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Is granulocyte colony-stimulating factor level predictive for human IVF outcome?

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BACKGROUND: We investigated granulocyte colony-stimulating factor (G-CSF) in human reproduction. METHODS: From a total sample of 93 patients, we analysed in group 1 ($n = 82$) the level of G-CSF and estradiol (E_2) in serum and follicular fluid (FF) on day of follicular puncture (FP). Furthermore, in response to ovarian stimulation, G-CSF levels in serum were compared between low ($n = 11$), moderate ($n = 53$) and high ($n = 18$) response patients. In group 2 ($n = 23$) serum for G-CSF assessment was collected throughout menstrual cycle until gestation. Group 3 ($n = 11$) patients with endometriosis were assessed for G-CSF in serum and FF on day of FP without further differentiation. RESULTS: G-CSF in FF was higher than in serum ($P < 0.01$). G-CSF in serum increased from low through moderate to high response ($P < 0.001$); pregnancy rates were 0, 24.5 and 33.5% respectively. G-CSF in serum increased throughout stimulation, reached a peak with ovulation induction ($P = 0.01$) and decreased until embryo transfer ($P = 0.001$). G-CSF level only in pregnant patients ($n = 11$) increased from embryo transfer to implantation to gestation ($P = 0.005$). In endometriosis patients G-CSF in serum and FF was lower than in non-endometriosis patients ($P \leq 0.03$) and corresponded with low response patients. CONCLUSIONS: G-CSF is involved in follicle development and may be a predictor of IVF outcome.

Key words: granulocyte colony-stimulating factor/ICSI/IVF/pregnancy response

Introduction

Cytokines are increasingly recognized as potentially important local regulators of ovarian function (Erickson and Danforth, 1995). Granulocyte colony-stimulating factor (G-CSF) belongs to the family of haemopoietic growth factors (Clark and Kamen, 1987) and is produced primarily by haemopoietic cells, although several non-haemopoietic cell types, such as osteoblast, smooth muscle, endothelial, epithelial cells and human ovary (Zhao *et al.*, 1995), human endometrium (Giacomini *et al.*, 1995), as well as reproductive tissue cells have also been shown to produce G-CSF (Morstyn and Burgess, 1988; Duan, 1990; Brannstrom *et al.*, 1994; Giacomini *et al.*, 1995). Calhoun *et al.* (1999) reported the presence and distribution of G-CSF and its receptor in various human fetal tissues. Recently, we (Salmassi *et al.*, 2004) detected the expression of G-CSF and its receptor by luteinized granulosa cells. Some authors have reported that serum G-CSF concentration significantly increases during the ovulatory phase compared with all other phases, suggesting that G-CSF may play an important role in ovulation (Makinoda *et al.*, 1995, 1996). Hock *et al.* (1997) reported that in ovarian-stimulated patients the white blood cell counts and G-CSF levels in serum rose significantly during ovarian stimulation.

After ovulation the endometrium acquires the ability to implant the developing embryo within a specific time-window, termed the 'receptive phase'. It has been reported that some cytokines may have a more important function in achieving or maintaining pregnancy and may be essential members of the 'implantation window', whereas others may be supportive and/or redundant during this phase of the menstrual cycle. For example, animal experimentation showed that implantation of the blastocyst can proceed in the absence of most individual cytokines, although leukaemia inhibitory factor (LIF) and interleukin-11 (IL-11) have indisputable roles in this process. In other cases, such as macrophage colony-stimulating factor (M-CSF or CSF1), granulocyte macrophage colony-stimulating factor (GM-CSF), IL-1 and IL-6, the numbers of implantation sites or litter sizes are reduced when the cytokine is absent (Stewart *et al.*, 1992; Simon *et al.*, 1998; Smith *et al.*, 1998; Salamonsen *et al.*, 2000; von Wolff *et al.*, 2000).

In the present study, we describe the important role of the changes in serum G-CSF levels during the menstrual

cycle, in the process of follicular maturation, ovulation, implantation, pregnancy and their response to ovarian stimulation with recombinant (r)FSH.

Material and methods

Patients

From an original sample of 93 patients, serum and follicular fluid (FF) were collected on the day of follicular puncture (FP). The age of the patients ranged from 20 to 42 years, median 33 years, and the size of the lead follicle on the day of follicular puncture (FP) from 19 to 24 mm. These patients were divided into three groups as follows.

Group 1

Patients (n = 82) with the aetiology of tubal or male factor infertility were analysed for: (i) correlation between serum and FF with respect to G-CSF and correlation between G-CSF and estradiol (E_2) in serum; and (ii) comparison of G-CSF level in serum in response to ovarian stimulation and comparison of G-CSF level in serum between pregnant and non-pregnant patients.

Group 2

Patients (n = 23 of the 82 patients in group 1 with moderate response to ovarian stimulation) were monitored throughout the menstrual cycle until 4 weeks after embryo transfer: stimulation phase including stimulation days 6–8 (st. 6–8), st. 9–11 and day of hCG (Predalon; Organon, München, Germany) injection; oocyte retrieval: day of FP; post-retrieval days including the day of embryo transfer (ET; 2–3 days post-FP); 1 week post-FP, time of embryo implantation (FP +1w); 2 weeks post-ET, day of β-hCG evaluation and confirmation of pregnancy (ET +2w). One to three embryos at the 4–6-cell stage were transferred. Gestation: 3 and 4 weeks post-ET (ET +3w and ET +4w). Eleven of the 23 patients became pregnant.

In this group G-CSF levels in serum were analysed throughout the different ovarian cycle phases and gestation (nine analyses for every pregnant patient and eight analyses for every non-pregnant patient). The serum of all 23 patients was measured with the same enzyme-linked immunosorbant assay (ELISA) G-CSF kit from the same lot number to guarantee low intra- and inter-assay variances.

Group 3

Patients (n = 11) with endometriosis were assessed for G-CSF and estradiol in serum and FF on the day of FP. One of the 11 patients became pregnant. These patients were not monitored for low, moderate or high response to stimulation.

IVF stimulation

Patients undergoing IVF were stimulated with rFSH (Serono, Munich, Germany) after down-regulation with GnRH agonists Synarela (Pharmacia, Erlangen, Germany) or Enantone Gyn (Takeda, Aachen, Germany). Monitoring of follicle development by real-time ultrasound scans and serum E_2 levels was performed from day 5 until the day of FP. Once the leading follicle measured >17 mm in diameter and the 17β-E_2 level was adequately increased, but still <3000 pg/ml in serum, 5000–10 000 IU of hCG were administered s.c. Follicles were aspirated 36 h after administration of hCG. After embryo transfer the patients were treated with progesterone (Utrogest, 600 mg daily; Dr Kade/Besins, Berlin, Germany) until confirmation of pregnancy by β-hCG determination, 16 days after FP.

Biochemical analyses

G-CSF assay in serum and in FF

Blood and FF were taken from all IVF patients, centrifuged for 10 min at 350 *g* and 5°C, shock-frozen and kept at −80°C. After retrieval of the oocytes, the FF underwent the same treatment as the blood.

G-CSF levels in serum and FF were measured in duplicate by a solid phase ELISA using Quantikine G-CSF kit (R&D, Wiesbaden, Germany). A 4-fold dilution for serum and FF was performed with the Calibrator Diluent RD6P. This assay employs the quantitative sandwich enzyme immunoassay technique. G-CSF levels ranged between 1.25 and 40 pg/ml, with a sensitivity of 0.8 pg/ml. Precision was <5% for intra-assay and <8.5% for inter-assay.

Only those cases in which both FF and serum could be simultaneously collected on the day of oocyte retrieval were included in this study.

E_2 assay in serum and FF

E_2 levels were measured by a solid phase, competitive chemiluminescent enzyme immunoassay with the Immulite 2000 auto system (DPC-Biermann, Bad Nauheim, Germany) within the range of 0–2000 pg/ml for E_2 (sensitivity 15 pg/ml).

Statistical evaluation

Statistical analysis was performed using the statistical programme SPSS. Pearson's correlation coefficient (r) was applied to investigate the correlation between serum and FF with respect to G-CSF and between G-CSF and E_2 in serum. To differentiate between groups we used non-parametric procedures.

We performed a Kruskal–Wallis test to analyse differences in G-CSF levels between more than two groups: between patients with low, moderate and high response to ovarian stimulation. A Friedman test was applied for samples with repeated measurements and more than two groups, such as between G-CSF levels in serum throughout different ovarian cycle phases and gestation.

Paired comparisons were analysed by the Mann–Whitney U-test for unpaired and by the Wilcoxon signed rank test for paired samples. The differences in pregnancy rates between low, moderate and high response patients were analysed according to a χ^2-test. $P < 0.05$ was considered to be statistically significant throughout the manuscript.

Results

G-CSF level in serum and FF on the day of oocyte retrieval

The levels of G-CSF in serum and FF were detected in 82 infertile patients (group 1) undergoing IVF treatment. On the day of oocyte retrieval the median G-CSF level in FF (116.3 ± 32.6 pg/ml) was significantly higher than that in serum (57.2 ± 24.3 pg/ml) ($P < 0.01$, Wilcoxon signed rank test). Based on normally distributed values of G-CSF levels in serum and FF, we found a positive correlation (Pearson $r = 0.44$, regression coefficient $b_1 = 0.25$, $P < 0.01$, Figure 1). A significant and positive correlation was found between the levels of G-CSF and E_2 in serum on the day of FP (Pearson $r = 0.37$, $P < 0.05$).

There were no significant differences in G-CSF concentration in FF between follicles with fertilized oocytes and follicles with unfertilized oocytes. On the other hand, the level of G-CSF in follicular aspirates with oocytes (n = 76) was

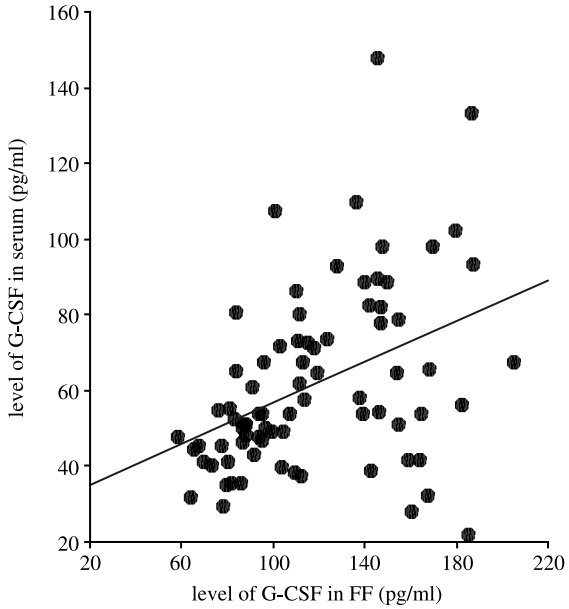

Figure 1. Correlation between granulocyte colony-stimulating factor (G-CSF) levels in serum and in follicular fluid (FF) on the day of oocyte retrieval ($n = 82$, $r = 0.44$, $P < 0.01$, regression coefficient $b_1 = 0.25$).

higher than in those without ($n = 6$), but it was not significant.

The mean G-CSF level in FF of patients who underwent ICSI treatment (126.1 ± 31 pg/ml, $n = 42$) was significantly higher than in those who underwent IVF treatment (106.8 ± 35.4 pg/ml, $n = 40$) ($P = 0.02$, Mann–Whitney *U*-test).

Relationship between G-CSF levels in serum and response to stimulation

G-CSF levels in serum ($n = 82$, group 1) express the response to ovarian stimulation with rFSH (Figure 2). The patients

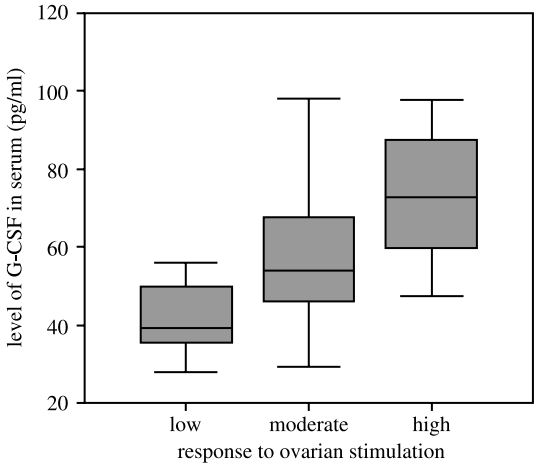

Figure 2. Relation between granulocyte colony-stimulating factor (G-CSF) levels in serum ($n = 82$ patients) and low, moderate and high response to ovarian stimulation. The differences between low, moderate and high responders were significant according to the Kruskal–Wallis test, $P < 0.001$. Paired comparison Mann–Whitney *U*-test: high/moderate $P = 0.012$, high/poor $P \leq 0.001$, moderate/poor $P < 0.02$.

Table I. Eighty-two IVF patients divided according to their response to ovarian stimulation into low, moderate and high response

Response	*N*	Total rFSH dose[a] (pg/ml)	Estradiol[a] (pg/ml)	Oocytes (*n*)
Low	11	2859.4 ± 890.5	422.3 ± 147.2	<5
Moderate	53	2664.1 ± 773.8	874.3 ± 448.6	6–10
High	18	2026.1 ± 555.5	1295.8 ± 576.6	>11
P		0.006	0.01	

[a]Values are mean ± SD. The differences in mean rFSH and mean estradiol between patients with low, moderate and high response to ovarian stimulation were statistically significant according to the Kruskal–Wallis test, $P = 0.006$ and 0.01.

were divided into low, moderate and high responders, according to the total dose of rFSH (± SD) up to the day of hCG injection. In Table I the E_2 levels and the number of oocytes identified on the day of FP are given. The differences in mean rFSH and mean E_2 between patients with low, moderate and high response to ovarian stimulation were statistically significant according to the Kruskal–Wallis test ($P = 0.006$ and 0.01).

Patients with a low response had the highest injected dose of rFSH but the lowest mean level of E_2 and the lowest number of oocytes (<5). For these patients the lowest level of G-CSF (40.7 ± 14.3 pg/ml, $n = 11$) was determined. Patients with a moderate response had a medium injected dose of rFSH, a medium level of E_2 and the number of retrieved oocytes ranged between 6 and 10. The level of G-CSF was determined at 59.3 ± 19.3 pg/ml ($n = 53$). Patients with a good response had the lowest injected dose of rFSH, the highest level of E_2 and >11 oocytes. The level of G-CSF in these patients was the highest at 72.4 ± 16.4 pg/ml ($n = 18$). The differences in G-CSF levels between patients with low, moderate and high response was statistically significant according to the Kruskal–Wallis test ($P < 0.001$).

Gestation and outcome

Patients with a good response showed the highest pregnancy rate of 33.3% (Table II). The pregnancy rate among patients with a moderate response was 24.5% and no pregnancy resulted in patients with a low response. The differences in the pregnancy rates between low, moderate and high responders were found to be significant at $P = 0.05$, according to the χ^2-test. The 82 patients showed a total pregnancy rate of 23.2%.

Table II. Relationship between pregnancy rate and response to ovarian stimulation with rFSH

Patients	Response			Total
	Low (*n*)	Moderate (*n*)	High (*n*)	(*n*)
Non-pregnant	11	40	12	63
Pregnant	0	13	6	19
Total	11	53	18	82
Pregnancy rate (%)	0	24.5	33.3	23.2

The differences in pregnancy rates between patients with low, moderate or high response were found to be significant at $P = 0.05$ according to the χ^2-test.

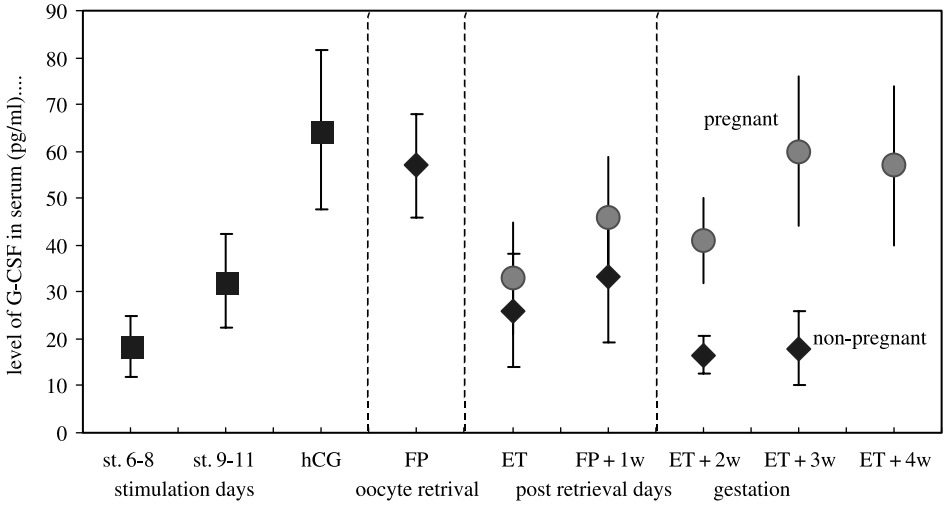

Figure 3. Comparison of the level of granulocyte colony-stimulating factor (G-CSF) in the serum of patients throughout ovarian cycle and gestation. Stimulation days: st. 6–8, st. 9–11 and the day of hCG injection (hCG). Oocyte retrieval: the day of follicular puncture (FP). Post-retrieval days: the day of embryo transfer (ET), 1 week post-FP (FP +1w) and 2 weeks post-ET (ET +2w). Gestation: 3 and 4 weeks after ET (ET +3w) and (ET +4w). Statistical analysis is shown in Tables III and IV. = pregnant (n = 11); = non-pregnant (n = 12); = up to the day of FP, pregnant and non-pregnant together (n = 23).

G-CSF expression during the menstrual cycle

The evaluation of G-CSF levels in serum of 23 patients (group 2) throughout the ovarian stimulation cycle and up to 3–4 weeks later is shown in Figure 3. A gradual increase of G-CSF from st. 6–8 through st. 9–11 is demonstrated, reaching a peak on the day of hCG injection. On the day of oocyte retrieval the G-CSF levels dropped slightly but not significantly. The results are summarized in Table III. The differences within these groups, as analysed by the Friedman test, were significant (P = 0.001). The levels of G-CSF on the day of hCG injection and on the day of oocyte retrieval were significantly higher than during st. 6–8 and st. 9–11 (P = 0.001, P = 0.01, Wilcoxon signed rank test).

The expression levels of G-CSF decreased from the day of hCG injection through FP to approximately the level of st. 9–11 on the day of embryo transfer (ET) (P = 0.001, Wilcoxon signed rank test).

In the post-retrieval days, from the day of ET to the day of implantation (FP +1w) and from the day of confirmation of pregnancy (ET +2w) to gestation (3 and 4 weeks after ET), the G-CSF levels of those patients who became pregnant (n = 11) increased continuously and reached the height of the G-CSF level on the day of hCG injection (Table IV). Those patients who did not become pregnant (n = 12) also showed an increase in G-CSF from ET to implantation, but at a lower level. If implantation failed, G-CSF decreased to the level of G-CSF in the early follicular phase (ET+2w and ET+3w). The differences in G-CSF levels among pregnant patients was significant according to the Friedman test (P = 0.006) in contrast to non-significant differences among non-pregnant patients.

Up to the day of FP there were no significant differences in the G-CSF levels of all 23 patients, whether they later became pregnant or not. For this reason, no distinction is made between pregnant and non-pregnant patients until after the day of ET.

The serum G-CSF levels and endometriosis

The serum and FF levels of G-CSF in endometriosis patients (Table V) were significantly lower than those in

Table III. The mean ± SD level of granulocyte colony-stimulating factor (G-CSF) of patients (n = 23) during stimulation (st.) phase and on day of follicular puncture (FP)

Cycle phase	Total G-CSF (pg/ml)	P
st. day 6–8	18.5 ± 6.9	
st. day 9–11	32.7 ± 10.4	0.02[a]
hCG injection	64.6 ± 20.3	0.003[b]
FP	57.7 ± 11.7	0.026[b]
P	0.001	NS[c]

The differences within these cycle phases, as analysed by the Friedman test, were P = 0.001.
[a]st. 9–11 related to st. 6–8.
[b]hCG injection and FP related to st. 9–11.
[c]FP related to hCG injection.
NS = non-significant (paired comparison by Wilcoxon signed rank test).

Table IV. Comparison of the mean ± SD level of granulocyte colony-stimulating factor (G-CSF) of patients during post-retrieval days and gestation

Post-retrieval days and gestation	Pregnant total G-CSF (pg/ml)	P[a]	Non-pregnant total G-CSF (pg/ml)	P[b]
ET	30.5 ± 13.7		26.4 ± 14.7	NS
FP +1w	42.1 ± 17.1	0.05	36.5 ± 16.4	NS
ET +2w	32.1 ± 13.9	NS	16.8 ± 4.0	0.017
ET +3w	53.1 ± 25.2	0.002	18.5 ± 8.20	0.036
ET +4w	56.4 ± 19.8	0.005		
P	0.006		NS	

The differences within post-retrieval days and gestation, as analysed by the Friedman test, was P = 0.006.
[a]Wilcoxon signed rank test related to embryo transfer (ET).
[b]Comparison between pregnant and non-pregnant patients by Mann–Whitney U-test.
FP = follicular puncture; w = weeks; NS = non-significant.

Table V. Comparison of mean ± SD granulocyte colony-stimulating factor (G-CSF) levels in serum and follicular fluid between endometriosis and non-endometriosis patients (Mann–Whitney *U*-test)

Patients	*N*	Total (serum) G-CSF (pg/ml)	Total (FF) G-CSF (pg/ml)
Non-endometriosis patients	82	57.7 ± 24.6	116.3 ± 33.6
Endometriosis patients	11	45.5 ± 12.4	95.8 ± 31.6
P		0.03	0.024

non-endometriosis patients ($P = 0.03$ and $P = 0.024$, Mann–Whitney *U*-test).

In patients with endometriosis, a higher dose of rFSH was injected (mean = 3416 ± 867 IU) compared to patients without endometriosis (mean = 2612 ± 643 IU, $P = 0.05$). Only one of 11 patients became pregnant. In a comparison of G-CSF levels in serum between patients with endometriosis (45 ± 12.4 pg/ml) and low response patients (40.7 ± 14.3), no significant differences were observed ($P > 0.05$, Mann–Whitney *U*-test).

Discussion

In the present study, in patients with the aetiology of tubal or male factor infertility, we measured the G-CSF concentration in FF in comparison to serum on the day of oocyte retrieval. Our results showed that G-CSF concentration in FF is significantly higher than in serum. Higher levels of macrophage (M)-CSF in FF compared to serum were reported by Witt and Pollard (1997) and Kawano *et al.* (2001). This implies an intrafollicular production and a potential autocrine or paracrine role of G-CSF/M-CSF within the follicular environment. Follicles have proven to be one of the major production sites of G-CSF and one of the contributors to the level of G-CSF in serum during follicular development. Some authors (Yanagi *et al.*, 2002; Salmassi *et al.*, 2004) have recently reported that G-CSF is produced in the human ovary mainly by granulosa, theca and stroma cells.

The mean G-CSF level in FF of patients who underwent ICSI treatment was significantly higher than in those who underwent IVF treatment ($P = 0.02$). The reason for higher levels of G-CSF in ICSI patients may be that ICSI patients (mainly females with healthy ovaries, males with pathological spermiogram) produce more G-CSF in FF than IVF patients (female cause of infertility).

With regard to the response to ovarian stimulation with rFSH, patients with a good response showed the highest G-CSF level in serum on the day of FP and the highest pregnancy rate (33.5%). Patients with a moderate response had a mid-G-CSF level in serum and a pregnancy rate of 24.5%. Patients with a low response showed the lowest G-CSF level in serum and no pregnancy occurred.

Similar results (Brannstrom and Norman, 1993; Nishimura *et al.*, 1998) showed that ovarian stimulation with hMG leads to a gradual increase in M-CSF levels in patients with >20 follicles (good response) but not in those with ≤2 (poor response). Thus, G-CSF and M-CSF levels in serum may reflect a successful stimulation and ample follicle maturation.

Although the cause of poor response to gonadotrophins is complicated and may consist of several dysfunctions of cytokines or growth factor networks, the defect in the mechanism of local (intrafollicular) G-CSF production could be one of the causes or results of poor ovarian response to gonadotrophins.

Ours is the first study to measure the G-CSF level in serum in all cycle phases until 4 weeks after ET, to the time of gestation or new menstruation. In our results G-CSF levels in serum increased gradually throughout the ovarian stimulation cycle from st. 6–8 through st. 9–11 and reached a peak on the day of hCG injection, indicating that gonadotrophins influence the G-CSF release. These results demonstrate that G-CSF is produced in the human follicular phase, immediately prior to the ovulatory phase, and plays an important role in folliculogenesis and in the mechanism of ovulation.

Our results correspond with the results of Hock *et al.* (1997) who also reported that in ovarian-stimulated patients the white blood cell counts and G-CSF levels in serum rose significantly from EF to late follicular phase (LF). Yanagi *et al.* (2002) described similar results in their study on the cyclic changes of G-CSF mRNA in the human follicle during the normal menstrual cycle. They found that the expression level of G-CSF mRNA in the LF phase was greater than in other phases.

After stopping ovarian stimulation with rFSH and administration of hCG, the level of G-CSF decreased significantly from the day of hCG to the day of embryo transfer. It appears that gonadotrophins alone—not hCG—influence G-CSF release. This corresponds with the studies of Brannstrom *et al.* (1994) on pre-ovulatory ovaries (prior to hCG injection). They revealed that GM-CSF release was not influenced by LH (hCG).

Our results show clearly that those patients who became pregnant revealed a continuous increase of G-CSF from the day of embryo transfer to the day of implantation and from the day of confirmation of pregnancy to gestation. In contrast, those patients who did not become pregnant also showed a slight increase in G-CSF from embryo transfer to implantation, but the level then decreased to the level of G-CSF on st. 6–8, indicating the beginning of a new cycle.

The characteristic expression profile of the G-CSF cytokine in the post-retrieval days suggests that G-CSF plays an important role in the implantation process and in the maintenance of pregnancy. If implantation does not occur or fails, the G-CSF level decreases significantly.

Other authors have also reported that serum G-CSF concentration significantly increases in the ovulatory phase and throughout the pregnancy, and suggest that G-CSF plays an important role in ovulation and the maintenance of pregnancy (Makinoda *et al.*, 1995).

Further data indicate that, in the case of GM-CSF, a member of the CSF growth-factor family, the addition of this cytokine to embryo culture media may improve the yield of implantation-competent blastocysts in human IVF programmes (Sjoblom *et al.*, 1999).

Interestingly, in contrast to non-pregnant patients, the G-CSF level of pregnant patients was significantly higher during the post-retrieval phase (FP +1w, embryo implantation) compared to the day of embryo transfer.

In this connection, it has been described that some cytokines such as LIF, IL-11, CSF-1, GM-CSF, IL-1 and IL-6 may have a more important function in achieving or maintaining pregnancy and may be essential members of the 'implantation window' (Stewart *et al.*, 1992; Tabibzadeh *et al.*, 1995; Salamonsen *et al.*, 2000; von Wolff, 2000). The same cytokines that are implicated in implantation in mice are generally maximally expressed in human endometrium with maximal production in the secretory phase, particularly during the 'implantation window'. Therefore, the high level of G-CSF at the time of implantation indicates that this cytokine could also be a member of the 'implantation window'. Its steady increase during the early pregnancy phase could also be used as a pregnancy biomarker.

Miyama *et al.* (1998) showed that during pregnancy decidual tissue produces G-CSF and the receptor for G-CSF is expressed on chorionic villous tissues. In Miyama's study, G-CSF induced greater proliferation of trophoblasts than that of control. It is concluded that the decidual cells and macrophages were sources of G-CSF in the decidual tissue and that G-CSF promoted trophoblast cell proliferation. Recently, G-CSF and G-CSF receptors have also been shown to be produced by placental, decidual and endometrial gland cells during pregnancy, suggesting that G-CSF may play a role in decidual and placental functions (Uzumaki *et al.*, 1989; Miyama *et al.*, 1998; McCracken *et al.*, 1999).

Similar results (Bhatnagar *et al.*, 1995) showed that M-CSF (i.e. CSF1)-treated embryos have significantly more trophoblast cells than control embryos. Futhermore, Guleria and Pollard (2000) reported that trophoblast cells can take over M-CSF-regulated functions from the macrophages for the modulation of immune responses to invading pathogenes at the maternal–fetus interface. The increase in G-CSF in the post-retrieval phase and at the time of gestation may have effects similar to those of M-CSF.

Moreover, the characteristic expression profile of the G-CSF cytokine during the menstrual cycle suggests that this cytokine is under the control of steroid hormones. In fact, stimulation of cytokine mRNA in endometrial cells by steroid hormones has been reported for M-CSF (Azuma *et al.*, 1990; Hatayama *et al.*, 1994; Kariya *et al.*, 1994), transforming growth factor β1 (Arici *et al.*, 1996), and vascular endothelial growth (VEGF) (Huang *et al.*, 1998). FSH levels in serum have little value for the assessment of pregnancy outcome; however, E_2, together with G-CSF, give a better recognition of the beginning of pregnancy. Similar results of Kligman and Rosenwaks (2001) show that markers of ovarian reserve (day 3 FSH, inhibin and E_2) are particularly predicitive and useful in guiding the choice of the optimal protocol for assisted reproductive treatment.

Our results showed for the first time that serum and FF levels of G-CSF in endometriosis patients were significantly lower than in non-endometriosis patients. In view of the low level of G-CSF and E_2 in the serum of endometriosis patients (only one of 11 patients became pregnant) and the need for a higher dose of rFSH for ovarian stimulation (response), endometriosis patients can be compared to patients with a low response. In this regard, low G-CSF levels in serum and in FF of endometriosis patients could be an indicator for poor follicle development and embryo implantation.

The data of Pellicer *et al.* (2000), Garrido *et al.* (2000) and Matalliotakis *et al.* (2003) demonstrate that cytokines are regulated differently in patients with endometriosis. Similar to our results, they found a significantly lower concentration of VEGF; however, they found a higher level of IL-6 and no significant changes in the level of IL-1β, GM-CSF, insulin-like growth factor-1 and interferon-γ in the follicular fluid of endometriosis patients. Furthermore, they reported that implantation rates were significantly decreased in patients with endometriosis. Their observations show that the follicular environment is different in cases with endometriosis and suggest that infertility in patients with endometriosis may be related to alterations within the oocyte which, in turn, result in embryos of lower quality and with a reduced ability to implant. These results support our lower pregnancy rate in endometriosis patients.

Additionally, the data of Kao *et al.* (2003) support dysregulation of select genes leading to an inhospitable environment for implantation, including genes involved in embryonic attachment, embryo toxicity, immune dysfunction, and apoptotic responses, as well as genes likely contributing to the pathogenesis of endometriosis, including aromatase, progesterone receptor, angiogenic factors and others.

In conclusion, our data showed that G-CSF is involved in follicle development and ovulation. It could be also a predictor of embryo implantation for IVF outcome.

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