Ovine follicular fluid inhibits thymidine incorporation by 3T3 fibroblasts *in vitro*

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Summary. The ability of antral follicular fluid obtained from sheep follicles to inhibit 3T3 fibroblasts maintained for 48 h in concentrations of fetal calf serum optimal for cell growth was examined. Addition of pooled follicular fluid to cultures resulted in a dose-dependent and reversible inhibition of [^3H]thymidine incorporation. Serum from ovariectomized ewes, fetal calf serum, bovine inhibin, oestradiol-17 β , testosterone, cortisol or progesterone were without effect over a range of doses. Treatment of pooled follicular fluid with charcoal–dextran did not reduce inhibitory activity which was only partly removed by heating at 85°C. Fluid obtained from large follicles (> 5 mm) was more potent as an inhibitor than was fluid obtained from smaller (< 5 mm) follicles. Gel chromatography of pooled fluid resolved two peaks of inhibitory activity associated with material of M_r ~180 000 and <10 000 respectively. No inhibitory activity was evident in fractions of serum from ovariectomized ewes chromatographed in an identical manner. These results indicate that ovine follicular fluid contains two components able to inhibit reversibly mitosis of 3T3 fibroblasts *in vitro*.

Keywords: fibroblast mitosis *in vitro*; inhibition; ovarian follicular fluid

Introduction

Marked changes in functional capacity and mitotic activity of thecal and granulosa cells occur as ovarian follicles grow towards ovulation. Although the exact relationship, if any, between mitosis and differentiation of follicle cells is uncertain, increased cell mass is thought to be necessary for adequate follicular and luteal steroid secretion. In women (McNatty *et al.*, 1979), as in sheep (Tsonis *et al.*, 1984) and mice (Pedersen, 1970), granulosa cell proliferation takes place in the late pre-antral and early antral phases of development with very little increase in granulosa cell number in healthy follicles beyond those stages. This is said to reflect reduced mitotic activity of granulosa cells (Hirshfield & Midgley, 1978) and suggests that mitotic activity of granulosa cells might be regulated by factors present in antral fluid.

One possible explanation of reduced mitotic activity of granulosa cells is the presence of an inhibitor of serum-derived mitogens in the antral fluid of large preovulatory follicles. We therefore examined the ability of antral fluid obtained from sheep follicles to inhibit [^3H]thymidine incorporation by 3T3 cells maintained in concentrations of serum optimal for cell growth.

Materials and Methods

Collection and processing of ovine follicular fluid (oFF). Ovaries were obtained from adult sheep within 10 min of slaughter and placed in ice-cold phosphate-buffered saline (PBS: 0.154 M-NaCl, 0.05 M-phosphate, 100 units kanamycin sulphate/ml). All subsequent processing was carried out aseptically at ice temperature. Ovaries were rinsed briefly in ethanol (70% v/v; 30 sec) and subsequently in fresh PBS. Follicular fluid was aspirated from all visible

follicles over a 2-h period using a 21-gauge hypodermic needle under gentle vacuum, pooled according to diameter of follicle (< 5 mm; > 5 mm) and centrifuged to remove granulosa cells (250 g, 15 min, 4°C). Cell-free follicular fluid was stored at -20°C for various periods before thawing, filter sterilization (0.22 µm) and addition to 3T3 cell cultures.

As indicated, 1-ml samples of unfractionated oFF or chromatographic fractions of oFF were heated at 85°C for 15 min, cooled to ice temperature and centrifuged (2500 g) to remove heat-precipitated material before addition to 3T3 cells.

Cells and culture. Swiss mouse embryonic lung fibroblasts (3T3; Flow Laboratories, North Ryde, NSW) were maintained in tissue culture flasks (Costar, Cambridge, MA, U.S.A.) containing medium (DMEM; Dulbecco's minimal essential medium) supplemented with fetal calf serum (FCS; 2.5% v/v), non-essential amino acids, glutamine (2 mM) and antibiotics (100 units streptomycin sulphate/ml; 100 µg penicillin/ml) and passaged at intervals which did not permit confluence to be reached. Cells were harvested by trypsinization, washed twice and resuspended at 5×10^4 cells/ml medium containing FCS (5% v/v) and [3 H]thymidine (20 nM; sp.act. 0.4 Ci/ml; Amersham International, Amersham, U.K.). Aliquants (100 µl) of this suspension were transferred to individual culture wells and the total volume of each well was adjusted to 200 µl by addition of test substance or medium alone (0% FCS). After 48 h at 37°C in an humidified atmosphere of CO₂ (5%) in air, media were removed by aspiration and cells lysed in 10% (w/v) trichloroacetic acid (TCA). Radioactivity in TCA-insoluble material was determined and expressed as a percentage of that in cultures to which no test substance had been added (control). Estimates of relative potency were based on comparison of dose-response curves obtained from at least 3 doses of test substance using regression analysis (Finney, 1964; Borth, 1976).

To examine whether the inhibitory effect of oFF on [3 H]thymidine incorporation by 3T3 cells was reversible, cells were grown for 48 h in complete culture medium (2.5% FCS DMEM) supplemented with oFF (20% v/v), washed, and subsequently grown for a further 48 h in complete medium supplemented with [3 H]thymidine only. Thymidine incorporation was compared to that in cells grown for 96 h in the presence of oFF (20% v/v) or for 96 h in complete medium alone. In all cases, [3 H]thymidine was present for the final 48 h of culture.

Chromatography. Pools (50 ml) of serum from ovariectomized ewes or oFF from follicles of all sizes were chromatographed on Sephacryl S-200 (5 × 100 cm) in ammonium acetate (0.1 M, pH 7.0) at 4°C. Fractions of 10 ml were collected, combined to form pools of 10 consecutive fractions and lyophilized. Absorbance of the eluant was monitored continuously at 280 nm. Lyophilized material was reconstituted in culture medium and the pH adjusted to 7.2 when necessary before addition to 3T3 cells. For studies of heat and trypsin sensitivity, fractions 20-60 (pool I), 80-120 (pool II), and 140-170 (pool III) were combined to form three separate pools.

Heat and charcoal-dextran treatment. Pooled oFF was divided into aliquants and subjected to charcoal-dextran (0.2% Norit-A, 0.02% Dextran-T70, w/v) or heating at 85°C for 5-30 min. Pools I, II and III from S-200 chromatography of oFF were similarly heated or exposed to agarose-immobilized trypsin at 37°C for 1 h. All treated material was centrifuged to remove charcoal, heat-precipitated matter or agarose-trypsin before addition to 3T3 cell cultures. Inhibitory activity in treated samples was compared to that in untreated oFF or untreated chromatographic fractions as appropriate.

Hormones. Purified inhibin (*M*, 31 000) was isolated from pooled bovine follicular fluid by gel chromatography, reversed-phase high-pressure liquid chromatography and preparative polyacrylamide gel electrophoresis (Robertson *et al.*, 1986). Biological activity of this preparation was confirmed by suppression of follicle-stimulating hormone secretion by rat pituitary cells *in vitro* (data not shown).

Steroids were purchased commercially (Steraloids, Wilton, NH, U.S.A.) and dissolved in ethanol to give stock solutions of 2.5 mM. Serial dilutions of stock solutions in culture medium were prepared such that the final concentrations of ethanol in cell cultures was less than 0.002% (v/v).

Results

Addition of FCS to cells plated in the absence of FCS resulted in a dose-dependent stimulation of [3 H]thymidine incorporation (Fig. 1). Over 3 similar tests, maximal [3 H]thymidine incorporation was observed at FCS concentrations of 2.5% (v/v). For this reason, all subsequent experiments were performed in this concentration of FCS.

When oFF pooled from large (> 5 mm) follicles was added to 3T3 cells maintained throughout the experiment in medium containing 2.5% FCS, a dose-dependent inhibition of [3 H]thymidine incorporation was evident (Fig. 2). Further addition of equivalent amounts of serum from ovariectomized ewes or FCS to cultures initiated in 2.5% FCS were without significant effect. A survey of 5 separate pools of oFF indicated that variable levels of inhibitory activity were present in each (Fig. 3). Potency was not apparently related to the month in which oFF was collected.

When cells were grown in the presence of oFF (20% v/v) for 96 h, [3 H]thymidine incorporation over the final 48 h was significantly lower than that by cells to which no oFF was added ($P < 0.001$;

Fig. 1

Fig. 1. Determination of FCS concentrations optimal for [³H]thymidine incorporation. Cells maintained in 5% FCS were exposed to 1% FCS for 24 h, harvested by trypsinization and plated in medium without FCS. Cells were grown for 48 h in the presence of [³H]thymidine and a range of doses of FCS. [³H]Thymidine incorporation is expressed as a percentage of that in cultures to which no FCS was added. Each point represents the mean of triplicate determinations ($\pm$ s.d.).

Fig. 2

Fig. 2. Inhibition of [³H]thymidine incorporation by oFF. Cells were grown for 48 h in the presence of oFF (●), serum from ovariectomized ewes (■) or FCS (▲) at a range of concentrations. Values represent the mean of triplicate determinations ($\pm$ s.d.).

Fig. 4). After removal of oFF from culture medium at 48 h, [³H]thymidine incorporation was not significantly different from control.

Incorporation of [³H]thymidine by cells exposed to a range of doses of purified bovine inhibin (M_r 31 000), oestradiol-17 β , testosterone, cortisol or progesterone was not significantly different from that incorporated by cells grown in the absence of test substance (Fig. 5). In this same series of experiments, oFF inhibited, and 8-bromo cyclic AMP stimulated [³H]thymidine incorporation in a dose-dependent manner (data not shown).

Although pre-treatment of pooled oFF with charcoal-dextran did not reduce inhibitory activity relative to untreated oFF of the same pool, heating at 85°C resulted in partial but significant reduction of inhibitory activity. Dose-dependent suppression of [³H]thymidine incorporation by residual activity in heated oFF was parallel to that in untreated oFF. When heated for 5, 15 and 30 min, potencies relative to untreated oFF were 0.38 (0.33–0.43), 0.43 (0.38–0.49) and 0.45 (0.39–0.51) respectively. The 95% confidence limits of each estimate of relative potency are stated in parentheses.

Fig. 3. Seasonal variation of inhibitory activity of oFF. Pools of oFF were collected during the months of February (★), June (▲), July (●, ◆) and October (■) and added to cultures of 3T3 cells to give final concentrations of 5–40% (v/v). Each point represents the mean of quadruplicate determinations. Variance around each point was less than 15% and no significant differences between pools was evident at any given dose.

Fig. 4. Reversible inhibition of mitosis by oFF. Cells were grown for 96 h in the absence of oFF (control), or in the presence of oFF (20% v/v) for 48 h (oFF: wash) or 96 h (oFF) as indicated. [³H]Thymidine was added to all cultures at 48 h. Results are expressed as c.p.m./culture and represent the mean of quadruplicate measurements (± s.d.).

Fig. 5. Effects of constituents of oFF on mitosis of 3T3 cells. Cells were grown for 48 h in the presence of a range of concentrations of purified bovine inhibin (M_r 31 000), oestradiol-17 β , testosterone, cortisol or progesterone. Values are mean ($\pm$ s.d. for quadruplicate estimates) [3 H]thymidine incorporation expressed as a percentage of that in cultures to which no inhibin or steroid was added (control). Divisions on each vertical axis represent an interval of 10% control.

Addition of oFF from follicles of <5 mm in diameter to cultures of 3T3 cells did not alter [3 H]thymidine incorporation significantly when compared to that by cells cultured in the absence of oFF. Addition of oFF pooled from larger follicles (>5 mm diameter) resulted in a dose-dependent suppression of [3 H]thymidine incorporation (Table 1).

When subjected to gel chromatography at pH 7.0, two peaks of inhibitory activity were eluted (Fig. 6). Of 50 000 units of activity loaded (1 μ l starting material = 1 unit of inhibitory activity), 17 646 units were recovered as material of M_r ~180 000 (12 194 units) and M_r <10 000 (5452 units). No inhibitory activity was evident in serum from ovariectomized ewes or in any eluted fractions of such serum chromatographed in an identical manner.

Neither heating at 85°C nor exposure to trypsin resulted in loss of inhibitory activity from the material of lower molecular weight (Fig. 7; pool III). Although the inhibitory activity associated

Table 1. Inhibition of mitosis by oFF from small and large antral follicles

Follicle	Conc. (% , v/v)			
	0.5	1.6	5.0	16.0
Small (< 5 mm)	101 (6)	—	92 (16)	119 (18)
Large (> 5 mm)	102 (18)	89 (3)	44 (22)	34 (6)

Pools of oFF were prepared from small antral follicles (< 5 mm) and large antral follicles (> 5 mm) and added to 48-h cultures of 3T3 cells at a range of concentrations. Results are expressed as a percentage of [³H]thymidine incorporation by cells grown in the absence of oFF (control). Values are the mean of triplicate estimates and s.d. (in parentheses).

Fig. 6. S-200 chromatography of oFF. Pooled oFF (50 ml) was chromatographed on Sephacryl (S-200) equilibrated in ammonium acetate (pH 7.0). Fractions of 10 ml were collected and absorbance (280 nm) measured. Inhibitory activity was measured in consecutive pools of 10 fractions (total volume = 100 ml) after lyophilization and reconstitution in culture medium. Activity in reconstituted pools of eluted fractions was measured relative to that in starting material (1 µl starting material = 1 unit of inhibitory activity). Molecular weight markers were IgG (180 000), bovine serum albumin (BSA; 67 000), myoglobin (Myo; 17 200) and cytochrome C (CytC; 11 700). Void volume (V₀) and total column volume (V_t) are indicated. Pooled serum from ovariectomized ewes (50 ml) was treated in an identical manner with no inhibitory activity being evident in starting material or eluted fractions (data not shown).

Fig. 7. Sensitivity of resolved S-200 chromatographic fractions to heating and trypsin. After S-200 chromatography of 50 ml oFF (see Fig. 6), fractions 20–60 (pool I), 80–120 (pool II) and 140–170 (pool III) were pooled. Each pool was assayed for inhibitory activity following no further treatment (○), heating at 85°C for 15 min (●), or exposure to trypsin at 37°C for 20 min (■). Each point represents the mean of quadruplicate determinations.

with material of higher molecular weight (pool I) was reduced significantly after exposure to trypsin, heating of this material was without significant effect. As control, non-inhibitory material of intermediate molecular weight (pool II) remained apparently inactive following heating or exposure to trypsin (Fig. 7).

Discussion

We have used [^3H]thymidine incorporation rather than cell number or DNA content as an estimate of mitotic activity *in vitro*. Although this does not necessarily correlate with changes in cell number, [^3H]thymidine incorporation does reflect accurately the number of cells which have progressed through the 'S' phase, a necessary requisite of cell division. Based on [^3H]thymidine incorporation, our data indicate that oFF inhibits serum-induced mitosis of 3T3 fibroblasts *in vitro*. This effect is both dose-dependent and reversible in that washing of oFF from cultures resulted in complete restoration of serum-induced mitotic activity.

Inhibitory activity recovered from oFF was associated with material of $M_r \sim 180\,000$ and $< 10\,000$, suggesting that either two very distinct components of oFF are each capable of inhibiting mitosis, or that the material of higher molecular weight represents active material bound to a carrier protein. Incomplete loss of activity at 85°C suggested that oFF contained both heat-labile and heat-stable inhibitory components. This would be consistent with the observed chromatographic heterogeneity of inhibitory activity. Given that the component of lower molecular weight was stable to both heating and exposure to trypsin, the material of higher molecular weight would be expected to be heat-sensitive to explain the partial reduction in inhibitory activity after heating unfractionated oFF. However, although the component of higher molecular weight was trypsin-sensitive, heating had little or no effect. The reduction in inhibitory activity after heating of unfractionated oFF must therefore have been due to interaction with other components of follicular fluid, possibly endogenous proteases.

Failure of charcoal treatment of oFF to remove inhibitory activity and an inability of exogenous steroids to reduce mitotic activity further argue against the inhibitory components being

steroidal in nature. In view of the concentrations of steroids (Carson *et al.*, 1981), and of inhibin in oFF (Tsonis *et al.*, 1983), and the manner in which the present experiments were conducted, it is estimated that the final concentrations of steroids and inhibin in culture media to which oFF had been added would have been of the order 10^{-8} M. Concentrations of inhibin and exogenous steroids studied in these experiments were several orders of magnitude above and below this level.

Purified bovine inhibin of M_r 31 000 prepared in this laboratory (Robertson *et al.*, 1986) did not alter mitotic activity of 3T3 fibroblasts. It is established that the alpha subunit of inhibin has extensive homology with the constituent subunit of homodimeric transforming growth factor type beta (TGF- β ; Mason *et al.*, 1985), a factor capable of both stimulation and inhibition of mitosis in normal tissues (see Moses *et al.*, 1985, for review). Our data suggest that, despite extensive structural homology, inhibin does not share any functional domain with TGF- β or that each is similarly active and 3T3 cells under these experimental conditions do not respond to TGF- β -like activity.

Tissue-specific endogenous inhibitors of mitosis are said to exist in a variety of non-neoplastic tissues including liver (Verley *et al.*, 1971), cerebral cortex (Johnson *et al.*, 1985), serum (Del'Aquila *et al.*, 1984) and testis (Thumann & Bustos-Obregon, 1982). We are unable to state the relationship, if any, of the activity described in the present study with any of these inhibitors from other tissues or the inhibitors of embryo cleavage (Hung & Millard, 1985), oocyte maturation (Tsafriri & Channing, 1975), pituitary FSH secretion (Robertson *et al.*, 1986), gonadotrophin binding (Yang *et al.*, 1976; Darga & Reichert, 1978), granulosa cell luteinization (Ledwitz-Rigby *et al.*, 1977) or gonadotrophin-induced follicular development (diZerega *et al.*, 1982, 1983) said to be present in follicular fluid. Because mitosis of granulosa cells is said to be independent of pituitary gonadotrophins (Gospodarowicz & Bialecki, 1979) it is unlikely that, in the ovary, inhibitors of mitosis are related to inhibitors of gonadotrophin binding to ovarian cells.

It is possible that inhibition of mitosis by oFF reflects the presence in oFF of factors capable of blocking the effects of mitogens present in FCS. Possible mechanisms of action at this level include binding and inactivation of mitogens by components of oFF, inhibition of interaction of mitogens with cellular receptors, or disruption of mitogen-induced subcellular responses. There is evidence for the testis that endogenous inhibitors of mitosis block the binding of epidermal growth factor (EGF) to its receptor and thus EGF-induced mitosis of testicular cells (Holmes *et al.*, 1986). Alternatively, inhibition of serum-induced mitosis may be due to the presence in oFF of inhibitors which interact directly with 3T3 cells to block thymidine uptake or metabolism rather than blocking the effects of mitogens in FCS.

In addition to 3T3 cells (Makris *et al.*, 1983), cells derived from the mouse embryo (Hung & Millard, 1985), human ovarian tumour (Kikuchi *et al.*, 1986) and bovine aortic endothelium (Koos, 1986) have been used to study regulation of mitotic activity *in vitro* by ovarian factors. There is also experimental evidence in support of regulation of testis cell proliferation by seminiferous tubule growth factor (Bellve & Feig, 1984) which is based on studies of 3T3 rather than testis cells. Attempts to compare the effect of an endogenous ovarian growth factor on proliferation of 3T3 and granulosa cells have as yet proved inconclusive (Makris *et al.*, 1983).

Even though 3T3 fibroblasts have been used extensively as a bioassay for growth factor activity and in studies of the control of mammalian cell proliferation, confirmation of an inhibitory factor in oFF is entirely contingent on demonstration of an inhibition of oFF of granulosa cell rather than 3T3 fibroblast mitosis *in vitro*. Despite this reservation, it remains possible that the ability of oFF to inhibit serum-induced mitosis may account for the observed inhibition of gonadotrophin-induced follicular development when charcoal-treated oFF was administered to sheep (Cahill *et al.*, 1985).

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