Laminin- $\alpha_6\beta_1$ integrin interaction enhances survival and proliferation and modulates steroidogenesis of ovine granulosa cells

F Le Bellego, C Pisselet, C Huet, P Monget and D Monniaux

Physiologie de la Reproduction et des Comportements, UMR 6073 INRA-CNRS-Université François Rabelais de Tours, INRA 37380 Nouzilly, France

(Requests for offprints should be addressed to D Monniaux; Email: monniaux@tours.inra.fr)

(C Huet is now at The Burnham Institute, La Jolla, California 92037, USA)

Abstract

This study aimed to determine the physiological role of laminin (LN) and its receptor, $\alpha_6\beta_1$ integrin, in controlling the functions of granulosa cells (GC) during follicular development in sheep ovary. Immunohistochemistry experiments showed the presence of increasing levels of LN ($P<0.0001$), and high levels of mature $\alpha_6\beta_1$ integrin in GC layers of healthy antral follicles during the follicular and the preovulatory phases of the estrous cycle. *In vitro*, the addition of a function-blocking antibody raised against α_6 subunit (anti- α_6 IgG) to the medium of ovine GC cultured on LN impaired cell spreading ($P<0.0001$), decreased the proliferation rate ($P<0.05$) and increased the apoptosis rate ($P<0.05$). Furthermore, addition of anti- α_6 IgG enhanced estradiol (E2) secretion by GC in the presence or absence of follicle-stimulating hormone (FSH), luteinizing hormone or insulin-like growth factor-I in

culture medium ($P<0.0001$), and inhibited progesterone (P4) secretion in basal conditions or in the presence of low (0.5 ng/ml) FSH concentrations only ($P<0.0001$). The anti- α_6 IgG effect was specific to an interaction of LN with $\alpha_6\beta_1$ integrin since it was ineffective on GC cultured on heat-denatured LN, RGD (arginine-glycine-aspartic acid) peptides and non-coated substratum. Hence, this study established that $\alpha_6\beta_1$ integrin 1) was expressed in GC of antral follicles, 2) mediated the actions of LN on survival, proliferation and steroidogenesis of GC, and 3) was able to dramatically modulate P4 and E2 secretion by GC *in vitro*. It is suggested that during the follicular and the preovulatory phases of the estrous cycle, the increasing levels of LN in GC of large antral follicles might support their final development to ovulation.

Journal of Endocrinology (2002) **172**, 45–59

Introduction

In the ovary, terminal development of antral follicles to ovulation is characterized by important transitions in the functions of granulosa cells (GC). First, GC from antral follicles gradually lose their proliferative activity and differentiate into estradiol (E2)-secreting cells. Thereafter, in preovulatory follicles, GC luteinize into progesterone (P4)-secreting cells in response to the preovulatory surge of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH). It has previously been hypothesized that extracellular matrix (ECM) components present in ovarian follicles might modulate the actions of gonadotropins on these transitions (Gospodarowicz *et al.* 1980, Savion *et al.* 1981, Amsterdam *et al.* 1989, Aten *et al.* 1995, Aharoni *et al.* 1996, Huet *et al.* 1997, Rodgers *et al.* 1999). Within the ovarian follicles, the outer layer of GC rests on a basement membrane separating the intra-follicular compartment from the theca (Luck 1994). This basement membrane is believed to play a role in influencing proliferation and differentiation of GC (Amsterdam *et al.* 1989, Luck 1994) and in protecting them from

entering apoptosis in early atretic follicles (Monniaux *et al.* 1999). Laminin (LN) has previously been shown to be one of the most abundant ECM components within the basement membrane (Wordinger *et al.* 1983, Leardkamolkarn & Abrahamson 1992, Zhao & Luck 1995, Lee *et al.* 1996, Huet *et al.* 1997, van Wezel *et al.* 1998, Rodgers *et al.* 1999, Alexopoulos *et al.* 2000). *In vitro*, purified LN used as a culture substratum improves GC survival in rat (Aharoni *et al.* 1996) and sheep (Huet *et al.* 2001) and stimulates their proliferation in sheep (Huet *et al.* 2001). However, the effects of LN on GC steroidogenesis are still the object of controversy since LN enhances P4 secretion in rat (Aten *et al.* 1995, Aharoni *et al.* 1996) and pig (Sites *et al.* 1996), but inhibits it in human (Fujiwara *et al.* 1997). Moreover, the functional importance of LN in GC differentiation to an E2-secreting cell type during terminal follicular development, and then to a P4-secreting cell type during the luteinization process has not been established.

LN exerts its biological effects acting on target cells via specific cell surface $\alpha\beta$ heterodimeric receptors named integrins (Vuori 1998, Giancotti & Ruoslahti 1999). At

least nine different integrins have been described as LN receptors (Kühn & Eble 1994, Mercurio 1995). Interestingly, GC from different animal species express very high levels of α_6 integrin subunit (human: Honda *et al.* 1995; marmoset: Giebel *et al.* 1996, Giebel and Rune 1997; pig: Fujiwara *et al.* 1995; mouse: Nakamura *et al.* 1997) which has been reported to associate with β_1 or β_4 integrin subunit, forming highly specific LN receptors in various cell types (Sonnenberg *et al.* 1988). *In vivo*, GC of primates strongly express β_1 integrin subunit (Giebel *et al.* 1996, Giebel & Rune 1997), suggesting that a functional $\alpha_6\beta_1$ LN receptor may be present in these cells. However, the role of $\alpha_6\beta_1$ integrin in mediating the actions of LN on GC survival, proliferation and steroidogenesis during follicular development and luteinization remains to be established.

The present study aimed to assess the role of both LN and its receptor, $\alpha_6\beta_1$ integrin, in controlling GC functions during terminal follicular development in sheep ovary. For this purpose, changes in LN and $\alpha_6\beta_1$ integrin levels were studied in sheep antral follicles larger than 1 mm diameter during the follicular and preovulatory phases of the estrous cycle *in vivo*. In further experiments *in vitro*, the effects of the addition of a function-blocking antibody specifically raised against α_6 integrin, that has been reported to inhibit interaction between $\alpha_6\beta_1$ integrin and all LN isoforms (Sonnenberg *et al.* 1990), were investigated on GC shape, apoptosis, proliferation, and secretion of E2 and P4. Results suggest that LN–integrin $\alpha_6\beta_1$ interaction has an important role to play in modulating survival, growth and steroidogenesis of GC in sheep antral follicles and thereby might participate in the regulation of terminal follicular development to the preovulatory stage.

Materials and Methods

Reagents and chemicals

Fluorogestone acetate sponges used to synchronize estrous cycles, and human chorionic gonadotropin (hCG) used for injections to animals were obtained from Intervet (Angers, France). Porcine FSH (pFSH) from pituitary extract (pFSH activity=1.15 times activity of NIH pFSH-P1) used for injections to animals, and purified ovine LH (oLH) (CY 1085, oLH activity=1.38 times activity of NIH S21) were obtained from Dr Y Combarnous (Nouzilly, France). Purified ovine FSH-20 (oFSH) (lot no. AFP-7028D, 4453 IU/mg, FSH activity=75 times activity of oFSH-S1) used for culture treatment was a gift from NIDDK, National Hormone Pituitary Program, Bethesda, MD, USA. Recombinant human insulin-like growth factor-I (IGF-I) was a gift from Dr P Swift (Ciba-Geigy, Saint Aubin, Switzerland). B2 medium was prepared according to Menezo (1976). Rat monoclonal antibody GoH3 raised against human α_6 integrin subunit (anti- α_6 IgG) was purchased from Serotec (Oxford, Oxon, UK), and rabbit polyclonal antibody anti-LN-1 (anti-LN)

from Engelbreth-Holm-Swarm (EHS) mouse sarcoma was from the Institut Pasteur (Lyon, France). Mouse monoclonal antibody raised against human integrin β_1 subunit (anti- β_1 IgG) was a gift from Dr C Boucheix (Paris, France). Anti-rat and anti-rabbit IgG antibodies coupled to horseradish peroxidase were purchased from Interchim (Montluçon, France), and anti-mouse IgG antibody coupled to horseradish peroxidase was obtained from Diagnostic Pasteur (Marnes-la-Coquette, France). The following reagents were purchased from Sigma (L'Isle d'Abeau Chesnes, France): McCoy's 5a medium with bicarbonate, penicillin/streptomycin, bovine serum albumin (BSA tissue culture grade) used for culture medium, transferrin, selenium, bovine insulin, androstenedione, LN from EHS tumor (mainly LN-1; Mercurio 1995), anti-rat IgG (whole molecule)-agarose, Igepal, phenylmethylsulfonyl fluoride (PMSF), L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E64), pepstatin A and aprotinin. Hepes, L-glutamine, fungizone and trypsin were purchased from GIBCO BRL (Cergy-Pontoise, France). Pronectin F (RGD peptides: arginine-glycine-aspartic acid sequence) was obtained from Interchim, Tissue-Tek from Miles Laboratories (Elkhart, IN, USA), 3-aminopropyltriethoxy-silane (silane) and saponin from Fluka Biochemika (Mulhouse, France), BSA used for immunochemistry from Bayer (Puteaux, France), Vectastain Elite ABC kit from Vector Laboratories (AbCys, Paris, France), 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB) from Aldrich Chimie (L'Isle d'Abeau Chesnes, France) and Schiff reagent for Feulgen staining from Merck (Schuchardt, Germany). ECL (enhanced chemiluminescence) reagents were obtained from Amersham Pharmacia Biotech (Les Ulis, France). Sterile 96-well plates (Nuncion Delta) were obtained from Nunc (Naperville, IL, USA) and plastic tissue culture chamber slides from Poly-Labo (Strasbourg, France). [³H]Thymidine (specific activity 6.7 Ci/nmol) was obtained from Dupont De Nemours (Les Ulis, France) and NTB2 emulsion for autoradiography from Integra Bioscience (Cergy-Pontoise, France). Nitrocellulose filter (0.2 µm pore size) was purchased from Schleicher and Schuell (Ecqueville, France). The BioRad Protein Assay kit was purchased from BioRad (Ivry s/Seine, France).

Animals

All procedures were approved by the Agricultural Agency and the Scientific Research Agency (approval number A 37801) and conducted in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching. Seventy-eight adult Romanov ewes were treated with intravaginal sponges impregnated with progestagen (fluorogestone acetate, 40 mg) for 15 days to mimic a luteal phase.

For immunohistochemistry experiments, 10 ewes were slaughtered at different times of the follicular phase. A first

group of 3 ewes was slaughtered in the early follicular phase (12 h after sponge removal). A second group of 3 ewes was slaughtered in the late follicular phase (36 h after sponge removal). A third group of 4 ewes was slaughtered in the preovulatory phase (20 h after an intramuscular injection of 1000 IU hCG performed 36 h after sponge removal to mimic a preovulatory LH surge). After slaughter, ovaries were dissected and immediately embedded in Tissue-Tek and frozen in liquid nitrogen for subsequent immunohistochemical analysis.

Alternatively, cells for culture and immunoblotting were collected from animals in the luteal phase of the estrous cycle (10 days after sponge removal, plus intramuscular injections of 6 IU and 5 IU pFSH 24 h and 12 h prior to slaughter respectively). This treatment allowed recovery of GC with a viability ranging between 60 and 80%, as estimated by trypan blue exclusion. Ovaries from 3 ewes were used for each culture experiment. A total number of 12 and 18 cultures of GC from small and large follicles, respectively, were performed.

Immunohistochemistry

Ovarian sections (10 µm) were prepared with a cryostat and attached to silane-coated slides. The size and the degree of atresia of each follicle were estimated on serial sections fixed in methanol-formaldehyde-acetic acid (80:15:5) and subsequently stained with Feulgen. Follicles were classified according to their size: small follicles (1–3 mm in diameter) and large follicles (3.5–7 mm in diameter). The degree of atresia of each follicle was assessed by microscopic examination of GC: healthy follicles with frequent mitosis and no pyknosis, atretic follicles with pyknosis and no mitosis, and late atretic follicles with rare healthy GC and widespread pyknosis.

Sections were fixed for 10 min in ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS) containing 0.1% saponin (PBS/saponin) and washed in PBS/saponin. Immunohistochemistry was then carried out using the Vectastain ABC kit according to the manufacturer's instructions with few modifications. Briefly, sections were treated with 0.3% H₂O₂ for 30 min, saturated in rabbit or goat serum, and incubated overnight with primary antibodies in PBS/saponin containing 0.1% BSA (final dilutions were 10 ng/ml for anti- α_6 IgG, 1:100 000 for anti-LN and 1.5 ng/ml for anti- β_1 IgG). Control sections were incubated with the same dilution of purified rat IgG or rabbit non-immune serum diluted in the same buffer. All sections were subsequently incubated for 4 h at room temperature with biotinylated rabbit anti-rat or goat anti-rabbit IgG (final dilution 1:800 in PBS/saponin/BSA), washed in PBS/saponin, and finally incubated for 30 min with the avidin-biotin-horseradish peroxidase complex. The staining was obtained by incubating sections in Tris-HCl (50 mM, pH 7.8) containing

0.2 mg/ml DAB and 0.0036% H₂O₂ for 4 min at room temperature.

For LN and integrin α_6 subunit, quantification of immunostaining was performed by densitometric analysis using a microscope-linked personal computer-based image analyzer (SAMBA TM 2005, Alcatel TITN Meylan, France) as previously described (Huet *et al.* 1997). Each section was analyzed with a $\times 100$ objective. For each follicle, the mean optical density of staining (expressed in arbitrary units) was estimated from measurements of 20 microscopical square fields of constant area (45 µm²) chosen at random in the wall of GC. Quantitative analysis was performed on follicles larger than 1 mm in diameter present in one ovary from each of the 10 ewes. In total, 150 follicles were analyzed: 73 healthy follicles, 41 atretic follicles and 36 late atretic follicles. To assess the specificity of staining, additional measurements were performed in follicular antrum, in non-vascularized areas of interstitial tissue close to the studied follicles, and in ovarian control sections incubated with rat IgG or rabbit non-immune serum.

Isolation of GC

Immediately after slaughter, ovaries from 3 ewes were immersed for 15 min in isotonic solution containing fungizone and antibiotics (penicillin and streptomycin). Then ovaries were placed in B2 medium and follicles larger than 1 mm diameter were dissected within 1 h of slaughter. Follicular fluid from large follicles (>4 mm) was aspirated with a 26-gauge needle. Each follicle was then slit open in B2 medium and GC were removed by gently scraping the interior surface of the follicle with a platinum loop. GC suspensions were pooled according to the size class (small: 1–3 mm, or large: >4 mm) of follicles. The two resulting cell suspensions were centrifuged at 300 $\times g$ for 7 min and re-suspended in culture medium (McCoy's 5a containing bicarbonate supplemented with 20 mmol/l Hepes, 100 kUI/l penicillin, 0.1 g/l streptomycin, 3 mmol/l L-glutamine, 0.1% BSA (w/v), 100 µg/l insulin, 0.1 µmol/l androstenedione, 5 mg/l transferrin, 20 µg/l selenium). Cells were counted and the viability was estimated by trypan blue exclusion. For immunoprecipitation and western blotting experiments, suspensions were centrifuged at 300 $\times g$ for 7 min. The pellets were stored at -20°C until experiments.

Immunoprecipitation

GC were lysed for 30 min on ice in 1 ml RIPA buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors (2 mM PMSF, 10 µM aprotinin, 29.2 µM pepstatin A). Insoluble material was removed by a quick centrifugation at 20 000 $\times g$. Anti- α_6 IgG (25 µg/ml) and anti-rat IgG agarose beads (1/10 v/v) were added to the

supernatant and samples were incubated for 5 h at 4 °C under constant agitation. Agarose beads were pelleted, washed 5 times in RIPA buffer, and re-suspended in 20 µl Laemmli buffer without β-mercaptoethanol.

Western immunoblotting

Whole ovaries and GC were crushed at 4 °C in 25 mM Tris-HCl (pH 7.5) containing 250 mM sucrose and protease inhibitors (1 mM PMSF, 2 mM E64 and 10 µM aprotinin). Cell membranes were precipitated by centrifugation at $30\,000 \times g$, re-suspended in the same buffer without protease inhibitors and stored at -20 °C. Protein concentration was determined with the BioRad Protein Assay.

Ovary homogenates and GC membranes were respectively submitted to electrophoresis on 8% and 10% (w:v) SDS-polyacrylamide gel under reducing conditions. The proteins were then electrotransferred onto nitrocellulose filters overnight at 4 °C. Filters were incubated for 2 h at room temperature with 20 mM Tris-buffered saline (TBS) (pH 7.6), containing 10% nonfat dry milk powder (NFDMP) and 0.2% Tween-20 to saturate non-specific sites. Thereafter, filters were incubated for 1 h at 37 °C with anti- α_6 IgG (final dilution 1 µg/ml), anti- β_1 IgG (final dilution 1.5 µg/ml) or anti-LN (final dilution 1:1000) antibodies in TBS/5% NFDMP/0.05% Tween-20. After washing in TBS/1% NFDMP/0.2% Tween-20, nitrocellulose filters were incubated for 1 h at 37 °C with a horseradish peroxidase-conjugated anti-rat, anti-mouse or anti-rabbit IgG (final dilution 1:10 000) in TBS/5% NFDMP/0.05% Tween-20. After washing in TBS/1% NFDMP/0.2% Tween-20, the signal was detected by ECL.

Granulosa cell culture

GC culture was performed according to the method of Campbell *et al.* (1996). Cells were cultured in 96-well tissue-culture plates coated with LN, heat-denatured LN, RGD peptides, or non-coated plates (plastic). The different substrata were prepared 72 h before use and allowed to dry at room temperature. LN was used at 5 µg/cm² in distilled water. Heat-denatured LN was used at the same concentration after a 1-h heating treatment at 80 °C. This treatment induced LN fragmentation, as assessed by western immunoblotting (data not shown). RGD peptides were used at 0.4 µg/cm² in PBS. GC suspensions from small and large follicles were seeded at 10^5 viable cells/well and cultured at 37 °C in a humidified atmosphere with 5% CO₂, in serum-free culture medium (see Isolation of GC). The effects of anti- α_6 IgG or control rat IgG were tested at different doses in the range 0.01 to 5 µg/ml, in combination with oFSH (0.5 or 5 ng/ml), IGF-I (100 ng/ml) and oLH (5 ng/ml). Each condition was tested in triplicate in each culture. Culture media were partially replaced

(175 µl over 250 µl) every 48 h and the spent medium was stored at -20 °C until assay. At 48 h or 144 h of culture, cells were detached with trypsin and counted.

The time required for GC isolation was such that, to maintain GC viability, each culture was performed with pooled GC recovered from only 3 ewes. As this did not provide a sufficient number of cells to allow comparison of all experimental conditions within a single culture, distinct culture experiments were designed to investigate the effect of the substrata (LN, heat-denatured LN, RGD peptides, plastic) and the effect of the treatments (FSH, IGF-I, LH). However, the effects of anti- α_6 IgG were always compared with the control rat IgG within the same culture experiment.

Determination of thymidine labeling index, pyknotic index and cell nuclear diameter

GC proliferation and survival were assessed by measuring the thymidine labeling index (percentage of labeled cells) and the pyknotic index (percentage of pyknotic cells) respectively, after 48 h of culture. In parallel, the spreading of GC on LN was estimated by measuring the diameter of the cellular nuclei after 72 h of culture.

To determine the thymidine labeling index, cells were washed with B2 medium without thymine, then incubated with [³H]thymidine (0.25 µCi/ml) at 37 °C for 2 h. After 2 washes with B2 medium (with thymine), cells were detached with 1% trypsin, pelleted and fixed in 3% glutaraldehyde for 1.5 h at room temperature. Cells were then smeared onto histological slides by cytocentrifugation. Smears were stained with Feulgen, dipped in NTB2 emulsion, air-dried, and exposed for autoradiography for 6 days at 4 °C. The thymidine labeling index was estimated by counting the number of labeled and unlabeled cells in 20 different microscopic fields (objective $\times 100$).

To determine the pyknotic index, cells were detached by trypsin, fixed in glutaraldehyde, submitted to cytocentrifugation and smears were stained with Feulgen as described above. The pyknotic index was estimated by counting the number of pyknotic cells, i.e. cells with condensed or fragmented nuclear chromatin (Aharoni *et al.* 1995), and non-pyknotic cells in 20 different microscopic fields (objective $\times 100$). Previous results established that the presence of pyknotic cells was associated with DNA fragmentation characteristic of the apoptotic process in cultured GC (Huet *et al.* 2001).

For both thymidine labeling index and pyknotic index, calculations were made on 500–1000 cells per slide.

To evaluate cell spreading, GC were grown on plastic culture chamber slides coated with LN in the presence or absence of anti- α_6 IgG. Cell nuclear diameter was measured on 40 Feulgen-stained cells per well with a reticule under a microscope (objective $\times 100$).

Estradiol-17 β and progesterone radioimmunoassays

The concentrations of E2 and P4 in the culture medium of GC from large follicles were measured after 144 h of culture by radioimmunoassay protocols previously described (Saumande 1981, Saumande *et al.* 1985, Saumande 1991) and adapted to directly measure steroids in cell culture media. The limit of detection of E2 was 1.5 pg/tube (7.5 pg/well) and the intra- and interassay coefficients of variation were less than 7% and 9% respectively. The limit of detection of P4 was 12 pg/tube (60 pg/well) and the intra- and interassay coefficients of variation were less than 10% and 11% respectively. Results are expressed as the amount of steroids secreted between 96 h and 144 h of culture per 50 000 cells recovered at the end of the culture period.

Statistical analysis

All experimental data are presented as the mean $\pm$ S.E.M. In immunohistochemistry experiments, statistical comparisons of means were performed using ANOVA followed by Tukey-Kramer tests. In GC culture experiments, the effects of increasing doses of anti- α_6 IgG were analyzed using one-way ANOVA for repeated measures followed by Tukey-Kramer tests. Data were fitted to sigmoidal curves with GraphPad PRISM software (San Diego, CA, USA). For a given substratum and combination of treatments, the effects of anti- α_6 IgG on cell numbers, thymidine labeling index and pyknotic index were analyzed by paired *t*-test. The effects of anti- α_6 IgG on cell spreading were analyzed by *t*-test. The effects of anti- α_6 IgG on GC steroidogenesis were analyzed using two-way ANOVA in order to appreciate the effect of anti- α_6 IgG as well as the culture effect resulting from variations between both animals and quality of the ovarian follicles dissected for each culture.

Results

LN and its receptor, $\alpha_6\beta_1$ integrin, are expressed in GC of antral ovarian follicles in vivo

LN, α_6 and β_1 integrin subunits were detected by western immunoblotting in the whole ovary and GC (Fig. 1). LN was detected as a heterotrimer consisting of a heavy chain (band at 400 kDa) and 2 light chains (2 bands at approximately 200 kDa) (Fig. 1A, lanes 1 and 2). The α_6 integrin subunit was detected as a single band at 120 kDa (Fig. 1A, lanes 3 and 4) and the β_1 integrin subunit as two bands corresponding to the mature (125 kDa) and the precursor (110 kDa) forms as previously described (Rubinstein *et al.* 1997) (Fig. 1A, lanes 5 and 6). In immunoprecipitation experiments performed on GC, the α_6 integrin subunit co-immunoprecipitated with the mature β_1 integrin subunit but not with the precursor form (Fig. 1B).

Figure 1 (A) Western immunoblotting analysis of LN, α_6 and β_1 integrin subunits in GC (lanes 2, 4, 6) and in whole ovary homogenates (lanes 1, 3, 5). Lanes 1 and 2: detection of the 3 chains of LN; lanes 3 and 4: detection of α_6 integrin subunit; lanes 5 and 6: detection of β_1 integrin subunit (upper band) and its precursor (lower band). (B) Association between α_6 and β_1 integrin subunits. GC lysates were submitted to immunoprecipitation (IP) with anti- α_6 IgG, then western immunoblotting analysis was performed with anti- β_1 IgG, as described in Materials and Methods. Lane 1: without immunoprecipitation, the two bands corresponding to the mature (upper band) and the precursor (lower band) forms of β_1 integrin subunit were detected. Lane 2: immunoprecipitation with anti- α_6 IgG, only the mature form of β_1 integrin subunit was detected. Lane 3: control immunoprecipitation with non-specific rat IgG, β_1 integrin subunit was undetectable.

By immunohistochemistry, LN was detected in the follicular basement membrane, theca and GC layers, and interstitial tissue (Fig. 2A,B,C) as previously described (Huet *et al.* 1997). In all follicles, the basement membrane was strongly stained. In contrast, the intensity of staining was very low in follicular antrum (Fig. 3B) and similar to values obtained in ovarian tissue of control sections incubated with non-immune serum (data not shown). In GC layers, the intensity of LN staining did not vary with the size of healthy antral follicles (Fig. 3A), but increased with atresia (Fig. 3B) ($P < 0.01$, late atretic vs healthy small follicles) as previously described (Huet *et al.* 1997). In addition, regardless of the follicular size, the intensity of LN staining in GC of healthy follicles increased ($P < 0.0001$) during the follicular phase and after hCG administration in the preovulatory phase of the cycle (Fig. 3A).

The α_6 integrin subunit was clearly detected in GC and endothelial cells of blood vessels present in theca and interstitium (Fig. 2D,E,F). The intensity of the α_6 integrin subunit staining was high in GC, compared with staining in non-vascularized areas of interstitial tissue (Fig. 3D). In contrast, the intensity of staining was very low in follicular antrum (Fig. 3D) and similar to values obtained on ovarian tissue in control sections incubated with non-immune IgG (data not shown). In GC layers, the intensity of staining was lower in large, compared with small healthy follicles

Figure 2 Immunohistochemical localization of LN (A, B, C), α_6 (D, E, F) and β_1 integrin subunits (G, H, I) in a small healthy follicle (A, D, G), in a large healthy preovulatory follicle after hCG treatment (B, E, H) and in an atretic follicle (C, F, I). a: antrum; bl: basal lamina; g: granulosa cells; t: theca; ★: blood vessels. Bar=30 μ m.

(Fig. 3C, $P<0.0001$), and it decreased in atretic, compared with healthy, small follicles (Fig. 3D, $P<0.001$). No change in the α_6 integrin subunit staining was observed during the follicular phase and after hCG administration in the preovulatory phase of the cycle (Fig. 3C).

The β_1 integrin subunit was detected in endothelial cells close to the follicular basement membrane, in the theca and in GC layers (Fig. 2G,H,I).

Anti- α_6 IgG impairs GC spreading on LN in vitro

To study the role of the $\alpha_6\beta_1$ integrin in GC function, GC cultured in serum-free medium on LN, heat-denatured LN, RGD peptides-coated wells or on non-coated wells were treated with anti- α_6 IgG or control rat IgG. When cultured for 48 h to 144 h on LN or RGD peptides, GC lose the round shape that they have *in vivo* and adopted a flattened shape by spreading on these substrata

Figure 3 Quantitative changes in immunostaining intensity of LN (A, B) and of α_6 integrin subunit (C, D) in GC during follicular growth and atresia. Ovaries were collected at different times of a synchronized estrous cycle, i.e. in the early follicular phase, the late follicular phase or in the preovulatory phase. Panels A and C represent quantitative changes in immunostaining intensity of LN and α_6 integrin subunit, respectively, in GC from healthy small (<3 mm) and large (>3 mm) follicles, at different stages of the estrous cycle. Panels B and D represent quantitative changes in immunostaining intensity of LN and α_6 integrin subunit, respectively, in GC from healthy small and large follicles during atresia, in non-vascularized areas of interstitial tissue (Int) and in follicular antrum (Ant). Data represent mean optical densities (expressed in arbitrary units) $\pm$ S.E.M. Within each panel and each follicular size class, different letters indicate values significantly different ($P < 0.01$). (n): number of studied follicles per class, or number of ovarian sections with measurements in interstitial tissue or follicular antrum.

(Fig. 4B,D). On heat-denatured LN or on non-coated substratum, GC cultures exhibited a heterogeneous mix of spread and rounded cells (Fig. 4F,H). The addition of anti- α_6 IgG (0.5 to 5 μ g/ml) to the culture medium impaired cell spreading and enlargement on LN (nuclear diameter of GC: small follicles $9.5 \pm 0.2 \mu$ m vs $13.7 \pm 0.3 \mu$ m, large follicles $8.1 \pm 0.1 \mu$ m vs $10.8 \pm 0.2 \mu$ m, with vs without anti- α_6 IgG, both $P < 0.0001$). Upon anti- α_6 IgG treatment, cells aggregated to form clusters of rounded cells (Fig. 4A compared with 4B). The anti- α_6 IgG did not induce any morphological change in GC cultured on heat-denatured LN, RGD peptides or non-coated substratum (Fig. 4C compared with 4D, 4E compared with 4F, 4G compared with 4H). Control rat IgG had no effect on GC morphology, regardless of the substratum. These results were obtained in cultures of GC from both small and large follicles.

Anti- α_6 IgG decreases proliferation and enhances apoptosis of GC cultured on LN

When GC from both large and small follicles were cultured on LN, the addition of increasing concentrations of anti- α_6 IgG (0.01 to 5 μ g/ml) in the culture medium led to a decrease in the number of cells counted after 144 h of culture ($P < 0.01$ and $P < 0.001$, for small and large follicles respectively, Fig. 5A,B). To further understand the effects of anti- α_6 IgG, GC proliferation and apoptosis were evaluated separately after 48 h of culture. On LN-coated wells, the proliferation rate of GC, estimated by the thymidine labeling index, was higher in cells from small compared with large follicles (Fig. 5C,D). It decreased in GC from both small and large follicles with increasing concentrations of anti- α_6 IgG in the culture medium (both $P < 0.05$, Fig. 5C,D). The apoptosis rate of GC, estimated by the pyknotic index (Huet *et al.* 2001), was higher in

Figure 4 Morphological effects of addition of anti- α_6 IgG or control rat IgG (0.5 $\mu\text{g/ml}$) on GC cultured on different substrata. Cells were cultured on LN (A, B), RGD peptides (C, D), heat-denatured LN (E, F), or plastic (non-coated substratum) (G, H) for 144 h in the presence of anti- α_6 IgG (A, C, E, G) or control rat IgG (B, D, F, H). Dots which appear in E and F are erythrocytes. Addition of anti- α_6 IgG impaired cell spreading on LN, but not on other substrata. Bar = 50 μm .

Figure 5 Effects of increasing concentrations of anti- α_6 IgG on cell numbers, proliferation rate and apoptosis rate of GC cultured on LN-coated wells. GC from small (A, C, E) and large (B, D, F) follicles were cultured during 144 h in serum-free medium in the presence of increasing concentrations of anti- α_6 IgG. (A, B) Numbers of cells after 144 h of culture; (C, D) percentage of thymidine-labeled cells after 48 h of culture; (E, F) percentage of pyknotic cells after 48 h of culture. Data represent means $\pm$ S.E.M. of 3 independent cultures. Different letters indicate values that differ significantly ($P < 0.05$).

cells from small compared with large follicles (Fig. 5E,F). It increased in GC from large follicles ($P < 0.05$) but not small follicles, with increasing concentrations of anti- α_6 IgG in the culture medium (Fig. 5E,F).

From these results, addition of 0.5 $\mu\text{g/ml}$ anti- α_6 IgG to the culture medium was shown to be maximally effective in decreasing proliferation and increasing apoptosis of GC cultured on LN-coated wells in basal culture conditions.

This dose was therefore chosen to test, in further experiments, the effect of anti- α_6 IgG on GC cultured on LN-coated wells in FSH-, LH-, IGF-I- or FSH+IGF-I-stimulated conditions. Addition of anti- α_6 IgG at this dose led to a significant decrease in the number of GC counted after 144 h of culture on LN-coated wells, in all tested culture conditions (except in presence of 0.5 ng/ml FSH, and 100 ng/ml IGF-I for GC from small follicles) (Fig. 6A,B). The proliferation rate of GC was also significantly decreased in the presence of anti- α_6 IgG ($P<0.05$) (except in the presence of 5 ng/ml FSH for GC from large follicles) (Fig. 6C,D). The apoptosis rate was significantly increased in the presence of anti- α_6 IgG ($P<0.05$) when GC were cultured with IGF-I (100 ng/ml), LH (5 ng/ml), or FSH (5 ng/ml, not significant for GC from small follicles) (Fig. 6E,F).

The addition of control rat IgG to the medium of GC cultured in LN-coated wells had no effect on cell numbers, proliferation and apoptosis. Moreover, anti- α_6 IgG had no effect on GC cultured on heat-denatured LN, RGD peptides and non-coated substratum (data not shown).

Anti- α_6 IgG enhances E2 production and decreases P4 production by GC from large follicles cultured on LN

When GC from large follicles were cultured on LN, E2 secretion by GC decreased dramatically throughout the culture (3.31 ± 1.04 ng/ 50×10^3 cells/48 h vs 0.16 ± 0.06 ng/ 50×10^3 cells/48 h, 48 h vs 144 h of culture, $P<0.0001$), whereas P4 secretion increased (1.34 ± 0.45 ng/ 50×10^3 cells/48 h vs 84.31 ± 38.48 ng/ 50×10^3 cells/48 h, 48 h vs 144 h of culture, $P<0.0001$), indicating that GC progressively luteinized when cultured on LN substratum. Addition of anti- α_6 IgG at 5 μ g/ml in the culture medium partially reversed these changes since it enhanced E2 (Fig. 7A) and decreased P4 (Fig. 7B) secretion after 96 h and 144 h of culture. At 144 h of culture, addition of increasing concentrations of anti- α_6 IgG in culture medium (0.01 to 5 μ g/ml) enhanced E2 ($P<0.05$; Fig. 7C) and decreased P4 secretion ($P<0.05$; Fig. 7D) in a dose-dependent manner.

From these results, the addition of 0.5 μ g/ml anti- α_6 IgG to the culture medium was maximally effective in increasing E2 secretion and decreasing P4 secretion by GC cultured in LN-coated wells. Addition of anti- α_6 IgG at this dose enhanced E2 secretion by GC cultured in LN-coated wells in the presence of FSH (0.5 and 5 ng/ml), LH (5 ng/ml), IGF-I (100 ng/ml) or FSH+IGF-I ($P<0.0001$) (Fig. 7E). The effects of anti- α_6 IgG on P4 secretion varied with culture conditions. In basal conditions or in the presence of FSH at 0.5 ng/ml, P4 secretion by GC cultured on LN substratum was inhibited ($P<0.0001$) by anti- α_6 IgG. In contrast, in the presence of highly stimulating conditions (with FSH or LH at 5 ng/ml, or with IGF-I at 100 ng/ml or with

FSH+IGF-I), P4 secretion was high and remained unchanged overall by the addition of anti- α_6 IgG (Fig. 7F).

The addition of control rat IgG in the medium of GC cultured on LN had no effect on steroidogenesis. Moreover, anti- α_6 IgG was ineffective on GC cultured on heat-denatured LN, RGD peptides and non-coated substratum (data not shown).

Discussion

This study established that a functional LN receptor, the integrin $\alpha_6\beta_1$, was expressed in GC of antral growing follicles in sheep *in vivo*. *In vitro*, we have shown that integrin $\alpha_6\beta_1$ is able to mediate most actions of LN on proliferation, survival and steroidogenesis of ovine GC.

By immunohistochemistry using antibodies raised against LN-1, we have confirmed the presence of LN in basement membrane, granulosa and theca cells of ovine antral follicles, as shown previously in various animal species (Bagavandoss *et al.* 1983, Wordinger *et al.* 1983, Bortolussi *et al.* 1989, Leardkamolkarn & Abrahamson 1992, Zhao & Luck 1995, Lee *et al.* 1996, Huet *et al.* 1997, van Wezel *et al.* 1998, Rodgers *et al.* 1999, Alexopoulos *et al.* 2000). Laminins are heterotrimeric proteins containing α , β and γ subunits, and the association of structural isoforms of these subunits gives rise to different LN (Mercurio 1995). Results of this study did not allow the determination of which type of LN is present in ovarian follicles, since the antibodies used can identify any LN containing the $\alpha 1$, $\beta 1$ or $\gamma 1$ chains. The presence of $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$ and $\gamma 1$ LN chains has recently been reported in bovine ovarian follicles, and high levels of the $\alpha 1$, $\beta 2$ and $\gamma 1$ chains have been observed in basement membrane and Call-Exner bodies of healthy follicles (van Wezel *et al.* 1998, 1999, Rodgers *et al.* 1999), suggesting the presence of LN-3 (S-LN). However, the physiological role of intrafollicular LN in regulating follicular development has not yet been established. In the present study, we reported that the α_6 and β_1 integrin subunits of the specific $\alpha_6\beta_1$ LN receptor are both highly expressed in GC of growing follicles in sheep. As in the human (Honda *et al.* 1995) and marmoset (Giebel *et al.* 1996, Giebel & Rune 1997), the β_1 integrin subunit was also detected in theca cells, endothelial cells and ovarian stroma. Expression of α_6 integrin subunit was essentially observed in GC of healthy growing antral follicles, in agreement with previous observations (human: Honda *et al.* 1995; marmoset: Giebel *et al.* 1996, Giebel & Rune 1997; pig: Fujiwara *et al.* 1995; mouse: Nakamura *et al.* 1997) as well as in endothelial cells. In addition, for the first time we showed by immunoprecipitation that the α_6 integrin subunit is structurally associated with the mature form of the β_1 integrin subunit in ovine GC, suggesting that the integrin $\alpha_6\beta_1$ may constitute a functional LN receptor in these cells (Sonnenberg *et al.* 1988).

Figure 6 Effects of addition of anti- α_6 IgG on cell numbers, proliferation rate and apoptosis rate of GC cultured on LN-coated wells in FSH-, LH- and/or IGF-I-stimulated conditions. GC from small (A, C, E) and large (B, D, F) follicles were cultured during 144 h in serum-free medium in the presence or in absence of anti- α_6 IgG (0.5 $\mu\text{g}/\text{ml}$) and in the presence of FSH (0.5 or 5 ng/ml), LH (5 ng/ml) and/or IGF-I (100 ng/ml). (A, B) Numbers of cells after 144 h of culture; (C, D) percentage of thymidine-labeled cells after 48 h of culture; (E, F) percentage of pyknotic cells after 48 h of culture. Data represent means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, with vs without anti- α_6 IgG. (n): number of independent cultures per condition.

To determine the potential role of integrin $\alpha_6\beta_1$ in ovine GC functions, we studied the effects of addition of the GoH3 antibody, a function-blocking antibody

specifically raised against α_6 integrin subunit, to the culture medium of ovine GC. This anti- α_6 IgG was reported to be highly specific in inhibiting interaction

Figure 7 Effects of addition of anti- α_6 IgG on E2 (A, C, E) and P4 (B, D, F) secretion by GC from large follicles cultured on LN substratum. (A, B) Time-course effects of anti- α_6 IgG. GC were cultured during 144 h on LN-coated wells in serum-free medium in the presence or absence of anti- α_6 IgG (0.05 μ g/ml or 5 μ g/ml). At each culture time (48 h, 96 h and 144 h), the amount of E2 and P4 secreted by 50×10^3 cells during the past 48 h was measured. Results are expressed as a percentage of control, i.e. basal conditions without anti- α_6 IgG at each culture time. (C, D) Dose-dependent effects of anti- α_6 IgG. GC were cultured during 144 h on LN-coated wells in serum-free medium in the presence or absence of increasing concentrations of anti- α_6 IgG (0.01 to 5 μ g/ml). Results are expressed in ng steroids secreted by 50×10^3 cells between 96 h and 144 h of culture. (E, F) Effects of anti- α_6 IgG in FSH-, LH- and IGF-I-stimulated culture conditions. GC were cultured during 144 h on LN-coated wells in serum-free medium in the presence or absence of anti- α_6 IgG (0.5 μ g/ml) and in the presence of FSH (0.5 or 5 ng/ml), LH (5 ng/ml) and/or IGF-I (100 ng/ml). Results are expressed in ng steroids secreted by 50×10^3 cells between 96 h and 144 h of culture. Data represent means $\pm$ S.E.M. of 3 (panels A, B, C, D) and 5–9 (panels E and F) independent cultures. In each of the panels A–D, different letters indicate values that differ significantly ($P < 0.05$). In E and F, *** $P < 0.0001$ with vs without anti- α_6 IgG.

between integrin $\alpha_6\beta_1$ and all the LN isoforms (Sonnenberg *et al.* 1990). From our results, this antibody dramatically impaired cell spreading on LN-1 and modulated cell survival, proliferation and steroidogenesis of GC cultured on LN-1. Accordingly, the GoH3 antibody has been shown to inhibit adhesion of pancreatic carcinoma cells (Vogelmann *et al.* 1999) and hepatoma cells (Torimura *et al.* 1999), and to impair migration of gingival epithelial cells (Gräber *et al.* 1999) and hepatocarcinoma cells (Nejjari *et al.* 1999) cultured on a LN substratum. In ovine GC, no effect of this antibody was observed when cells were cultured on non-coated substratum, RGD peptides, or heat-denatured LN, indicating that all the observed effects on ovine GC were specific of an interaction between $\alpha_6\beta_1$ and structurally intact LN. From these results, it is suggested that the $\alpha_6\beta_1$ integrin plays a crucial role in mediating the interaction between ovine GC and LN.

The addition of the anti- α_6 IgG to the culture medium severely impaired the spreading of ovine GC, particularly cells from large follicles, on LN. Cells remained attached to the LN substratum, suggesting that $\alpha_6\beta_1$ was not the sole integrin involved in the interaction between GC and LN. In addition to the α_6 and β_1 integrin subunits, the presence of the subunits α_2 (Giebel *et al.* 1996, Yamada *et al.* 1999) and α_3 (Giebel *et al.* 1996) and the absence of the β_4 subunit (Honda *et al.* 1995) have been reported in GC. In sheep GC, we have detected the presence of the $\alpha_v\beta_3$ integrin by immunohistochemistry and immunoblotting (our unpublished observations). The $\alpha_v\beta_3$ integrin is also able to bind LN as well as other ECM components such as fibronectin, collagens and tenascin. Its role in mediating the actions of these components, and in particular LN on GC, has to be investigated.

In our *in vitro* conditions, the loss of contacts between GC of large antral follicles and LN, following the addition of the anti- α_6 IgG, was accompanied by an increase in pyknotic rate. It is well established that the loss of cell attachment to ECM causes apoptosis, according to a process named anoikis (Ruoslahti & Reed 1994, Giancotti & Ruoslahti 1999). The present result is consistent with previous data showing that LN enhances cell survival in rat (Aharoni *et al.* 1996) and ovine GC (Huet *et al.* 2001). *In vitro*, histological observations have established that GC adjacent to the follicular basement membrane are protected from entering apoptosis in early atretic follicles (Monniaux *et al.* 1999). Thus, one can hypothesize that LN present in high concentrations in the basement membrane is able to maintain GC survival during follicular development. In addition, our results indicate that the integrin $\alpha_6\beta_1$ might have a more important role in activating survival signaling pathways in GC from large compared with small antral follicles. In small follicles, other integrins such as $\alpha_v\beta_3$ may be able to mediate the cell survival effect of LN.

From our results, LN- $\alpha_6\beta_1$ interaction would promote proliferation of GC. Accordingly, we have shown previously that LN, compared with non-coated substratum, enhances proliferation of sheep GC (Huet *et al.* 2001). In other cell types, the effect of LN on cell proliferation is the object of controversy. For example, it has been reported that LN enhances proliferation in full-term fetal liver cells (Hirata *et al.* 1983) and skeletal myoblasts (Öcalan *et al.* 1988), but inhibits it in airway smooth muscle cells (Hirst *et al.* 2000). It is established that the cytoplasmic domain of the β subunit is required for integrins to trigger signaling events that modulate cellular functions (Hemler & Lobb 1995). Different isoforms of β_1 integrin subunit, corresponding to spliced variants of the integrin cytoplasmic domain, have been shown recently to exert opposing effects on proliferation of CHO cell lines (Fornaro *et al.* 2000). Further investigations are needed now to determine which β_1 isoforms are complexed with the α_6 subunit in ovine GC.

In our experiments, LN enhanced P4 and inhibited E2 secretion by GC from large follicles in sheep, suggesting that LN exerted a luteinizing action on GC *in vitro*. In agreement, LN was previously shown to enhance P4 secretion by rat (Aten *et al.* 1995, Aharoni *et al.* 1996) and pig (Sites *et al.* 1996) GC. In addition, we report for the first time that this luteinizing effect of LN was, at least partly, mediated by integrin $\alpha_6\beta_1$. The mechanisms by which LN influences the steroidogenesis pathways of GC are unknown. It is established that integrin binding to ECM components promotes focal adhesion kinase activation and changes in mechanical tensions of the cytoskeleton and thereby induces multiple signaling pathways (Kumar 1998, Vuori 1998). In our model, we can suggest that the drastic cell rounding triggered by addition of the anti- α_6 IgG directly affected GC steroidogenesis. Accordingly, previous studies have also reported an enhancement of E2 secretion by bovine or human GC exhibiting a rounded rather than a flattened shape (Ben-Rafael *et al.* 1988, Bussenot *et al.* 1993, Gutiérrez *et al.* 1997). However, in rat (Carnegie & Tsang 1988), human (Ben-Rafael *et al.* 1988) and pig (Sites *et al.* 1996), in contrast to sheep, a rounded cell shape has also been associated with an enhancement of P4 secretion by GC. Alternatively, one can hypothesize that activation of the integrin $\alpha_6\beta_1$ by LN would have promoted activation of signaling pathways modulating E2 and P4 secretion differently, independently of modulation of cell shape. In GC, activation of the protein kinase A or C pathways by gonadotropins or growth factors has an important role in modulating the P4/E2 secretion ratio (Legault *et al.* 1999). In the present study, the inhibiting effect of the anti- α_6 IgG on P4 secretion observed in basal or low-stimulating culture conditions was completely abolished in the presence of highly luteinizing concentrations of gonadotropins or/and IGF-I. Altogether, these results suggest that cross-talks in signaling pathways of LN,

FSH, and IGF-I exist between LN and gonadotropins in GC.

We report for the first time the presence of increasing amounts of LN in GC of antral follicles during the follicular and the preovulatory phases of the estrous cycle *in vivo*. Within the membrana granulosa of rat and bovine follicles, LN has been mainly localized to Call-Exner bodies (Leardkamolkarn & Abrahamson 1992, van Wezel *et al.* 1999), suggesting that GC can synthesize LN, but a contribution from theca cells in the synthesis of at least one LN chain is not excluded (Rodgers *et al.* 1999). It can be proposed that the increased LH concentration at the end of the follicular phase is leading to high levels of LN in preovulatory follicles. From our results, this increase in LN intrafollicular levels may have important physiological consequences. Particularly, the involvement of LN in initiating luteinization of mature GC in preovulatory follicles is strongly suggested.

In conclusion, our results established that LN, acting via the integrin $\alpha_6\beta_1$, had an important role in modulating GC functions in sheep antral follicles. It is suggested that during the follicular and preovulatory phases of the estrous cycle, LN present in the basement membrane and GC of the growing antral follicles would sustain follicular growth by enhancing GC proliferation and maintaining cell survival in the dominant follicle(s) destined to ovulate. Moreover, LN might contribute both to the regulation of follicular steroidogenesis by increasing the intrafollicular P4/E2 concentration ratio in preovulatory follicles before the preovulatory gonadotropin surge, and to the luteinization of GC.

Acknowledgements

The authors thank Drs C Boucheix, J L Duband, E Reiter and W Hornebeck for helpful discussions, A Beguey for photographic work and all the members of the ovine experimental unit for animal management.

References

- Aharoni D, Dantes A, Oren M & Amsterdam A 1995 cAMP-mediated signals as determinants for apoptosis in primary granulosa cells. *Experimental Cell Research* **218** 271–282.
- Aharoni D, Meiri I, Atzmon R, Vlodavsky I & Amsterdam A 1996 Differential effect of components of the extracellular matrix on differentiation and apoptosis. *Current Biology* **7** 43–51.
- Alexopoulos E, Shahid J, Ongley HZ & Richardson MC 2000 Luteinized human granulosa cells are associated with endogenous basement membrane-like components in culture. *Molecular Human Reproduction* **6** 324–330.
- Amsterdam A, Rotmensch S, Furman A, Venter EA & Vlodavsky I 1989 Synergistic effect of human chorionic gonadotropin and extracellular matrix on *in vitro* differentiation of human granulosa cells: progesterone production and gap junction formation. *Endocrinology* **124** 1956–1964.
- Aten RF, Kolodecik TR & Behrman HR 1995 A cell adhesion receptor antiserum abolishes, whereas laminin and fibronectin glycoprotein components of extracellular matrix promote, luteinization of cultured rat granulosa cells. *Endocrinology* **136** 1753–1758.
- Bagavandoss P, Midgley AR & Wicha M 1983 Developmental changes in the ovarian follicular basal lamina detected by immunofluorescence and electron microscopy. *Journal of Histochemistry and Cytochemistry* **31** 633–640.
- Ben-Rafael Z, Benadiva CA, Mastroianni L, Garcia CJ, Minda JM, Iozzo RV & Flickinger GL 1988 Collagen matrix influences the morphologic features and steroid secretion of human granulosa cells. *American Journal of Obstetrics and Gynecology* **159** 1570–1574.
- Bortolussi M, Zanchetta R, Doliana R, Castellani I, Bressan GM & Lauria A 1989 Changes in the organization of the extracellular matrix in ovarian follicles during the preovulatory phase and atresia. An immunofluorescence study. *Basic and Applied Histochemistry* **33** 31–38.
- Bussenot I, Ferre G, Azoulay-Barjonet C, Murgo C, Vieitez G & Parinaud J 1993 Culture of human preovulatory granulosa cells: effect of extracellular matrix on steroidogenesis. *Biology of the Cell* **77** 181–186.
- Campbell BK, Scaramuzzi RJ & Webb R 1996 Induction and maintenance of oestradiol and immunoreactive inhibin production with FSH by ovine granulosa cells cultured in serum-free media. *Journal of Reproduction and Fertility* **106** 7–16.
- Carnegie JA & Tsang BK 1988 The cytoskeleton and rat granulosa cell steroidogenesis: possible involvement of microtubules and microfilaments. *Biology of Reproduction* **38** 100–108.
- Fornaro M, Steger CA, Bennett AM, Wu JJ & Languino LR 2000 Differential role of β_{1C} and β_{1A} integrin cytoplasmic variants in modulating focal adhesion kinase, protein kinase B/AKT, and Ras/mitogen-activated protein kinase pathways. *Molecular Biology of the Cell* **11** 2235–2249.
- Fujiwara H, Ueda M, Takakura K, Mori T & Maeda M 1995 A porcine homolog of human integrin α_6 is a differentiation antigen of granulosa cells. *Biology of Reproduction* **53** 407–417.
- Fujiwara H, Honda T, Ueda M, Nakamura K, Yamada S, Maeda M & Mori T 1997 Laminin suppresses progesterone production by human luteinizing granulosa cells via interaction with integrin $\alpha_6\beta_1$. *Journal of Clinical Endocrinology and Metabolism* **82** 2122–2128.
- Giancotti FG & Ruoslahti E 1999 Integrin signaling. *Science* **285** 1028–1032.
- Giebel J & Rune GM 1997 Relationship between expression of integrins and granulosa cell apoptosis in ovarian follicles of the marmoset (*Callithrix jacchus*). *Tissue and Cell* **29** 525–531.
- Giebel J, de Souza P & Rune GM 1996 Expression of integrins in marmoset (*Callithrix jacchus*) ovary during folliculogenesis. *Tissue and Cell* **28** 379–385.
- Gospodarowicz D, Delgado D & Vlodavsky I 1980 Permissive effect of the extracellular matrix on cell proliferation *in vitro*. *PNAS* **77** 4094–4098.
- Gräber HG, Wilharm J & Conrads G 1999 Monoclonal antibodies against integrin subunits α_6 and β_1 inhibit migration of gingival epithelium in organ culture. *Journal of Periodontology* **70** 388–393.
- Gutierrez CG, Glazyrin AL, Robertson GW, Campbell BK, Gong JG, Bramley TA & Webb R 1997 Ultra-structural characteristics of bovine granulosa cells associated with maintenance of oestradiol production *in vitro*. *Molecular and Cellular Endocrinology* **134** 51–58.
- Hemler ME & Lobb RR 1995 The leukocyte β_1 integrins. *Current Opinion in Hematology* **2** 61–67.
- Hirata K, Yoshida Y, Shiramatsu K, Freeman AE & Hayasaka H 1983 Effects of laminin, fibronectin and type IV collagen on liver cell cultures. *Experimental Cell Biology* **51** 121–129.
- Hirst SJ, Twort CH & Lee TH 2000 Differential effects of extracellular matrix proteins on human airway smooth muscle cell proliferation and phenotype. *American Journal of Respiratory Cell and Molecular Biology* **23** 335–344.

- Honda T, Fujiwara H, Ueda M, Maeda M & Mori T 1995 Integrin α_6 is a differentiation antigen of human granulosa cells. *Journal of Clinical Endocrinology and Metabolism* **80** 2899–2905.
- Huet C, Monget P, Pisselet C & Monniaux D 1997 Changes in extracellular matrix components and steroidogenic enzymes during growth and atresia of antral ovarian follicles in the sheep. *Biology of Reproduction* **56** 1025–1034.
- Huet C, Pisselet C, Mandon-Pépin B, Monget P & Monniaux D 2001 Extracellular matrix regulates ovine granulosa cell survival, proliferation and steroidogenesis: relationships between cell shape and function. *Journal of Endocrinology* **169** 347–360.
- Kühn K & Eble J 1994 The structural bases of integrin–ligand interactions. *Trends in Cell Biology* **4** 256–261.
- Kumar CC 1998 Signaling by integrin receptors. *Oncogene* **17** 1365–1373.
- Leardkamolkarn V & Abrahamson DR 1992 Immunoelectron microscopic localization of laminin in rat ovarian follicles. *Anatomical Record* **233** 41–52.
- Lee VH, Britt JH & Dunbar BS 1996 Localization of laminin proteins during early follicular development in pig and rabbit ovaries. *Journal of Reproduction and Fertility* **108** 115–122.
- Legault S, Bailey JL, Fortier MA, Rouillier P & Guilbault LA 1999 Intracellular regulation of estradiol and progesterone production by cultured bovine granulosa cells. *Molecular Reproduction and Development* **54** 371–378.
- Luck MR 1994 The gonadal extracellular matrix. *Oxford Reviews in Reproductive Biology* **16** 33–85.
- Menezo Y 1976 Milieu synthétique pour la survie et la maturation des gamètes et pour la culture de l'oeuf fécondé. *Compte Rendu de l'Académie des Sciences Paris D* **282** 1967–1970.
- Mercurio AR 1995 Laminin receptors: achieving specificity through cooperation. *Trends in Cell Biology* **5** 419–423.
- Monniaux D, Mandon-Pépin B & Monget P 1999 L'atrésie folliculaire, un gaspillage programmé. *Medecine/Sciences* **15** 157–166.
- Nakamura K, Fujiwara H, Higuchi T, Honda T, Nakayama T, Kataoka N, Fujita K, Ueda M, Maeda M & Mori T 1997 Integrin α_6 is involved in follicular growth in mice. *Biochemical and Biophysical Research Communications* **235** 524–528.
- Nejjari M, Hafdi Z, Dumortier J, Bringuier A-F, Feldmann G & Scoazec J-Y 1999 $\alpha_6\beta_1$ Integrin expression in hepatocarcinoma cells: regulation and role in cell adhesion and migration. *International Journal of Cancer* **83** 518–525.
- Öcalan M, Goodman SL, Köhl U, Hauschka SD & von der Mark K 1988 Laminin alters cell shape and stimulates motility and proliferation of murine skeletal myoblasts. *Developmental Biology* **125** 158–167.
- Rodgers RJ, van Wezel IL, Irving-Rodgers HF, Lavranos TC, Irvine CM & Krupa M 1999 Roles of extracellular matrix in follicular development. *Journal of Reproduction and Fertility* **54** 343–352.
- Rubinstein E, Poindessous-Jazat V, Le Naour F, Billard M & Boucheix C 1997 CD9, but not other tetraspans, associates with the β_1 integrin precursor. *European Journal of Immunology* **27** 1919–1927.
- Ruoslahti E & Reed JC 1994 Anchorage dependence, integrins, and apoptosis. *Cell* **77** 477–478.
- Saumande J 1981 Radioimmunoassay of estradiol-17 β in unextracted ewe plasma. *Steroids* **38** 425–437.
- Saumande J 1991 Culture of bovine granulosa cells in a chemically defined serum-free medium: the effect of insulin and fibronectin on the response to FSH. *Journal of Steroid Biochemistry and Molecular Biology* **38** 189–196.
- Saumande J, Tamboura D & Chupin D 1985 Changes in milk and plasma concentrations of progesterone in cows after treatment to induce superovulation and their relationships with number of ovulations and of embryos collected. *Theriogenology* **23** 719–731.
- Savion N, Lui GM, Laherty R & Gospodarowicz D 1981 Factors controlling proliferation and progesterone production by bovine granulosa cells in serum-free medium. *Endocrinology* **109** 409–420.
- Sites CK, Kessel B & LaBarbera AR 1996 Adhesion proteins increase cellular attachment, follicle-stimulating hormone receptors, and progesterone production in cultured porcine granulosa cells. *Proceedings of the Society for Experimental Biology and Medicine* **212** 78–83.
- Sonnenberg A, Modderman PW & Hogervorst F 1988 Laminin receptor on platelets is the integrin VLA-6. *Nature* **336** 487–489.
- Sonnenberg A, Linders CJT, Modderman PW, Damsky CH, Aumailley M & Timpl R 1990 Integrin recognition of different cell-binding fragments of laminin (P1, E3, E8) and evidence that $\alpha_6\beta_1$ but not $\alpha_6\beta_4$ functions as a major receptor for fragment E8. *Journal of Cell Biology* **110** 2145–2155.
- Torimura T, Ueno T, Kin M, Ogata R, Inuzuka S, Sugawara H, Kurotatsu R, Shimada M, Yano H, Kojiro M, Tanikawa K & Sata M 1999 Integrin $\alpha_6\beta_1$ plays a significant role in the attachment of hepatoma cells to laminin. *Journal of Hepatology* **31** 734–740.
- Vogelmann R, Kreuser ED, Adler G & Lutz MP 1999 Integrin $\alpha_6\beta_1$ role in metastatic behavior of human pancreatic carcinoma cells. *International Journal of Cancer* **80** 791–795.
- Vuori K 1998 Integrin signaling: tyrosine phosphorylation events in focal adhesions. *Journal of Membrane Biology* **65** 191–199.
- van Wezel IL, Rodgers HF & Rodgers RJ 1998 Differential localization of laminin chains in bovine follicles. *Journal of Reproduction and Fertility* **112** 267–278.
- van Wezel IL, Irving-Rodgers HF, Sado Y, Ninomiya Y & Rodgers RJ 1999 Ultrastructure and composition of Call-Exner bodies in bovine follicles. *Cell and Tissue Research* **296** 385–394.
- Wordinger RJ, Rudick VL & Rudick MJ 1983 Immunohistochemical localization of laminin within the mouse. *Journal of Experimental Zoology* **228** 141–143.
- Yamada S, Fujiwara H, Honda T, Higuchi T, Nakayama T, Inoue T, Maeda M & Fujii S 1999 Human granulosa cells express integrin α_2 and collagen type IV: possible involvement of collagen type IV in granulosa cell luteinization. *Molecular Human Reproduction* **5** 607–617.
- Zhao Y & Luck MR 1995 Gene expression and protein distribution of collagen, fibronectin and laminin in bovine follicles and corpora lutea. *Journal of Reproduction and Fertility* **104** 115–123.

Received 31 August 2001
Accepted 19 September 2001