an increased risk of severe OHSS (Agrawal
et al., 1999; Ludwig
et al., 1999). However, measurements of
VEGF in serum are compromised by VEGF contributed from
platelets during blood clotting (Maloney
et al., 1998). Also
VEGF plasma concentrations after ovulation induction with
HCG have been correlated with the risk of OHSS (Abramov
et al., 1997). Again, in these patients the VEGF concentration
in follicular fluids is lower than in control patients without
risk of OHSS (Pellicer
et al., 1999).
The importance of human growth hormone for follicular
recruitment and selection has not yet been decided (Stone and
Levron
et al., 1993). Obviously, only patients
with human growth hormone deficit profit from an additional
human growth hormone therapy along with conventional
gonadotrophin ovarian stimulation (de Boer
et al., 1997).
Both VEGF as well as VEGFR-1 are up-regulated by
hypoxia (Shweiki
et al., 1992; Gerber
et al., 1997). Oocyte
growth is sensitive to hypoxic damage (Van Blerkom, 1998).
In contrast, high oxygen content of follicular fluid has been
positively correlated with VEGF concentrations (Doldi
et al., 1997; Van Blerkom
et al., 1999). Other results show that high
VEGF concentrations in follicular fluids indicate inferior
oocyte quality with poor fertilization rates. It is postulated
that elevated VEGF concentrations are a warning sign for
insufficient oxygen supply (Barroso
et al., 1999).

An index to estimate the biological activity of VEGF by
dividing VEGF/sVEGFR-1 revealed an increasing availability of
VEGF with higher ovarian response to gonadotrophin
therapy. In group A this index was 1.03, in group B 1.71, and
in group C 3.21 (Figure 5).

Discussion

Data presented in this study indicate that availability of
biologically active VEGF is associated with the individual
response to gonadotrophin therapy. A negative correlation can
be assumed with the amount of sVEGFR-1 in follicular fluids.
No free sVEGFR-1 was detected in follicular fluid suggesting that
the intrafollicular amount reflected the capacity of
sVEGFR-1 production by surrounding capillary endothelia. In
contrast to previous data (Otani
et al., 1999), no production of
VEGFR-1 in theca interna or granulosa cells could be
demonstrated either by immunohistochemistry or by Northern
blot technique (Yan
et al., 1998).
Several trials were undertaken to unravel causes of low
ovarian reaction to gonadotrophin therapy. There is evidence
that diminished ovarian reserve might result in low response
(Pellicer
et al., 1998). Obviously different from these patients
are women with elevated FSH concentrations reflecting a
reduced inhibin production by granulosa cells. This condition is
mainly observed in the perimenopausal phase (Muasher, 1992).
Vegetation of bio-available VEGF in follicular fluids from patients undergoing ovarian stimulation adapted by low,
average, or high response to gonadotrophin therapy.

![Figure 4](image-url)

![Figure 5](image-url)

It is a matter of debate whether therapy regimes with high
doses of FSH (>450 IU FSH/day) result in more mature
follicles in low responders (Hofmann
et al., 1989; Karande
et al., 1990). Intravenous administration of FSH in a pulsatile
manner did not improve IVF outcome in low responders
(Edelstein
et al., 1990).

The overall pregnancy rate in patients with low ovarian
response is reduced. Therefore, IVF was ensured by intracyto-
plasmic sperm injection. However, no benefit from this
procedure could be demonstrated (Moreno
et al., 1998).

Glucocorticoid treatment for patients with low ovarian
response was applied to increase the number of mature oocytes
in IVF cycles. There was no improvement in any clinical
aspect (Bider
et al., 1997). Accordingly, follicular fluid
concentrations of cortisol showed no correlation with oocyte
number or quality (Bider
et al., 1998). Finally, neither
follicular fluid concentrations of cortisol and cortisone nor the
cortisol/cortisone ratio reflect the quality of ovarian response
to gonadotrophin therapy (Andersen
et al., 1999).

The importance of human growth hormone for follicular
recruitment and selection has not yet been decided (Stone and
Marrs, 1992; Levron
et al., 1993). Obviously, only patients
with human growth hormone deficit profit from an additional
human growth hormone therapy along with conventional
gonadotrophin ovarian stimulation (de Boer
et al., 1999).

Elevated serum concentrations of free VEGF have been
correlated with an increased risk of severe OHSS (Agrawal
et al., 1999; Ludwig
et al., 1999). However, measurements of
VEGF in serum are compromised by VEGF contributed from
platelets during blood clotting (Maloney
et al., 1998). Also
VEGF plasma concentrations after ovulation induction with
HCG have been correlated with the risk of OHSS (Abramov
et al., 1997). Again, in these patients the VEGF concentration
in follicular fluids is lower than in control patients without
risk of OHSS (Pellicer
et al., 1999).
The results presented here are in good agreement with data
indicating that high concentrations of bio-available VEGF in
follicular fluids lead to the pronounced induction of endothelial
permeability during OHSS. Consistently, blocking of VEGF
action by antibodies or in biological systems by sVEGFR-1
reduces the endothelial permeability (Ferrara
et al., 1998; Levin
et al., 1998).
Poor ovarian response to gonadotrophin stimulation might
be the result of inhibited VEGF bio-availability. Hypoxemia,
or oxygen demand, enhances VEGF expression (Gerber
et al., 1997) in maturing follicles and augments VEGFR-1 production
in corresponding endothelial cells (Shweiki
et al., 1992) concomitantly. Alternative splicing of the flt-1 mRNA to
produce sVEGFR-1 significantly contributes to the regulation of VEGF activity (He et al., 1999). Rising sVEGFR-1 production and secretion in the vicinity of maturing follicles is detrimental for further development. Thereby, excessive sVEGFR-1 production in ovarian blood vessels results in poor ovarian response due to diminished bio-active VEGF supply. High follicular VEGF production cannot overcome the blocking effects of sVEGFR-1. VEGF concentrations in follicles from those patients may consequently be higher than in women with pronounced ovarian response to gonadotrophin stimuli.

In summary, the data presented here indicate that a delicate balance between VEGF and its natural occurring antagonist sVEGFR-1 is necessary to allow an adequate ovarian reaction to gonadotrophin therapy. Excess of bio-active VEGF increases the risk for OHSS. Excess of sVEGFR-1 results in poor response and reduced chances of conception.

Acknowledgements
This study was supported by the Deutsche Forschungsgemeinschaft, grant number Ne 388/5–1 and WE 1211/3–2.

References


Received on June 23, 2000; accepted on January 9, 2001