Molecular Human Reproduction vol.4 no.3 pp. 203–206, 1998

Secretion of vascular endothelial growth factor/vascular permeability factor from human luteinized granulosa cells is human chorionic gonadotrophin dependent

Joseph Neulen[1,5], Sonja Raczek[2], Martin Pogorzelski[2], Klaus Grunwald[1], T.K.Yeo[3], Harold F.Dvorak[3], Herbert A.Weich[4] and Meinert Breckwoldt[2]

[1]Department of Gynaecology, Endocrinology and Reproductive Medicine, University Clinic RWTH D-52074 Aachen, [2]Universität Frauenklinik, D-79106 Freiburg, Germany, [3]Beth Israel Hospital, Harvard Medical School, Boston, MA, USA and [4]Gesellschaft für Biotechnologische Forschung, D-38124 Braunschweig, Germany

[5]To whom correspondence should be addressed

Vascularization is a prominent event during corpus luteum formation, providing low density lipoproteins for steroid biosynthesis and enabling transport of secreted steroids. The process of vascularization is controlled by specific regulators. Vascular endothelial growth factor (VEGF), otherwise named vascular permeability factor (VPF), induces endothelial cell proliferation as well as angiogenesis *in vivo* and increases capillary permeability. Here we report the expression of VEGF/VPF mRNA by cultured human luteinized granulosa cells (GC) for at least 10 days. Without HCG VEGF/VPF expression declined after day 4 and by day 10 was reduced to ~30% of the value at day 4. However, after culture in the presence of 1 U/ml human chorionic gonadotrophin (HCG), expression of VEGF/VPF mRNA by GC was four times greater than control experiments by day 10, and increased 100% from day 4 to day 10. Simultaneously, HCG supplementation increased VEGF/VPF secretion by GC. Medium VEGF/VPF on day 3 was 13 pM without and 11 pM with HCG. Medium VEGF/VPF on day 10 was 6 pM without HCG and 29 pM with HCG. These results suggest that vascularization of the corpus luteum is induced by HCG-mediated effects of VEGF/VPF.

Key words: granulosa cells/ovarian hyperstimulation syndrome/vascular endothelial growth factor/vascular permeability factor

Introduction

Vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF) occurs in different forms. Molecular cloning reveals the existence of five subtypes of VEGF/VPF that are generated by alternate splicing (Ferrara *et al*., 1991; Charnock-Jones *et al*., 1993). VEGF/VPF_{165} is the most abundant form of this growth factor inducing endothelial cell proliferation as well as angiogenesis *in vivo* associated with increased capillary permeability (Ferrara and Henzel, 1989; Leung *et al*., 1989; Connolly, 1991; Ferrara *et al*., 1991; Benifla *et al*., 1994). VEGF/VPF_{165} is an ~46 kDa dimeric glycoprotein that dissociates upon reduction into two identical 23 kDa subunits (Ferrara and Henzel, 1989).

Recently, VEGF/VPF mRNA was detected in rat (Phillips *et al*., 1990; Dissen *et al*., 1994), bovine (Garrido *et al*., 1993) and monkey (Ravindranath *et al*., 1992) corpus luteum and in luteinized human granulosa cells (GC) (Yan *et al*., 1993; Kamat *et al*., 1995) using Northern blot analysis and in-situ hybridization techniques. In GC, human chorionic gonadotrophin (HCG) augments VEGF/VPF mRNA expression in a dose- and time-dependent manner. VEGF/VPF production by GC has been demonstrated by immuno-histochemical staining procedures (Neulen *et al*., 1995). Moreover, medium conditioned by rat granulosa cells promoted proliferation of vascular endothelial cells *in vitro* (Koos, 1986). In human follicular fluid from pre-ovulatory follicles a factor was identified which enhanced vascular permeability (Goldsman *et al*., 1995).

Vascularization and adequate blood flow are essential requirements for normal corpus luteum function (Carr *et al*., 1981; Jones, 1991; Richardson *et al*., 1992). In rats, diabetic vessel damage was shown to lead to reduced progesterone production and consequently to an inadequate endometrial transformation (Garris, 1988). Changes in peak systolic blood flow velocity demonstrated by ultrasound techniques have been suggested as a useful monitor of corpus luteum function (Alcazar *et al*., 1996; Bourne *et al*., 1996).

Abundant VEGF/VPF generation, however, is reported to elicit ovarian hyperstimulation syndrome, a severe complication of the use of ovarian stimulation in infertility treatment (McClure *et al*., 1994; Neulen *et al*., 1995).

This study was designed to demonstrate secretion of VEGF/VPF by human GC *in vitro* and to describe the effects of HCG on VEGF/VPF gene expression and secretion by GC during extended culture.

Materials and methods

Luteinized GC were obtained from 11 women undergoing oocyte retrieval for in-vitro fertilization. Reasons for infertility were tubal occlusion (seven couples), male factor infertility (three couples), and 'unexplained infertility' (one couple). Written consent was obtained and the experimental design was approved by the local ethical committee. Mean age of these patients was 31.1 years (range: 24–38 years). In these patients, multiple follicular development was achieved

with pure follicle stimulating hormone (FSH) (225 IU per day; Fertinorm HP®; Serono, Munich, Germany) until follicular maturity. Gonadotrophin therapy was preceded by complete desensitization of the pituitary gland with 0.1 mg per day of triptorelin (Decapeptyl®; Ferring, Kiel, Germany). Mean total FSH dose administered per patient was 2025 IU (range: 1125–2925 IU). An average of eight follicles >13 mm per patient were induced (range: four to 17 follicles). For ovulation induction 10 000 IU HCG (Pregnesin®; Serono, Munich, Germany) were injected 36 h prior to ultrasound-guided transvaginal follicle aspiration. Before ovulation induction mean oestradiol concentrations were determined in the range of 3.2–14.7 nM.

Granulosa cell preparation and culture conditions were as described (Neulen *et al*., 1995). Culture was continued for 10 days. To study the effects of HCG, medium was supplemented with 1 IU/ml HCG continuously during cell culture. Media were completely replaced by fresh media at day 3, 5, 7 and 10, and the removed media were used for VEGF/VPF quantification (seven individual cell cultures). For slot blot analysis cells were harvested after 4, 7 and 10 days of culture (four individual cell cultures). In the remaining cell cultures media were replaced after the indicated time intervals.

For slot blot determination, total cellular RNA from GC was prepared by acid guanidinium thiocyanate–phenol–chloroform extraction and hybridization was performed under stringent conditions as described elsewhere (Weindel *et al*., 1992). Specificity of the VEGF/VPF gene probe has been previously demonstrated by Northern blotting (Yan *et al*., 1993). After autoradiogram laser densitometry of slot blots, VEGF/VPF expression rates were related to Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) expression which is expected to be constant. Experiments were repeated four times.

VEGF/VPF concentrations were determined in culture media by DELPHIA technique as described by Yeo *et al*. (Yeo *et al*., 1992) employing polyclonal rabbit VEGF/VPF antibodies.

Values were compared by Student's *t*-test for unpaired data.

All chemicals were obtained from Sigma, Deisenhofen, Germany.

Results

Slot blot analysis showed that VEGF/VPF gene expression in GC was ~4-fold enhanced after 7 and 10 days by culture in the presence of continuous HCG stimulation when compared to control cultures [four individual cell cultures; $P < 0.05$ (control versus 1 U HCG/ml medium at day 7 and day 10)]. VEGF/VPF expression increased 100% from day 4 of culture to day 10 in the presence of 1 U/ml HCG. After only 4 successive days of treatment with HCG there was no difference in VEGF/VPF gene expression between HCG-treated and control GC.

In control GC VEGF/VPF gene expression declined by day 10 to ~30% of the value on day 4 (Figures 1 and 2).

GC secreted VEGF/VPF into the culture medium. In control GC cultures VEGF/VPF concentrations decreased from 13 pM on day 3 to 6 pM by day 10. In cultures supplemented with 1 U/ml HCG VEGF/VPF concentrations increased to 27 pM at day 7 and 29 pM at day 10 [$P < 0.01$ (control versus 1 IU HCG/ml medium); seven individual GC] (Figure 3).

Discussion

The data presented here demonstrate that HCG augments the VEGF/VPF mRNA content of human GC during extended cell culture. Moreover, HCG enhanced VEGF/VPF secretion by human GC simultaneously.

Thus, the physiological angiogenesis which occurs during corpus luteum formation represents another effect mediated by HCG. It appears that not only is steroidogenesis supported by exogenous or endogenous trophoblastic HCG, but also that anatomical differential and functioning of the corpus luteum are regulated by this hormone.

The data suggest that HCG effects on VEGF/VPF production are maintained for a prolonged period. Lack of HCG causes GC depletion of VEGF/VPF. It can be assumed that vascularization

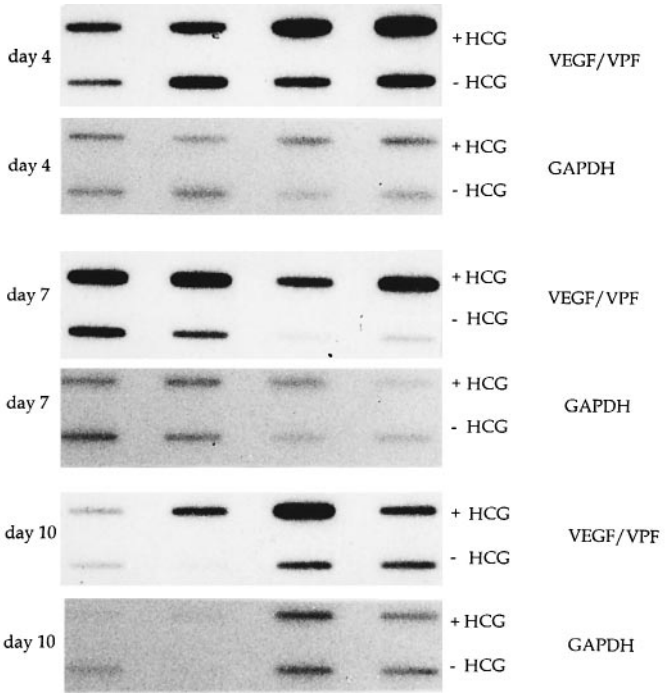

Figure 1. Slot blot analysis of vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF) gene expression by human luteinized granulosa cells compared with GAPDH gene expression. Each lane represents the results of one individual cell culture.

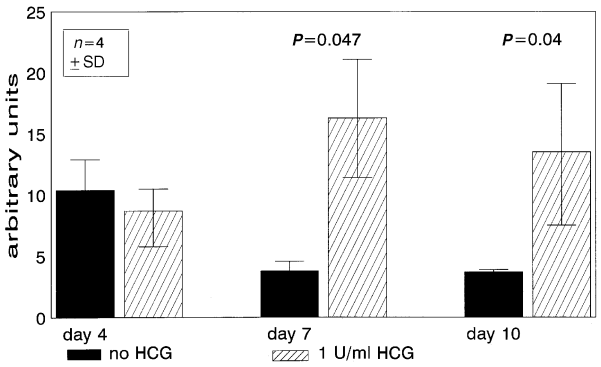

Figure 2. Vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF) gene expression in human luteinized granulosa cells (GC) after 7 and 10 days of cell culture in the presence of human chorionic gonadotrophin (HCG) compared with expression in unstimulated GC. The VEGF/VPF bands in Figure 1 were analysed by laser densitometry and normalized using GAPDH gene expression. Results are expressed as mean ± SD. *P* values indicate the difference between stimulated and control cultures.

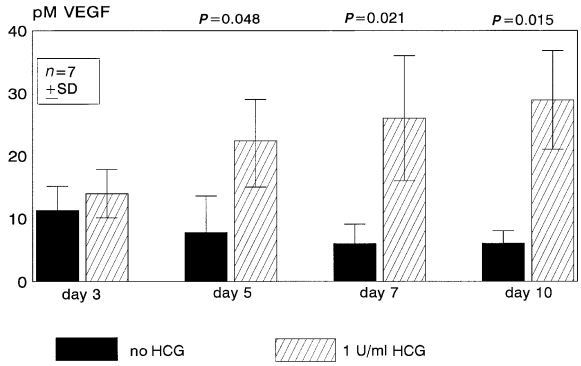

Figure 3. Secretion of vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF) into the culture medium by luteinized granulosa cell cultures in the presence or absence of human chorionic gonadotrophin (HCG). Results are expressed as mean ± SD. *P* values indicate the differences between stimulated and control cultures.

remains inadequate in corpora lutea without sufficient HCG or luteinizing hormone (LH) support.

Recent experiments (Neulen *et al*., 1995) demonstrated in cultured human GC that interruption of HCG supplementation for 72 h resulted in an intensified response of GC to HCG. 1 U/ml HCG increased VEGF/VPF mRNA expression after 72 h in culture. In those cells specific HCG binding could be confirmed. In the experiments reported here the culture conditions were changed to continuous HCG supplementation *in vitro*, and this led to a prolonged insensitivity to HCG which prevailed for at least 4 days. *In vivo*, primate corpus luteum responsiveness to HCG depends upon luteal age (Benyo *et al*., 1993). HCG causes an increase in luteal progesterone production after ovarian stimulation during the mid-luteal and late luteal phases, but not at the early luteal phase (Auletta *et al*., 1995; Saunders *et al*., 1996). In our experiments the effects of HCG on VEGF/VPF gene expression and secretion *in vitro* could be confirmed after only 4 days of continuous HCG exposure. Culture in the absence of HCG supplementation appeared to sensitize GC towards HCG stimulation.

A previous paper (Yan *et al*., 1993) demonstrated that the VEGF/VPF mRNA expression by GC from individual patients was highly variable, despite similarities of their oestradiol concentrations and number of follicles.

Physiologically, VEGF/VPF effects have been suggested to initiate vascular growth into the corpus luteum and to maintain vascular support. Production and secretion of VEGF/VPF by luteinized GC are regulated by LH or HCG.

With an increase in the mass of GC and/or intensified sensitivity towards LH/HCG, larger amounts of VEGF/VPF become effective, resulting in conditions analogous to ovarian hyperstimulation syndrome (OHSS). In urine samples obtained from patients undergoing ovarian stimulation for infertility treatment during the corpus luteal phase VEGF/VPF concentrations rose above the values seen in the pre-ovulatory phases (Robertson *et al*., 1995). This increase was accentuated in patients developing ovarian hyperstimulation syndrome. Accordingly, VEGF/VPF concentrations in serum from patients with OHSS were greater than those from healthy women (Krasnow *et al*., 1996). VEGF/VPF provokes capillary leakage resulting in massive third space fluid accumulation (Rabau *et al*., 1967; Senger *et al*., 1983). Ascites from patients with OHSS contains large amounts of VEGF/VPF (McClure *et al*., 1994). VEGF/VPF stimulates secretion of von Willebrand factor (vWF) in vascular endothelial cells (Brock *et al*., 1991). In serum from patients with OHSS very high concentrations of vWF were observed (Todorow *et al*., 1993). These results indicate that during corpus luteum activity high amounts of VEGF/VPF are produced, the quantity being dependent, to some extent, on the number of luteinized GC. It can be assumed that human GC are a major source of VEGF/VPF production and secretion (Yan *et al*., 1993; Neulen *et al*., 1995). These results suggest that in GC VEGF/VPF production might not be regulated by product inhibition. Therefore, high HCG concentrations provoke even greater production of VEGF/VPF. Consequently, patients with multiple pregnancies are at higher risk for OHSS than patients with singleton pregnancies (Mathur *et al*., 1995).

Two major groups of patients suffering from OHSS can be distinguished. Some patients will develop OHSS immediately after follicular puncture and embryo transfer irrespective of whether a pregnancy is achieved or not. Another group of patients will develop OHSS only at ~7 days after follicular puncture, around the time of embryo implantation (Manaka *et al*., 1995). There is obviously a higher risk of developing severe OHSS during early pregnancy (McClure *et al*., 1992; Morris *et al*., 1995). Since early onset of OHSS is probably elicited by exogenous HCG application it can be avoided by lowering the HCG dosage or by replacing it by GnRH analogues for ovulation induction (Shalev *et al*., 1994; Brinsden *et al*., 1995). Late onset OHSS, however, is provoked by endogenous HCG of trophoblastic origin due to an exponential increase in HCG (Zalel *et al*., 1995).

The impact of HCG on GC can persist until the corpora lutea become insensitive to HCG, after the ninth week of gestation. Accordingly, in clinical studies OHSS symptoms gradually diminish after the first trimester of pregnancy (Marsepoil *et al*., 1992; Benifla *et al*., 1994).

The present data are in accordance with a growing body of evidence that VEGF/VPF may represent a significant pathophysiological promotor for OHSS.

Acknowledgements

Supported by the Deutsche Forschungsgemeinschaft Grant Ne 388/4-1.

References

Alcazar, J.L., Laparte, C. and Lopez-Garcia, G. (1996) Corpus luteum blood flow in abnormal early pregnancy. *J. Ultrasound Med*., **15**, 645–649.

Auletta, F.J., Kelm, L.B. and Schofield, M.J. (1995) Responsiveness of the corpus luteum of the rhesus monkey (Macaca mulatta) to gonadotrophin *in vitro* during spontaneous and prostaglandin F(2a)-induced luteolysis. *J. Reprod. Fertil*., **103**, 107–113.

Benifla, J.L., Conard, J., Naouri, M. *et al.* (1994) Syndrome d'hyperstimulation ovarienne et thrombose. A propos d'un cas de thrombose de la veine jugulaire interne. Revue de la litterature. *J. Gynecol. Obstet. Biol. Reprod*., **23**, 778–783.

Benyo, D.F., Little-Ihrig, L. and Zeleznik, A.J. (1993) Noncoordinated expression of luteal cell messenger ribonucleic acids during human chorionic gonadotropin stimulation of the primate corpus luteum. *Endocrinology*, **133**, 699–704.

Bourne, T.H., Hagstrom, H.G., Hahlin, M. *et al*. (1996) Ultrasound studies of vascular and morphological changes in the human corpus luteum during the menstrual cycle. *Fertil. Steril*., **65**, 753–758.

Brinsden, P.R., Wada, I., Tan, S.L. *et al*. (1995) Diagnosis, prevention and managment of ovarian hyperstimulation syndrome. *Br. J. Obstet. Gynaecol*., **102**, 767–772.

Brock, T.A., Dvorak, H.F. and Senger, D.R. (1991) Tumor secreted vascular permeability factor increases cytosolic Ca^{2+} and von Willebrand factor release in human endothelial cells. *Am. J. Pathol*., **138**, 213–221.

Carr, B.R., McDonald, P.C. and Simpson, E.R. (1981) The role of lipoproteins in the regulation of progesterone secretion by the corpus luteum. *Fertil. Steril*., **38**, 303–311.

Charnock-Jones, D.S.C., Sharkey, A.M., Rajput-Williams, J. *et al*. (1993) Identification and localization of alternately spliced mRNAs for vascular endothelial growth factor in human uterus and estrogen regulation in endometrial carcinoma cell lines. *Biol. Reprod*., **48**, 1120–1128.

Connolly, D.T. (1991) Vascular permeability factor: a unique regulator of blood vessel function. *J. Cell. Biochem*., **47**, 219–233.

Dissen, G.A., Lara, H.E., Fahrenbach, W.H. *et al*. (1994) Immature rat ovaries become revascularized rapidly after autotransplantation and show a gonadotropin-dependent increase in angiogenic factor gene expression. *Endocrinology*, **134**, 1146–1154.

Ferrara, N. and Henzel, W.J. (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem. Biophys. Res. Commun*., **161**, 851–858.

Ferrara, N., Houck, K.A., Jakeman, L.B. *et al*. (1991) The vascular endothelial growth factor family of polypeptides. *J. Cell. Biochem*., **47**, 211–218

Garrido, C., Saule, S. and Gospodarowicz, D. (1993) Transcriptional regulation of vascular endothelial growth factor gene expression in ovarian bovine granulosa cells. *Growth Factors*, **8**, 109–117.

Garris, D.R. (1988) Effects of diabetes on uterine conditions, decidualization, vascularization, and corpus luteum function in the pseudopregnant rat. *Endocrinology*, **122**, 665–672.

Goldsman, M., Pedram, A., Dominguez, C.E. *et al*. (1995) Increased capillary permeability induced by human follicular fluid: hypothesis for an ovarian origin of the hyperstimulation syndrome. *Fertil. Steril*., **63**, 268–272.

Jones, G.S. (1991) The luteal phase defect: a review of pathophysiology. *Curr. Opin. Obstet. Gynecol*., **3**, 641–648.

Kamat, B.R., Brown, L.F., Manseau, E.J. *et al*. (1995) Expression of vascular endothelial growth factor by human granulosa and theca lutein cells. Role in corpus luteum development. *Am. J. Pathol*., **146**, 157–165.

Koos, A.D. (1986) Stimulation of endothelial cell proliferation by rat granulosa cell conditioned medium. *Endocrinology*, **119**, 481–489.

Krasnow, J.S., Zeleznik, A.J., Berga, S.L. *et al*. (1996) Vascular permeability factor and vascular endothelial growth factor in ovarian hyperstimulation syndrome: A preliminary report. *Fertil. Steril*., **65**, 552–555.

Leung, D.W., Cachianes, G., Kuang, W.J. *et al*. (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science*, **246**, 1306–1309.

Manaka, C., Araki, S., Motoyama, M. *et al*. (1995) Management of ovarian hyperstimulation syndrome. *Jpn. J. Fertil. Steril*., **40**, 427–432.

Marsepoil, T., Cordesse, A., Varguy, P. *et al*. (1992) Atteinte hepatique au cours d'un syndrome d'hyperstimulation ovarienne. *Rev. Fr. Gynecol. Obstet*., **87**, 148–149.

McClure, N., Leya, J., Radwanska, E. *et al*. (1992) Luteal phase support and severe ovarian hyperstimulation syndrome. *Hum. Reprod*., **7**, 758–764

McClure, N., Healy, D.L., Rogers, P.A.W. *et al*. (1994) Vascular endothelial growth factor as capillary permeability agent in ovarian hyperstimulation syndrome. *Lancet*, **344**, 235–236.

Mathur, R.S., Joels, L.A. and Jenkins, J.M. (1995) Ovarian hyperstimulation syndrome may be more likely if multiple pregnancy occurs following assisted conception. *Acta Genet. Med. Gem*., **44**, 233–235.

Morris, R.S., Paulson, R.J., Sauer, M.V. and Lobo, R.A. (1995) Predictive value of serum oestradiol concentrations and oocyte number in severe ovarian hyperstimulation syndrome. *Hum. Reprod*., **10**, 811–814.

Neulen, J., Yan, Z., Raczek, S. *et al*. (1995) HCG-dependent expression of vascular endothelial growth factor/vascular permeability factor in human granulosa cells: Importance in ovarian hyperstimulation syndrome. *J. Clin. Endocrinol. Metab*., **80**, 1967–1971.

Phillips, H.S., Hains, J., Leung, D.W. and Ferrara, N. (1990) Vascular endothelial growth factor is expressed in rat corpus luteum. *Endocrinology*, **127**, 965–967.

Rabau, E., Serr, D.M., David, A. *et al.* (1967) Human menopausal gonadotropins for anovulation and sterility. *Am. J. Obstet. Gynecol*., **98**, 92–98.

Ravindranath, N., Little-Ihrig, L., Phillips, H.S. *et al*. (1992) Vascular endothelial growth factor messenger ribonucleic acid expression in the primate ovary. *Endocrinology*, **131**, 254–260.

Richardson, M.C., Davies, D.W., Watson, R.H. *et al*. (1992) Cultured human granulosa cells as a model for corpus luteum function: Relative roles of gonadotrophin and low density lipoprotein studies under defined culture conditions. *Hum. Reprod*., **7**, 12–18.

Robertson, D., Selleck, K., Suikkari, A.M. *et al*. (1995) Urinary vascular endothelial growth factor concentrations in women undergoing gonadotropin treatment. *Hum. Reprod*., **10**, 2478–2482.

Sanders, S.L., Stouffer, R.L. and Brannian, J.D. (1996) Androgen production by monkey luteal cell subpopulations at different stages of the menstrual cycle. *J. Clin. Endocrinol. Metab*., **81**, 591–596.

Senger, D.R., Galli, S.J., Dvorak, A.M. *et al*. (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science*, **219**, 983–985.

Shalev, E., Geslevich, Y. and Ben-Ami, M. (1994) Induction of pre-ovulatory luteinizing hormone surge by gonadotrophin-releasing hormone agonist for women at risk for developing the ovarian hyperstimulation syndrome. *Hum. Reprod*., **9**, 417–419.

Todorow, S., Schrick, S.T., Siebzehnruebl, E.R. *et al*. (1993) Von Willebrand factor: an endothelial marker to monitor *in vitro* fertilization patients with ovarian hyperstimulation syndrome. *Hum. Reprod*., **8**, 2039–2046.

Weindel, K., Marmé, D. and Weich, H. (1992) AIDS-associated Kaposi's sarcoma cells in culture express vascular endothelial growth factor. *Biochem. Biophys. Res. Commun*., **183**, 1167–1174.

Yan, Z., Weich, H.A., Bernart, W. *et al*. (1993) Vascular endothelial growth factor (VEGF) messenger ribonucleic acid (mRNA) expression in luteinized human granulosa cells *in vitro*. *J. Clin. Endocrinol. Metab*., **77**, 1723–1725.

Yeo, K.T., Sioussat, T.M., Faix, J.D. *et al*. (1992) Development of time-resolved immunofluorometric assay of vascular permeability factor. *Clin. Chem*., **38**, 71–75.

Zalel, Y., Orvieto, R., Ben-Raphael, Z. *et al*. (1995) Recurrent spontaneous ovarian hyperstimulation syndrome associated with polycystic ovary syndrome. *Gynecol. Endocrinol*., **9**, 313–315.

Received on February 25, 1997; accepted on January 8, 1998