Effects of insulin-like growth factors and binding protein 1 on bovine granulosa cell division in anchorage-independent culture

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Granulosa cells can exhibit the properties of stem cells and tumour cells. Contact with neighbouring cells does not inhibit their replication *in vivo* and they can divide *in vitro* while embedded in agar and thus without anchorage on a substratum. By culturing granulosa cells without anchorage, those cells that do not require anchorage, and thus exhibit at least one property of stem cells, divide. The effects of insulin-like growth factors (IGFs) and insulin-like growth factor binding protein 1 (IGFBP-1) on the replication of such cells was investigated by isolating granulosa cells from follicles (3–5 mm diameter) from cyclic cows and culturing them in soft agar-methylcellulose solution. Cell division was measured as [³H]thymidine incorporation into DNA, total DNA, or as the amount of the nuclear La antigen. This antigen is involved in RNA synthesis and is expressed ubiquitously; here it was used to estimate the numbers of cells indirectly. Both IGF-I (100 ng ml⁻¹) and IGF-II (100 ng ml⁻¹) and their respective analogues, as well as insulin, all at the same concentration, significantly increased DNA synthesis as determined by [³H]thymidine incorporation ($n = 5$). An increase in the number of cells in the presence of IGF-I was also confirmed by DNA measurement ($P < 0.05$, $n = 5$) and by western immunoblotting analyses of La antigen ($n = 3$). IGFBP-1 significantly inhibited cell division stimulated by IGF-I ($P < 0.001$) and IGF-II ($P < 0.001$), but not that stimulated by the analogue, LR³IGF-I, which has low affinity for IGFBPs. Other factors also known to affect IGF synthesis or effectiveness (FSH, oestradiol, growth hormone) did not appear to influence division of granulosa cells when cultured under anchorage-independent conditions, while dibutyryl cAMP significantly inhibited cell division ($P < 0.01$, $n = 5$). In conclusion, IGFs have a role to play in stimulating division of stem-cell-like granulosa cells during follicular development and IGFBP-1 can specifically inhibit the mitogenic effects of IGFs.

Introduction

A growing body of evidence suggests that a number of growth factors control proliferation and maturation of ovarian granulosa cells, among which are the insulin-like growth factor family, their receptors and their binding proteins (reviewed in Adashi *et al.*, 1991). Ovarian granulosa cells have been shown to produce insulin-like growth factors I or II (IGF-I, IGF-II) in a number of species including rats, pigs, cows and humans (Adashi *et al.*, 1991). Furthermore, these cells can produce specific IGF-binding proteins (IGFBPs) (Hsu and Hammond, 1987; Ramasharma and Li, 1987; Voutilainen and Miller, 1987; Mondschein *et al.*, 1990; Adashi *et al.*, 1991; Stanko *et al.*, 1994). IGFBPs consist of at least six identified proteins that can bind IGFs and have the ability to not only regulate the transport of IGFs, but also the presentation of IGFs to cell surface receptors. Several studies have identified various IGFBPs in follicular fluid.

IGFBP-3 has been purified from pig follicular fluid (Ui *et al.*, 1989), while others found it was present in follicular fluid from both small and large follicles. IGFBP-2 has been found in fluid predominantly from small follicles (Mondschein *et al.*, 1991). Shimasaki *et al.* (1991) detected IGFBP-2 to -6 in pig follicular fluid and Stanko *et al.* (1994) have detected IGFBP-2, -3 and -5 in bovine follicular fluid. In *in vitro* studies it has been shown that mRNA encoding IGFBP-1 is expressed in human ovulatory granulosa cells (Koistinen *et al.*, 1990) and that these cells synthesize and secrete IGFBP-1 (Suikkari *et al.*, 1989). The production of one or more of the IGFBPs during either the follicular phase or the luteal phase of the cycle suggests a role in the regulation of IGFs. In particular IGFBPs may mediate IGF action in granulosa cell proliferation and differentiation.

Proliferation of granulosa cells and their specialization into different cell types (basal mural granulosa cells, antral granulosa cells, cumulus cells and corona radiata cells) is an important part of ovarian follicle development. However, many aspects of these processes are unclear. During development from primary to preovulatory stages, granulosa cells proliferate, with their

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mitotic index first increasing and then decreasing (Lussier *et al.*, 1987). It is not known whether either nondividing cells are recruited to divide, or fewer cells cease dividing during early stages of folliculogenesis, to account for the observed increase in the mitotic index. Clearly not all granulosa cells are equal with respect to replication. Granulosa cells also divide while in contact with each other, whereas most cell types in organs of adults, excluding tumour cells and stem cells, are inhibited by contact with neighbouring cells. The only granulosa cells that have anchorage on a substratum are those aligning the basal lamina, and yet mitosis occurs in the inner layers of the membrana granulosa. The ability of some granulosa cells to divide without anchorage and without contact inhibition *in vivo* suggests that some granulosa cells have some properties of stem cells; with the remaining property of pluripotency still to be demonstrated. In support of the idea that some granulosa cells have properties of stem cells, we have shown that they can survive and divide *in vitro* without anchorage (Lavranos and Rodgers, 1994; Lavranos *et al.*, 1994; Rodgers *et al.*, 1995).

Very little is known of the behaviour and regulation of the stem-cell-like granulosa cells. Initial studies have shown that these cells can produce a basal lamina and proteoglycans (Rodgers *et al.*, 1995). Their proliferation can be enhanced by basic fibroblast growth factor (bFGF) and they can be induced to differentiate into luteal cells (Lavranos *et al.*, 1994). Since IGFs and their associated binding proteins are an important component of follicles, the aims of this study were to determine the effects of IGFs, their analogues and IGFBP-1 on the replication of bovine granulosa cells that have the properties of stem cells.

Materials and Methods

Isolation of granulosa cells

The procedure for the isolation of bovine granulosa cells and their subsequent culture under anchorage-independent conditions used in this study was a modification of the method described by Lavranos *et al.* (1994). Briefly, bovine ovaries were collected into ice-cold Hepes-buffered Earle's balanced salt solution without calcium or magnesium (EBSS) from animals killed at a local abattoir. On the basis of the gross appearance of ovarian structures and the reproductive tract, the animals were judged to be cyclic and nonpregnant. Granulosa cells were gently scraped from small and medium-sized follicles (3–5 mm diameter) into Hepes-buffered α -modification minimal essential medium (α -MEM; Code 50-112-PA; Cytosystems, Castle Hill, NSW), containing 5 μ g DNase ml⁻¹ (DN-25; Sigma Chemical Co., St Louis, MO). The cells were washed twice, centrifuged at 200 g for 10 min and then resuspended in 2 ml medium and mechanically dispersed by syringing gently through a 27 gauge needle and then filtered through a sterile gauze filter. Cells were washed twice in fresh media without DNase and resuspended in 1–2 ml medium. The proportion of viable cells and yields were determined by counting cells in a haemocytometer in 0.2% (w/v) Trypan blue. Cells were then placed into anchorage-independent culture as described below.

Anchorage-independent culture

For anchorage-independent culture in 96-well plates (Falcon; Becton Dickinson and Co., NJ), 100 μ l 0.5% (w/v) agar in

culture medium (α -MEM with 10% fetal calf serum) was added to each well at 42°C and allowed to gel at room temperature. To this a 10 μ l aliquot of isolated granulosa cells in suspension (2×10^4 viable cells) was added and overlaid with 50 μ l 1% (w/v) methylcellulose (Methylcell; Dow Chemical Co., MI) solution in culture medium. For anchorage-independent culture in 35 mm plates (Corning; Corning Glass Works, Corning, NY), the plates were set up as described by Lavranos *et al.* (1994). The cells were cultured under control conditions or with the addition of various growth factors alone or in combination and included: 1 mmol dibutyryl cAMP l⁻¹ (Sigma), 100 ng insulin ml⁻¹ (crystalline porcine; Eli Lilly Pty Ltd, Indianapolis, IN), 10, 30, 100 or 300 ng human IGF-I ml⁻¹, IGF-I analogues with low affinity for IGFBPs (Francis *et al.*, 1992): 100 ng longR³insulin-like growth factor I ml⁻¹ and 100 ng des(1-3) IGF-I ml⁻¹ or 10, 30, 100 or 300 ng human IGF-II ml⁻¹ and its analogue (also with low affinity for IGFBPs) des(1-6) IGF-II at 100 ng ml⁻¹ (IGF-I, IGF-II and analogues were from Gropep Pty Ltd, Adelaide, SA), 2 μ g IGFBP-1 ml⁻¹ (purification of IGFBP-1 is described below), 100 ng ovine FSH ml⁻¹ (NIADDK-oFSH-17; NIADDK-NIH Bethesda, MD), 100 ng ovine growth hormone ml⁻¹ (GH; NIADDK-oGH-15), or 100 ng oestradiol ml⁻¹ (Sigma Chemical Co). Maximum effects of IGF-I were observed at 100 ng ml⁻¹ and so this dose was used for the other related factors. The dose of oestradiol was chosen as being a maximum concentration found in follicular fluid, and high doses of FSH and GH were examined since the half lives of these proteins in culture have not been defined. The granulosa cells were cultured for a total of 3–4 days (96-well plates, for the [³H]thymidine incorporation experiments) or 7 days (35-mm plates, for the total DNA experiments) at 37°C in a humidified atmosphere of 7% O₂:10% CO₂:83% N₂. Cell morphology during culture was examined by light microscopy.

Purification of human insulin-like growth factor binding protein 1

The purification of human IGFBP-1 from amniotic fluid, collected for genetic amniocenteses between 16 and 18 weeks' gestation, was adapted from procedures described by Busby *et al.* (1988) and Wang *et al.* (1990). An initial precipitation with ammonium sulfate (66 g added to 460 ml, 27 000 g, 20 min, 4°C) was discarded. A second precipitation (55% (w/v) ammonium sulfate) was suspended in 0.025 mol sodium phosphate buffer l⁻¹, pH 7.1, and subjected to ion exchange chromatography (DEAE-Sepharcel 2.2 cm $\times$ 15 cm column; sequentially eluted with 0.1 mol l⁻¹, 0.2 mol l⁻¹ and 0.5 mol l⁻¹ NaCl in 0.25 mol sodium phosphate buffer l⁻¹, pH 7.1; Amrad Pharmacia Biotech, North Ryde, NSW). The eluate was monitored at an absorbance of 280 nm, and IGF binding capacity was measured by the method of Clemmons *et al.* (1986) and immunoreactive IGFBP-1 was measured by radioimmunoassay (O'Leary and Longley, 1994). Fractions containing IGFBP-1 in the 0.2 NaCl mol l⁻¹ eluate were pooled, and the concentrations of sodium phosphate, pH 7.1, and NaCl were adjusted to 0.2 mol l⁻¹ and 0.25 mol l⁻¹, respectively. The IGFBP-1 was further purified by hydrophobic interaction chromatography (phenyl-Sepharose CL-4B 2.2 cm $\times$ 30 cm column; Amrad Pharmacia Biotech). The column was washed with 15 ml 0.01 mol sodium phosphate l⁻¹/5% (v/v) isopropanol and then developed with distilled

water. Fractions containing immunoreactive IGFBP-1 were resuspended in 0.01 mol sodium phosphate l^{-1} , pH 7.1, before a final purification by gel chromatography (Sephacryl S-200 0.7 cm $\times$ 60 cm column; Amrad Pharmacia Biotech). The recovery of IGFBP-1 from amniotic fluid was 12%. The isoelectric point was determined to be 4.3. SDS-polyacrylamide gel electrophoresis after reduction of samples with 2-mercaptoethanol (5% v/v) showed only a single band of purified protein with a molecular mass of 32–34 kDa.

[^3H]thymidine incorporation assay

The procedure for the assay of [^3H]thymidine incorporation into DNA under anchorage-independent conditions was performed as detailed by Lavranos and Rodgers (1994). Briefly, after 48 h of culture, 0.1 μCi [^3H]thymidine (ICN) in a volume of 10 μl was added to each well of a 96-well plate, under sterile conditions and the cells were cultured for a further 24 h. The cells were harvested by first solubilizing the agar by a modification of the method of Vogelstein and Gillespie (1979) originally used to recover DNA fragments from electrophoresis agarose gels. A solution (100 μl) saturated with both sodium iodide and sodium sulfite (167 g NaI, 31 g Na_2SO_3 , 92 ml H_2O , stirred and filtered) and also containing 10 mmol EDTA l^{-1} (diluted from 1 mol l^{-1} stock, pH 8), was added to each well and incubated at 55°C for 1 h to solubilize the agar. The cells were then harvested onto glass filter paper (Titertek, ICN Biomedicals Inc., Costa Mesa, CA) using a 96-well cell harvester (INOTECH, Biosystems International, Lansing, MI) and the radioactivity measured using a programmable counter (Matrix 9600, Direct Beta Counter, Packard Instrument Co. Inc., Meriden, CT). Each treatment was performed in replicates of eight and each experiment was performed three to five times as indicated. The results are reported as c.p.m. of [^3H]thymidine incorporated into DNA over 24 h per 6×10^4 cells originally seeded.

Total DNA estimation

The total DNA content per 35 mm dish was determined using the method of Labarca and Paigen (1980) and as described by Lavranos *et al.* (1994). Briefly, recovered cell pellets were resuspended in 1 ml EDTA (2 mmol l^{-1}) in 0.05 mol l^{-1} phosphate buffer, pH 7.4, then sonicated for 30 s. An aliquot of this mixture was added to an equal volume of 1 μg Hoechst dye 33258 ml $^{-1}$ (Code B288; Sigma) in the same phosphate buffer. After equilibration, the fluorescence of each sample was recorded at a wavelength of 455 nm, using an excitation wavelength of 355 nm. The standard used was calf thymus DNA (Code D1501, Type 1; Sigma).

Western immunoblotting of La antigen

Granulosa cells were collected before culture (day 0) or after culture for 7 days under control conditions or in the presence of 1 mmol dibutyl cAMP l^{-1} or 100 ng IGF-I ml $^{-1}$. Cells were homogenized in 200 μl buffer (containing: 58.35 mmol Tris l^{-1} , pH 6.8, 2% (w/v) SDS, 2% (v/v) 2- β mercaptoethanol, 1 mmol EDTA l^{-1} , 6 mol urea l^{-1} and 1 mmol phenylmethylsulfonylfluoride l^{-1}). Particulate matter was removed by centrifugation (12 000 g for 3 min) and the supernatants (equal volumes from each treatment) were subjected to SDS-PAGE under reducing conditions (Laemmli, 1970). The separated

proteins were electroblotted onto nitrocellulose-coated nylon membrane (Hybond C-Extra; Amersham, North Ryde, NSW) (20% (v/v) methanol, 10 mmol Tris l^{-1} , 150 mmol glycine l^{-1} solution, 4°C, 175 mA, 16 h). Membranes were incubated in buffer (0.01 mol Tris-buffered saline l^{-1} , 0.2% (v/v) nonidet P40, 5% (w/v) BSA) for 45 min at 37°C, and then for 2.5 h at room temperature with specific monoclonal antiserum to the bovine La antigen, and then for 45 min with ^{125}I -radiolabelled goat-anti-rabbit IgG (Sigma), 10^6 c.p.m. ml $^{-1}$, labelled by the method of Thorell and Johansson (1971). Autoradiograms of the immunoblots were developed using Kodak XAR film.

Statistical analyses

Data were analysed by analysis of variance (ANOVA) and Student–Newman–Keuls' tests. Linear regression analyses were also carried out to show a positive relationship between log of the dose of growth factor and the mitotic response of the cells. All statistical analyses were carried out using the 'Statistical Package for the Social Sciences' (SPSS International BV, The Netherlands).

Results

Cell behaviour

All increases in [^3H]thymidine incorporation, DNA content or La antigen in response to IGFs were observed by microscopy to be accompanied by increases in size of colonies and hence number of cells. In the presence of dibutyl cAMP the colonies had very few cells and these cells hypertrophied.

Effects of insulin-like growth factor I under anchorage-independent conditions

Time course and dose of insulin-like growth factor I. Granulosa cells were cultured in anchorage-independent conditions with or without treatments (100 ng IGF-I ml $^{-1}$, 1 mmol dibutyl cAMP l^{-1}) and the amount of [^3H]thymidine incorporated into DNA was determined for each 24 h period ($n = 2$). Since the absolute values of [^3H]thymidine incorporation varied between the two experiments the data were not combined, and the results of one experiment are shown (Fig. 1). The results of the other experiment were similar. The rate of [^3H]thymidine incorporation by granulosa cells under anchorage-independent conditions was constant for the first 48 h of culture and then increased (Fig. 1). IGF-I enhanced the rate above that of controls after 48 h of culture (Fig. 1). In subsequent experiments, the granulosa cells were radiolabelled with the [^3H]thymidine between 48 h and 72 h. Dibutyl cAMP inhibited [^3H]thymidine incorporation after day 3 of culture. In three separate experiments, there was a significant stimulatory effect of IGF-I on the amount of [^3H]thymidine incorporated by granulosa cells (Fig. 2). This was dose-dependent and linearly related to the log of the dose of IGF-I (linear regression $P < 0.01$). Near maximum stimulation was achieved at 100 ng ml $^{-1}$ (Fig. 2). This dose was therefore used in subsequent experiments.

Insulin-like growth factor I, insulin-like growth factor I analogues and insulin-like growth factor binding protein 1. The results from five experiments examining the effects of these factors on

Fig. 1. Time course of [^3H]thymidine incorporation into DNA by bovine granulosa cells cultured *in vitro* in either control medium or with the addition of dibutyryl cAMP (dbcAMP; 1 mmol l^{-1}) or insulin-like growth factor I (IGF-I; 100 ng ml^{-1}). The results are from one representative experiment of two carried out and the values are means $\pm$ SD of eight replicates of c.p.m. of [^3H]thymidine incorporation per 6×10^4 cells seeded at 0 h. The radiolabelling period is the time period in culture that the cells were exposed to [^3H]thymidine.

[^3H]thymidine incorporation are shown (Fig. 3). The dose used for all the IGFs and analogues was 100 ng ml^{-1} as this dose gave near maximum stimulation of IGF-I and IGF-II. Both IGF-I and long 3 IGF-I stimulated a significant ($P < 0.01$) three- to fivefold increase in [^3H]thymidine incorporation above that of controls. IGFBP-1 significantly ($P < 0.001$) inhibited the stimulatory effect of IGF-I, its inhibitory effect by itself was small and not significant. To demonstrate that the inhibitory effect of IGFBP-1 on IGF-I action was specific, its effect on long 3 IGF-I, an analogue of IGF-I with minimal binding to IGFBPs (Francis *et al.*, 1992), was examined. IGFBP-1 had no effect on the stimulatory effect of long 3 IGF-I ($108 \pm 10\%$ of the mean $\pm$ SEM concentrations observed with long 3 IGF-I alone, $n = 3$), suggesting that the inhibitory effect of IGFBP-1 on IGF-stimulated cell division was via its ability to bind IGF-I. In another set of experiments (Fig. 4), IGF-I increased the total content of DNA (per dish) but the effect was not significant. The analogues des(1-3)IGF-I and long 3 IGF-I significantly ($P < 0.05$) increased the total content of DNA.

La antigen is a nuclear enzyme involved in RNA synthesis (Chan and Tan, 1987) and thus is ubiquitously expressed, and can be used indirectly to measure the number of cells or nuclei. In all three experiments (Fig. 5) the amounts (per dish) of La antigen were substantially increased in the presence of IGF-I above that of controls.

The effects of insulin-like growth factor II on granulosa cells cultured under anchorage-independent conditions

IGF-II stimulated [^3H]thymidine incorporation by granulosa cells cultured under anchorage-independent conditions in a dose-dependent manner (ANOVA, $P < 0.0001$) linearly related to the log of the dose of IGF-II (linear regression $P < 0.05$). Near maximum stimulation was observed at 100 ng ml^{-1} (Fig. 2). This dose was used in subsequent experiments. Both IGF-II ($P < 0.001$) and des(1-6)IGF-II ($P < 0.01$) significantly

Fig. 2. The dose-response effects of IGF-I or IGF-II on [^3H]thymidine incorporation into DNA by bovine granulosa cells cultured under anchorage-independent conditions *in vitro*. Cells were cultured for 3 days under control conditions or with the addition of either insulin-like growth factor I (IGF-I; 10, 30, 100 or 300 ng ml^{-1}) or IGF-II (10, 30, 100 or 300 ng ml^{-1}). [^3H]thymidine was present from 48 h to 72 h of culture and the results are expressed as c.p.m. incorporated per 6×10^4 cells seeded at 0 h. The results from three separate experiments (1, 2, and 3) are shown and the values are means $\pm$ SD of eight replicates.

stimulated threefold the incorporation of [^3H]thymidine into DNA by granulosa cells cultured under anchorage-independent conditions (Fig. 3). IGFBP-1 significantly ($P < 0.001$) inhibited the stimulatory effect of IGF-II (Fig. 3).

Effects of other factors

Insulin stimulated a significant ($P < 0.05$) twofold increase in the amount of [^3H]thymidine incorporation into DNA (Fig. 3). A high dose of FSH either alone or in combination with oestradiol had no significant effect ($P > 0.05$) (Fig. 6). FSH did not augment the stimulatory effect of IGF-I (Fig. 6). Oestradiol or ovine GH also had no significant effect ($P > 0.05$) on the incorporation of [^3H]thymidine by granulosa cells cultured under anchorage-independent conditions (Fig. 6).

Dibutyryl cAMP significantly ($P < 0.01$) and substantially inhibited [^3H]thymidine incorporation into DNA (Fig. 3), particularly later in culture (Fig. 1). The concentrations of La antigen were similar to those in control groups (Fig. 5).

Discussion

Stem cells and tumour cells can divide under anchorage-independent culture conditions, and we have shown that a

Fig. 3. The effects of factors on [³H]thymidine incorporation into DNA by bovine granulosa cells cultured under anchorage-independent conditions *in vitro*. Cells were cultured for 3 days in either control medium or with the addition of growth factors or other peptides including dibutyl cAMP (dbcAMP; 1 mmol l⁻¹), insulin (100 ng ml⁻¹), insulin-like growth factor I (IGF-I; 100 ng ml⁻¹), long³insulin-like growth factor I (LR³IGF-I; 100 ng ml⁻¹), insulin-like growth factor II (IGF-II; 100 ng ml⁻¹), des(1-6)insulin-like growth factor II (Des(1-6)IGF-II; 100 ng ml⁻¹), and insulin-like growth factor binding protein 1 (BP1; 2 µg ml⁻¹). [³H]thymidine was present from 48 h to 72 h of culture and the results are expressed as c.p.m. of [³H]thymidine incorporated per 6 × 10⁴ cells seeded at 0 h. Results are from five experiments (eight replicates per experiment), means ± SEM; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, significantly different from controls.

proportion of bovine granulosa cells from small antral follicles can also divide under these conditions. IGF-I, in a dose- and time-dependent manner, increased division of these cells as measured by the amount of [³H]thymidine incorporated into DNA, the concentration of DNA and the concentration of nuclear La antigen. This effect was inhibited by IGFBP-1. IGF-II and insulin also stimulated mitosis of granulosa cells under anchorage-independent conditions, whereas other hormones, including FSH, GH and oestradiol, at set doses, had no effect on division of these cells under anchorage-independent conditions.

The current state of knowledge of the granulosa cells that have properties of stem cells is rudimentary. Granulosa cell replication is not contact inhibited, certainly before antrum formation, because all the cells are tightly packed together and yet still divide. After antrum formation, there is the potential for granulosa cells that would otherwise be inhibited by cell contact to divide, because there is more intercellular space, particularly in the layers closest to the antrum. Whether this occurs has still to be determined. The progression from contact-uninhibited to contact-inhibited cell division is the natural progression (except during tumourogenesis) in fetal development and in adult organs where stem cells reside. It is therefore possible that a similar progression of cell maturation occurs during folliculogenesis. The availability of anchorage is

also limited in follicles to the most basal layer of granulosa cells that align the basal lamina. Since, in cattle, the membrana granulosa is not pseudostratified, none of the inner layers of granulosa cells have anchorage, yet cells in these areas divide. When most cells are denied anchorage they either cease dividing (fibroblasts) or undergo apoptosis (endothelial cells, epithelial cells), but clearly a proportion of granulosa cells have the ability to divide without anchorage both *in vivo* and *in vitro*. On the basis of the results presented here and elsewhere, it is known that both bFGF (Lavrano *et al.*, 1994), a growth factor found in bovine oocytes (Van Wezel *et al.*, 1995), and IGF-I and IGF-II can stimulate the replication of these cells under anchorage-independent conditions. The results reported here show that both IGF-I and IGF-II stimulate division of granulosa cells under anchorage-independent conditions, and these effects are likely to be mediated via IGF-I receptors. IGF-I receptors have similar affinities for both IGF-I and IGF-II, but much lower affinity for insulin (Baxter, 1988). In a similar ranking of activities, we observed that IGF-I and IGF-II were approximately equipotent and insulin less potent at equal doses. IGF-I receptors are expressed on bovine granulosa cells, although the role of these receptors is probably twofold: to increase mitogenesis and to increase steroidogenesis. IGF receptor concentrations are some 20 times greater in preovulatory follicles (Spicer *et al.*, 1994) when steroidogenesis is

Fig. 4. The mean $\pm$ SEM amounts as a percentage of control amounts per dish of DNA after culture of bovine granulosa cells under anchorage-independent conditions. The control DNA content was $5.35 \pm 0.8 \mu\text{g}$ per dish. Granulosa cells were cultured for 7 days in 35 mm dishes under either control conditions or with the addition of insulin-like growth factor I (IGF-I; 100 ng ml^{-1}), or the IGF-I analogues: des(1-3)IGF-I (100 ng ml^{-1}), or LR³IGF-I (100 ng ml^{-1}). * $P < 0.05$, significantly different from controls ($n = 5$, three replicates per experiment).

maximal and mitogenesis of granulosa cells is declining on a per cell number basis (Lussier *et al.*, 1987). A role for insulin receptors in regulating mitosis of granulosa cells under anchorage-independent conditions cannot be entirely ruled out, even though insulin was much less effective than the IGFs. Gong *et al.* (1993) observed small mitogenic effects at low doses of insulin (0.05 ng ml^{-1}) on bovine granulosa cells cultured on a substratum. These results suggest that granulosa cells express insulin receptors. Whether the mitogenic effect of activating these insulin receptors is direct, or occurs via enhanced production of IGFs or their receptors requires further investigation.

The ligand that activates bovine granulosa cell IGF type I receptors *in vivo* is most likely IGF-I, at least at some stages of follicular development, since IGF-I is produced by bovine granulosa cells (Spicer *et al.*, 1988). However, a role for IGF-II during follicular development and the luteal phase remains to be determined. The presence of IGF-II in follicular fluid in a number of species including humans (Ramasharma *et al.*, 1986) and sheep (Monget *et al.*, 1993) strongly suggests a role for IGF-II. In human ovaries IGF-II is produced by granulosa cells (Ramasharma and Li, 1987) rather than the theca cells as in most other species studied. Evidence in rats suggests that IGF-II plays a role in granulosa differentiation (Davoren *et al.*, 1986; Adashi *et al.*, 1990). Teissier *et al.* (1994) have identified

IGF-II receptors at high concentrations in granulosa cells of atretic follicles and also in theca of healthy follicles in sheep. In addition, they found low concentrations of IGF-II receptors in granulosa cells of healthy follicles and in theca cells of atretic follicles. This information provides support for a role of IGF-II in follicular development. The presence of IGF-II in bovine follicles and its ability to traverse the basal lamina remains to be demonstrated in bovine follicles.

Various IGFBPs have been identified within follicular fluid. Of the six IGFBPs identified to date, IGFBP-2 to -6 have been identified in pig follicular fluid (Shimasaki *et al.*, 1991) and IGFBP-2, -3 and -5 are found in bovine follicular fluid (Stanko *et al.*, 1994), although the origin of these binding proteins remains to be determined. In rat ovaries, mRNAs encoding IGFBP-2 and IGFBP-3 were detected in the thecal interstitial cells and mRNA encoding IGFBP-4 was localized almost solely to the granulosa cells of atretic follicles (Nakatani *et al.*, 1991). We investigated the effect of IGFBP-1 as this is expressed by human granulosa cells just before ovulation and by luteinizing human granulosa cells *in vitro* (Suikkari *et al.*, 1989; Koistinen *et al.*, 1990). Since granulosa cells cease dividing at ovulation as they luteinize (Lussier *et al.*, 1987), IGFBP-1 has been postulated to inhibit the mitogenic effect of IGF-I at ovulation. The expression of IGFBP-1 has not been examined in bovine ovaries but it was found to inhibit the mitogenic effect of IGF-I. Moreover, the mitogenic effect of IGF-I was specific as it failed to inhibit the effect of the IGF-I analogue longR³IGF-I. Further studies, awaiting the isolation of probes for bovine IGFBP-1, are required to investigate the physiological role of IGFBP-1 in bovine ovaries.

These investigations into the effects of FSH, GH and oestradiol on proliferation of granulosa cells under anchorage-independent conditions were not exhaustive, using only one dose of each. However, no effects of FSH, alone or in combination with IGF-I, GH or oestradiol were observed. Other research suggests that gonadotrophins may condition the cell to respond maximally to IGF-I. In rats, for example, FSH can upregulate IGF-I binding and this was shown to be amplified by GH but not prolactin, both *in vitro* (Adashi *et al.*, 1986) and *in vivo* (Adashi *et al.*, 1988). Others have postulated a synergistic interaction between IGF-I and FSH in inducing proliferation of granulosa cells from small follicles *in vitro* (Gong *et al.*, 1993); however, our results showed that there was no synergy under anchorage-independent conditions. Using bovine granulosa cells, Langhout *et al.* (1991) have shown, as we have under anchorage-independent conditions, that oestradiol, FSH and GH alone have no effect on granulosa cell proliferation *in vitro*.

Dibutyryl cAMP substantially inhibited the incorporation of [³H]thymidine into DNA by granulosa cells cultured without anchorage and induced the cells to hypertrophy. Under similar culture conditions, the cells developed the ultrastructural morphology of large luteal cells (Lavarano *et al.*, 1994) and the concentrations of steroidogenic enzymes increased 50-fold (Rodgers *et al.*, 1996). Taken together these observations suggest that the cells stopped dividing and differentiated in response to dibutyryl cAMP.

In summary, our findings support the view that there is a role for IGFs in stimulating the division of bovine stem-cell-like granulosa cells during follicular development. In addition,

Fig. 5. Western immunoblots from three replicate experiments (1, 2, 3) developed using antiserum against La antigen. Bovine granulosa cells were collected before incubation (P) or after incubation under control conditions (C) or in the presence of dibutyl cAMP (cA; 1 mmol l^{-1}) or insulin-like growth factor I (I; 100 ng ml^{-1}) for 7 days. The cells were then homogenized and equal volumes of each homogenate were loaded onto each lane. The size (kDa) and positions of the molecular weight standards are indicated. The arrowhead indicates the position of the La antigen.

Fig. 6. The effects of FSH, oestradiol (E_2) and growth hormone (GH) on [^3H]thymidine incorporation into DNA by bovine granulosa cells cultured under anchorage-independent conditions *in vitro*. Cells were cultured for 3 days either in control medium or with the addition of insulin-like growth factor I (IGF-I; 100 ng ml^{-1}), FSH (100 ng ml^{-1}) alone or FSH with IGF-I or oestradiol (E_2 ; 100 ng ml^{-1}) or with oestradiol alone or growth hormone (GH; 100 ng ml^{-1}) alone. [^3H]thymidine was present from 48 h to 72 h of culture and the results are expressed as c.p.m. of [^3H]thymidine incorporation per 6×10^4 cells seeded at 0 h. Results are from three experiments (eight replicates per experiment), means $\pm$ SEM. Bars with different superscripts (a, b) are significantly different from each other, $P < 0.05$.

IGFBP-1 has the ability to inhibit these mitotic effects of IGFs, further suggesting that IGFBPs are intricately involved with the regulation of the effects of IGF-I.

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