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Different protein patterns derived from follicular fluid of mature and immature human follicles

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The purpose of our study was to compare the protein patterns originating from fluids of mature and immature human follicles in order to gain further insight into their biochemical composition. A total of 10 patients were stimulated for in-vitro fertilization (IVF) using different stimulation protocols. Follicular fluids were aspirated transvaginally and analysed microscopically for the presence of oocytes. Follicular fluids were stored at –18°C. Samples of 500 µl were processed for two-dimensional gel electrophoresis. Up to 60 proteins in various groups could be detected. Seven protein spots were selected for chemical analysis by cutting them out of the gels and subjecting them to internal amino acid sequencing procedures. Our results can be summarized as follows: (i) major differences were not detected between the protein patterns from the various mature follicles of a particular patient, nor were significant differences observed in the proteins derived from follicular fluids collected from the seven patients with mature follicles; (ii) considerable differences were observed in the protein patterns derived from fluids of immature compared with mature follicles. Fluid from the three patients with immature follicles contained many fewer proteins, some of which were expressed at low levels. We conclude that the observed variations in protein composition of follicles of different developmental age reflect their physiological condition and serve as biomedical markers for follicular maturity.

Key words: amino acid sequencing/follicular fluid/in-vitro fertilization–embryo transfer/proteins/two-dimensional gel electrophoresis

Introduction

Since the pioneering studies on follicular fluid about 20 years ago (Shalgi *et al.*, 1973; Edwards, 1974), increasing knowledge with respect to protein composition (Nagy *et al.*, 1989; Gonzales *et al.*, 1992), protein concentrations (Suchanek *et al.*, 1990), and particular proteins (Urdl, 1991; Andersen *et al.*, 1992) originating from follicular fluid has greatly contributed to a more detailed insight into the physiological processes related to follicular growth and oocyte maturity.

Spectrophotometric analysis of proteins or the protein content has been introduced to study human follicular fluid (Bayer *et al.*, 1990; Huyser *et al.*, 1993). Various hormones such as oestradiol and progesterone (Franchimont *et al.*, 1989; Andersen, 1991, 1993; Tarlatzis *et al.*, 1993) and their binding proteins (Campo *et al.*, 1989), follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Hillier, 1994) have been estimated and related to follicular maturation and oocyte development. Growth factor proteins such as insulin-like growth factors (IGF) and their binding proteins (Cataldo and Giudice, 1992; Kubota *et al.*, 1993; Eden *et al.*, 1993; Schuller *et al.*, 1993), epidermal growth factor (EGF) (Angervo *et al.*, 1992), growth hormone and its binding proteins (Amit *et al.*, 1993; Tarlatzis *et al.*, 1993), as well as tumour necrosis factor (Wang *et al.*, 1992), transforming growth factor (TGF) (Chegini and Williams, 1992; Mulheron *et al.*, 1992), plasma digoxin-like immunoreactive factor (Jakobi *et al.*, 1991), and three pregnancy proteins (Bischof, 1989) have been detected in follicular fluid.

Cytokines such as interleukin-1, -2, -6 (Wang and Norman, 1992; Machelon *et al.*, 1994), macroglobulins and immunoglobulins (Vaughan and Vale, 1993; Papale *et al.*, 1994), lysophospholipids (Lepage *et al.*, 1993) such as lipid transfer protein (Ravnik et al., 1992, 1993), protein kinases (Yang *et al.*, 1993), endothelin-1 (Kamada *et al.*, 1993), fibrinogen and plasminogen (Gulamali-Majid *et al.*, 1987; Lobb and Dorrington, 1987), superoxide dismutase (Shiotani *et al.*, 1991), inhibin (De Jong et al., 1990; Robertson *et al.*, 1990), activin (Sadatsuki et al., 1993) and follistatin (Hillier and Miro, 1993; Cataldo et al., 1994) as binding protein (Krummen *et al.*, 1993) have been added to the steadily growing list of proteins which are included in the follicular fluid of mammals and have been investigated at the molecular level concerning their functional role during folliculogenesis and their respective potential in normal and abnormal mechanisms of reproductive physiology (reviewed by Giudice *et al.*, 1993).

In order further to increase our knowledge regarding the protein composition of follicular fluid, we have started to analyse and compare proteins derived from mature and immature follicles via two-dimensional gel electrophoresis. The procedure, which, to our knowledge is applied to this kind of biological material for the first time, enables the analysis of a large number of proteins and provides a necessary prerequisite for the isolation and identification of particular proteins. In addition, differences in protein composition between mature and immature follicles detected by two-dimensional gel electrophoresis are most likely to be related to their different physiological states. A preliminary report on this subject has been published recently (Spitzer *et al.*, 1994). In this study,

we have selected seven proteins for identification because they have been shown to be quite differently expressed in mature versus immature follicular fluids. They have been identified by internal amino acid sequence analysis and by its comparison with already known sequences stored in a protein data base. These proteins and probably others as yet unidentified may be useful biomedical markers for follicular maturity.

Material and methods

IVF

Ten patients with tubal infertility were stimulated for in-vitro fertilization (IVF) and embryo transfer with three different stimulation protocols. We used human menopausal gonadotrophin (HMG) monotherapy, HMG in combination with clomiphene citrate, or gonadotrophins (HMG or FSH) in combination with gonadotrophin-releasing hormone agonists (GnRHa). When using a pituitary down-regulation we applied buserelin 900 mg per day from the mid-luteal phase onwards (long protocol). Ovarian stimulation was monitored by oestradiol and LH measurements as well as ultrasound investigations. Human chorionic gonadotrophin (HCG) was administered when three or more follicles with a diameter of >18 mm had developed. Follicle puncture was performed transvaginally under ultrasonographic guidance. With regard to the dimensions used for classifying mature versus immature follicles during ultrasonographic observation, these were in accordance with worldwide common practice in IVF programmes, i.e. the diameter of mature follicles ranged between 18 and 20 mm, and that for immature ones between 10 and 14 mm. From the fluid of mature follicles, oocytes with the surrounding cumulus complex were isolated, assessed for their morphological maturity, and selected ones were further processed for IVF. About 48 h after insemination of the oocytes, the embryos were transferred into the uterus. Corpus luteum phase was supplemented three times with HCG administration.

Two-dimensional gel electrophoresis

For electrophoretic analysis, the following nine stock solutions were required: (i) 28.5 g urea, 1 g Nonidet P-40, 2 ml Servalyt (Serva, Heidelberg, Germany) pH 5–7, 0.5 ml two-dimensional-Pharmalyte pH 3–10, and distilled H_2O to 47.5 ml: 0.95 ml aliquots were stored at –18°C; 50 µl 2-mercaptoethanol was added before use; (ii) 14.2 g acrylamide, 0.8 g Bis, and distilled H_2O to 50 ml; (iii) 9.6 g urea, 0.4 ml Servalyt pH 5–7, 0.1 ml two-dimensional-Pharmalyte pH 3–10, and distilled H_2O to 20 ml: 0.5 ml aliquots were stored at –18°C; (iv) 100 g glycerol, 23 g sodium dodecyl sulphate (SDS) (Serva), and 125 ml 0.5 M Tris–HCl (United States Biochemical Corp., Cleveland, Ohio, USA) pH 6.8, and distilled H_2O up to 950 ml; 5% 2-mercaptoethanol was added before use; (v) 75 g acrylamide, 2 g Bis and distilled H_2O added to 250 ml; (vi) 300 g acrylamide, 1.5 g Bis, and distilled H_2O to 1 l; (vii) 90.9 g Tris, 80 ml SDS 10% w/v, pH 8.8 adjusted with HCl, and H_2O added to 1 l; (viii) 30 g Tris, 20 ml SDS 10% w/v, pH 6.8 adjusted with HCl, and H_2O added to 500 ml; (ix) 3 g Tris, 14.4 g glycine (United States Biochemical Corp.), 10 g SDS, trace amount of Bromophenol Blue, and H_2O added to 1 l.

Acetic acid, hydrochloric acid (37%), methanol, ortho-phosphoric acid (85%) and sodium hydroxide were all obtained from Merck (Darmstadt, Germany) and all other reagents, e.g. acrylamide agarose type V high gelling temperature, from Sigma (St Louis, MO, USA).

The two-dimensional gel electrophoresis procedure was specifically modified for the present study according to Murach *et al.* (1990). Before isoelectric focusing (IEF), 1 ml samples of follicular fluid were centrifuged for 10 min at 16 000 *g*. Volumes of 500 µl from the supernatants were mixed with 500 µl of stock solution (i) and stored as sample material at –18°C. For IEF, the gel mix (containing 6.6 g urea, 2.4 ml distilled H_2O, 2.4 ml Nonidet P-40 w/v, 1.6 ml stock solution (ii), 480 µl Servalyt pH 5–7, 160 µl Pharmalyte pH 3–10, 10 µl Temed, and 12 µl ammonium persulphate 10% w/v) was poured into the gel tubes and allowed to polymerize for 2 h. After rinsing the gel surfaces, the gels were placed in a standard electrophoresis apparatus, the lower chamber being filled with 10 mM phosphoric acid. The tops of the gels were layered with 10 µl of stock solution (i) and 10 µl of stock solution (iii); 0.02 N NaOH was added very carefully to the upper chamber. The gels were prerun at 200 V for 15 min, 300 V for 30 min, and 400 V for 60 min. The surfaces were washed and loaded with 40 µl of sample material. The gels were then run for 19 h at 400 V. The IEF gels were stored in stock solution (iv) at –18°C. For the second dimension, two clamped glass plates were sealed with a 1% agarose and 0.1% SDS solution. The degassed slab gel mix, containing 15 ml stock solution (vi), 14.8 ml stock solution (vii), 7 µl Temed, and 140 µl ammonium persulphate 10% w/v, was covered with an SDS solution (0.1 % w/v) and allowed to polymerize for 2 h. The stacking gel (containing 0.75 ml stock solution (v), 1.25 ml stock solution (viii), 3 ml distilled H_2O, 10 µl Temed, and 20 µl ammonium persulphate 10% w/v) was poured on top of the plates. Agarose [0.2 g melted in 20 ml of stock solution (iv)] was rapidly filled into the groove above the stacking gel. After having added the IEF gel, again some agarose was added on top of it to cover the gel system completely. Electrode buffer [stock solution (ix)] was poured into the buffer trays; the anode was connected to the lower chamber and the cathode to the upper one. Until the dye front reached the lower gel, a constant voltage of 100 V was used, thereafter 200 V.

Gels were stained in 0.1% Coomassie Brilliant Blue R-250, 50% methanol, and 10% acetic acid. A total of 265 gels were produced for protein analysis and 80 gels were provided for amino acid sequencing procedures.

Amino acid sequence analysis

Internal amino acid sequence analyses were performed essentially according to Eckerskorn and Lottspeich (1989). For this analysis, seven proteins (nos 1–7) were selected because of their distinct differences in activity between mature and immature follicles. Protein spots 1–7 individually collected from the 10 two-dimensional gels were excised, washed extensively with water, dried for 1 h in a speedvac concentrator (Bachhofer, München, Germany) and incubated with 200 µl cleavage buffer containing endoproteinase LysC (Boehringer, Tutzing, Germany) in 25 mM Tris/HCl pH 8.5. Enzyme to protein ratio was about 1:10. After 6 h at 37°C, the reaction was stopped by adding to the sample 400 µl 0.1% trifluoroacetic acid in acetonitrile. After 2–3 h at 37°C, the sample was filtered through an anatop filter (Merck), 400 µl water added to the filtrate and reduced in volume by evaporation on a speedvac concentrator. The gel pieces were incubated again with 400 µl 0.1% trifluoroacetic acid in acetonitrile overnight at 37°C, filtered through the anatop filter and reduced in volume. The combined filtrates were acidified with formic acid and applied into reversed phase high performance liquid chromatography (RP-HPLC). RP-HPLC was performed using a supersphere 60 RP select B column (Merck). The solvent system was A: 0.1% trifluoroacetic acid in water and B: 0.1% trifluoroacetic acid in acetonitrile. For peptide elution a gradient was performed from 0% B to 70% B in A during 65 min with a flow rate of 200 µl/min. Detection wavelength was 206 nm. Fractions were collected manually and analysed using a 477A pulsed liquid phase sequencer equipped with a 120A PTH analyser (both from Applied Biosystems,

Table I. In-vitro fertilization (IVF) patients with mature and immature follicles from which follicular fluid was analysed for protein patterns

Patient no.	Stimulation protocol[b]	Follicle maturity	Number of		Pregnancy
			follicles	oocytes	
1 (Fig. 4)	CC+HMG	i	13	6	no
2 (Fig. 3)	HMG	m	9	4	yes
3 (Fig. 1)	HMG + GnRHa-LP	m	5	4	yes
4 (Fig. 5)	HMG + GnRHa-LP	m	12	8	no[a]
5 (Fig. 2)	CC+HMG	m	13	5	no[a]
6 (Fig. 4)	FSH + GnRHa-LP	i	19	12	no
7 (Fig. 3)	HMG	m	7	6	no
8 (Fig. 3)	HMG	m	12	9	no
9 (Fig. 4)	FSH + GnRHa-LP	i	8	5	no
10 (Fig. 3)	CC+HMG	m	8	4	no

HMG = human chorionic gonadotrophin, GnRHa = gonadotrophin-releasing hormone agonists, CC = clomiphene citrate, FSH = follicle stimulating hormone, LP = long protocol, i = immature (10–14 mm), m = mature (18–20 mm).
[a]Pregnancy in the next IVF cycle.
[b]We deliberately used different hormonal stimulation protocols to avoid any influence on the outcome of the protein patterns derived from follicular fluid.

Foster City, CA, USA). Amino acid sequences were compared with the PIR protein sequence database (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin 53711, USA). Position numbers were assigned to correspond with the position of amino acid residues in the protein as given in the database.

Results

From 10 IVF patients, follicular fluid was collected from 106 follicles of which 63 (59.4%) contained oocytes. The different stimulation protocols administered for superovulation induction are summarized in Table I. Pregnancy occurred in two patients (pregnancy rate per cycle 20%); another two patients became pregnant in the following IVF cycle.

Follicular fluid derived from mature and immature follicles was analysed for protein patterns using two-dimensional gel electrophoresis. About 60 proteins in various groups could be detected reproducibly on the two-dimensional gels (see Figure 1). These proteins ranged in molecular weight between 100 and 10 kDa and were positioned via IEF according to their charge between pH 10 and 3.

For further investigation, seven proteins were selected from the total protein spots because of their characteristic and well defined positions on the gels, a necessary prerequisite for their biochemical analysis. Moreover, they were useful proteins to distinguish between mature and immature follicular fluid. They were subjected to partial amino acid sequencing procedures and could be identified by comparing their specific sequences with those obtained from a protein data base (Table II). The seven proteins have been determined as transferrin, serum albumin, α_1-antitrypsin, haptoglobulin-1, immunoglobulin Ig-κ chain C region, apolipoprotein A-1, and a subunit or fragment of serum albumin.

These proteins and all the other ones detected on the two-dimensional gels were taken for comparing the protein patterns derived from various follicular fluids.

(i) Mature follicles from a particular patient. The overall pattern of proteins derived from follicular fluid of four different mature follicles of patient no. 5 were quite similar when compared to each other, although minor quantitative and qualitative differences existed, as indicated in Figure 2. Furthermore, there were no noticeable differences in protein composition observed between follicles with or without oocytes detected. A common protein pattern emerged from different follicles of a particular patient.

(ii) Mature follicles from different patients. The analysis of proteins originating from follicular fluid of mature follicles of four different patients (nos 2, 7, 8, and 10; see Table I) showed that their patterns corresponded to a high degree and were very similar, irrespective of the presence or absence of oocytes isolated from the follicular fluid (see Figure 3). Again, however, some small differences in intensity and position of some protein spots were found (Figure 3C, D). For the second dimension, a slightly increased time for electrophoresis was used to achieve, if possible, an increased resolution of protein separation and additional spots in the higher molecular weight region (for comparison, see also Figure 1). As a consequence of this, some proteins with low molecular weight were no longer detectable on the bottom of the gels. However, no increased resolution was observed, thus leading to the conclusion that the basic pattern was consistent for follicles from different patients.

(iii) Immature follicles from different patients. Conversely, the patterns of proteins obtained from follicular fluid of immature follicles of the three patients nos 1, 6, and 9 (see Table 1) differed significantly from those derived from mature follicles. In particular, protein spots 1 and 2 (transferrin and serum albumin, respectively) were detectable at a very low degree or were nearly absent and spot 7 (a subunit or fragment of albumin) could not be detected at all. Spots 4, 5, and 6 (haptoglobulin-1, Ig-κ chain C region, and apolipoprotein A-1 respectively) showed variable expression, mostly at low levels. Spot 3 (α_1-antitrypsin), on the other hand, was present consistently and served as a suitable marker for positional orientation in the gels. In addition to the quantitative and qualitative alterations of specific proteins, there were many fewer proteins detected on the gels (see Figure 4 in comparison with Figures 1–3).

(iv) Physiologically intermediate follicles from a particular patient. The analysis of proteins from follicular fluids of four different follicles of patient no. 4 (see Table I) showed that these patterns lay between the ones originating from mature and immature follicles with respect to number and spot intensity of proteins (Figure 5). In particular, spots 1 and 2 were significantly reduced in their quantity as judged from inferior staining employed to 'visualize' proteins on the gels. The follicles of this patient were determined to be 'mature' with respect to their size estimated during ultrasonographic examination. However, our protein analysis revealed that these follicles, most likely, were not yet in a physiologically mature state.

Discussion

Ovarian stimulation protocols for IVF and embryo transfer have changed in the last few years since GnRHa were

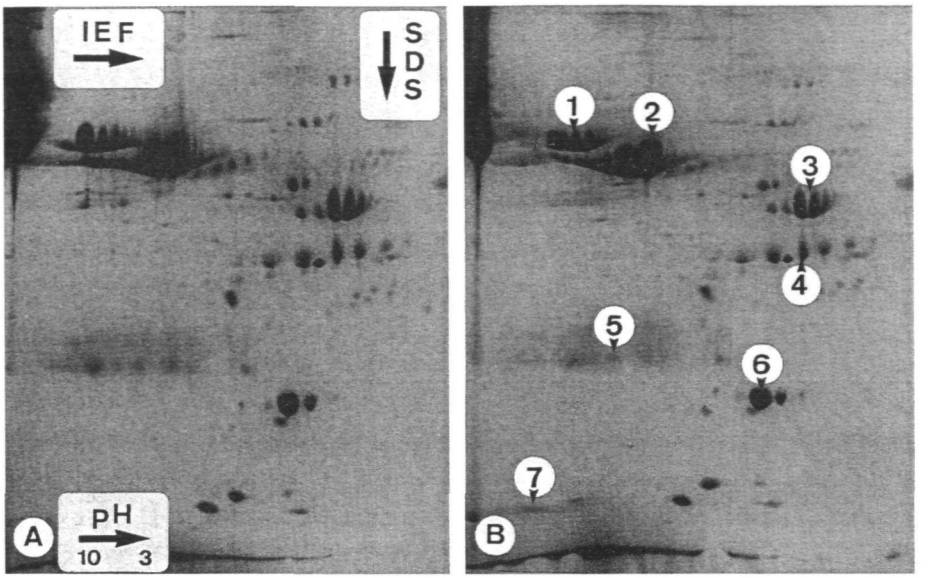

Figure 1. Coomassie-stained two-dimensional gels of proteins in follicular fluid obtained from two different mature follicles of patient no. 3 (see Table I) who underwent hormonal treatment for in-vitro fertilization (IVF). (**A**) Without an oocyte; (**B**) with oocyte. These two pictures demonstrate the reproducibility of the methods used in this study. (**A**) First dimension: isoelectric focusing (IEF) ranging from pH 10 to 3; second dimension: sodium dodecyl sulphate (SDS) separating from 100 to 10 kDa. (**B**) The following proteins have been identified via partial amino acid sequencing procedures: spot 1 = transferrin, spot 2 = serum albumin, spot 3 = α_1-antitrypsin, spot 4 = haptoglobulin-1, spot 5 = Ig-κ chain C region, spot 6 = apolipoprotein A-1, spot 7 = serum albumin subunit or fragment.

Table II. Proteins identified by internal amino acid sequence analysis

Spot no.[a]	Sequences found	Protein identified in PIR protein database
1	LXMGSGLNLXEPNNK	Transferrin (pos. 516-)
	DGAGDVAFVK	Transferrin (pos. 216-)
	DLLFK	Transferrin (pos. 311-)
	XGLVPVLAENYNXK	Transferrin (pos. 421-)
2	LVNEVTE	Serum albumin (pos. 66-)
	VHTEXXH	Serum albumin (pos. 265-)
3	IVDLVK	α_1-Antitrypsin (pos. 193-)
	AVLTIDEK	α_1-Antitrypsin (pos. 360-)
4	FTDHLK	Haptoglobin-1 (pos. 292-)
	SXAVAEYGVYVK	Haptoglobin-1 (pos. 380-)
5	VYAXEVTHQ	Ig-κ chain C region (pos. 83-)
	DSTYSLSS	Ig-κ chain C region (pos. 62-)
	VQWK	Ig-κ chain C region (pos. 38-)
6	VQPYLDD	Apolipoprotein A-1 (pos. 121-)
	VEPLRAE	Apolipoprotein A-1 (pos. 143-)
	DSGRDYV	Apolipoprotein A-1 (pos. 48-)
7[b]	LVAASQAALG	Serum albumin (pos. 599-)
	QTALVELVK	Serum albumin (pos. 550-)
	TPVS	Serum albumin (pos. 491-)

[a]See Figure 1.
[b]Due to its position at low molecular weight on the two-dimensional gels, this protein spot has to be considered as a subunit or fragment of spot 2.
X = no amino acid identified, pos. = position numbers correspond to the position of amino acid residues in the protein as given in the data base (see Materials and methods).

introduced. In large series of IVF and embryo transfer in France and the USA, the clinical pregnancy rate increased in GnRHa treated cycles. In addition, there was a reduced cancellation rate using analogues. However, contradictory effects of various GnRHa on steroid synthesis by human follicle cells have been reported. Oestradiol and progesterone concentrations were found to be either similar (Stone *et al.*, 1988), lower (Brzyski *et al.*, 1990) or higher (Hartshorne, 1989) in GnRHa cycles compared with those without GnRHa. We therefore decided to stimulate patients by applying different protocols and to rely on the parameter of follicle maturation, which is independent from the stimulation protocol and therefore should not influence the outcome of the protein patterns derived from follicular fluid. Despite the variability of different hormonal treatments used, the observed protein patterns were not influenced by this, and remained reproducible and showed a high degree of conformity.

Although the quality of embryos following GnRHa stimulation may be inferior when compared with those obtained after clomiphene and HMG stimulation, uterine receptivity is better and implantation rates increase when GnRHa are used (Testart *et al.*, 1993). Follicles show a more optimal maturation with higher inhibin and progesterone concentrations and lower oestradiol during a GnRHa regime compared with clomiphene stimulation (Andersen *et al.*, 1992). Mantzavinos *et al.* (1983) were able to identify a correlation between follicular growth on ultrasound and oestradiol blood concentrations and the volume of aspirated fluid. The ultrasonographically measured size of the follicle is still the most common clinical parameter of follicular maturity. Follicles with a diameter of ~18–20 mm and a corresponding volume of 3–4 ml show the highest oocyte recovery rates and more mature oocytes (Simonetti *et al.*, 1985; Wittmaack *et al.*, 1994). Furthermore, the fertilization and cleavage rates of oocytes derived from follicles with volumes of >2 ml are relatively constant with respect to the success rate.

The content and concentration of particular proteins in follicular fluid related to the acrosome reaction and sperm capacitation have been associated with successful IVF outcome (Anderson *et al.*, 1994; Miska *et al.*, 1994). Free oestradiol concentrations and oestradiol/androgen ratio of follicular fluids have been used as another parameter for oocyte maturity and

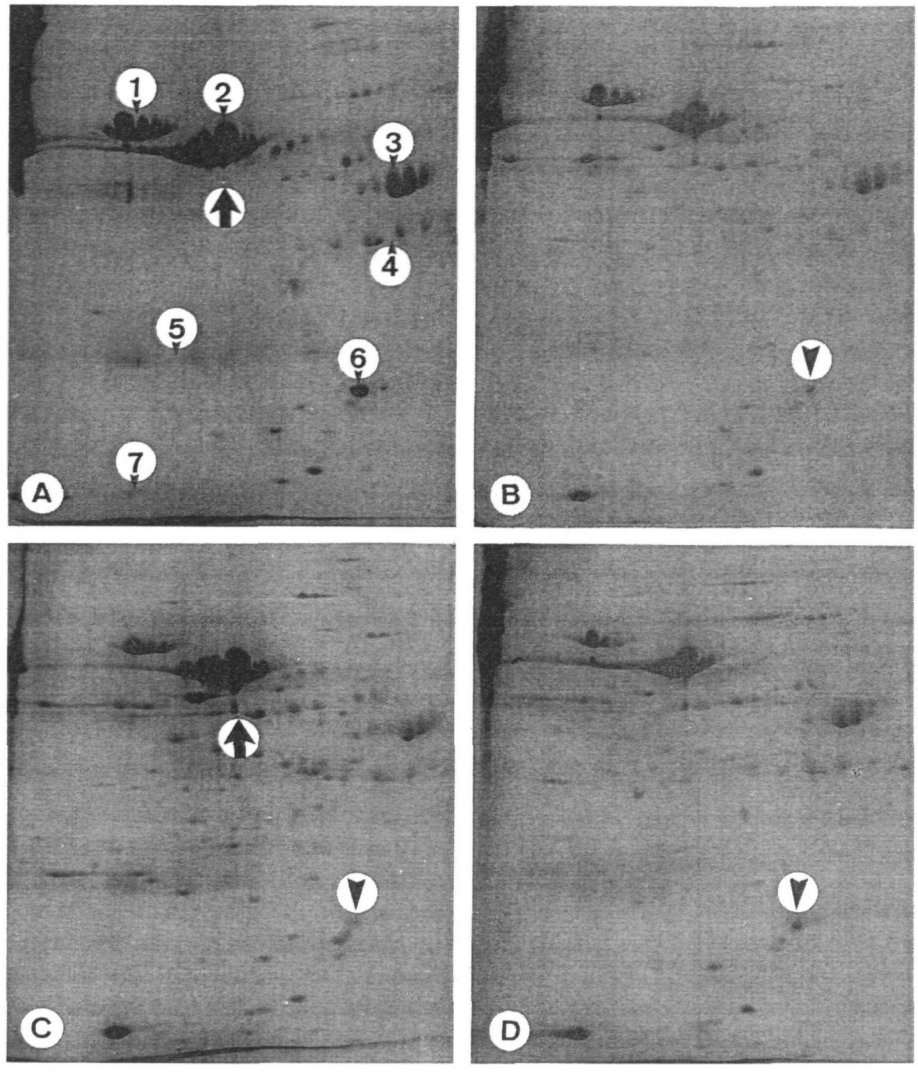

Figure 2. Coomassie-stained two-dimensional gels of proteins in follicular fluid obtained from four mature follicles of patient no. 5 listed in Table I. (**A**) and (**B**) with oocytes, (**C**) and (**D**) without oocytes detected. Spots 1–7 label the position of the seven proteins identified. Note that the overall patterns were quite similar when compared to each other, although minor differences were observed. For example, in region labelled with arrow in (**A**) and (**B**), spot 6 was less strongly expressed in (**B**) compared with (**D**), and spot 7 was present at low intensity in (**A**).

pregnancy potential of oocytes (Andersen, 1991, 1993). In addition, the concentration of prolactin together with steroid hormones in follicular fluid seems to be crucial for obtaining mature stage-II oocytes. These findings emphasize that differences in the hormonal milieu surrounding oocytes may have profound effects on the success of IVF (Lee *et al.*, 1987). Fukuda *et al.* (1995) have found that the endocrine health status, i.e. concentrations of oestradiol, progesterone, testosterone and androstendione, of the follicle is associated with its morphological characteristics observed during ultrasound examination. It is suggested that concentrations of specific proteins in follicular fluid may reflect the physiological condition of the follicle (Nayudu *et al.*, 1983, 1989). Permeability of follicular epithelium to plasma proteins depends on the relative molecular mass of plasma proteins and developmental state of the follicle. As the follicle matures it becomes gradually more permeable to plasma proteins. The number and amount of those proteins transported via the blood–follicle barrier into the follicular fluid increases steadily during folliculogenesis. In this respect, protease inhibitor concentrations increase in developing follicles to prevent unrestrained proteolysis within the follicle, and as a consequence of this, ovulation may be compared to an inflammatory response (Espey, 1980). In addition, other proteins are secreted by human granulosa cells and theca cells into the follicular fluid and play an important role in the regulation of follicular maturation and ovulation (Lobb and Dorrington, 1987; Nandecar *et al.*, 1992). Growth factors such as IGF-1 are considered to promote follicular maturation and differentiation of granulosa cells. They potentiate the steroidogenic effects of FSH in these cells and are responsible for the accumulation of progesterone and oestradiol as well as the biosynthesis of inhibin and enhance granulosa cell proliferation. In the theca cells, IGF-1 enhances LH-stimulated androgen synthesis. Growth factors in conjunction with growth hormone are responsible for a balanced molecular interaction during ovarian response to FSH and LH stimulation (Lunenfeld *et al.*, 1991). The IGF-1 binding protein modulates the action of this growth factor by competing for IGF-1 binding

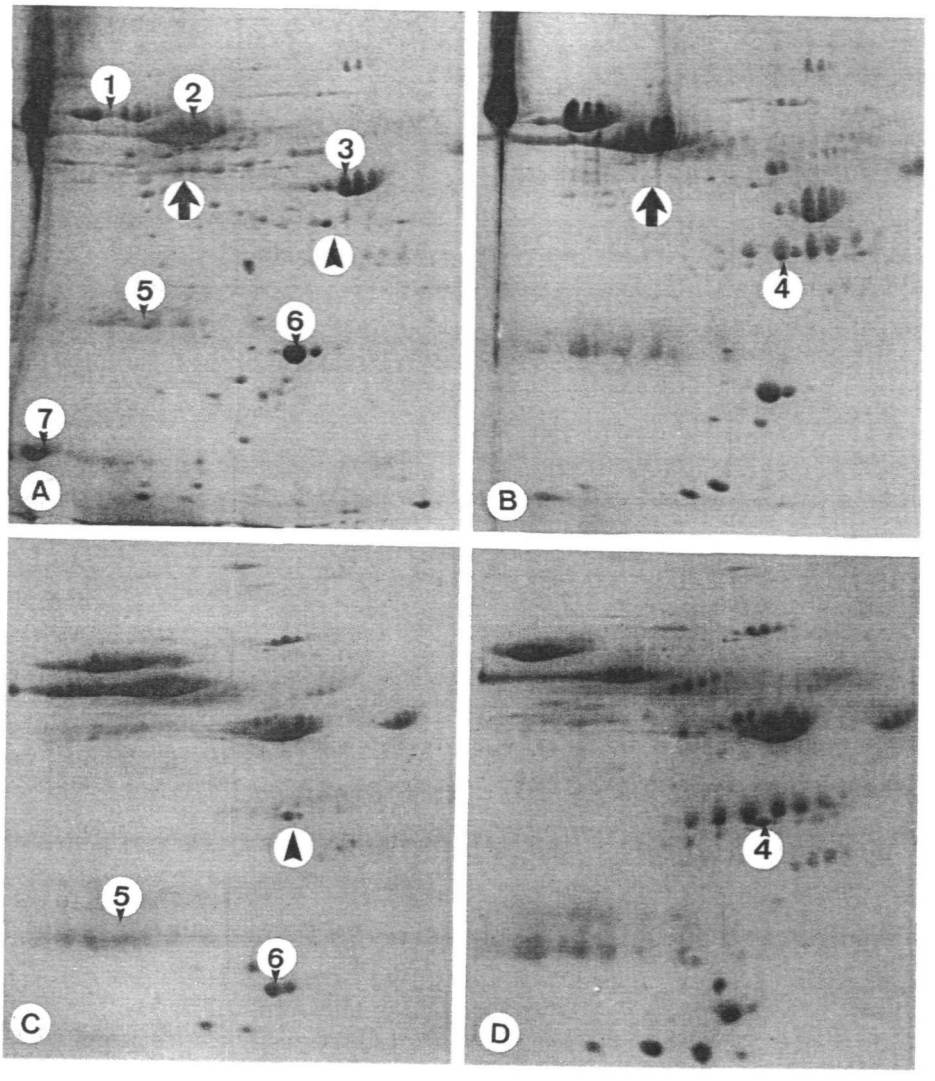

Figure 3. Coomassie-stained two-dimensional gels of proteins in follicular fluid obtained from mature follicles of patients nos 2 (**A**), 7 (**B**), 8 (**C**) and 10 (**D**). (**A**) to (**C**) with oocytes, (**D**) without an oocyte detected. The position of the identified proteins 1–7 is labelled. Note that among the four patients analysed, the overall protein patterns were comparable to each other. Some minor differences between protein profiles of the different patients were detectable. For example, see area indicated by arrow in (**A**) and (**B**) or spot 4 which is well expressed in (**B**) and (**D**) when compared to (**A**) and (**C**). In (**C**) and (**D**), a slightly longer time for two-dimensional electrophoresis was used in an attempt to obtain a higher resolution of protein separation. As a consequence of this, proteins with low molecular weight were no longer present on these gels. Compare the positions of spots 5 and 6 in (**A**) with (**C**).

sites (Suikkari *et al.*, 1991). Sarvas *et al.* (1994) have reported that a positive correlation exists between the amount of IGF-1 binding proteins and the follicular size and therefore can be used as another criteria for the maturity of follicles. The production of IGF-1 binding protein in granulosa–luteal cells is stimulated by EGF, a 6 kDa peptide, which is most likely derived from serum and appears in follicular fluid by passing the blood–follicle barrier. This growth factor, that also stimulates progesterone production and participates in regulating FSH-dependent processes, serves as another important molecular marker for follicular development (Terranova, 1991).

Similarly, transforming growth factors such as TGF-α and -β, a homodimer of 25 kDa, are multifunctional peptides that are synthesized by a variety of different somatic cells but also produced in the ovary by granulosa and theca cells. They are found in follicular fluid and modulate steroidogenesis, granulosa mitosis, mucification of cumulus cells and oocyte maturation (Chegini and Williams, 1992). Immunoreactive TGF and its specific messenger RNA increase with follicle size and have been useful for determining follicular growth (Mulheron *et al.*, 1992). On the other hand, abnormal amounts of certain growth factors have been associated with the polycystic ovarian syndrome (PCOS) and used to clinically characterize and medically treat it (Insler and Lunenfeld, 1991).

Concentrations of sex steroids and FSH and LH in follicular fluid have been employed as hormonal markers for normal or abnormal follicular maturity (Hillier, 1994). Various cytokines detected in follicular fluid have been shown to be extremely useful biochemical markers, because of their involvement in ovarian function and regulation of steroidogenesis (reviewed by Ben-Rafael and Orvieto, 1992). Bioactive and immunoactive interleukin-1, -2, and -6 have been detected at high concentrations in human follicles and associated with follicular maturity (reviewed by Giudice *et al.*, 1993). Inhibin and activin have

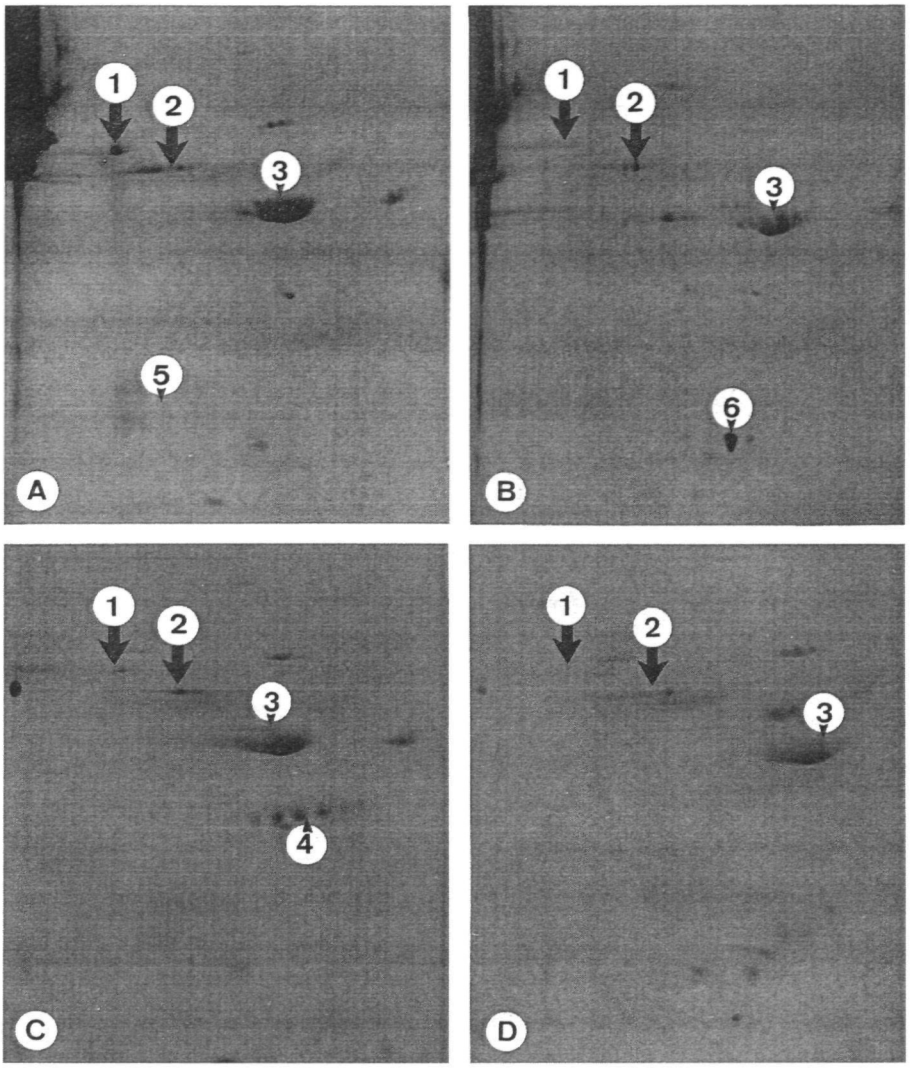

Figure 4. Coomassie-stained two-dimensional gels of proteins in follicular fluid obtained from immature follicles of patients nos 1 (**A**), 6 (**B**) and 9 (**C**) and (**D**). There were significantly fewer proteins on these gels when compared to those patterns originating from mature follicles (Figures 1–3). In particular, spots 1 and 2 were either hardly detectable or almost absent. This holds true, to some extent, also for spots 4–6. Spot 4 was well expressed only in (**C**), spot 6 only in (**B**). Spot 7 could not be detected, whereas spot 3 was clearly present in all four samples.

been added to the list of proteins that are found in follicular fluid and related to follicle growth. Small follicles produce only activin whereas mature follicles produce both activin and inhibin. These proteins are synthesized by granulosa cells, secreted into the follicular fluid and act antagonistically, i.e. activin inhibits and inhibin augments LH-stimulated androgen production (Hillier and Miro, 1993).

Gulamali-Majid *et al.* (1987) estimated protein concentrations in follicular fluid, and for their study selected six specific and already known proteins (α_1-antitrypsin, α_2-macroglobulin, antithrombin III, fibrinogen, plasminogen, and ceruloplasmin) as potential indicators of oocyte maturity. The concentrations of all six proteins were significantly higher in the fluid of mature follicles as were the follicle fluid:plasma ratios for α_1-antitrypsin, antithrombin III and ceruloplasmin. The authors assumed that these non-steroidal markers could aid in oocyte selection and in the timing of insemination for IVF.

Gonzales *et al.* (1992) found significantly higher concentrations of six proteins (C3 complement fraction, ceruloplasmin, α_1-antitrypsin, transferrin, α_2-macroglobulin, and β_2-microglobulin) in follicular fluids from which oocytes were taken that cleaved after IVF in comparison with those from which oocytes were taken that did not cleave or fertilize. These differences in protein concentrations can be used as biochemical markers of IVF outcome and demonstrate the importance of particular proteins for follicular maturation.

In our study, presented here, we have applied two-dimensional electrophoresis to investigate proteins derived from mature and immature follicles of different patients in order to gain further insight into the complex changes in protein synthesis that occur during follicular development. To our knowledge, this is the first study on the protein composition of follicular fluid using two-dimensional electrophoresis. The great advantage of this method over others lies in the simultaneous detection of many proteins that can be separated from each other by molecular weight and electrical charge. In addition, proteins of interest can be directly

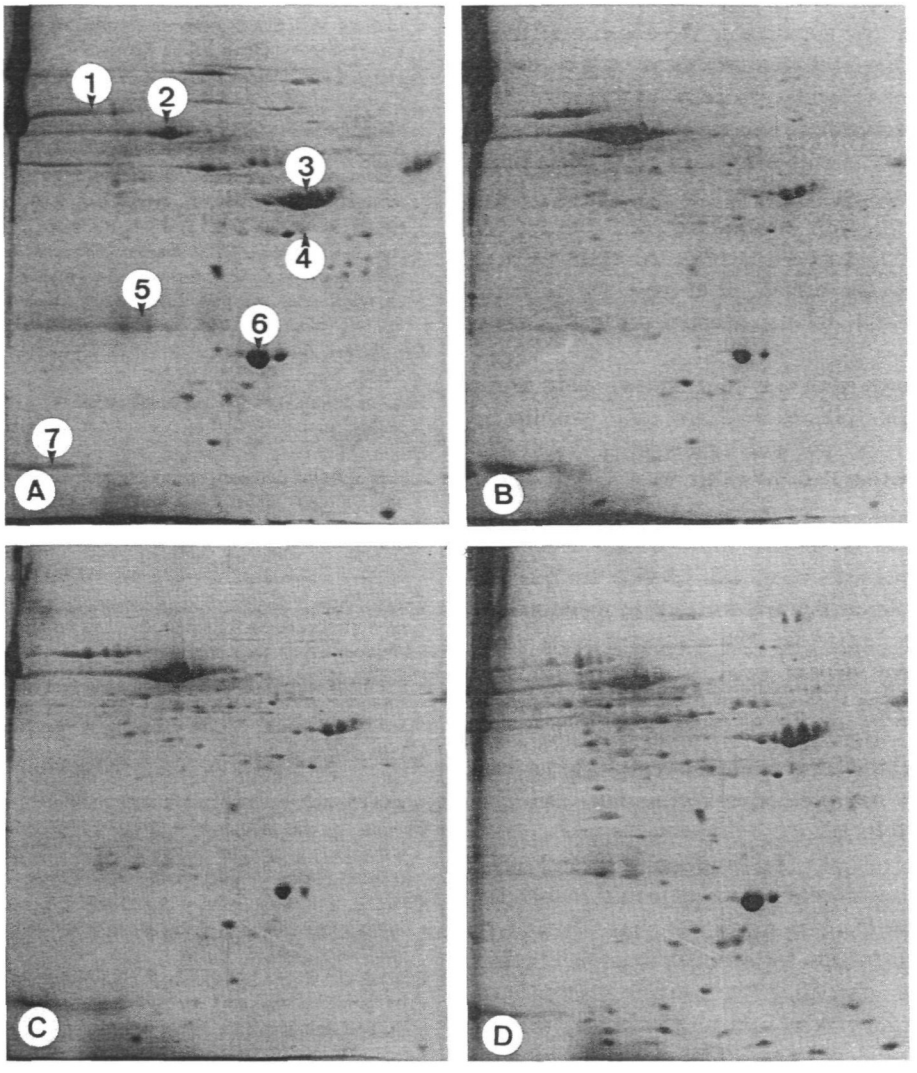

Figure 5. Coomassie-stained two-dimensional gels of proteins in follicular fluid obtained from four follicles of patient no. 4. They were considered to be 'mature' according to their size during ultrasonographic observation. (**A**) to (**C**) with oocytes, (**D**) without an oocyte. The overall pattern of protein intensity reflects an intermediate state between a truly mature (Figures 1–3) and immature (Figure 4) protein pattern. Note the reduced expression of spots 1 and 2, but spots 4, 5, and 7 were also less pronounced. Spots 3 and 6 were expressed quite normally.

isolated by cutting the protein spot in question out of the gel and subjecting it to partial amino acid sequencing procedure for molecular identification.

Nevertheless, it should be realized that only a part of the whole range of proteins originating from follicular fluid is visualized by this technique. By comparing many two-dimensional gels with each other, we have focused our attention on seven proteins that appear in a characteristic pattern on the gels and show distinct and reproducible differences between samples derived from mature and immature follicles. These proteins have been identified via internal amino acid sequence analysis and with the aid of a protein database. Spot no. 1 protein, identified as transferrin, is a glycoprotein with two repetitive domains required for iron transport and metal binding. This globin may also have a further role in stimulating cell proliferation (Uzan *et al.*, 1984). Spot no. 2 protein, identified as albumin, is a globular and unglycosylated serum protein and shows exceptional binding capacities for a variety of bioactive molecules, in particular for free fatty acids (Minghetti *et al.*, 1986). Spot no. 3 protein is found to be α_1-antitrypsin, a single polypeptide chain, that is one of several protease inhibitors and involved primarily in the inhibition of lysosomal proteases (Long *et al.*, 1984). Spot no. 4 protein is haptoglobulin 1, a glycoprotein with tetrachain structure that is involved in molecular transport mechanisms (Brune *et al.*, 1984). Spot no. 5 protein was identified as Ig-κ chain C region that is encoded in the human germline constant region gene and is functionally part of the immune system (Hieter *et al.*, 1980). Spot no. 6 protein was identified as apolipoprotein A-1, a polypeptide that interacts with specific cellular receptors and many of the enzymes involved in the regulation of lipid metabolism (Sharpe *et al.*, 1984). Spot no. 7 protein is found to be a subunit or fraction of serum albumin due to its low molecular weight estimated from the position on the two-dimensional gels. However, we do not know whether this smaller molecule results from artificial fragmentation or is a processed form of the entire serum albumin.

Our findings, that high amounts of these proteins are related

to mature follicles and low amounts or the absence of them related to immature follicular fluid, make these proteins useful biochemical markers for follicular maturity. In this respect, it is of great interest to observe more intermediate protein patterns obtained from patient no. 4 whose follicles were determined as mature by ultrasonographic examination. But infering from our protein investigation, they do not represent the typical pattern derived from mature follicles. We therefore assume that this may have been the reason, or at least one of them, why this particular patient did not become pregnant during this IVF cycle.

We have clearly shown with our comparative study using mature versus immature follicles that not only meaningful conclusions but also important new information about the complex protein composition of follicular fluid have resulted from this study. Moreover, the molecular identification of several proteins, present in mature follicles and absent or weakly expressed in immature ones, will provide the basis of a clinically applicable screening test for IVF programmes to improve IVF outcome further. In this context it is of great interest that one patient in our study (see Figure 5) was considered to have mature follicles estimated by size during ultrasonographic observation. However, when follicular fluid was analysed for its protein composition, we found that this pattern did not clearly represent nor correspond with that expected from mature follicles.

On the two-dimensional gels, we have found several other as yet unidentified proteins which are present in mature follicles and absent or at a low level in immature ones. They may emerge as potential candidates for specific functions during folliculogenesis. On the other hand, the consistent absence of a particular protein or proteins in follicular fluid may lead to the analysis of genetic defects linked to inferior maturation of follicles and oocytes. Follicle maturity is an important factor and an absolutely necessary prerequisite for the optimal physiological state of the oocyte utilized for IVF. To rely on specific follicular proteins as criteria for follicular maturation is of utmost practical importance for evaluating those follicles from which oocytes have been aspirated for IVF. Subsequent application of specific protein tests to screen follicular fluid should contribute to an improvement in IVF outcome.

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