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Relationship of ovarian stimulation response with vascular endothelial growth factor and degree of granulosa cell apoptosis

R.Quintana[1,3], L.Kopcow[1], G.Marconi[1], C.Sueldo[1], G.Speranza[1] and R.I.Barañao[2]

[1]Instituto de Ginecología y Fertilidad (IFER), Buenos Aires 1122 and [2]Instituto de Biología y Medicina Experimental (IByME)–CONICET, Buenos Aires 1428, Argentina

[3]To whom correspondence should be addressed. E-mail: ifer@velocom.com.ar

BACKGROUND: The aim of this study was to evaluate the concentration of vascular endothelial growth factor (VEGF) in follicular fluid and in granulosa cell cultures in relation to the degree of apoptosis in granulosa cells from patients with different types of ovarian response to controlled ovarian hyperstimulation. METHODS: We studied 30 women who underwent controlled ovarian hyperstimulation and oocyte retrieval. Group A comprised patients with 1–4 follicles ($n = 10$), group B patients with 5–14 follicles ($n = 10$) and group C patients with >15 follicles ($n = 10$). RESULTS: Mean (±SD) VEGF concentrations in follicular fluid were 1232 ± 209, 813 ± 198 and 396 ± 103 pg/ml for groups A, B and C respectively ($P > 0.01$). Concentrations of VEGF in granulosa cell supernatant were 684 ± 316, 1101 ± 295 and 1596 ± 227 pg/ml respectively ($P < 0.05$). Percentages of apoptotic cells in granulosa cells culture was 55.02 ± 7.5, 23.98 ± 4.4 and 14.2 ± 2.3% respectively (A versus B, $P < 0.01$, A versus C, $P < 0.006$, B versus C, NS). CONCLUSIONS: Our findings showed that in patients with decreased ovarian response to controlled ovarian hyperstimulation, follicular fluid VEGF concentration is elevated, the concentration from granulosa cells culture supernatant is decreased and the percentage of apoptotic granulosa cells is increased, while opposite findings occurred in patients with normal or hyper-responses.

Key words: apoptosis/controlled ovarian hyperstimulation (COH)/granulosa cells (GC)/IVF/vascular endothelial growth factor (VEGF)

Introduction

Vascular endothelial growth factor (VEGF) is a cytokine with a dimeric glycoprotein structure, that shows potent angiogenic and endothelial cell mitogenic properties (Ferrara *et al.*, 1992). In the ovary, VEGF is produced by both granulosa and theca cells (Ravindranath *et al.*, 1992; Friedman *et al.*, 1997); the granulosa cells (GC) generate higher production of VEGF in response to FSH, LH, human chorionic gonadotrophin (HCG) and hypoxia (Shweiki *et al.*, 1992; Christenson and Stouffer, 1997; Neulen *et al.*, 1998), and is instrumental in the conversion of the avascular preovulatory follicle into the corpus luteum, a very vascular organ.

Since VEGF stimulates vascular permeability, a number of studies have evaluated its role in the ovarian hyperstimulation syndrome (OHSS) (Rizk *et al.*, 1997; Ludwig *et al.* 1999; Pellicer *et al.*, 1999), with increasing serum levels of VEGF after HCG administration being predictive for the risk of developing OHSS (Artini *et al.*, 1998; Agrawal *et al.*, 1999). Furthermore, the elevated concentrations of VEGF in follicular fluid correlated negatively with conception rates in assisted reproductive technologies (Friedman *et al.*, 1998).

Apoptosis or programmed cell death has been described in the ovary (Tilly, 1996). This physiological process is induced by androgens, gonadotrophin-releasing hormone (GnRH) and GnRH analogues, but inhibited by oestrogens, FSH, LH, and HCG (Billig *et al.*, 1993; Chun *et al.*, 1994; Goodman *et al.*, 1998; Papadopoulos *et al.*, 1999; Matsubara *et al.*, 2000).

The degree of apoptosis has predictive value on the outcome of assisted reproductive technology cycles (Seifer *et al.*, 1996; Nakahara *et al.*, 1997; Oosterhuis *et al.*, 1998). A higher degree of apoptosis in GC correlates with a lower number of oocytes aspirated during assisted reproductive technologies (Oosterhuis *et al.*, 1998). Although the relationship between VEGF and apoptosis has been described in other organs (Tran *et al.*, 1999; Volm *et al.*, 1999; Kanellis *et al.*, 2000; Li *et al.*, 2000), this interaction has not been studied in the human ovary. Therefore, the purpose of this report is to evaluate the concentration of VEGF in follicular fluid and in the conditioned media of GC culture in relationship with the degree of apoptosis in GC from assisted reproductive technology patients with different types of ovarian response to controlled ovarian hyperstimulation (COH).

Materials and methods

Patients

We studied 30 women who underwent oocyte retrieval for IVF procedures at our Institute between July and December of 1999.

Patients were selected in a consecutive way until groups described as follows were completed.

All patients received leuprolide acetate (leuprolide acetate, Lupron®; Abbot Laboratories, Buenos Aires, Argentina) for pituitary desensitization. Beginning on day 21 of the cycle before gonadotrophin stimulation, leuprolide acetate was given daily for a minimum of 2 weeks, after which serum oestradiol concentration was determined to assess the adequacy of suppression. When the serum oestradiol concentration was <50 pg/ml, two ampoules of rFSH (rFSH-Gonal-F®; Serono Laboratories, Buenos Aires, Argentina) containing 150 IU of FSH were given for 3 days i.m. in addition to the daily dose of leuprolide acetate. From day 4 of stimulation, two to four ampoules of HMG (Pergonal®; Serono), containing 75 IU of FSH and 75 IU of LH, were given i.m. in addition to the daily dose of leuprolide acetate for COH.

Follicular development was monitored by vaginal ultrasound using a 5 MHz transvaginal probe and serum oestradiol concentration. When the serum oestradiol concentration was ⩾450 pg/ml and at least two follicles measured ⩾17 mm in mean diameter, 10 000 IU of HCG (Profasi; Serono) was injected i.m. Transvaginal follicular aspiration was performed 34–36 h after HCG.

Patients were divided as follows: group A or hyporesponders: patients who had 1–4 follicles [with an average (± SD) of 3.50 ± 0.35, $n = 10$]; group B or normoresponders: patients who had 5–14 follicles (with an average of 10.43 ± 0.90, $n = 10$); group C or hyperresponders: patients who had ⩾15 follicles (with an average of 19.71 ± 1.21, $n = 10$) (A versus B and B versus C, $P < 0.0001$).

Follicular fluid

Follicular fluid was collected from each patient from the first follicle punctured. This was selected on the basis of being the most regular and biggest follicle (follicular diameter: 20.90 ± 1.37 mm in group A, 21.80 ± 1.54 mm in group B and 21 ± 1.34 mm in group C) present in each patient undergoing oocyte retrieval. We chose this option in order to avoid a dilution effect caused by follicle irrigation, which could alter VEGF concentration artificially.

Isolation and culture of GC

Pre-luteinized GC from each patient were isolated from pooled follicular fluid by centrifugation at 400 *g* for 10 min and suspended in RPMI 1640 (Gibco BRL, Life Technologies, Rockville, MD, USA) media supplemented with 1% of fetal calf serum (Gibco BRL) and 50 µg/ml antibiotic-antimycotic (Sigma Chemical Co., St Louis, MO, USA).

Granulosa cells were purified by centrifugation through a Histopaque gradient (Sigma) with its density adjusted to 1.065 g/l, during 40 min at 400 *g*.

1×10^6 purified GC were cultured in each of eight wells (Permanox Lab-Teck; Nalge Nunc International, Naperville, MD, USA), for 48 h at 37°C. After incubation conditioned media were collected, centrifuged and stored at –20°C until their use for cytokine determinations.

Apoptosis

In each culture percentage of apoptotic cells were counted by the acridine orange–ethidium bromide technique described previously (Duke and Cohen, 1991). In accordance with this technique, the viable cells were stained entirely green, while early apoptotic cells were observed with the nucleus stained orange and the cytoplasm green, and later apoptotic cells were stained orange completely. A total of 200 cells was counted per sample using a fluorescence microscope, and the percentage of apoptotic cells was calculated as the number of cells with fluorescent orange nucleus/total cells counted.

Quantification of VEGF

VEGF concentration in follicular fluid and in GC culture supernatants was quantified using enzyme-linked immunosorbent assay commercial (ELISA) kits (Cytimmune Sciences Inc., USA). The sensitivity for VEGF ELISA kits was 18.6 pg/ml, the intra-assay variability was 8.9% and the inter-assay variability was 11.1%. All samples were assessed in triplicate.

Statistical analysis

Values are expressed as mean ± SD. Comparisons among the different groups were assessed by Student's *t*-test and Bonferroni ad hoc *t*-tests. A *P*-value ⩽ 0.05 was considered statistically significant.

Results

The results are shown in Table I. The mean age for the different groups was 36.0 ± 1.17, 34.87 ± 1.4 and 33.40 ± 1.5 years for hypo-, normo- and hyper-responders respectively (NS). The total amount of gonadotrophin ampoules administered was: 54.2 ± 9.3 for group A, 25.5 ± 6.8 for group B, and 24.7 ± 5.5 for group C. Serum oestradiol concentrations on HCG administration day were: 1076 ± 175 pg/ml in group A, 3486 ± 615 pg/ml in group B, and 5805 ± 2390 pg/ml in group C (A versus B, $P < 0.0001$; B versus C, $P = 0.01$). The mean number of oocytes retrieved was 2.9 ± 0.35 for group A, 8.5 ± 0.8 for group B, and 15.3 ± 1.39 for group C) (A versus B and B versus C, $P < 0.0001$).

We found significantly increased VEGF concentrations in follicular fluid obtained from hyporesponder patients with respect to hyperresponder patients. Values obtained were 1232 ± 209 pg/ml in group A, and 396 ± 103 pg/ml in group C ($P < 0.01$) (Figure 1). Group B, with an average value of 813 ± 198 pg/ml, was also lower than group A, but this difference did not achieve statistical significance.

In contrast to this, we observed that in the supernatants of GC cultures from group A patients, VEGF concentrations were significantly diminished with respect to group C patients. Mean VEGF concentration in GC supernatant was 684 ± 316 pg/ml for group A, and 1596 ± 227 pg/ml for group C ($P < 0.05$) (Figure 2). Group B with an average value of 1101 ± 295 pg/ml was also higher than group A, but this difference did not achieve statistical significance.

In the assessment of apoptotic GC we found a significant difference between hyporesponder patients and normoresponder patients ($P < 0.01$) and between hyporesponders and hyper-responder patients ($P < 0.006$).

The percentages of apoptotic cells in GC cultures were 55.02 ± 7.50 for group A, 23.98 ± 4.40 for group B and 14.20 ± 2.30% for group C (Figure 3). (A versus B, $P = 0.01$; A versus C, $P = 0.006$).

Linear regression analyses were performed between the number of ampoules of gonadotrophins used and VEGF concentrations for each group in follicular fluid. No statistical correlations were obtained. Similarly, no significant correlations were observed when regression analyses were performed between VEGF in CG supernatants and ampoules (data not shown).

Table I. Comparison of treatment characteristics from three groups of women showing different responses to controlled ovarian hyperstimulation. Group A = hyporresponders, group B = normoresponders, group C = hyperresponders. Data are mean ± SD, $n = 10$

Group	A	B	C
Age (years)[a]	36.00 ± 1.17	34.87 ± 1.40	33.40 ± 1.50
No. of ampoules administered	54.20 ± 9.30	25.50 ± 6.80	24.70 ± 5.50
Oestradiol concentrations on HCG administration day (pg/ml)[b]	1076 ± 175	3486 ± 615	5805 ± 2390
Number of oocytes retrieved[c]	2.87 ± 0.35	8.5 ± 0.82	15.29 ± 1.39

[a]Not significant.
[b]Group A versus B, $P < 0.001$; group B versus C, $P < 0.01$.
[c]Groups A versus B and B versus C, $P < 0.001$.
HCG = human chorionic gonadotrophin.

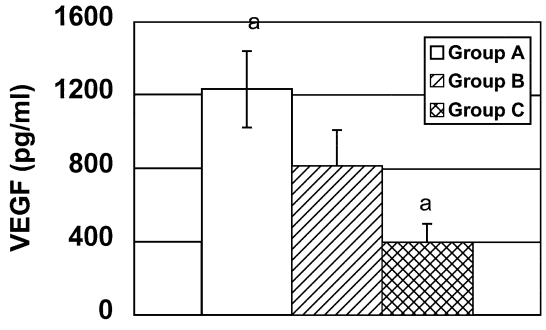

Figure 1. Vascular endothelial growth factor (VEGF) concentrations in follicular fluid of three groups of women categorized by their responses to controlled hyperstimulation. Group A = hyporresponders, group B = normoresponders and group C = hyperresponders. Data are mean ± SD, $n = 10$. [a]$P < 0.01$.

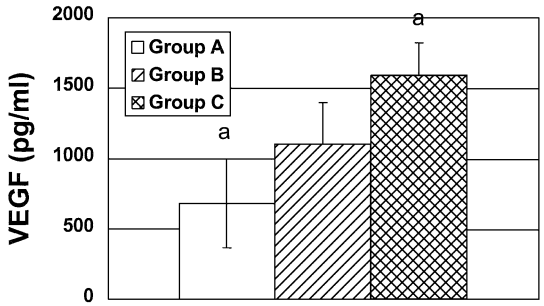

Figure 2. Vascular endothelial growth factor (VEGF) concentrations in the supernatant of granulosa cell cultures from three groups of women categorized according to their responses to controlled hyperstimulation, as described in Figure 1. Data are mean ± SD, $n = 10$. [a]$P < 0.05$.

Discussion

Clinical and experimental evidence highlights VEGF as a cytokine with a powerful effect on vascular permeability, and as an angiogenic agent through the proliferation of endothelial cells and formation of new vessels (Ferrara *et al.*, 1992). It also increases cell survival by inhibiting apoptosis in endothelial cells (Tran *et al.*, 1999; Volm *et al.*, 1999; Kanellis *et al.*, 2000; Li *et al.*, 2000).

VEGF has been found to be an important mediator in OHSS, and serum concentrations after HCG administration in assisted

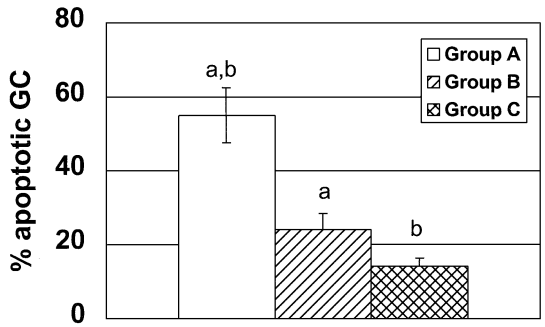

Figure 3. Percentages of apoptotis in the supernatant of granulosa cell (GC) cultures from three groups of women categorized according to their responses to controlled hyperstimulation, as described in Figure 1. Data are mean ± SD, $n = 10$. [a]$P < 0.01$, [b]$P < 0.006$.

reproductive technology patients seem to predict the risk of developing the syndrome (Rizk *et al.*, 1997).

VEGF concentrations in follicular fluid can be measured in only one follicle or in the pool obtained from the follicular fluid from all aspirated follicles. We chose to measure VEGF concentrations in follicular fluid in only the biggest and most regular follicles in order to avoid the dilution effect caused by lavage with medium performed during the aspiration. This influence on VEGF concentration would be dependent on the follicle volume and the number of lavages performed for each follicle. We usually use multiple lavage on hyporesponders to obtain as many oocytes as possible (Waterstone and Parsons, 1992), and on hyper-responders to aspirate granulosa cells in order to try to prevent OHSS (Tomazevic and Meden-Vrtovec, 1996). So follicular fluid–VEGF dilution would be present in different proportions if we had chosen to measure the concentration in the pool of follicular fluid.

In contrast, we used the pool of follicular fluid for GC culture as we studied the percentage of apoptotic cells (with fluorescent orange nucleus)/total cells counted. Dilution with medium does not affect this relationship.

Several investigators researched the relationship between VEGF concentration in follicular fluid and embryo quality and pregnancy potential in assisted reproductive technology cycles. A recent study (Friedman *et al.*, 1997) found an inverse relationship between VEGF concentrations in follicular fluid and conception rates, number of oocytes retrieved and peak

serum oestradiol concentrations whereas they found a positive correlation between VEGF concentrations in follicular fluid and chronological age and number of gonadotrophin ampoules administered. These authors concluded that elevated concentration of VEGF in follicular fluid is a marker of diminished pregnancy potential. More recently, it was shown (Barroso *et al.*, 1999) that the concentration of VEGF in follicular fluid is negatively correlated with embryo quality as determined by morphology scoring on day 3 of in-vitro culture.

Our findings, of higher follicular fluid–VEGF concentration in those patients with poor ovarian response, in comparison with those that showed a hyper-response to COH, seem to agree with the findings listed above. Furthermore, not only did the poor responders show a lower peak serum oestradiol concentration and number of oocytes, but the pregnancy rate was also significantly lower (data not shown) in comparison with patients with better ovarian response to COH.

Hypoxia is a well-recognized stimulant for VEGF production in many cell types (Shweiki *et al.*, 1992; Goldberg and Schneider, 1994; Levy *et al.*, 1995). Friedman *et al.* demonstrated elevated VEGF concentrations in follicular fluid in response to hypoxia (Friedman *et al.*, 1997). On the other hand, Van Berklom *et al.* did not find an association between individual VEGF concentrations in follicular fluid and the percentage of dissolved oxygen in a young population of optimally responding women (Van Berklom *et al.*, 1997). Since the VEGF concentration in follicular fluid depends not only on the induction of VEGF production but also on the number and quality of GC responding, we studied the VEGF produced in the supernatant of GC culture *in vitro* from all three types of ovarian response. Our findings of lower concentration of VEGF in GC culture from hyporesponders, in comparison with hyper-responders, led us to focus on the degree of GC apoptosis present in the different groups.

Apoptosis is a physiological process that leads in a controlled fashion to cell death and plays an important role in maintaining homeostasis in tissues such as liver and ovary. Seifer *et al.* demonstrated increased apoptotic GC in pre-ovulatory follicles of women with decreased ovarian reserve (Seifer *et al.*, 1996). In addition, Nakahara *et al.* showed an increased number of apoptotic bodies in GC from patients with diminished ovarian response and ultimately lower pregnancy potential (Nakahara *et al.*, 1997). In our population with poor ovarian response, we encountered a significantly higher percentage of apoptotic cells in comparison with patients with better ovarian response, in agreement with the authors listed above. The inverse relationship between VEGF and apoptosis has been described in tumour tissue (Volm *et al.*, 1999). To our knowledge, this is the first study looking at the relationship of VEGF and apoptosis in GC from patients with different types of ovarian response.

Ovarian folliculogenesis requires a dynamic interaction between the maturing oocyte, the nurturing GC and regulatory factors present in follicular fluid. An inverse relationship between VEGF and apoptosis has been described in tissues other than ovary (Tran *et al.*, 1999; Volm *et al.*, 1999; Kanellis *et al.*, 2000; Li *et al.*, 2000). In this study, we found that in hyporesponders, the concentrations of VEGF in follicular fluid and the degree of apoptosis present in GC were high whereas there were low concentrations of VEGF in GC supernatants. The opposite was found in hyper-responders to COH. In order to explain our findings, it is tempting to speculate that poorly developed perifollicular microvasculature, as seen in patients with poor ovarian response, leads to severe hypoxia in pre-ovulatory follicles and subsequently to reactive production of compensating VEGF by GC from the same follicles. Yet in a recent study, Van Berklom *et al.* found higher concentrations of VEGF in follicular fluid of follicles with higher dissolved oxygen content and better perifollicular vascularization in a group of good responders (Van Berklom *et al.*, 1997). Whether these findings can be applied to patients with poor ovarian response or whether the variability in VEGF concentration may be a reflection of the number of apoptotic GC and/or the levels of other regulatory follicular factors known to be angiogenic (i.e. leptin, nitric oxide) or GC survival factors (i.e. IGF-1, EGF, etc) remains to be determined.

We found concentrations of VEGF in follicular fluid and in the supernatant of GC cultures to be reversed. Different publications evaluated concentrations of VEGF in both follicular fluid and supernatants of GC cultures separately, but we could not find any article comparing both. Most authors agree that low concentrations of VEGF in follicular fluid are found in 'good prognosis' patients (as determined by peak serum oestradiol concentrations, number of gonadotrophin ampoules administered, number of oocytes retrieved, embryo quality and pregnancy rate) (Friedman *et al.*, 1997; Barroso *et al.*, 1999). In contrast, high levels of VEGF production by GC cultures are found in patients with elevated concentrations of serum oestradiol, higher number of follicles, oocytes retrieved, fertilization rate and pregnancy rate (Doldi *et al.*, 1997,1999). These opposite findings in prognosis between concentrations of VEGF in follicular fluid and in supernatants of GC cultures remain to be explained. One possible hypothesis could be that GC exposed to hypoxia *in vivo* produce a reactive secretion of VEGF, while in GC cultured *in vitro* and exposed to different oxygen conditions, the production of VEGF may be inhibited. We are currently studying VEGF secretion in GC culture under different levels of oxygen saturation.

In conclusion, our findings showed that in patients with decreased ovarian response to COH in assisted reproductive technologies, the concentrations of VEGF in follicular fluid are elevated, the concentrations in the supernatant from GC culture are decreased and the percentage of apoptotic GC is increased, whilst opposite findings were encountered in patients with normal or exaggerated response to COH.

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