Effects of epidermal growth factor on the growth and differentiation of cultured mouse ovarian follicles

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The aim of this investigation was to determine the influence of epidermal growth factor (EGF) on follicular growth and steroidogenesis in mice. Follicles were cultured in medium containing human recombinant EGF at concentrations of 1–20 ng ml⁻¹. Oestradiol production was assayed immunoenzymatically, and growth was measured by recording follicle diameter daily and by analysing the total DNA content of follicles. The effect of EGF on cumulus–oocyte complexes isolated from cultured follicles was also assessed. Results showed that EGF inhibited oestradiol production in a dose-dependent manner, but had no mitogenic effect. Despite almost complete inhibition of oestradiol production at concentrations of EGF ≥ 10 ng ml⁻¹, follicles were still able to achieve preovulatory size and morphology, although the incidence of atresia was increased over controls. Conversely, at a concentration of only 1 ng EGF ml⁻¹, a significantly greater number of follicles reached the Graafian stage compared with control follicles. Cumulus expansion and meiotic maturation by isolated cumulus–oocyte complexes from cultured follicles was dramatically stimulated in the presence of EGF and FSH, but not by FSH alone. These findings suggest that EGF may have a modulatory effect on oestradiol production *in vivo*, and that follicular growth and differentiation may be uncoupled from steroidogenesis. Finally, ovulatory changes in the cumulus–oocyte complex may require the presence of this factor.

Introduction

Epidermal growth factor (EGF) is a single-chain polypeptide, which has been shown to have a potent mitogenic effect on a variety of different cell types from a number of different species (for review, see Carpenter and Cohen, 1979). Most of the work that has been done on follicular cell responses to EGF has made use of granulosa monolayer cultures, in which the granulosa cells have been removed from interactions with other cell types (for reviews, see May and Schomberg, 1989; Ackland *et al.*, 1992). Other work has focused on the short-term culture of hamster follicles that were isolated at different stages of development and maintained for only a few hours (Roy and Greenwald, 1991a, b; Roy, 1993a, b). All these studies have shown that the effects of EGF on granulosa cells are very diverse, and it has been suggested that EGF may have a paracrine role in follicular growth and differentiation.

New culture methods capable of supporting murine follicle development from preantral to preovulatory stages provide an opportunity to study the effects of EGF under more physiological conditions (for review, see Gosden *et al.*, 1993). The following study describes the effects of EGF on follicular growth, morphology and steroidogenesis using a culture system modified for the study of murine follicle metabolism (Boland *et al.*, 1993). Follicles were exposed to concentrations of human recombinant EGF ranging from 1 to 20 ng ml⁻¹. The lower limit of the range represents the concentration of

EGF that mouse follicles would normally be exposed to *in vivo* from the circulation (Byyny *et al.*, 1974). Higher concentrations were also tested because there is an increasing body of evidence that ovarian cells are sites of production of an EGF-like substance (Rall *et al.*, 1985; Hsu *et al.*, 1987; Hiramatsu *et al.*, 1992), and concentrations of EGF between 10 and 20 ng ml⁻¹ have been recorded in porcine follicular fluid (Hsu *et al.*, 1987).

In addition, unlike their counterparts *in vivo*, cumulus–oocyte complexes isolated from cultured follicles exhibit a low incidence of cumulus expansion and first polar body formation in the presence of FSH, which may partly explain why attempts at fertilization have been unsuccessful. As EGF has been reported to stimulate cumulus expansion and meiotic maturation of cumulus–oocyte complexes isolated from mouse ovaries (Downs *et al.*, 1988; Downs, 1989), a further study was undertaken to investigate its effect on cumulus–oocyte complexes from cultured follicles in the hope of improving maturation and facilitating subsequent fertilization.

Materials and Methods

Animals

Twenty-four-day old, prepubertal, C57BL/6 $\times$ CBA/ca F₁ hybrid, female mice were housed in a room controlled for temperature and light on a 14 h light:10 h dark photoperiod, and were given free access to food and water.

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Follicle isolation and culture

Mice were killed by cervical dislocation and their ovaries removed into watchglasses containing Leibovitz medium (L-15; Gibco-BRL, Irvine) at 37°C. Preantral mouse follicles $180 \pm 10 \mu\text{m}$ in diameter were isolated by microdissection using fine needles (Boland *et al.*, 1993), and placed in 20 μl of α -minimal essential medium (α -MEM; Gibco-BRL) under 50 μl mineral oil in the V-wells of a microtitre plate (Bibby-Sterilin Ltd, Staffs), according to the method of Boland *et al.* (1993). Medium was simplified from that originally published and contained only 1 iu human FSH ml^{-1} (hFSH: NIDDK, Bethesda, MD), 10 μg human transferrin ml^{-1} (Sigma Chemical Co, Poole) and 5% serum from hypogonadal (*hpg:hpg*) mice. The original supplements of pyruvate, glutamine and insulin were omitted as they are not required for normal follicle development *in vitro* (Boland *et al.*, 1993, 1994; Spears *et al.*, 1994).

Experiment 1: effect of EGF on follicle development

The culture medium was additionally supplemented with human recombinant EGF (1376454: Boehringer-Mannheim, Lewes) at 0, 1, 5, 10 and 20 ng ml^{-1} . For each experiment, ten follicles were cultured in control medium (no EGF) and ten follicles in each of the EGF-treated groups (1–20 ng EGF ml^{-1}). Every 24 h, follicles were transferred to fresh wells of medium, and 5 μl of media from the previous day's incubation was sampled for oestradiol (see below). Follicle growth and morphology were also assessed every 24 h. On day 5 of culture, two representative preovulatory follicles were removed for histology from each control and EGF-treated group. The remaining antral follicles were enzymatically digested for measurement of DNA content (see below). Each experiment was repeated four times.

Experiment 2: effect of EGF on cumulus expansion and meiotic maturation of cumulus–oocyte complexes from cultured follicles

Fifty follicles were cultured as described above except that EGF was omitted from the culture medium. Follicles were transferred to fresh medium every 24 h and follicle growth was measured using a calibrated grid. On day 5 of culture, follicles were ruptured using fine needles and the cumulus–oocyte complexes removed. The cumulus–oocyte complexes were then divided into two groups and cultured overnight at 37°C in α -MEM containing 1 iu hFSH ml^{-1} , 10 μg transferrin ml^{-1} and 5% fetal calf serum with or without 10 ng EGF ml^{-1} .

The following day, germinal vesicle breakdown and first polar body formation were scored, and cumulus expansion was measured subjectively according to the criteria described by Vanderhyden *et al.* (1990). A score of 0 was given for cumulus–oocyte complexes with completely compacted cumulus cells that formed a monolayer on the surface of the culture dish; +1 for the minimum observable response to EGF, which was no monolayer formation and cumulus–oocyte complexes with a glistening appearance; +2 for cumulus–oocyte complexes with an expanded outer layer of cells; +3 for expansion of all layers except the corona radiata; and +4 for cumulus–oocyte complexes exhibiting fully expanded and mucified

cumulus cells, including the corona radiata. Both groups of cumulus–oocyte complexes (with and without EGF) were scored 'blind' by the same investigator after covering the lids of the culture dishes and assigning codes 'A' and 'B' randomly. After scoring, the codes were checked against the experimental treatment and the procedure was repeated twice after shuffling the culture dishes.

Steroidogenesis

Oestradiol production was measured immunoenzymatically for individual follicles on each day of culture using a Serozyme II photometer (Serono Diagnostics, Woking) and oestradiol assay kit (sensitivity $\geq 18 \text{ pmol l}^{-1}$) according to the manufacturer's instructions. The interassay and intra-assay coefficients of variation were $\leq 5\%$.

Follicle growth and morphology

The number of preovulatory follicles and the total number of antral stages were recorded at the end of each culture. Preovulatory follicles were classified as having (i) a diameter $\geq 400 \mu\text{m}$, (ii) a large antral cavity, (iii) an acentrically placed cumulus–oocyte complex, and (iv) no signs of atresia (visible as dark patches of dead cells within the membrana granulosa). Antral follicles included all follicles with a clearly defined antral cavity of any size and no signs of atresia in the membrana granulosa.

Follicle growth was monitored in two ways: (i) measuring follicle diameter on each day of the culture using a precalibrated ocular micrometer at $\times 40$ magnification; or (ii) by assessing DNA accumulation in non-atretic, preovulatory follicles on day 5 with a fluorochrome dye, Hoechst 33258 (bisbenzimidazole; Sigma Chemical Co), and a Dynatech 'Microfluor' automatic microplate fluorescence reader (365 nm excitation, 450 nm emission; Dynatech, Billingshurst), using a method modified from West *et al.* (1985).

For measurement of DNA, follicles were washed in two changes of double-distilled, deionized water and placed individually in 50 μl of $10 \times$ trypsin solution (25 mg ml^{-1} ; Sigma Chemical Co) in the U-wells of a microtitre plate (Bibby-Sterilin). The follicles in each well were then crudely teased apart with needles and incubated at 37°C. After 60 min of digestion, the contents of each well were pipetted up and down three times to break up the cells and the plate was then returned to the incubator for a further 15 min. Digestion was stopped with 50 μl of trypsin inhibitor (4.4 mg ml^{-1} in double-distilled deionized water) and wells were adjusted to a final pH of 7.0 using 100 μl of alkaline double-distilled deionized water (with pH adjusted to 11.2 using NaOH). The contents of each well (200 μl) were then mixed thoroughly before being transferred to a 96-well, white-background plate (Dynatech). Ten wells containing 100 μl double-distilled deionized water (pH 11.2), 50 μl trypsin and 50 μl trypsin inhibitor acted as the negative control to quantify background fluorescence.

A second plate was set up with 100 μl standards of salmon testes DNA (Type III; Sigma Chemical Co) diluted to give a

Fig. 1. Oestradiol profiles for mouse follicles cultured in (●) control conditions and (■) 1 ng EGF ml⁻¹, (▲) 5 ng EGF ml⁻¹, (□) 10 ng EGF ml⁻¹, and (△) 20 ng EGF ml⁻¹.

Fig. 2. Growth profiles for mouse follicles cultured in (●) control conditions, and in (■) 1 ng EGF ml⁻¹, (▲) 5 ng EGF ml⁻¹, (□) 10 ng EGF ml⁻¹, and (△) 20 ng EGF ml⁻¹.

range of concentrations from 0 to 1000 ng per 100 μl double-distilled deionized water. The volume of each well was then made up to 200 μl with a mixture of 100 mmol NaCl l⁻¹ and 10 mmol Tris l⁻¹ (pH 7.0). Ten wells containing 100 μl double-distilled deionized water and 100 μl NaCl-Tris buffer acted as a negative control for the DNA standards. A stock solution of 200 μg Hoechst 33258 ml⁻¹ (Sigma Chemical Co) was diluted 1:500 with NaCl-Tris buffer (pH 7.0), and 50 μl was pipetted rapidly into each well containing the digested follicles, negative controls and DNA standards. The final volume of each well was 250 μl and the final pH of the wells was 7.0, which is the optimum pH for Hoechst dye fluorescence. Measurements of fluorescence for individual follicles were converted to nanograms of DNA by extrapolation from the salmon testes DNA calibration curve after subtraction of the reagent blank fluorescence values.

Histology

Follicles were fixed in 5% paraformaldehyde overnight, dehydrated through a graded series of ethanol concentrations and then embedded in 2-hydroxyethylmethacrylate resin (Sigma Chemical Co). Blocks were sectioned at a thickness of 1 μm on to polylysine-coated slides, and stained with haematoxylin and eosin before mounting.

Statistical analysis

Probability values (*P* value) between the different groups were determined by the unpaired Student's *t* test and the Kruskal-Wallis ANOVA.

Results

Experiment 1: effect of EGF on follicle development

Steroidogenesis. Epidermal growth factor caused a dose-dependent decline in oestradiol production over the 5-day culture (Fig. 1). A concentration of only 1 ng EGF ml⁻¹ significantly reduced oestradiol production to approximately half that of control follicles (control, 115 ± 10 nmol oestradiol l⁻¹ in 24 h; 1 ng EGF ml⁻¹, 51 ± 7 nmol oestradiol l⁻¹ in 24 h on day 5 of culture; *P* < 0.001 for days 2–5). At 5 ng ml⁻¹, EGF severely inhibited oestradiol production (12.7 ± 1 nmol oestradiol l⁻¹ in 24 h on day 5; *P* < 0.001 for days 2–5), and between 10 and 20 ng ml⁻¹, production was only just detectable (6.1 ± 0.3 and 3.3 ± 0.1 nmol oestradiol l⁻¹ in 24 h on day 5, respectively; *P* < 0.001 for days 2–5).

Growth and morphology. There was no significant difference in diameter between control follicles and those cultured in the presence of 1 and 5 ng EGF ml⁻¹ (*P* > 0.05 for each day of culture; Fig. 2). However, follicles cultured with 10 and 20 ng EGF ml⁻¹ showed a slight, but significant, decline in diameter on day 5 of culture compared with control follicles (control, 439 ± 7 μm; 10 ng EGF ml⁻¹, 416 ± 8 μm, *P* < 0.05; 20 ng EGF ml⁻¹, 398 ± 7 μm, *P* < 0.02). The absence of any EGF-stimulated increase in follicle growth was supported by the finding that there was no significant difference in the DNA content of control follicles on day 5 and those cultured with EGF at concentrations ranging from 1 to 20 ng ml⁻¹ when analysis of variance was determined by the Kruskal-Wallis test (*P* > 0.1; Table 1). Freshly isolated follicles had a DNA content of only 30 ± 2 ng, demonstrating the capability of the system to sustain cell proliferation. The DNA

Table 1. Amount of DNA in mouse follicles after 5 days of culture in different concentrations of EGF

	Concentration of EGF (ng ml ⁻¹)				
	0	1	5	10	20
Amount of DNA in follicles	580 ± 46	610 ± 51	640 ± 35	670 ± 32	585 ± 64

Fig. 3. Percentage of healthy antral (□) and preovulatory (■) mouse follicles on day 5 of culture in control and epidermal growth factor (EGF)-treated groups.

calibration curve was linear from 10 to 1000 ng DNA and the intra-assay and interassay coefficients of variation were < 5%.

Follicles in all of the EGF-stimulated groups were able to reach the terminal stages of development and exhibited normal Graafian morphology despite attenuation of oestradiol production. However, EGF appeared to have a biphasic effect on the rate of follicle atresia (Fig. 3). At a concentration of 1 ng ml⁻¹, EGF had increased the number of antral and Graafian follicles by the end of the culture period compared with control groups ($P < 0.01$ and $P < 0.05$, respectively). Conversely, at higher concentrations of EGF, the number of antral follicles was significantly reduced compared with controls ($P < 0.01$ for 10 and 20 ng EGF ml⁻¹), and at a concentration of 20 ng EGF ml⁻¹, there was also a decline in the number of Graafian follicles ($P < 0.05$). However, there was no difference in the total number of follicles at the antral and Graafian stages cultured in control conditions and in those cultured with 5 ng EGF ml⁻¹ ($P > 0.5$ and $P > 0.2$, respectively) (Fig. 3). Light microscopy showed that the morphology of follicles reaching the Graafian stages of development was similar among all the experimental groups and there were few, if any, cells that could be described as atretic.

Experiment 2: effect of EGF on cumulus expansion and meiotic maturation of cumulus-oocyte complexes isolated from cultured follicles

Cumulus-oocyte complexes cultured in the presence of 10 ng EGF ml⁻¹ overnight were significantly more expanded and mucified than were cumulus-oocyte complexes cultured in the presence of FSH alone ($P < 0.001$). Although the incidence of germinal vesicle breakdown was similar between the two groups ($P > 0.05$), first polar body formation was significantly higher in the EGF-treated group ($P < 0.001$) (Table 2).

Discussion

Previous studies with granulosa cell monolayer cultures from humans (Steinkampf *et al.*, 1988; Mason *et al.*, 1990), pigs (Hiramatsu *et al.*, 1992) and rats (Hsueh *et al.*, 1981) have shown that EGF inhibits oestradiol production. This finding has been confirmed in mice under more physiological conditions by the present study, which shows that EGF causes a dose-dependent decline in oestradiol production by isolated follicles developing *in vitro*. Murine ovarian follicles would normally be exposed to 1.2 ± 0.2 ng EGF ml⁻¹ from the circulation (Byyny *et al.*, 1974). A concentration of approximately 1 ng ml⁻¹ (including the serum contribution) reduces oestradiol production by cultured follicles to almost half that of control values, suggesting that follicular oestradiol production could be modulated by EGF *in vivo*.

Although follicles probably come into contact with EGF through the blood stream, the mouse ovary is also thought to be a site of EGF synthesis (Rall *et al.*, 1985), and concentrations of > 10 ng ml⁻¹ have been detected in the follicular fluid of other species (Hsu *et al.*, 1987). The present study demonstrates that there is a significant decline in the number of cultured follicles reaching the antral stages of development above a concentration of 5 ng EGF ml⁻¹. In pigs, the concentration of EGF is higher in the follicular fluid of small antral follicles than it is in the larger Graafian types (13.6 ± 1 and < 6 ng ml⁻¹, respectively; Hsu *et al.*, 1987), and, as the majority of small antral follicles become atretic *in vivo* ($> 99\%$), it is possible that high concentrations of EGF in the follicular fluid may reflect incipient atresia. Despite high concentrations of EGF favouring atresia, low concentrations appear to enhance antral development, indicating that EGF may have a biphasic effect on follicle survival.

It has been shown that mouse follicles can grow to preovulatory sizes in a concentration of FSH (100 mIU ml⁻¹) that is insufficient to stimulate more than transient oestradiol production (Nayudu and Osborn, 1992). The present study confirms

Table 2. Maturation of mouse cumulus–oocyte complexes isolated from antral follicles grown *in vitro* and cultured with or without 10 ng EGF ml⁻¹

Culture conditions	Cumulus expansion (score 3–4)	Germinal vesicle breakdown	First polar body formation
Control	0/19 (0%)	18/19 (95%)	6/19 (32%)
EGF (10 ng ml ⁻¹)	19/19 (100%)	19/19 (100%)	17/19 (90%)

this finding by demonstrating that at high concentrations of FSH (1 iu ml⁻¹) normal follicular oestradiol production can be almost completely inhibited by EGF without preventing pre-ovulatory development. The observation that normal follicular growth and differentiation can be uncoupled from oestradiol synthesis supports the findings from a number of studies, which have indicated that this steroid may not be as important within the ovary as was originally thought. It was reported that follicle development and ovulation could be achieved in gonadotrophin-deficient women given recombinant FSH, despite low concentrations of oestradiol in the serum of these patients (Schoot *et al.*, 1992). Subsequently, Uilenbroek *et al.* (in press) demonstrated the same phenomenon in hypophysectomized rats.

Despite the dramatic inhibitory action of EGF on steroidogenesis, this growth factor does not appear to have an obvious mitogenic effect on mouse follicular cells. Although this finding is inconsistent with the behaviour of ovarian cells from a number of other species (Gospodarowicz *et al.*, 1977; Gospodarowicz and Bialecki, 1979), the granulosa cells of both guinea-pigs and rats also fail to proliferate in response to EGF (Gospodarowicz and Bialecki, 1979).

In addition to the effects of EGF on developing follicles *in vitro*, isolated cumulus–oocyte complexes from cultured follicles exhibit a large capacity for cumulus expansion and a high rate of meiotic maturation to the first polar body stage when cultured in the presence of this growth factor. This finding is in agreement with previous work that showed that EGF can promote cumulus cell expansion and oocyte maturation in cumulus–oocyte complexes isolated from mouse ovaries (Downs, 1989). However, unlike isolated cumulus–oocyte complexes grown *in vivo*, the cumulus–oocyte complexes from follicles grown *in vitro* achieved only a low rate of first polar body formation and completely failed to undergo cumulus expansion when cultured with FSH in the absence of EGF. This point is important because, under normal conditions, FSH alone should be able to stimulate the expansion of mouse cumulus–oocyte complexes as long as serum is also present (Eppig 1979a, b; 1980; Schroeder and Eppig, 1984; Salustri *et al.*, 1989; Chen *et al.*, 1990). The inability of FSH to induce expansion and oocyte maturation in the cumulus–oocyte complexes from the cultured follicles suggests a role for EGF in preparing the oocyte for ovulation and fertilization, a possibility that Downs *et al.* (1988) have already alluded to. As it is likely that mouse follicles are exposed to EGF *in vivo* (Byyny *et al.*, 1974; Rall *et al.*, 1985), the absence of adequate EGF under follicle culture conditions could, perhaps, explain why attempts at oocyte fertilization have been unsuccessful in the past. Subsequent to this observation, we have demonstrated that the oocytes from

follicles grown *in vitro* are not only fertile, but have the capacity to be fertilized and produce live pups when cultured in the presence of EGF before insemination (Spears *et al.*, 1994).

In summary, EGF has been shown to have a profound modulatory effect on follicular steroidogenesis in mice at physiological concentrations, but a mitogenic role for this growth factor in the mouse follicle was not observed. At the blood plasma concentration, EGF caused a decline in oestradiol production but increased the number of follicles reaching the antral and Graafian stages of development. At higher concentrations, oestradiol production was severely attenuated, but follicles still continued to grow to the preovulatory stage, although the incidence of atresia was increased. The ability of cumulus–oocyte complexes isolated from cultured follicles to expand and undergo high rates of meiotic maturation in the presence of EGF, but not in the presence of FSH alone, indicates that this factor could influence oocyte fertility. Together, these findings provide further evidence to suggest that EGF has a role during follicular development and oocyte maturation *in vivo*.

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