Extracellular matrix regulates ovine granulosa cell survival, proliferation and steroidogenesis: relationships between cell shape and function

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Abstract

The extracellular matrix (ECM), constituting the follicular basal lamina and present also between follicular cells and in the follicular fluid, is believed to regulate granulosa cell (GC) function during follicular development. Ovine GCs isolated from small (1–3 mm in diameter) or large (4–7 mm in diameter) antral follicles were cultured on various pure ECM components (type I collagen, fibronectin, laminin), synthetic substrata enhancing (RGD peptides) or impairing (poly 2-hydroxyethylmethacrylate (poly-hema)) cell adhesion, or in the presence of heparin. The effects of these factors, used alone or in combination with IGF-I and/or FSH, were evaluated in terms of GC spread, survival, proliferation and steroidogenesis.

When grown on type I collagen (CI) gel, poly-hema or heparin, GCs from both large and small follicles exhibited a round shape and a low proliferation rate. Compared with non-coated plastic substratum as a control, these ECM or synthetic compounds enhanced estradiol secretion and reduced progesterone secretion by large-follicle GCs. In contrast, GCs from both large and small follicles spread extensively on CI coating, fibronectin, laminin and RGD peptides. Fibronectin and laminin dramatically increased the proliferation rate and enhanced survival of GCs from

both origins. Moreover, fibronectin, laminin and RGD peptides reduced estradiol secretion by large-follicle GCs. Unexpectedly, CI coating increased estradiol secretion and reduced progesterone secretion by large-follicle GCs, suggesting that type I collagen was able to maintain estradiol secretion independently of GC shape. Finally, GC responsiveness to IGF-I and FSH, in terms of proliferation and steroidogenesis, was generally maintained when cells were grown on ECM components, RGD peptides and in the presence of heparin. However, when large-follicle GCs were grown as non-adherent clusters (as observed on poly-hema) basal and IGF-I- and/or FSH-stimulated progesterone secretions were totally abolished.

Overall, this study shows that GC shape, survival, proliferation and steroidogenesis can be modulated *in vitro* by pure ECM components in a specific and coordinated manner. It is suggested that, *in vivo*, fibronectin and laminin would sustain follicular development by enhancing the survival and proliferation of GCs, whereas type I collagen might participate in the maintenance of estradiol secretion in large antral follicles.

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Introduction

During terminal development of mammalian ovarian follicles, granulosa cells (GCs) lose their proliferative activity, differentiate into estradiol-secreting cells, and finally luteinize into progesterone-secreting cells in response to the luteinizing hormone (LH) pre-ovulatory surge (Hirshfield 1991, Monniaux *et al.* 1997). These transitions in GC activity are under the control of endocrine and paracrine factors such as pituitary gonadotrophins and ovarian growth factors (Fortune 1994, Greenwald & Roy 1994, Richards 1994). Adhesion to the extracellular matrix (ECM) regulates the survival, proliferation and differentiation of numerous cell types in many tissues (Ruoslahti

1990, Adams & Watt 1993, Lelièvre *et al.* 1996, Streuli 1999) and may also regulate GC function. *In vivo*, ovarian follicles are surrounded by a typical basement membrane containing ECM components such as laminin, type IV collagen, heparan sulfate proteoglycans and fibronectin. Importantly, the composition of the follicular basement membrane has been shown to change during follicular growth and atresia (for a review, see Rodgers *et al.* 1999). The follicular basement membrane is believed to influence GC shape and function. Indeed, the outer GCs, which are in direct contact with the basement membrane, display some specific properties, such as a cuboid shape, a lower level of proliferative activity, better survival during atresia, and a higher degree of differentiation, as assessed by the

level of expression of steroidogenic enzymes and gonadotrophin receptors (Amsterdam *et al.* 1975, Zlotkin *et al.* 1986, Amsterdam & Rotmensch 1987, Ishimura *et al.* 1989, Amsterdam *et al.* 1998). Moreover, ECM components are not only present in the follicular basement membrane but have also been detected around GCs, thecal cells and in the follicular fluid (Ax & Bellin 1988, Peter *et al.* 1995, Zhao & Luck 1995, Huet *et al.* 1997, Eriksen *et al.* 1999). We previously showed that follicular growth and atresia in the sheep are accompanied by changes in follicular ECM content and localization (Huet *et al.* 1997). This remodeling of the ECM is likely to regulate follicular cell function.

In vitro studies have previously shown that ECM can modulate GC survival and function in various mammalian species. For example, ECM derived from bovine corneal endothelial cells (a basement-membrane-like substratum) stimulates bovine GC proliferation and progesterone secretion in response to follicle-stimulating hormone (FSH) (Gospodarowicz *et al.* 1980, Savion *et al.* 1981). Similarly, rat and human luteinized GCs require a basement-like substratum in order to retain their structural and functional characteristics in culture (for a review, see Amsterdam *et al.* 1998). However, the mechanism of regulation of GC steroidogenesis by the ECM is unclear from those studies because growth factors, known to be sequestered in these complex matrices, may have played a role (Aharoni *et al.* 1997). Moreover, no extensive study comparing the effects of various pure ECM components on GC function has been reported before.

Relationships between the shape and the function of GCs have been suggested in previous studies: one reported an enhancement of estradiol secretion in rounded bovine GCs cultured on plastic substratum (Gutiérrez *et al.* 1997). Similarly, luteinized human GCs exhibiting a rounded shape when cultured on a collagen matrix show increased estradiol secretion (Ben-Rafael *et al.* 1988, Bussenot *et al.* 1993). Notably, the rounded shape has been also associated with an enhancement of progesterone secretion in rat (Carnegie & Tsang 1988), human (Ben-Rafael *et al.* 1988) and pig (Sites *et al.* 1996) GCs cultured on ECM, suggesting that the relationship between cell shape and steroidogenesis is not unequivocal.

The present study aimed to determine the combined effects of cell shape and adhesion to pure ECM components on the survival, proliferation and steroidogenesis of ovine GCs *in vitro*. Experiments were carried out with highly proliferative GCs from small antral follicles (1–3 mm in diameter) and highly steroidogenic GCs from large antral follicles (4–7 mm in diameter) cultured in the presence of various purified ECM components that are present *in vivo* in ovarian follicles (laminin, fibronectin, type I collagen, heparin), or in the presence of synthetic compounds with adhesive (RGD peptides) or anti-adhesive (poly 2-hydroxyethylmethacrylate (poly-hema)) properties. Importantly, ECM and synthetic compounds

were tested either alone or in combination with insulin-like growth factor-I (IGF-I) and/or FSH for their ability to regulate GC function.

Materials and Methods

Reagents and chemicals

Fluorogestone acetate sponges, used to synchronize estrous cycles, were obtained from Intervet (Angers, France). The porcine FSH from pituitary extract (pFSH activity = $1.15 \times$ NIH pFSH-P1 activity) used for animal injections was obtained from Dr Y. Combarnous (Nouzilly, France). Purified ovine FSH-20 (lot no. AFP-7028D, 4453 IU/mg, FSH activity = $175 \times$ oFSH-S1 activity) used for culture treatment was a gift from the NIDDK, National Hormone Pituitary Program. Recombinant human IGF-I was a gift from Dr P. Swift (Ciba-Geigy, Saint-Aubin, Switzerland). B2 medium for the isolation of GCs was prepared according to Menezo (Menezo 1976). McCoy's 5a medium with bicarbonate, penicillin, streptomycin, BSA (tissue-culture grade), transferrin, selenium, bovine insulin, androstenedione, trypsin, collagenase A, laminin from the basement membrane of Engelbreth-Holm-swarm mouse sarcoma (laminin-1; Mercurio 1995), fibronectin from bovine plasma, poly-hema and trypan blue were purchased from Sigma (L'Isle d'Abeau Chesnes, France). Hepes, L-glutamine and Fungizone were purchased from Gibco BRL (Cergy-Pontoise, France). Type I collagen (Vitrogen 100) was purchased from Celtrix (Santa Clara, CA, USA), RGD peptides (ProNectin F) from Interchim (Montluçon, France) and heparin from Leo Laboratories (Saint Quentin en Yvelines, France). [3 H]thymidine (specific activity 6.7 Ci/mmol) was purchased from Du Pont De Nemours (Les Ulis, France), and K5 emulsion for autoradiography was obtained from Ilford (St Priest, France). Schiff reagent for Feulgen staining was purchased from Merck (Schuchardt, Germany). Sterile, 96-well culture plates (Nuncclon delta) were obtained from Nunc (Naperville, IL, USA), and plastic tissue-culture chamber slides were obtained from Poly Labo (Strasbourg, France).

Animals

All procedures involving animals were approved by the Agricultural Agency and the Scientific Research Agency (approval number A37801) and conducted in accordance with the guidelines for the care and use of agricultural animals in agricultural research and teaching. Cyclic adult Romanov ewes ($n=92$) were treated with intravaginal sponges impregnated with progestagen (fluorogestone acetate, 40 mg) for 15 days (D1 to D15) to mimic a luteal phase. For the recovery of GCs from large antral follicles (4–7 mm in diameter), ewes were primed with pFSH,

administered intramuscularly 24 h (6 IU) and 12 h (5 IU) before slaughter at D15. Following this treatment, GCs were isolated from four to five healthy large antral follicles per ewe. For the recovery of GCs from small antral follicles (1–3 mm in diameter), ewes were slaughtered in the luteal phase of the following estrous cycle (sponge removal at D15, ovulation at D18 and slaughter at D22), and GCs could be isolated from 10–15 healthy small antral follicles per ewe. Both treatments allowed the recovery of GCs with a viability ranging between 60 and 80%, as estimated by trypan blue exclusion.

Isolation of GCs

Immediately after slaughter, ovaries from three to four ewes were washed in sterile PBS supplemented with Fungizone and antibiotics. The ovaries were then immersed in B2 medium, and follicles were dissected within 1 h after slaughter. Follicular fluid from large follicles was aspirated with a 26-gauge needle, follicles were slit open in B2 medium, and GCs were removed by gently scraping the interior surfaces of the follicles with a platinum loop. GC suspensions were pooled according to the size of the follicles (small and large). Cells from the two resulting cell suspensions were pelleted and resuspended in culture medium (McCoy's 5a containing bicarbonate, supplemented with Hepes (20 mmol/l), penicillin (100 kIU/l), streptomycin (0.1 g/l), L-glutamine (3 mmol/l), BSA (0.1%, w/v), insulin (100 µg/l), androstenedione (10^{-7} mol/l), transferrin (5 mg/l) and selenium (20 µg/l)). The number of living cells in each suspension was estimated with a hemocytometer and trypan blue exclusion.

GC culture

GC culture was performed according to the method described by Campbell *et al.* (1996). Cultures were performed in 96-well plates or in chamber slides that were pre-coated with ECM or synthetic compounds. Uncoated plates or culture chamber slides were used as controls. Cells 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 containing either no exogenous factors or 0.5 or 5 ng oFSH/ml and/or 100 ng IGF-I/ml. Each combination of treatments was tested in triplicate in each culture. Cultures of GCs from large and small follicles were performed for 144 and 192 h respectively. Culture media were partially changed every 48 h, by replacing 175 µl of the total (250 µl) with pre-equilibrated medium. The spent medium was stored at –20 °C prior to assay.

ECM and synthetic compounds tested

Freshly isolated GCs were seeded on pre-prepared substrata of pure ECM components (type I collagen, fibro-

nectin, laminin) or synthetic compounds, such as RGD peptides (Ruoslahti & Pierschbacher 1987, Goxe *et al.* 1995) and poly-hema (Folkman & Moscona 1978), known to (respectively) enhance and impair cell adhesion. Thirty microliters of a solution of fibronectin (diluted in distilled water), laminin (diluted in distilled water), RGD peptides (diluted in PBS) or poly-hema (undiluted) were used to coat culture wells; the coatings were then allowed to dry at room temperature for 48 h and (in the case of the RGD peptides) washed before use. The concentrations of ECM or synthetic compounds were chosen according to preliminary titration experiments (data not shown). In current use, substrata contained 2.8 µg/cm² of fibronectin, 5.0 µg/cm² of laminin, 120 µg/cm² of type I collagen (CI gel), 36 µg/cm² of type I collagen (CI coating), 0.4 µg/cm² of RGD peptides and 10 mg/cm² of poly-hema. The effect of heparin on GC function was assessed by adding 1 mg/ml heparin to the culture medium. Type I collagen (CI, 0.4 mg/ml, diluted in culture medium) was distributed in the culture wells (30 µl for the coating and 100 µl for the gel) and stored in a humidified incubator until use.

Determination of GC numbers

The numbers of cells in 96-well plates were determined 48 h after seeding and at the end of culture (144 and 192 h after seeding for small- and large-follicle GCs respectively). Cells were detached by trypsin treatment followed by collagenase treatment (10 min in 20 mg/ml collagenase A) when grown on type I collagen. The number of cells per well was estimated by counting an aliquot of each resulting cell suspension with a hemacytometer (PolyLabo, Strasbourg, France) under a phase-contrast microscope (Micromecanique, Evry, France).

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

The spreading of GCs on the substratum was estimated by measuring the diameter of the cellular nucleus after 72 h culture. GCs grown on chamber slides coated with the ECM or one of the synthetic compounds or in the presence of heparin were fixed with Bohm-Sprenger fixative (formaldehyde 15%, acetic acid 5%, methanol 80%) at room temperature for 10 min then stained with Feulgen. Cell nuclear diameter was measured on 80 Feulgen-stained cells per well with a reticule under a microscope ($\times 100$ objective).

Proliferation and survival were assessed by measuring the thymidine labeling index (the percentage of labeled cells), the mitotic index (the percentage of mitotic cells) and the pyknotic index (the percentage of pyknotic cells, i.e. cells with condensed or fragmented chromatin; Aharoni *et al.* 1995) after 48 h culture. For the [³H]thymidine-incorporation procedures, GCs grown in 96-well plates were incubated for 2 h with [³H]thymidine

(0.25 µCi/ml, 300 µl/well, prepared in thymidine-free B2 medium), washed with B2 medium, then detached by trypsin/collagenase treatment, fixed in 3% glutaraldehyde for 1–2 h and recovered as smears on histological slides by cytocentrifugation (Watson 1966). Finally, GCs were stained with Feulgen and slides were dipped in Ilford K5 emulsion, air-dried and exposed for autoradiography for 6 d at 4 °C. The thymidine labeling index, the mitotic index and the pyknotic index were determined on smears of GCs by counting the percentages of labeled, mitotic or pyknotic cells in 20 different microscopic fields ($\times 100$ objective) for each culture well, so that the calculations could be based on 1500–3000 cells per well.

Detection of DNA fragmentation

DNA fragmentation was analyzed to characterize the occurrence of apoptosis in our culture conditions. GCs grown on ECM or synthetic compounds were recovered, after 48 h culture, by trypsin/collagenase treatment, pelleted and then stored at –20 °C. DNA was extracted from the cells, subjected to 3'-end labeling with [32 P]-dideoxynucleotide ([32 P]-ddATP, specific activity 3000 Ci/mmol, 3 µCi/tube) by terminal transferase and then analyzed by agarose gel electrophoresis as described previously (Tilly & Hsueh 1993).

Steroid radioimmunoassay

The concentrations of estradiol-17 β and progesterone in culture medium of large-follicle GCs were measured, after 144 h culture, by radioimmunoassay according to a previously described protocol (Saumande 1981, 1985, 1991) adapted to cell-culture medium so that a direct assay of steroids could be performed. The limit of detection for estradiol was 1.5 pg/tube, corresponding to 7.5 pg/well, and intra-assay and interassay coefficients of variation were less than 7 and 9% respectively. The limit of detection for progesterone was 12 pg/tube, corresponding to 60 pg/well, and intra-assay and interassay coefficients of variation were less than 10 and 11% respectively. Steroid concentrations in culture media from each culture were measured in the same assay. Results are expressed as the amount of steroid secreted between 96 and 144 h culture on a per 10^4 cell basis, cell counting being performed after 144 h culture.

Culture protocol design

To maintain GC viability, the time required for GC isolation was such that each culture experiment was performed with pooled GCs recovered from only three to four ewes. As this did not provide a sufficient number of cells to allow comparison of all experimental conditions within a single culture, the different substrata had to be tested in distinct culture experiments. Because of the large 'between-culture' variations in the amounts of steroids

secreted by GCs and in their proliferation rates, uncoated wells were used in all cultures as an 'intraculture' reference. Consequently, the effect of each ECM or synthetic compound was estimated in comparison with uncoated wells (controls) within the same culture.

For analysis of steroidogenesis and changes in GC numbers during culture, a total number of 10 and 9 cultures of GCs from small and large follicles, respectively, were performed. Each ECM or synthetic compound was tested in at least three independent cultures (small-follicle GCs: $n=3$ for CI coating, fibronectin and poly-hema, $n=5$ for RGD peptides and heparin, $n=7$ for laminin, and $n=9$ for CI gel; large-follicle GCs: $n=3$ for RGD peptides and poly-hema, $n=4$ for CI coating, fibronectin and heparin, $n=5$ for laminin, and $n=8$ for CI gel).

Statistical analyses

For cell nuclear diameters, comparisons of means were performed by one-way ANOVA followed by a Dunnett test. For the thymidine labeling index, the mitotic index and the pyknotic index, chi-square analyses were performed to compare each substratum with the control within each culture. In this particular case, a given substratum was considered to have a significant effect (labeled with an asterisk in the figures) when the differences between values obtained with this substratum and control values were found to be statistically significant ($P<0.05$) in all cultures performed ($n=3$ to $n=4$).

For steroid concentrations in culture media and GC numbers, experimental data are presented as the means $\pm$ S.E.M. of measurements from three to nine independent cultures. As stated above, the amounts of steroids produced by GCs were very variable from one culture to another. This variation, considered as a 'culture effect', might result from variations between both animals and the quality of the ovarian follicles dissected for each culture. Consequently, statistical comparisons of means were performed by two-way ANOVA to allow for (1) the 'substratum effect', resulting from the specific regulation of GC steroidogenesis by each ECM or synthetic compound tested, and (2) the 'culture effect'. *Post-hoc* comparisons were performed with the Newman-Keuls test, to compare each substratum with the control.

Results

Modulation of GC shape by ECM

On uncoated substratum, GC cultures exhibited a heterogeneous mix of spread isolated cells and round clustered cells (Fig. 1A) as previously described by Campbell *et al.* (1996). GCs spread on coated type I collagen (Fig. 1D), fibronectin (Fig. 1E) and laminin (Fig. 1F), whereas they adhered to – but retained a rounded shape – on gel of

Figure 1 Morphology of GCs from small follicles cultured for 72 h in control conditions (A), with heparin in culture medium (B), on CI gel (C), on CI coating (D), on fibronectin (E), on laminin (F), on RGD (G) and on poly-hema (H). Bar = 25 μm.

type I collagen (Fig. 1C). The presence of heparin in the culture medium of GCs prevented efficient spreading of GCs on uncoated wells (Fig. 1B). When used as culture substratum, RGD peptides enhanced GC spread (Fig. 1 G), whereas GCs cultured on poly-hema aggregated to form large clusters of round cells resting on the bottoms on the wells (Fig. 1H).

When GCs were cultured on CI coating, fibronectin, laminin, and RGD peptides, their apparent spread was correlated with a significant increase in nuclear diameter measured after Feulgen staining at 72 h of culture (Fig. 2,

$P < 0.01$, except for large-follicle GCs grown on CI coating or RGD peptides). By contrast, when GCs were cultured on CI gel, poly-hema or in the presence of heparin in the culture medium, their apparent rounding was correlated with a decrease in nuclear diameter (Fig. 2, $P < 0.01$, except for small-follicle GCs grown on CI gel).

Modulation of GC proliferation and/or survival by ECM

Under all conditions tested, FSH had no effect on GC proliferation and/or survival (data not shown).

Figure 2 Nuclear diameter of small-follicle GCs (A) and large-follicle GCs (B) after 72 h culture in control conditions (C), on CI gel (CG), on CI coating (CC), on fibronectin (FN), on laminin (LN), on RGD peptides (RGD), on poly-hema (PH), or with heparin in the culture medium (HEP). The data represent means $\pm$ S.E.M. of nuclear diameters measured on 80 nuclei per culture condition in a representative culture. Significant increases (*) or decreases (#) in nuclear diameters compared with controls are indicated. Differences between a given substratum or heparin treatment and control were significant ($P < 0.01$).

Effect of IGF-I and ECM on the proliferation rate of GCs The effect of IGF-I and ECM on GC proliferation was assessed by measuring the DNA replication of subconfluent GCs after 48 h culture. A clear correlation was observed between the thymidine labeling index and the mitotic index (small-follicle GCs: $r = 0.80$, $n = 34$, $P < 0.001$; large-follicle GCs: $r = 0.79$, $n = 36$, $P < 0.001$ – both being measured after 48 h culture), ruling out the possibility that some thymidine incorporation was due to DNA repair.

The previously reported proliferative effect of IGF-I on GCs was confirmed in these experiments and was observed on most substrata, as shown by the twofold increase in the thymidine labeling index of both small- (Fig. 3A) and large-follicle GCs (Fig. 3B) ($P < 0.05$, except for small-follicle GCs grown on poly-hema and for large-

follicle GCs grown on CI gel or with heparin). Accordingly, on all substrata, IGF-I led to an increase in GC numbers at the end of culture, compared with values in IGF-I-free conditions ($P < 0.001$, except for small-follicle GCs grown on poly-hema or with heparin; data not shown).

Fibronectin and laminin dramatically raised the thymidine labeling index of small-follicle GCs in the presence and absence of IGF-I (Fig. 3A, $P < 0.001$), and, to a lesser extent, of large-follicle GCs in the absence (Fig. 3B, $P < 0.05$ on fibronectin, NS on laminin) and in the presence of IGF-I (Fig. 3B, $P < 0.001$). Poly-hema had no significant effect on the proliferation rates of GCs from both large and small follicles. The addition of heparin significantly reduced the thymidine labeling index of small-follicle GCs without IGF-I (Fig. 3A, $P < 0.05$), but had no effect under any other conditions. Accordingly, the numbers of GCs of both types recovered at the end of culture on laminin and fibronectin were higher than the numbers in control wells ($P < 0.001$; data not shown). In the presence of heparin, the numbers of GCs of both types recovered at the end of culture were reduced in comparison with control heparin-free conditions ($P < 0.001$; data not shown).

Effect of IGF-I and ECM on GC survival When small- and large-follicle GCs were cultured on uncoated wells, only about 25% of the seeded cells remained after 48 h culture, indicating either a poor initial adhesion rate or a high occurrence of cell death during the first 48 h culture. IGF-I had no effect on GC numbers during the first 48 h culture, but type I collagen used as gel or coating increased the initial adhesion or survival of GCs from both small ($P < 0.01$ on CI gel; $P < 0.05$ on CI coating) and large follicles ($P < 0.01$ on CI gel and coating), whereas other ECM or synthetic compounds had no effect (data not shown).

To establish whether IGF-I and ECM molecules could alter GC survival, we counted the percentage of pyknotic cells (pyknotic index) in GC culture after 48 h culture (Fig. 4). IGF-I had no effect on the GC pyknotic index under any conditions (data not shown). When compared with the control, laminin and, to a lesser extent, fibronectin reduced the pyknotic index of small- and large-follicle GCs ($P < 0.05$, NS for large-follicle GCs on fibronectin), indicating better survival of GCs when grown on these ECM substrata. By contrast, poly-hema raised the pyknotic index of small-follicle GCs only ($P < 0.05$), suggesting that small-follicle GCs specifically require adhesion to a substratum for their survival. Finally, type I collagen, RGD peptides and heparin had no effect on GC survival during the first 48 h culture (there was no significant change in the GC pyknotic index).

Analysis of DNA fragmentation in cultured GCs showed the presence of small DNA fragments, indicating that the presence of pyknotic cells was associated with apoptosis (Fig. 4C).

Figure 3 Thymidine labeling index (% thymidine-labeled cells) of small-follicle GCs (A) and large-follicle GCs (B) after 48 h culture with (right panel) or without (left panel) IGF-I, in control conditions (C), on CI gel (CG), on CI coating (CC), on fibronectin (FN), on laminin (LN), on RGD peptides (RGD), on poly-hema (PH), or with heparin in the culture medium (HEP). The data represent means $\pm$ S.E.M. of percentages of labeled cells measured from three to four independent cultures. Significant increases (*) or decreases (#) in the percentages of labeled cells compared with controls are indicated. Differences between a given substratum or heparin treatment and controls were considered significant when $P < 0.05$ in all cultures.

Modulation of GC steroidogenesis by ECM

Steroidogenesis of small-follicle GCs is much less active than that of large-follicle GCs (25-fold less estradiol and 100-fold less progesterone; data not shown) and is poorly regulated by FSH, IGF-I or ECM. Consequently, only those results concerning large-follicle GCs are presented here.

Effect of ECM on basal steroidogenesis of GCs GCs grown for 144 h on uncoated wells were able to produce detectable amounts of estradiol and progesterone in the absence of FSH and IGF-I (basal steroidogenesis). CI gel and, to a lesser extent, CI coating raised estradiol secretion (Fig. 5A, $P < 0.001$ for CI gel; Fig. 5C, $P < 0.05$ for CI coating) and reduced progesterone secretion (Fig. 5B, $P < 0.001$ for CI gel; Fig. 5D, $P < 0.01$ for CI coating). By contrast, fibronectin and laminin dramatically reduced estradiol secretion (Fig. 6A and C; $P < 0.001$) and slightly raised progesterone secretion (Fig. 6B, $P < 0.001$ for fibronectin; Fig. 6D, $P < 0.01$ for laminin). RGD peptides partially mimicked the effect of fibronectin and laminin by reducing estradiol secretion (Fig. 7A, $P < 0.001$), but had no effect on basal secretion of progesterone (Fig. 7B). As shown for type I collagen, poly-hema and heparin led to a significant increase in estradiol secretion (Fig. 7C and E, $P < 0.001$) and to a decrease in progesterone secretion

(Fig. 6D, $P < 0.001$ for poly-hema; Fig. 7F, $P < 0.01$ for heparin).

Effect of ECM on GC responsiveness to FSH and IGF-I On uncoated wells, FSH alone modulated estradiol and progesterone secretions in a dose-specific manner, by slightly – but consistently – stimulating estradiol secretion when used at 0.5 ng/ml ($P < 0.05$), and by strongly stimulating (by about 20-fold, $P < 0.001$) progesterone secretion when used at 5.0 ng/ml. IGF-I, alone or combined with FSH, was a strong stimulator of progesterone secretion ($P < 0.001$). IGF-I raised the basal production of estradiol ($P < 0.001$) and potentiated the effect of FSH on estradiol secretion ($P < 0.001$).

Overall, the GC steroidogenic response to FSH and IGF-I was maintained when GCs were grown on ECM or RGD peptides substrata, compared with controls. In contrast, poly-hema abolished the increase in progesterone secretion after FSH and IGF-I treatment ($P < 0.05$).

Discussion

To clarify the roles of ECM in the ovary, we investigated the effects of GC adhesion to ECM on their function *in vitro*. We showed that pure ECM components such as

type I collagen, fibronectin, laminin and heparin are able to alter GC function, even in absence of any exogenous growth factors. Moreover, ECM components regulated GC shape, survival, proliferation and steroidogenesis in a specific and coordinated manner, and these effects could

be at least partly mimicked by synthetic compounds in terms of promoting or impairing cell adhesion.

ECM components could be divided into two distinct groups according to their ability to influence GC shape and function. First, adhesion on fibronectin or laminin ensured GC spread and survival and simultaneously regulated GC function by stimulating proliferation and inhibiting estradiol secretion. RGD peptides were able to promote GC spread, and mimicked, at least partly, the effects of fibronectin and laminin on GC proliferation and steroidogenesis, suggesting the involvement of integrins. The integrin $\alpha 6 \beta 1$ laminin receptor and the integrin $\alpha 5 \beta 1$ fibronectin receptor are expressed at the surfaces of GCs in various species such as the mouse, the pig, the marmoset, the human and the sheep (Giebel *et al.* 1996, Fujiwara *et al.* 1998, Makrigiannakis *et al.* 1998, Le Bellego *et al.* 1999) and are thus potential candidates for the mediation of the effects of these ECM components.

In contrast to the flattened shape of GC spread on fibronectin and laminin, GCs grown on a gel of type I collagen exhibited a rounded shape and their function was simultaneously directed in a estradiol-secreting pathway, resulting in parallel inhibition of progesterone secretion as well as proliferation and survival. Previous studies have shown that type I collagen stimulates GC steroidogenesis *in vitro*, in terms of the secretion of estradiol (human, Bussenot *et al.* 1993), progesterone (rat, Carnegie *et al.* 1988; cow, Luck *et al.* 1991), or both steroids (human, Ben-Rafael *et al.* 1988). The discrepancies in the natures of the steroids induced by type I collagen may be due to differences between species or between the initial states of differentiation of GCs recovered for culture. Interestingly, inhibition of GC spread by poly-hema or heparin led to the same effects on GC function, indicating some close relationships between cell shape and function, as previously described for GCs (Amsterdam & Rotmensch 1987, Ben-Ze'ev & Amsterdam 1987, Ben-Ze'ev *et al.* 1987, Carnegie & Tsang 1988, Tsang *et al.* 1988, Amsterdam *et al.* 1989, Ben-Ze'ev & Amsterdam 1989, Gutiérrez *et al.* 1997) and other cell types (Folkman & Moscona 1978, Ben-Ze'ev 1986, Getzenberg *et al.* 1990,

Figure 4 Pyknotic index (% pyknotic cells) of small-follicle GCs (A) and large-follicle GCs (B) after 48 h culture in control conditions (C), on CI gel (CG), on CI coating (CC), on fibronectin (FN), on laminin (LN), on RGD peptides (RGD), on poly-hema (PH), or with heparin in the culture medium (HEP). IGF-I had no significant effects on the pyknotic index at 48 h of culture, so the 'with IGF-I' data and the 'without IGF-I' data were pooled. The data represent means $\pm$ S.E.M. of percentages of pyknotic cells measured from three independent cultures. Significant increases (*) or decreases (#) in the pyknotic index compared with controls are indicated. Differences between a given substratum or heparin treatment and controls were considered significant when $P < 0.05$ in all cultures. (C) Representative patterns of DNA fragmentation in GCs cultured in control conditions (C), on CI gel (CG), on CI coating (CC) and on poly-hema (PH).

Figure 5 Effects of CI gel (A, B) and CI coating (C, D) on secretion of estradiol (A, C) and progesterone (B, D) by large-follicle GCs, with or without IGF-I, in comparison with controls. The data are expressed as the amounts of steroid secreted in the culture medium per 10⁴ cells between 96 and 144 h culture. The data represent the means $\pm$ S.E.M. of measurements from eight (CI gel) and four (CI coating) independent cultures. An asterisk indicates that steroid levels on CI gel or CI coating are significantly different from those of the controls (see the text for *P* values).

Ingber 1997). For steroidogenic cells such as GCs, it is very likely that cell morphology influences cell polarization and organelle organization through the cytoskeleton, thereby controlling steroid production and secretion (Lawrence *et al.* 1979). Consequently, culture substrata that allow GCs to keep a round shape similar to that exhibited *in vivo* might help in the maintenance of estradiol-secreting activity.

Even if some of the effects of type I collagen gel might be mediated through a modification of cell shape, type I collagen used as a coating also stimulated estradiol secretion and inhibited GC proliferation, despite the fact that it provoked GC spread. This result suggests that, in GCs, type I collagen may promote differentiation signals independent of its effect on cell morphology. *In vivo*, the different type I collagen chains are present in sheep GCs, and levels of immunodetectable type I collagen increase in granulosa layers during terminal follicular growth (Huet *et al.* 1997). Moreover, the presence of both the $\alpha 2$ and $\beta 1$ integrin subunits has been reported in GCs of primates

(Giebel *et al.* 1996, Yamada *et al.* 1999), suggesting that $\alpha 2\beta 1$ integrin, a receptor for type I collagen, is probably functional in ovine GCs. Hence, we hypothesize that the increase in GC contacts with type I collagen that occurs during late follicular maturation might play a role in inducing or maintaining GC differentiation, i.e. in the inhibition of proliferation and in the enhancement of estradiol secretion. Further experiments are needed to determine the mechanisms of action of type I collagen on GCs, and, in particular, to assess the role of integrins in mediating its action.

As discussed above, ECM components were able to modulate basal steroidogenesis of GCs. In addition, the present study investigated whether GC-matrix interactions change GC responsiveness to IGF-I and FSH. A dramatic reduction in the progesterone response to both IGF-I and FSH was observed in GCs grown on polyhema. This loss of GC responsiveness might be the consequence of cell rounding, impaired cell adhesion to the substratum, or enhanced intercellular contacts through

Figure 6 Effects of fibronectin (A, B) and laminin (C, D) on the secretion of estradiol (A, C) and progesterone (B, D) by large-follicle GCs, with or without IGF-I, in comparison with controls. The data are expressed as the amounts of steroid secreted in the culture medium per 10^4 cells between 96 and 144 h culture. The data represent the means $\pm$ S.E.M. of measurements from four (fibronectin) and five (laminin) independent cultures. An asterisk indicates that steroid levels on fibronectin or laminin are significantly different from those of the controls (see the text for *P* values).

the formation of aggregates. Cell rounding induced by culture on CI gel or in the presence of heparin was not associated with such a loss of progesterone secretion by GCs in response to IGF-I and FSH, indicating that changes in cell shape are not critical for GC responsiveness. Interestingly, culture of GCs as aggregates, which enhances GC contacts through cadherins, has been previously shown to be associated with an increase in cell survival (Peluso *et al.* 1996, Makrigiannakis *et al.* 1999) and a desensitization to gonadotrophins (Harandian & Farookhi 1988). Therefore, it can be hypothesized that intercellular contacts have an important modulatory effect on GC luteinization in ovarian follicles.

Our results show that the addition of heparin to the culture medium of GCs induces cell rounding, reduces cell proliferation, enhances estradiol secretion and reduces progesterone secretion. The inhibition of GC spread by heparin indicates that GC adhesion to uncoated culture wells could involve glycosaminoglycans. However, it remains to be determined as to whether the changes in

GC function resulting from heparin treatment are directed by the change in cell shape, or involve different mechanisms. A previous study showed that an increase in glycosaminoglycan synthesis by cultured rabbit GCs, resulting from inhibition of proteoglycan synthesis by β -D-xyloside treatment, similarly reduced cell proliferation and progesterone secretion (Benhaim *et al.* 1995). We hypothesize that heparin treatment of GCs may alter the bioavailability of heparin-binding proteins such as fibroblast growth factor-2 (FGF-2) (Ruoslahti & Yamaguchi 1991). FGF-2 has been shown to stimulate GC proliferation (Savion *et al.* 1981, Gospodarowicz & Ferrara 1989) and progesterone secretion (Gospodarowicz & Ferrara 1989, Roy & Greenwald 1991), and to inhibit estradiol secretion (Adashi *et al.* 1988, Vernon & Spicer 1994), and it is likely that the addition of excess heparin to the medium of GCs would disturb the action of endogenous FGF-2.

The existence of different molecular forms of ECM components such as laminin or fibronectin raises the

Figure 7 Effects of RGD peptides (A, B), poly-hema (C, D) and heparin (E, F) on the secretion of estradiol (A, C, E) and progesterone (B, D, F) by large-follicle GCs, with or without IGF-I, in comparison with controls. The data are expressed as the amounts of steroid secreted in the culture medium per 10^4 cells between 96 and 144 h culture. The data represent the means $\pm$ S.E.M. of measurements from three to four independent cultures. An asterisk indicates that steroid levels on RGD peptides, poly-hema or with heparin are significantly different from those of the controls (see the text for *P* values).

question of the physiological significance of the present results obtained with GCs cultured on laminin-1 or plasma fibronectin. Indeed, for laminin, the association of dif-

ferent isoforms of the α -, β - and γ -subunits into hetero-trimers gives rise to different laminin molecules (Mercurio 1995). In the ovary, laminin-1 ($\alpha 1\beta 1\gamma 1$ structure)

has been immunolocalized in the basal lamina, theca and GCs of ovarian follicles from different animal species (Bagavandoss *et al.* 1983, Bortolussi *et al.* 1989, Leardkamolkarn & Abrahamson 1992, Huet *et al.* 1997, Van Wezel *et al.* 1998). The presence of two additional subunits, $\alpha 2$ and $\beta 2$, in the basal lamina of bovine ovarian follicles (Van Wezel *et al.* 1998), suggests that other laminins such as laminin-2 (merosin), laminin-3 (S-laminin), or/and laminin-4 (S-merosin) may also be expressed. Interestingly, GCs of various animal species express high levels of the specific laminin receptor $\alpha 6\beta 1$ integrin (human, Honda *et al.* 1995; marmoset, Giebel *et al.* 1996; mouse, Nakamura *et al.* 1997; pig, Fujiwara *et al.* 1998; sheep, Le Bellego *et al.* 1999), which binds laminin-1, laminin-2 and laminin-3 with high affinity (Mercurio 1995). Overall, it is suggested that laminin-1 might play an important physiological role in modulating GC functions during follicular development, but the roles of other laminins must now be investigated in the ovary. Fibronectin also exists in different forms arising as a consequence of mRNA alternative splicing occurring in three main regions called ED-A (extra domain A), ED-B (extra domain B) and V (variable) (Yamada 1991). *In vivo*, reverse transcriptase/PCR experiments have recently shown that bovine follicles express ED-A+, ED-A-, ED-B+, ED-B-, V+ and V-fibronectin isoforms (De Candia & Rodgers 2000). In the sheep ovary, immunodetectable plasma fibronectin, lacking the ED-A and ED-B regions, is present in basal lamina, theca and GCs, and the ED-A+ isoform has been found in theca, but not in GCs (Huet *et al.* 1997). The presence of soluble ED-A+ fibronectin has also been reported recently for bovine follicular fluid (Colman-Lerner *et al.* 1999). *In vitro*, both plasma fibronectin (Morley *et al.* 1987, Saumande 1991, Aten *et al.* 1995, Aharoni *et al.* 1997, Sites *et al.* 1996, and the present results) and ED-A+ fibronectin (Colman-Lerner *et al.* 1999) modulate GC functions. Interestingly, in a bovine granulosa cell line, ED-A peptides stimulate DNA synthesis when used in a soluble form, and inhibit cell adhesion on coated culture dishes (Colman-Lerner *et al.* 1999). Further experiments are needed to assess the physiological importance of the different fibronectin isoforms, as well as the respective roles of the soluble forms present in follicular fluid and the insoluble forms deposited in basal lamina and on cell membranes.

In conclusion, the present study provides strong evidence that adhesion of ovine GCs on various pure ECMs *in vitro* can direct GC survival, shape, proliferation and steroidogenesis, in a specific and coordinated manner. *In vivo*, the presence of type I collagen, fibronectin and laminin in GC layers of ovarian follicles suggests that they may play important roles in controlling follicular development. It can be hypothesized that fibronectin and laminin would sustain follicular development by enhancing the

survival and proliferation of GCs, whereas type I collagen might participate in the maintenance of estradiol secretion in large antral follicles.

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