Analysis by two-dimensional electrophoresis of proteins secreted by sheep ovarian follicles: effects of the *Fec^B* gene, follicle size and atresia

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Proteins secreted by sheep ovarian follicles at different stages of maturation (small healthy, large healthy or large atretic) and originating from ewes with different ovulation rates (homozygous carriers or noncarriers of the *Fec^B* gene) were analysed by two-dimensional electrophoresis (PAGE) followed by computerized image analysis. Follicles were incubated intact for 1 h to assess steroidogenesis, and then incubated for 24 h in the presence of [³⁵S]methionine. Secreted proteins were then resolved by iso-electric focusing followed by migration on 10% acrylamide slab gels and fluorography. Incorporation of label into proteins was similar irrespective of genetic type or health status of the follicles (4–6%). Small follicles incorporated significantly less ($P < 0.05$) label. Over 100 spots were detected on the fluorographs. The presence of the *Fec^B* gene induced qualitative and quantitative changes in the follicular protein patterns. Frequency of detection of spots 116 and 129 was significantly higher ($P < 0.01$) in *Fec⁺Fec⁺* compared with *Fec^BFec^B* follicles (80% versus 8%). In addition, amounts of proteins present in spots 2 and 12 were increased in *Fec^BFec^B* follicles, while those in spots 9 and 21 were decreased. Size and atresia affected protein patterns only quantitatively. Increased amounts of proteins in spots 1, 10, 38, 44 and 113 were associated with atresia ($P < 0.05$). Follicle enlargement was associated with increased ($P < 0.05$) amounts of proteins in spots 5 and 6 and decreased amounts of proteins in spot 16. Amounts of three proteins (33, 40 and 58) were related positively to oestradiol production *in vitro* before labelling.

Introduction

Follicles have two possible fates: growth towards ovulation or atresia. Large growing follicles display increased steroidogenesis (Tsonis *et al.*, 1984; Monniaux, 1987) and inhibin production (Tsonis *et al.*, 1983; Campbell *et al.*, 1991) together with limited granulosa cell proliferation (Turnbull *et al.*, 1977; Driancourt *et al.*, 1985). In atretic follicles, while inhibin production is maintained (Tsonis *et al.*, 1983; Campbell *et al.*, 1991), the ability to produce steroids is reduced (Tsonis *et al.*, 1984; Monniaux, 1987; Webb *et al.*, 1989), and the pattern of insulin-like growth factor (IGF) binding proteins (Monget *et al.*, 1983) and the amounts of glycosaminoglycans (Ax and Bellin, 1988) are altered. With the exception of these proteins (inhibin, IGF-binding proteins and glycosaminoglycans), very little is known about proteins that could be markers of follicular maturation or atresia. Information using one-dimensional (1D) PAGE is available to compare the ovarian protein patterns in large versus small follicles in cattle (Savion and Gospodarowicz, 1980; Skinner and Osteen, 1988). The effects of atresia on protein patterns are still unknown. Only 10–15 protein bands

are usually visualized on samples of ovarian proteins after 1D PAGE (Moor and Crosby, 1987). Each band may be generated by one, several or a large number of proteins. Two-dimensional (2D) electrophoresis is more accurate since each spot is a single protein. Hence the first aim of this study was to characterize the 2D pattern of proteins secreted by large versus small follicles, as well as from large healthy versus large atretic follicles.

Mature follicles ovulate after the LH surge. Ovulation rate in sheep ranges from 1 to > 10. Amongst the most prolific breeds is the Booroola, for which a major gene controlling ovulation rate has been identified (Davis *et al.*, 1982). Despite extensive research (for review see Montgomery *et al.*, 1992), the way the *Fec^B* gene acts to alter ovulation rate has not been fully clarified. Higher FSH support to follicles has been reported (McNatty *et al.*, 1987). However, there is also evidence that gonadotrophin-independent (that is, intraovarian) mechanisms are involved (Fry *et al.*, 1988; McNatty *et al.*, 1990). As 2D electrophoresis followed by image analysis is a powerful tool to study line differences and mutation effects (Aquadro and Avise, 1981; Bossis and Mugniery, 1993; Gormon and Phan Thanh, 1995), the second aim of this study was to compare the pattern of secreted proteins by follicles from homozygous *Fec^BFec^B* and *Fec⁺Fec⁺* ewes.

Materials and Methods

Materials

Culture medium, minimum essential medium (MEM) and methionine-free MEM was obtained from Sigma (St Quentin Fallavier). [^{35} S]methionine was purchased from NEN (Les Ulis); acrylamide, bisacrylamide and urea were obtained from BioRad (Ivry-sur-Seine). Ampholines were acquired from BDH (Poole, Dorset).

Animals

Experimental animals were generated by crossbreeding heterozygous $\text{Fec}^{\text{B}}\text{Fec}^+$ animals. The two rams used were born as crosses between $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$ rams obtained from New Zealand and Romanov ewes. Spermatozoa of these rams were used to fertilize two superovulated Australian Merino $\times$ Merinos d'Arles ewes. Embryos were collected and transferred to recipients. Twenty-three female ewe lambs were born from this superovulation-embryo transfer attempt.

The progeny of each ewe was submitted to repeated laparoscopies ($n = 3-4$) to assign it to a specific genotype using procedures described by Elsen and Le Roy (1991). Mean ovulation rates of the $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$ and $\text{Fec}^+ \text{Fec}^+$ were 5.1 ± 0.4 and 1.5 ± 0.2 , respectively ($n = 6$ per genotype). The oestrous cycles of these 12 ewes were synchronized by the intravaginal insertion of progestagen impregnated sponges (Chronogest, Intervet, Angers) for 14 days. At 30 h after sponge removal, the ewes were hemiovariectomized under general anaesthesia, induced by barbiturates (Nesdonal: Rhône-Mérieux, Lyon) (0.6 g i.v.) and maintained by halothane, to remove the ovary with the largest ($\text{Fec}^+ \text{Fec}^+$) or with the most numerous ($\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$) large follicles.

Follicle culture

All large follicles of each ewe were dissected from the ovaries. As size of the ovulatory follicles is markedly smaller in $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$ than in $\text{Fec}^+ \text{Fec}^+$ ewes (3 versus 7 mm), small $\text{Fec}^+ \text{Fec}^+$ follicles with a size similar to the largest $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$ follicles ($n = 10$) were also dissected. All intact follicles after dissection were transferred to 24-well plates filled with 1 ml MEM (Sigma, La Verpillière) incubated at 38°C under an oxygen: CO_2 (95%:5%) atmosphere. Incubation was for 1 h. Such an incubation was used to define healthy (producing over $500 \text{ pg ml}^{-1} \text{ h}^{-1}$) versus atretic (producing less than this threshold amount) large follicles as described and validated by Webb and Gauld (1985) and Webb *et al.* (1989). At the end of this 1 h incubation, the incubation media were stored at -20°C before assay for oestradiol and testosterone. All follicles were then transferred to new 24-well plates containing 1 ml MEM supplemented with 1% (w/v) polyvinylalcohol (Sigma) and devoid of methionine. [^{35} S]methionine (NEN) (50 μCi per well) was then added and follicles were then incubated for 24 h in the same conditions (38°C , 95% oxygen) as before.

At the end of culture, media were collected and processed for electrophoresis (see below). All small follicles were fixed

and processed for histological examination to detect atresia. A follicle was defined as atretic when it contained more than five pycnotic bodies in the section studied (Driancourt *et al.*, 1985). This histological approach was used because small healthy follicles cannot be discriminated from small atretic follicles by steroid production.

Electrophoresis

At medium collection, protease inhibitors (phenyl methyl sulfonyl fluoride (PMSF) to 1 mmol l^{-1} final concentration, benzamide to 5 mmol l^{-1} final concentration) were added and then the media were stored at -20°C until processed.

The secretion of synthesized polypeptides into culture medium was determined by measuring the incorporation of [^{35} S]methionine into non-dialysable macromolecules. Culture medium was dialysed extensively against deionized water using dialysis tubing with a molecular mass exclusion limit of 6–8 kDa. Radioactivity in the retentate was determined by scintillation spectrometry.

Polyacrylamide gel electrophoresis (PAGE) in the presence of SDS was performed on equal counts of culture medium (200 000 d.p.m.) to enable determination of quantitative and qualitative differences between follicle types. The procedures used were as described by Roberts *et al.* (1984).

Samples were dissolved in $1 \text{ mol urea l}^{-1}$, 2% (v/v) Nonidet P40 (NP40) and 0.5% (w/v) dithiothreitol. Proteins were resolved in the first dimension by isoelectric focusing in 4% (w/v) polyacrylamide tube gels containing 250 *N-N'*-diallyltartramide (DATD), $8.0 \text{ mol urea l}^{-1}$, 2% (v/v) NP40 and 5.1% (v/v) ampholytes (pH 3–10). This type of wide range ampholytes was selected to visualize as many proteins as possible using isoelectric focusing (IEF) as the first dimension separation. Tube gels were equilibrated in $50 \text{ mmol Tris-HCl l}^{-1}$, pH 6.8, containing 1% (w/v) SDS and 1% (v/v) β -mercaptoethanol before being subjected to electrophoresis in the second dimension on 10% (w/v) polyacrylamide gels in the presence of 0.5% (w/v) SDS. This acrylamide concentration permitted visualization of proteins between 110 and 20 kDa. Although gradient gels offer the separation of a wider range of proteins, they were not used in the present study because perfect repeatability (a key issue for image analysis) is difficult to achieve.

Slab gels were stained with Coomassie Blue R 250, destained in acetic acid, ethanol and water, soaked in water, equilibrated with $1 \text{ mol sodium salicylate l}^{-1}$ (30 min) and dried. Fluorographs were prepared with Kodak XAR X-ray film, and exposed for 3 weeks at -70°C . These conditions proved suitable to visualize small spots while not saturating the film with large spots.

Image analysis

The fluorographs of the 2D electrophoresis were subjected to qualitative and quantitative analysis using the Kepler Software (Large Scale Biology, Rockville, TN). Gels were digitized via a charge-coupled device camera (Kodak, Eikonix 1412) serving as scanner; care was taken that saturation was not reached for any of the spots. Scanned gels were then processed

for computerized analysis by a Digital workstation 3100. Spots were modelled as Gaussian least squares, so that features of each pattern (position, intensity) were reduced in a spot file. In an initial step, vertical streaks were removed. Matching was then achieved as follows. A general master pattern was obtained by comigration of all the samples studied. The investigator defined nine key points distributed evenly throughout the gels in the master and in each gel. Kepler used these key points to correct for distortion between gels. Automatic matching followed by investigator hand matching (for those spots that were not matched automatically) were then realized. In the final step, minor differences in overall load (as defined by the sum of the darkness of all spots) between gels were corrected automatically to produce accurate measurements of spot intensity (blackening density) in all gels.

Effects of blocking glycosylation or of using nonreducing conditions on protein patterns

Earlier work has demonstrated that most of the ovarian proteins were heavily glycosylated (Moor and Crosby, 1987; Smith and Moor, 1991). Whether proteins of interest identified after image analysis were indeed glycosylated was tested by incubating large intact follicles from three Fec^+Fec^+ ewes with $0.5 \mu\text{g}$ tunicamycin ml^{-1} (Sigma) (Smith and Moor, 1991), a compound that inhibits synthesis of *N*-glycosidically linked oligosaccharides. Two-dimensional fluorographs of tunicamycin-treated incubation medium (a pool of the three follicles) were then compared with those of the similar untreated (Fec^+Fec^+) samples.

A pool of all Fec^+Fec^+ large follicle samples was separated using nonreducing conditions (that is, by omitting dithiothreitol in the first dimension and β -mercaptoethanol in the second dimension), to check whether the proteins identified in the reducing conditions on the 2D fluorographs were subunits of larger molecules. This is known to alter markedly the protein pattern (Moor and Crosby, 1987) of ovarian proteins.

Steroid assays

Oestradiol and testosterone concentrations in culture media were quantified by radioimmunoassay (Terqui, 1978; Hochereau-de Reviers *et al.*, 1990). The intra-assay coefficients of variation were 7.7% and 11.5% for oestradiol and testosterone, respectively. The minimum detectable values for oestradiol and testosterone were 20 pg ml^{-1} and 0.2 ng ml^{-1} .

Statistical analysis

Each sample generated a fluorograph. For each spot, data such as frequency within a group, or darkness intensity in each individual sample were generated by Kepler. Frequencies were compared by chi-squared test. Mean darkness intensities for specific groups (Booroola effect, size effect, atresia effect) were calculated (from the individual data of each follicle contributing to the group) and compared by one-way ANOVA. Results are expressed as means $\pm$ SEM.

Results

Ovarian and follicular features

Mean ovulation rates at the previous laparoscopies of each Fec^BFec^B ewe were 4.5 ± 0.28 , 4.5 ± 0.62 , 4.5 ± 0.5 , 7.0 ± 0.5 , 5.25 ± 0.48 and 4.75 ± 0.85 , while those of each of the six Fec^+Fec^+ ewes were 1.0 ± 0 , 1.25 ± 0.25 , 1.25 ± 0.25 , 1.33 ± 0.28 , 2.0 ± 0 and 2.25 ± 0.25 . Four to six large follicles were dissected from Fec^BFec^B ovaries. Only one to two large follicles were obtained from Fec^+Fec^+ ovaries. After incubation for 1 h to assess health and steroid production, 17 of 23 Fec^BFec^B and six of seven Fec^+Fec^+ large follicles were healthy, as evidenced by an oestradiol output in culture medium exceeding $500 \text{ pg ml}^{-1} \text{ h}^{-1}$ (Webb *et al.*, 1989). Their respective sizes were 3.3 ± 0.5 and $5.5 \pm 0.7 \text{ mm}$ (Fec^BFec^B versus Fec^+Fec^+ ; $P < 0.01$). Oestradiol output of Fec^BFec^B follicles was marginally ($P < 0.1$) reduced ($3.81 \pm 2.44 \text{ ng ml}^{-1} \text{ h}^{-1}$) compared with Fec^+Fec^+ follicles ($4.8 \pm 1.1 \text{ ng ml}^{-1} \text{ h}^{-1}$). In contrast testosterone output by follicles of the two lines was similar (Fec^BFec^B , $3.31 \pm 3.24 \text{ ng ml}^{-1} \text{ h}^{-1}$) versus Fec^+Fec^+ , $3.6 \pm 2.69 \text{ ng ml}^{-1} \text{ h}^{-1}$). Atretic follicles of Fec^BFec^B ewes ($n = 6$) produced limited amounts of testosterone ($0.94 \pm 1.84 \text{ ng ml}^{-1} \text{ h}^{-1}$, $P < 0.02$ versus healthy Fec^BFec^B follicles) but their oestradiol output was low ($0.2 \pm 0 \text{ ng ml}^{-1} \text{ h}^{-1}$, $n = 2$) or undetectable ($n = 4$). Of the ten small (2.5 to 3.5 mm) follicles dissected from Fec^+Fec^+ ewes, four were healthy at histological examination.

Incorporation of radioactivity in newly synthesized proteins

No significant difference in the amounts of label incorporated by healthy Fec^BFec^B follicles ($6.2 \pm 0.6\%$) and Fec^+Fec^+ follicles ($4.9 \pm 0.4\%$) could be detected. In addition, healthy Fec^BFec^B ($n = 17$) and atretic Fec^BFec^B ($n = 6$) follicles displayed similar rates of incorporation ($6.2 \pm 0.6\%$ and $5.7 \pm 1\%$, respectively). In contrast, small Fec^+Fec^+ follicles incorporated markedly less radioactivity ($3.4 \pm 0.4\%$) than did large healthy Fec^+Fec^+ follicles ($4.9 \pm 0.4\%$) ($P < 0.05$). Healthy or atretic small follicles incorporated similar amounts of radioactivity (healthy $3.6 \pm 0.4\%$, $n = 4$; atretic $3.3 \pm 0.2\%$, $n = 6$).

Two-dimensional electrophoresis patterns

Comparison between a representative Coomassie blue stained gel (Fig. 1a) (master sample) and fluorographs from Fec^BFec^B and Fec^+Fec^+ (Fig. 1b and c, respectively) demonstrated that none of the ovarian proteins is present in amounts sufficient to be stained with this procedure. The master pattern after computerized matching analysis is also shown (Fig. 1d). Over 100 spots were detected in the master gel. Major ones were at the zone around 45 kDa (three spots pI 6.6) and 30 kDa (pI ranging from 6.8 to 7.6) (four–six spots). These spots at 45 and 30 kDa , together with 16 minor spots were present consistently in all the samples studied.

Genotypic differences in ovarian protein patterns

This comparison involved six Fec^+Fec^+ follicles and 13 Fec^BFec^B follicles, as four gels of Fec^BFec^B samples were not

Fig. 1. Representative two-dimensional patterns (after isoelectric focusing and migration on a 10% acrylamide slab gel) of a Coomassie blue stained sample of (a) secreted follicular proteins; 200 000 d.p.m. sample of proteins secreted by (b) a $Fec^B Fec^B$, or (c) a $Fec^+ Fec^+$ follicle and (d) of the image analysis map (master sample) presenting all spots detected at least once in a sample. Molecular mass markers are indicated on the left side of (a). Acidic and basic sides are shown at the top of the gel.

suitable for image analysis and were discarded from further study. Qualitative differences between genotypes were detected for two spots (116 and 129). Both spots are present with a significantly higher frequency ($P < 0.01$) (5/6 $Fec^+ Fec^+$ versus 1/13 $Fec^B Fec^B$ for both spots) in $Fec^+ Fec^+$ follicles. Furthermore, in the two $Fec^B Fec^B$ samples where these spots were detectable, their amount was always lower than in the $Fec^+ Fec^+$ follicles (spot 116 $Fec^B Fec^B$, 27 561 versus $Fec^+ Fec^+$, 53 086 $\pm$ 7180 volume units; spot 129, $Fec^B Fec^B$ 6189 versus $Fec^+ Fec^+$, 38 562 $\pm$ 8733).

Respective molecular masses and pIs were 36 kDa, pI = 6.4 (spot 116) and 96 kDa pI = 7.5 (spot 129) (Fig. 3). Quantitative differences between genotypes were also detected for four spots (Fig. 2). Amounts of proteins in spot 2 ($Fec^B Fec^B$, 421 561 $\pm$ 41 300 versus $Fec^+ Fec^+$, 256 300 $\pm$ 4700; $P < 0.05$) and in spot 12 ($Fec^B Fec^B$, 235 000 $\pm$ 39 800 versus $Fec^+ Fec^+$, 84 800 $\pm$ 17 500, $P < 0.05$) were increased in follicles from ewes carrying the Fec^B gene. In contrast, the converse was true for spot 9 ($Fec^B Fec^B$, 98 190 $\pm$ 9676 versus $Fec^+ Fec^+$, 172 290 $\pm$ 18 300; $P < 0.05$) and for

spot 21 ($Fec^B Fec^B$, 187 600 $\pm$ 50 300 versus $Fec^+ Fec^+$, 347 500 $\pm$ 38 200; $P < 0.05$). As the amounts of label incorporated per follicle were available, the actual production per follicle of proteins in specific spots could be calculated (concentration of the spot in the 200 000 d.p.m. sample $\times$ rate of incorporation). When this was achieved, the above findings were confirmed (spots 2 and 12: $Fec^B Fec^B > Fec^+ Fec^+$, $P < 0.05$; spots 9 and 21, $Fec^+ Fec^+ > Fec^B Fec^B$, $P < 0.05$).

Effects of follicle size on ovarian protein patterns

This comparison was undertaken using six large (≥ 5 mm) healthy $Fec^+ Fec^+$ follicles and four small (2.5 to 3.5 mm) healthy $Fec^+ Fec^+$ follicles.

No qualitative difference between the protein patterns of these samples could be detected. Quantitative differences were however found for three spots. Amount of protein in spot 16 was increased in small follicles ($P < 0.05$) (660 600 $\pm$ 90 300 versus 414 500 $\pm$ 57 000 for small and large follicles,

Fig. 2. Size effects of the pattern of proteins secreted by sheep follicles. Significant differences ($P < 0.05$) associated with small or large size are indicated by (●) and (⊙), respectively. Molecular mass markers are shown on the left of the map. Acidic and basic sides are indicated at the top of the gel.

respectively) (Fig. 3). In contrast, amounts of proteins in spots 5 and 6 were greater in large than in small follicles ($P < 0.02$ and $P < 0.05$, respectively) (spot 5: large $184\,300 \pm 31\,600$ versus small $47\,040 \pm 1020$; spot 6: large $19\,300 \pm 29\,000$ versus small $107\,600 \pm 11\,500$) (Fig. 3).

Follicle production (concentration $\times$ rate of incorporation) of spot 5 was also increased ($P < 0.05$) in large follicles.

Effects of atresia on ovarian protein patterns

The 13 healthy and six atretic $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$ follicles were used in this comparison. Atresia did not induce appearance or disappearance of specific proteins. In contrast, atresia increased the amounts of proteins measured in five spots (Fig. 4) (spots 1, 10, 38, 44 and 113) (Table 1). Furthermore, follicle production (concentration $\times$ rate of incorporation) of spot 38 (but not others) was marginally increased ($P < 0.07$) in atretic follicles.

Correlations between steroidogenesis and protein patterns in $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$ follicles

As information on *in vitro* steroidogenesis before labelling and the amounts of proteins in all detected spots were available

for the 13 healthy $\text{Fec}^{\text{B}}\text{Fec}^{\text{B}}$ follicles, correlations between these parameters were calculated. Positive correlations between oestradiol concentrations in incubation medium and specific spots were detected for spot 33 ($r = 0.65$, $P < 0.02$) spot 40 ($r = 0.61$, $P < 0.05$) and spot 58 ($r = 0.57$, $P < 0.05$). Testosterone concentration in medium was positively correlated with the amount of proteins in spot 126 ($r = 0.76$, $P < 0.01$) and negatively correlated with the amount of proteins in spot 22 ($r = -0.60$, $P < 0.05$).

Effects of tunicamycin and non-reducing conditions on protein patterns

Fifty-four spots were present on the fluorograph of the sample separated under nonreducing conditions. On the three samples, the glycosylation of which was prevented by tunicamycin administration, 57–75 spots were detected. Specific spots unaffected by blocking glycosylation or running the gels under nonreducing conditions compared with the pattern of ovarian proteins observed in $\text{Fec}^{\text{+}}\text{Fec}^{\text{+}}$ samples submitted to reducing conditions are shown (Fig. 4a and b, respectively).

Fig. 3. Qualitative differences in the pattern of proteins between $Fec^B Fec^B$ and $Fec^+ Fec^+$ follicles (●). Quantitative differences ($P < 0.05$) associated with greater (⊗) or smaller (⊙) amounts of protein in $Fec^B Fec^B$ versus $Fec^+ Fec^+$ follicles. Molecular mass markers and charge separation are indicated on the left and at the top of the gel, respectively.

Discussion

The first aim of this study was to detect ovarian proteins related to the physiological status of the follicle (small healthy, large healthy or large atretic). Three proteins were associated with changes in follicle size and five proteins were increased when follicles entered atresia. The second aim was to identify proteins that could be used as markers for the Fec^B gene. Two proteins were shown to be common in $Fec^+ Fec^+$ follicles and were seldom encountered in $Fec^B Fec^B$ follicles. In addition, quantitative differences between genotypes were found for four other proteins.

This study was prompted by the finding that very few studies attempting to characterize ovarian protein patterns (Knecht *et al.*, 1986; Moor and Crosby, 1987; Skinner and Osteen 1988; Smith and Moor, 1991) have tried to relate these protein patterns to the physiological status of the follicle. Furthermore, all studies have used 1D PAGE, a technique which may not be accurate enough to detect differences. Furthermore, despite the common belief that the Fec^B gene may be expressed in the ovary of adult sheep (Fry *et al.*, 1989; McNatty *et al.*, 1990), no attempt has been made to characterize ovarian proteins of each genotype. The approach used in

the present study relied on the comparison by image analysis of proteins secreted into incubation medium by intact follicles cultured in the presence of [^{35} S]methionine for 24 h. The use of intact follicles respects the three-dimensional structure of the follicle and maintains the complex relationships between the oocyte-granulosa cells and thecal cells. Heterogeneity within the somatic cell layers is also retained (granulosa: Lahtenmaki *et al.*, 1982; theca: Meduri *et al.*, 1992). Intact follicles cultured for 24 h remain viable, as evidenced, for example, by maintained aromatase activity throughout culture (Driancourt, 1994). Finally, culture of intact follicles prevents the occurrence of the alterations in protein patterns associated with culture systems (Moor and Crosby, 1987) or cell density (Savion and Gospodarowicz, 1980). The migration conditions combined isoelectric focusing and a separation in the second dimension using a constant 10% acrylamide gel. To maximize the chances of picking up as many spots as possible, wide range ampholytes (3.5–10) were used. It is, however, agreed that using non-equilibrium pH gradient electrophoresis (NEPHGE) instead of isoelectric focusing may have helped to visualize proteins with pI values more basic than the working limits of these ampholytes. The 10% acrylamide concentration for the second dimension was found suitable because the basal

Fig. 4. Two-dimensional pattern of proteins secreted by sheep follicles, the migration of which was not altered after incubation of the follicles with tunicamycin (● on (a)) or after separation under nonreducing conditions (○ on (b)).

Table 1. Quantitative differences in the amounts of proteins secreted by sheep follicles in five specific spots for which significant differences were detected between healthy and atretic follicles (arbitrary units) (mean $\pm$ SEM)

Follicle	1	10	Spot number 38	44	113
Healthy Fec ^B Fec ^B (n = 13)	169 200 $\pm$ 9700 ^a	63 200 $\pm$ 7500 ^a	44 200 $\pm$ 3900 ^a	96 900 $\pm$ 15 000 ^a	60 900 $\pm$ 9800 ^a
Atretic Fec ^B Fec ^B (n = 6)	237 900 $\pm$ 31 600 ^b	99 300 $\pm$ 6000 ^b	102 200 $\pm$ 17 000 ^c	175 100 $\pm$ 25 700 ^c	115 800 $\pm$ 18 000 ^c

^a versus ^b, $P < 0.05$; ^a versus ^c, $P < 0.02$.

membrane acts as a molecular sieve preventing compounds with very large molecular masses from crossing it (Shalgi *et al.*, 1973), and because many of the small molecular mass proteins (< 10 kDa) were removed during the dialysis step used to prepare the samples. Reducing conditions were used, as these increase the number of proteins that enter the gel (100 versus 50 spots in reducing and nonreducing conditions, respectively).

Incorporation of label into ovarian proteins was usually within 3–5% of the label provided. No difference was noted between Fec^BFec^B and Fec⁺Fec⁺ follicles despite their smaller size and reduced granulosa cell complement (Driancourt *et al.*, 1985; McNatty *et al.*, 1986). This implies that each somatic (granulosa or theca) cell of the Fec^BFec^B follicle is more active in terms of protein production than those of the Fec⁺Fec⁺ follicles. The observation of high rates of incorporation in atretic follicles was unexpected. This demonstrates that atretic follicles are not inactive and may still play roles in intraovarian regulation. This finding is in agreement with data on inhibin production by such follicles (Tsonis *et al.*, 1984; Campbell *et al.*, 1991) but is harder to reconcile with the widespread cell degeneration encountered in such follicles.

A total of over 100 spots was detected on the experimental gels. This compares favourably with the 10–15 bands visualized on samples of ovarian proteins following 1D PAGE (Moor and Crosby, 1987; Skinner and Osteen, 1988; Smith and Moor, 1991). There was good agreement between these 1D studies and the present one using 2D PAGE for the molecular masses of the major proteins (45 and 30 kDa). The present study also confirmed that most ovarian proteins were glycosylated (Moor and Crosby, 1987; Smith and Moor, 1991), as evidenced by altered molecular masses after follicle incubation in the presence of tunicamycin.

Some minor spots could be related to physiological status or genetic origin of the follicles. Two spots (116 and 129) were present at significantly different frequencies (80% in Fec⁺Fec⁺ versus 8% in Fec^BFec^B) in the genotypes studied. As follicles from Fec^BFec^B ewes are markedly smaller than those of Fec⁺Fec⁺ ewes, it was of key importance to check whether these spots were affected by follicle size. The spots were not affected by follicle size, so it is likely that the presence/absence of these spots did not vary in an all or none process, according to the presence/absence of the gene. This precludes use of these spots as markers of the Fec^B gene. No other qualitative differences related to small size or initiation of atresia could be observed. However, the concentration of certain spots was quantitatively affected by genotype (spots 2, 9, 12 and 21),

follicle size (spots 5, 6 and 16) and atresia (spots 1, 10, 38, 44 and 113).

The next step in this study was to check whether one or several of the above spots could be related to follicle function. As ovarian proteins may control aromatase activity of granulosa cells (Di Zerega *et al.*, 1982), oestradiol production of each Fec^BFec^B follicle was correlated with the intensities of the spots of its pattern. While positive correlations were detected for three spots (33, 40 and 58), none of these had been previously identified as affected by size, atresia or genotype. The same was true for testosterone production. Hence, the seven spots affected by physiological status or the six spots affected by genotype may be causing or altering other features of follicular function (for example, granulosa cell proliferation, gonadotrophin binding, angiogenesis; for review see Tsafri, 1988).

Identification of the proteins of interest would undoubtedly help to analyse their physiological effects. However, the fact that none of the ovarian proteins stained with Coomassie blue on 2D gels of ovarian proteins showed that these proteins are present in small amounts. Hence sequencing from 2D gels may not be feasible. Another option is to use western blotting on 2D gels to identify spots that correspond to a known antigen. Three protein families are known to be produced by ovarian follicles: tissue inhibitor of metallo-proteinases (TIMP) (Smith and Moore, 1991), insulin-like growth factor binding proteins (IGFBP) (Monget *et al.*, 1993) and inhibin (Hopko Ireland *et al.*, 1994). Preliminary results indicate that spot 16 and possibly spot 21 are recognized by an antibody against TIMP1 (M. A. Driancourt and W. Hornebeck, unpublished). There is also good agreement between the molecular mass and pI of IGFBP-4 (Fazleabas and Donnelly, 1992) and that of spot 38. In addition, spot 38 increases with atresia as does IGFBP-4 (Monget *et al.*, 1993). Western ligand blotting on 2D gels is required to confirm these findings.

Elucidation of the nature of the seven proteins involved in follicular maturation, of the six proteins associated with the Fec^B gene and of the five proteins associated with follicular steroidogenesis will undoubtedly lead to a better understanding of the control mechanisms of ovarian function.

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