Increase in transforming growth factor β1 in ovarian follicular fluid following ovarian stimulation and in-vitro fertilization correlates to pregnancy

Gabriel Fried1 and Håkan Wramsby

Department of Women and Child Health, Division of Obstetrics and Gynecology, Reproductive Medical Center, Karolinska Hospital, S-171 76 Stockholm, Sweden
1To whom correspondence should be addressed

We have analysed the content of the growth and differentiation regulating peptide, transforming growth factor β1 (TGFβ1), in follicular fluid from patients undergoing in-vitro fertilization (IVF), and correlated concentrations of TGFβ1 with the outcome of the IVF treatment and the concentrations of 17β oestradiol in serum at ovum retrieval. A total of 88 women with infertility of >3 years duration and age <38 years participated in the study. During IVF treatment, follicular fluid and matched serum samples were collected at ovum retrieval and analysed for TGFβ1, oestradiol, progesterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) using radioimmunoassay and enzyme-linked immunosorbent assay. We found that the TGFβ1 content in the follicular fluid at the time of oocyte retrieval correlated positively with subsequent pregnancy. In 29 women who became pregnant following IVF, follicular fluid TGFβ1 values were significantly higher (P = 0.005) than in 59 women where IVF was unsuccessful. In the pregnant group, TGFβ1 values correlated positively with oestradiol at ovum retrieval. TGFβ1 also correlated positively with the number of fertilized oocytes. TGFβ1 may thus be important for successful human pre-embryo development, contribute to successful embryo implantation and development and may be necessary for the establishment of pregnancy.

Key words: follicular fluid/IVF–embryo transfer/oestradiol/ovarian steroids/TGFβ

Introduction

The TGFβ proteins are a family of peptide growth factors that play an important role in regulating growth, differentiation and metabolism of many mammalian cell types. They belong to a superfamily of structurally related dimeric proteins including activins and inhibins, bone morphogenetic proteins and Mullerian inhibiting substance (Massagué et al., 1992; Sporn and Roberts, 1992). The five members of the TGFβ family (TGFβ1–5) show approximately 60–80% amino acid sequence identity with each other, and the mature forms of the five proteins are almost entirely conserved between species. They exert their effects through binding to specific cell surface receptors, which recently have been identified as transmembrane serine/threonine kinases (Lin and Lodish, 1993; Massagué et al., 1994). The multiple actions of the TGFβ proteins have been classified into three categories: (i) effects on the cell cycle; (ii) effects on the extracellular matrix; (iii) effects on other peptide growth factors and their receptors (Lin and Lodish, 1993).

In the human follicle, TGFβ1 and TGFβ2 have been found to be produced in both theca and granulosa cells in culture (Ruegsegger Veit and Assoian, 1988; Mulheron et al., 1992a). TGFβ1 might thus be involved in the regulation of follicular growth and oocyte maturation. We therefore hypothesized that TGFβ1 levels in the follicle may be correlated to fertilization and successful pregnancy. In order to investigate whether the levels of TGFβ1 present in the follicle at the time of ovum retrieval would correlate with the outcome of the in-vitro fertilization (IVF) treatment programme, we have analysed the levels of TGFβ1 in follicular fluid aspirated from patients undergoing IVF and embryo transfer.

Materials and methods

Study patients

A total of 88 women with infertility, treated in our clinic with IVF, participated in the present study. The criteria for acceptance in the IVF programme were the following: infertility of >3 years duration and age <38 years at the start of stimulation. Clinical data for the patients are given in Table I. The main indication for IVF was tubal factor (Table I). All patients followed our standard protocol for stimulation (Schematiczky et al., 1994). The cycle was started by initial suppression of ovarian function with a gonadotrophin-releasing hormone analogue (Suprefact, Hoechst, Stockholm, Sweden) taken six times daily for at least 14 days starting on day 21 of the menstrual cycle. After ultrasonographic verification of down-regulation of ovaries and endometrium, daily intramuscular injections of 75–300 IU human menopausal gonadotrophin (HMG) (Pergonal, Serono) were given. This was continued until the leading follicle reached a diameter of at least 17 mm and the serum concentrations of 17β oestradiol reached at least 2500 nmol/l. Then 5000 IU human choric gonadotrophin (Profasi, Serono Nordic AB, Solna) was given intramuscularly. 36 h later ovum pick-up was performed by aspirating follicles transvaginally using ultrasound monitoring. Aspirated oocytes were inseminated, and fertilization was assessed by the presence of pronuclei 16–18 h after insemination followed by cleavage observed 2 days after ovum retrieval. The ova were thoroughly denuded of granulosa cells. Pre-embryos were scored (Puissant et al., 1987), and those with the highest scores were selected for replacement.

Collection of follicular fluid

Follicles with a diameter of ≥13–14 mm were aspirated. Since it is necessary to avoid contamination by blood when analysing TGFβ1, only the initial clear fluid from aspirated follicles was saved for
analysis. Between six and 14 follicles were aspirated per patient. The fluids from a single patient were combined and centrifuged at 3000 g for 10 min to remove granulosa cells, and stored at −20°C until analysis.

**Analysis of TGFβ1**

Prior to analysis, the follicular fluid was thawed, and latent TGFβ1 in a 400 µl sample was activated by acidification with 40 µl 1.2 M HCl. Samples were vortexed and incubated at room temperature for 15 min. Then, samples were neutralized by addition of 80 µl 0.5 M HEPES/0.72 M NaOH, and vortexed. TGFβ1 content of neutralized samples of follicular fluid was determined by radioimmunoassay, with reagents in the NEK-071 kit from DuPont NEN (El du Pont de Nemours & Co., Boston, MA, USA). The volume of each sample was 100 µl. Corrections for dilutions of samples were carried out in duplicate. Sensitivity of the assay was 310 ng/l (12.5 pmol/l), and expressed as ng/l follicular fluid. All assays were performed in duplicate. The amount of TGFβ1 in follicular fluids varied between 1636–7871 ng/l. The median and range were 4170 ± 1636 and 2184 ± 8230 ng/l. Interassay reproducibility was 8.9% (n = 5).

**Analysis of 17β oestradiol, progesterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH)**

Serum sampling for 17β oestradiol was routinely performed 2 days prior to OPU and on the day of OPU. Oestradiol in serum was analysed by the routinely used enzyme-linked immunoassay at the Central Laboratory for Clinical Chemistry at the Karolinska Hospital. Oestradiol, progesterone, FSH and LH in follicular fluids were analysed using a random access chemiluminescent immunoassay system (Immulite analyser; Diagnostic Products Corporation, Los Angeles, CA, USA).

**Statistical analysis**

Data were analysed by the Mann–Whitney test, since the test for normality using the Kolmogorov–Smirnov test revealed that data were non-Gaussian. A two-tailed P value < 0.05 was considered to indicate statistical significance. Correlation analysis was performed according to the Pearson test. Comparison of percentages was performed with the χ² test. Results are presented as means ±SD, or as median and range, as appropriate.

**Results**

No significant difference was found between pregnant and non-pregnant patients with regard to age, total dose of HMG used during stimulation and days of stimulation until OPU (Table I).

The amount of TGFβ1 in the follicular fluids varied between 236 and 18 030 ng/l. When samples were grouped according to whether pregnancy occurred, the mean TGFβ1 value in the pregnant group was 4170 ± 1636 ng/l (SD, n = 29), whereas the value in the non-pregnant group was 2860 ± 1111 ng/l (SD, n = 59). The difference between the groups was significant (P = 0.005) (Figure 1, Table II). The difference remained

<table>
<thead>
<tr>
<th>Pregnant</th>
<th>Non-pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>32.5 ± 3.8</td>
</tr>
<tr>
<td>Total dose of HMG (IU)</td>
<td>2064 ± 463</td>
</tr>
<tr>
<td>Days until OPU</td>
<td>12.9 ± 1.8</td>
</tr>
<tr>
<td>Indication for IVF:</td>
<td></td>
</tr>
<tr>
<td>tubal factor</td>
<td>73.3%</td>
</tr>
<tr>
<td>male factor</td>
<td>3.3%</td>
</tr>
<tr>
<td>endometriosis</td>
<td>1.6%</td>
</tr>
<tr>
<td>cervical factor</td>
<td>6.6%</td>
</tr>
<tr>
<td>unexplained infertility</td>
<td>16.6%</td>
</tr>
</tbody>
</table>

IVF = in-vitro fertilization; HMG = human menopausal gonadotrophin; OPU = ovum pick-up.

Table I. Clinical data for the patients in the study. Data given as mean ± SD or as percentages

<table>
<thead>
<tr>
<th>Pregnant</th>
<th>Non-pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ1 (ng/l)</td>
<td>4170 ±1636</td>
</tr>
<tr>
<td>E2 (mmol/l)</td>
<td>1939 ± 922</td>
</tr>
<tr>
<td>P4 (µmol/l)</td>
<td>21.4 ± 10.1</td>
</tr>
<tr>
<td>FSH (mIU/ml)</td>
<td>3.7 ± 1.4</td>
</tr>
<tr>
<td>LH (mIU/ml)</td>
<td>1.5 ± 1.9</td>
</tr>
<tr>
<td>E2pre (mmol/l)</td>
<td>5086 ± 3508</td>
</tr>
<tr>
<td>E2opu (mmol/l)</td>
<td>3427 ± 1985</td>
</tr>
<tr>
<td>Leading follicle size (mm)</td>
<td>22.8 ± 2.3</td>
</tr>
<tr>
<td>Number of follicles (n)</td>
<td>10.4 ± 4.2</td>
</tr>
<tr>
<td>Oocytes retrieved (n)</td>
<td>7.2 ± 3.3</td>
</tr>
<tr>
<td>Oocytes fertilized (n)</td>
<td>5.3 ± 2.0</td>
</tr>
<tr>
<td>Pre-embryos replaced/OPU</td>
<td>2.13 ± 0.43</td>
</tr>
</tbody>
</table>

Table II. Levels of TGFβ1, 17β oestradiol (E2), progesterone (P4), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in follicular fluid at the day of ovum pick-up (OPU); 17β oestradiol in serum 2 days before OPU (E2pre); 17β oestradiol in serum at OPU (E2opu); number of oocytes retrieved and number of oocytes fertilized in the pregnant (n = 29) and non-pregnant (n = 59) group. Significant differences are indicated by symbols. Data given as mean ± SD. For the parameters where significant differences were detected using Mann–Whitney test, data are also given as median and range

**Figure 1.** Scatterplot showing concentrations of TGFβ1 in follicular fluid aspirated on the day of oocyte retrieval in the pregnant and non-pregnant group of women who participated in the in-vitro fertilization (IVF)–embryo transfer programme. Left panel = pregnant group; right panel = non-pregnant group; y axis: TGFβ1 (ng/l). The mean value is indicated by the horizontal line.
significant even if the outlier in the pregnant group was removed.

Further analysis revealed that although there was no difference in the total number of oocytes retrieved in the two groups (Table II), the number of oocytes that became fertilized was significantly higher in the pregnant group (Table II). In addition, the TGFβ1 levels were positively correlated with the number of fertilized oocytes in both groups (r = 0.24, P = 0.03). The percentage of fertilized oocytes in the pregnant group was 76% compared with 55% in the non-pregnant group (P = 0.001). This difference was only slightly influenced by presence of male factor. When we excluded patients where sperm counts after swim-up in the treatment cycle were <1 × 10⁹/ml and the percentages of rapidly progressive spermatozoa were <70%, the corresponding data were 76% fertilized oocytes in the pregnant group (n = 26) and 64% in the non-pregnant group (n = 44, P = 0.02).

In the whole population studied, the average number of transferred pre-embryos was lower in the non-pregnant group. We therefore selected a subset of patients (n = 67) where exactly two pre-embryos were replaced. This excluded six patients from the original pregnant group (one with one pre-embryo replaced, five with three pre-embryos replaced) and 14 patients from the original non-pregnant group (nine without embryo replacement, two with one pre-embryo replaced and three with three pre-embryos replaced). In this subset, TGFβ1 levels in the pregnant group were 4460 ± 1838 ng/l (SD, n = 23) and the levels in the non-pregnant group were 2963 ± 1472 ng/l (SD, n = 44). The difference was significant (P = 0.047). The number of fertilized oocytes was again higher in the pregnant group, 5.7 ± 1.9 as compared with 4.7 ± 2 in the non-pregnant group (P = 0.05).

We found no correlation either between mRNA levels and the size of the leading follicle, nor between TGFβ1 and the total number of follicles.

Since TGFβ1 may stimulate granulosa cells to produce oestrogen, we examined the relationship between TGFβ1 levels in the follicular fluid and circulating oestradiol. TGFβ1 levels at OPU correlated positively with serum oestradiol levels in the pregnant group 2 days before OPU (r = 0.52, P = 0.0014) and at OPU (r = 0.47, P = 0.01), whereas there was no correlation with serum oestradiol levels at OPU in the non-pregnant group.

The serum oestradiol levels at the day before OPU and at the day of OPU showed the expected profile in both the pregnant and non-pregnant group, with peak levels before OPU (Table II). Serum levels of oestradiol before OPU were strongly correlated to oestriadiol at OPU in both the pregnant (r = 0.79, P < 0.0001) and non-pregnant group (r = 0.56, P < 0.0001).

The levels of oestradiol, progesterone, FSH and LH in the follicular fluid at OPU were similar in the pregnant and non-pregnant groups (Table II).

Discussion

The present data show that follicular fluid aspirated at the time of oocyte retrieval from women undergoing stimulated cycles in an IVF/embryo transfer programme contain measurable amounts of TGFβ1, and that the average values of follicular fluid TGFβ1 were significantly higher in cycles where embryo transfer resulted in pregnancy. In the pregnant group, there were also significantly higher numbers of fertilized oocytes and transferred pre-embryos. When analysing data from patients who had exactly two pre-embryos replaced, we still found significantly higher levels of TGFβ1 in the pregnant group, as well as a significantly higher number of fertilized oocytes. This may indicate a relationship between TGFβ1 and oocyte quality.

It has previously been shown that TGFβ is measurable in human ovarian follicular fluid (Ruegsegger Veit and Assoian, 1988), and that it is produced in both granulosa and theca cells in the human ovary (Mulheron et al., 1992a). Immuno-cytochemical studies of the relative presence of TGFβ1 and TGFβ2 during the oestrous cycle in various species have shown that the dominant form present in granulosa cells is TGFβ1 (Mulheron et al., 1992b; Roy et al., 1992; Teers and Dorrington, 1992; Gangrade et al., 1993). TGFβ1 has been found to stimulate DNA synthesis in rat granulosa cells, and this stimulation was augmented by FSH. In addition, oestrogen stimulated TGFβ secretion, which was blocked by neutralizing antibodies to TGFβ (Dorrington et al., 1993). Moreover, TGFβ1 seems to have an inhibitory effect on androgen production by the theca cells (Hernandez et al., 1990). Thus, it is reasonable to assume that TGFβ may be involved in follicle development and the regulation of follicular oestrogen synthesis. It has been suggested that TGFβ is the missing link between FSH and oestradiol in follicle development (Dorrington et al., 1993). We found a strong correlation between TGFβ1 in the follicle at OPU and circulating oestradiol 2 days before OPU in the pregnant women, possibly indicating the existence of healthy follicles. The correlation between TGFβ1 and oestradiol at OPU was weaker, indicating that the relation between TGFβ1 and oestradiol may be most important before the onset of the LH peak.

TGFβ also influences production of extracellular matrix molecules as well as production of other growth factors. Therefore, it might, in addition to influencing oocyte maturation and fertilization, also play a role at implantation. TGFβ has indeed been suggested to regulate implantation (Lala and Graham, 1990). TGFβ is produced both by the embryo and the uterine decidua and can induce differentiation of human cytotrophoblast cells into non-invasive syncytiotrophoblasts, a mechanism that might restrict implantation. Furthermore, TGFβ can also regulate expression of proteases and protease inhibitors, which also may serve to control invasion of the embryo at implantation (Graham and Lala, 1991).

The availability of biologically active TGFβ is restricted by TGFβ binding proteins (LTBPs), which maintain TGFβ in latent form (Taiapale et al., 1994). Latent TGFβ can be activated by, for example, plasmin (Flaumenhaft et al., 1993) and the biological actions of the released TGFβ are mediated by TGFβ receptors, of which there are at least three different types described to date; TGFβ-type receptor I, II and III (Attisano et al., 1993; Franzén et al., 1993; López-Casillas et al., 1993; Massagué and Pandiella, 1993; Henis et al., 1994). Presence
of TGF-β-type II receptor was recently demonstrated in the hamster ovary, where its expression was critically and temporally influenced by gonadotrophins and steroid hormones (Roy and Kole, 1995).

Experiments with in-vitro fertilized mouse and bovine oocytes have shown that addition of TGFβ significantly increases inner cell mass (Lim et al., 1993) and also improves pre-embryo development beyond the 8-cell stage (Larson et al., 1992). Although targeted disruption of the TGFβ1 gene in mice showed that pregnancy and parturition was not disturbed, it also showed that the embryos received sufficient maternal TGFβ for prenatal rescue. However, the newborn TGFβ-deficient mice died after about 3 weeks (Letterio et al., 1994).

Our present results together with previous data indicate that TGFβ1, in addition to having a role in mediating endometrial–trophoblast interactions, also may be involved in the paracrine regulation of follicular growth. Our results show that higher levels of ovarian TGFβ1 in ovarian stimulation using HMG were associated with a higher number of fertilized oocytes and with pregnancy. This might possibly be mediated through promotion of oocyte quality. Further studies should address the molecular mechanisms whereby follicular fluid TGFβ1 may influence successful embryo development.

Acknowledgements

We thank Ms Birgitta Byström for excellent technical assistance, and Ms Marita Johansson and Ms Eva Andersson for collection and preparation of samples. This research was funded by the Swedish Medical Research Council (14X-07164), the Karolinska Institute and the Karolinska Hospital.

References


Received on July 2, 1997; accepted on December 4, 1997