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Rat oocytes induced to mature by epidermal growth factor are successfully fertilized

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Summary

Epidermal growth factor (EGF), which is a known mitogen, can also induce resumption of meiosis in the rat oocyte. The present study was designed in an attempt to elucidate whether oocytes, induced to mature by EGF in a follicle-enclosed oocyte culture, are fertilizable and can further develop into two-cell embryos. For further clarification of the effect of EGF on steroidogenesis in the ovarian follicle, progesterone concentrations were determined by radioimmunoassay. We found that oocytes matured by EGF (100 ng/ml) were successfully fertilized. Even though their rate of fertilization was relatively lower as compared to that of oocytes stimulated by luteinizing hormone (LH) both in vitro and in vivo (61%, 79%, and 83% respectively), once fertilized they exhibit an equal potential for further development (EGF: 48%, LH: 45%). On the other hand, EGF-induced progesterone production was very poor. These findings strongly support the idea that both mitogenesis and meiogenesis can be mediated by common signals. The results further suggest that progesterone production and oocyte maturation, in the rat, are independent events.

Introduction

The involvement of growth factors in ovarian physiology has been investigated actively in recent years, and studies from several laboratories suggest that such peptides may also play a role in the intrafollicular control of meiosis (Dekel and Sherizly, 1985; Downs, 1989). Meiosis of the mammalian oocyte is initiated during fetal life, and is arrested at the diplotene stage of the first meiotic prophase. Reinitiation of meiosis, also known as oocyte maturation, is triggered by the preovulatory surge of luteinizing hormone (LH) or by exogenous administration of this gonadotropin (Lindner and Bauminger, 1974).

Epidermal growth factor (EGF), which acts as a mitogen on a variety of somatic cells, also stimulates proliferation of granulosa cells in vitro (Skinner et al., 1987; Hill, 1989). Recent studies have shown that EGF can also induce resumption of meiosis in rat oocytes (Dekel and Sherizly, 1985; Downs, 1989). The effect of EGF on the oocyte is consistent with the theory that a common signal mediates both mitogenesis and the induction of meiosis.

Several reports demonstrate the existence of EGF-like activity in cultured granulosa, theca,

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and interstitial cells (rat: Skinner et al., 1987; hamster: Roy and Greenwald, 1990). Byyny et al. (1974) report that the concentration of EGF in the plasma of female mice is high enough to affect rat granulosa cells in a culture system. Other studies report that specific and high affinity receptors for EGF are present in rat and bovine granulosa and luteal cells. Furthermore, these receptor levels fluctuate during the estrous cycle and can also be modulated by exogenous gonadotropins (St-Arnaud et al., 1983; Feng et al., 1987; Rose et al., 1991). Taken together this evidence suggests that EGF could well play a physiological role in regulation of ovarian function.

EGF-induced oocyte maturation has been diagnosed by morphological markers, while neither fertilization nor embryonic development of these oocytes has yet been demonstrated. The present study was designed in an attempt to elucidate whether oocytes, induced to mature by EGF in a follicle-enclosed oocyte culture system, are fertilizable and can further develop into two-cell embryos. For further clarification of the effect of EGF on steroidogenesis, progesterone production by the ovarian follicle has also been analyzed.

Materials and methods

Animals

Wistar-derived rats were maintained in air-conditioned rooms (25°C) and supplied with laboratory animal pellets (Ambar, Israel) and tap water ad libitum. Artificial illumination was provided for 14 h daily.

Hormones and chemicals

Isolated intact ovarian follicles were incubated in vitro in the presence of: luteinizing hormone, ovine (oLH NIH LH S-20); follicle-stimulating hormone, ovine (oFSH NIH-13); and mouse epidermal growth factor (EGF, Sigma E-6135). For induction of oocyte maturation and ovulation in vivo, immature rats were injected with pregnant mares' serum gonadotropin (PMSG, Intervent) and human chorionic gonadotropin (hCG, Sigma CG-10).

Maturation of oocytes in vitro

Maturation in vitro was induced using the follicle-enclosed oocyte culture system. Immature, 25-day-old rats were primed by PMSG (15 IU) administered subcutaneously. The rats were killed 48 h after PMSG administration, and the ovaries were removed and placed in Leibovitz's L-15 tissue culture medium (L-15, Gibco, USA) supplemented with penicillin (100 U/ml), and 9% inactivated fetal calf serum (Bio-Lab, Israel). The large antral follicles were isolated and further incubated in the above medium (20 follicles/2 ml medium), in the presence or absence of either oLH (5 μg/ml) or EGF (100 ng/ml). These concentrations were previously demonstrated to induce a maximal proportion of matured oocytes in this culture system (Dekel et al., 1983; Dekel and Sherizly, 1985). Being incubated under our experimental conditions in hormone-free medium, the oocytes remain meiotically arrested (Dekel et al., 1983; Dekel and Sherizly, 1985). Incubation of follicles was carried out in 25 ml vials in N_2/O_2 (1 : 1) atmosphere in an oscillating water bath at 37°C. At the end of the incubation period, oocytes were recovered from the follicles and transferred to a sperm suspension for in vitro fertilization (IVF).

Viability and mucification of cumulus-oocyte complexes

Cumulus-oocyte complexes (COC) were recovered from the isolated follicles after culture with either EGF or LH. Viability of these complexes was evaluated morphologically using a stereoscopic microscope, at the two following time points: (1) upon isolation of the complexes from the follicles; (2) after 3 h of culture in fertilization medium, prior to their transfer to the sperm suspensions.

A viable COC was composed of a round oocyte with a clear and homogeneous ooplasm. This oocyte was surrounded by a homogeneous cumulus cell mass. Non-viable COCs were all composed of oocytes that were dark and flat, surrounded by a disaggregated cumulus mass.

Mucified cumuli displayed an expanded appearance while the non-mucified cumuli were composed of cells that were tightly packed. Sensitivity to hyaluronidase was used for confirmation

of cumulus mucification (Dekel et al., 1979). Dispersal of the cumulus cells was observed in the expanded, but not the tightly packed cumuli, following 10 min incubation in the presence of this enzyme (400 IU/ml, Sigma).

Maturation of oocytes in vivo

Oocytes that underwent maturation in vivo were used as controls. Immature, 27-day-old female rats were injected with 15 IU hCG 54 h following PMSG administration, and sacrificed 17–19 h later. Cumulus-enclosed oocytes were collected from the excised ampullae of the oviducts, and transferred to a sperm suspension for IVF. This group was also used for standardizing the results according to the daily variations in fertilization rates which may result from the use of different donors of spermatozoa.

In vitro fertilization

Spermatozoa were collected from the uteri of mature cyclic rats soon after mating and diluted in modified rat fertilization medium (RFMm; Shalgi, 1991) to a final concentration of $7–13 \times 10^5$ spermatozoa/ml. Aliquots of sperm suspension (100 μl) were incubated in Petri dishes under heavy paraffin oil (BDH) at 37°C for 5 h in a humidified atmosphere of 5% CO_2 in air to allow capacitation (Shalgi et al., 1983).

Eggs with associated cumuli were introduced to the sperm suspensions (10–15 per 100 μl drop). 40 h later the eggs were examined by Nomarsky interference-contrast microscopy for fertilization and first cleavage. Only eggs containing a sperm tail were classified as fertilized. The number of fragmented, polyspermic and parthenogenetic eggs was also recorded. Fragmented eggs are those with more than two fragments, yet a sperm tail could be observed. Polyspermic eggs are defined as 1- or 2-cell stage with more than one sperm tail in the ooplasm. Parthenogenetic eggs are 2-cell stage, yet no sperm tail could be observed.

Radioimmunoassay

To assess the effect of EGF on follicular steroidogenesis, follicles were incubated for different periods of time with or without EGF (1–500 ng/ml). Follicles incubated in the presence of oLH were used as positive controls. At the end of incubation 1 ml of medium was collected and progesterone concentrations were determined by radioimmunoassay (RIA), as described by Lindner and Bauminger (1974). These experiments were repeated at least twice with 2–4 replicates each, and the data of one representative experiment is presented.

Statistical analysis

An analysis of variance-ANOVA test was used to compare the recovery of oocytes under the different treatments. A Chi-squared test was used to compare fertilization and cleavage rate. Analysis was performed in the Blossom Stat Pack for Lotus 1-2-3.

Results

Viability of oocytes

The viability of oocytes matured in response to EGF was compared to that of oocytes induced to mature by LH. No significant difference was

TABLE 1

VIABILITY OF CUMULUS-OOCYTE COMPLEXES RECOVERED FROM FOLLICLES INCUBATED WITH EITHER EGF OR LH

Isolated follicles were incubated for 18 h with the indicated concentration of either EGF or LH. Cumulus-oocyte complexes (COC) were then recovered and their viability was evaluated, using the parameters described in Materials and methods, at the following two stages: (1) upon isolation of complexes from the follicles; (2) after 3 h of culture in fertilization medium, prior to their transfer to sperm suspensions. The results are expressed as the percentage of viable COC (1), or of oocytes transferred to IVF (2) out of the total number of follicles incubated. Each data point represents the mean ± standard error of 7 experimental days.

Treatment	Follicle No.	(1) Viable COC No./ Follicle No. (%)	(2) Viable oocyte No./ Follicle No. (%)
LH (5 μg/ml)	550	95.6 ± 1.9	83.0 ± 4.0
EGF (100 ng/ml)	817	94.5 ± 2.0	89.3 ± 2.0

found between the two groups (Table 1). This information allows us to further compare fertilizability and developmental potential of oocytes matured in response to both these hormones.

Fertilization and cleavage

We found that oocytes matured by EGF were successfully fertilized. However, their rate of fertilization was significantly lower than that found in the group of oocytes matured by LH, or in the control group (EGF: 61%, LH: 79%, control: 83%, $P < 0.05$, Fig. 1). On the other hand, the developmental potential of oocytes induced to mature by both LH and EGF was similar (EGF: 48%, LH: 45%), and different from the control group (71%, $P < 0.05$, Fig. 2). Follicle-enclosed oocytes incubated in hormone-free medium remained meiotically arrested. Similar to our previous study, fertilization rate of these oocytes was negligible (8%, Dekel et al., 1989). These oocytes served as a negative control for fertilization.

The rate of polyspermy (EGF: 19%, control: 10%), fragmentation (EGF: 19%, control: 14%), and parthenogenesis (EGF: 5%, control: 3%) was very low, with no significant difference between the treatment and the control groups (Table 2).

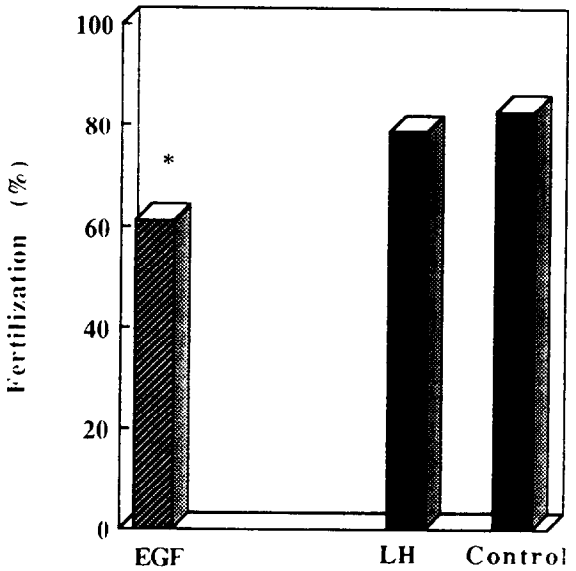

Fig. 1. Fertilization rate of oocytes induced to mature in vitro by EGF. Oocytes that underwent maturation in vitro were isolated from the follicles and transferred to sperm suspension for IVF. Oocytes that underwent maturation in vivo served as a control group. Each data point represents the mean of at least eight experiments including 150–250 follicles. * A Chi-squared test showed a significant difference ($P < 0.01$).

The effect of EGF on progesterone production

In addition to its effect on oocyte maturation EGF also stimulated the ovarian follicle to produce progesterone. Induction of progesterone production by EGF was dose dependent. The maximally effective dose was 100 ng/ml, with an ED_{50} at 2.5 ng/ml (Fig. 3). A similar dose was reported in a previous study (Dekel and Sherizly, 1985) to be maximally effective in inducing oocyte maturation.

In the absence of any inducer (control), progesterone concentrations remained at the baseline, with a very low level even after 8 h (33 pg progesterone/follicle, Fig. 4). However, EGF stimulation of progesterone production was quite moderate (100 ng/ml), 77 pg progesterone/follicle) as compared to that of LH (5 μg/ml, 484 pg progesterone/follicle). The effect of EGF on progesterone production could not be detected before 6 h of incubation. At this time point LH

TABLE 2

RATE OF EGGS EXHIBITING ABNORMAL DEVELOPMENT AFTER FERTILIZATION

Oocytes that underwent maturation in vitro under the influence of either EGF or LH were isolated from the follicles and transferred to sperm suspension for IVF and subsequent cleavage. Polyspermy, fragmentation and parthenogenesis were recorded. The results for polyspermy and fragmentation are expressed as the percentage of the indicated eggs out of the total number of fertilized eggs. Parthenogenesis is expressed as percentage of these eggs out of the total number transferred to IVF.

Treatment	Total No. (exp. days)	Polyspermic/ Fertilized (%)	Fragmented/ Fertilized (%)	Parthenogenetic/ Total (%)
EGF	259 (8)	19.1	19.1	5.0
Control	407 (18)	10.0	13.8	2.9

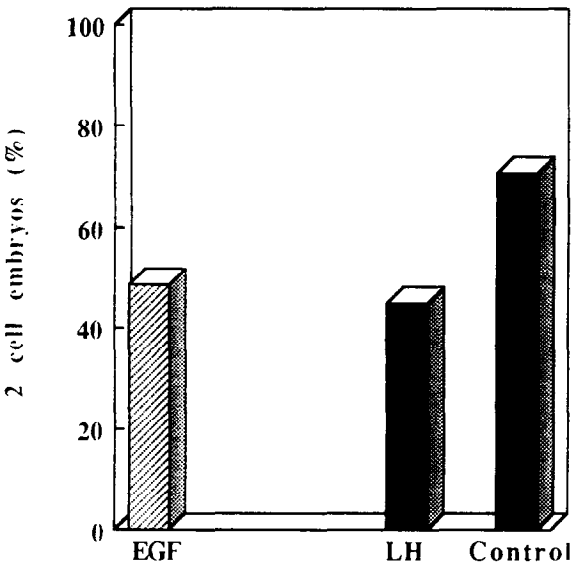

Fig. 2. Two-cell embryos developing from oocytes induced to mature in vitro by EGF. Oocytes that underwent maturation in vitro were isolated from the follicles and transferred to sperm suspension for IVF, and subsequent cleavage. Oocytes that underwent maturation in vivo served as a control group. The results are expressed as percentage of 2-cell embryos out of total fertilized eggs. Each data point represents the mean of at least eight experiments including 150–250 follicles.

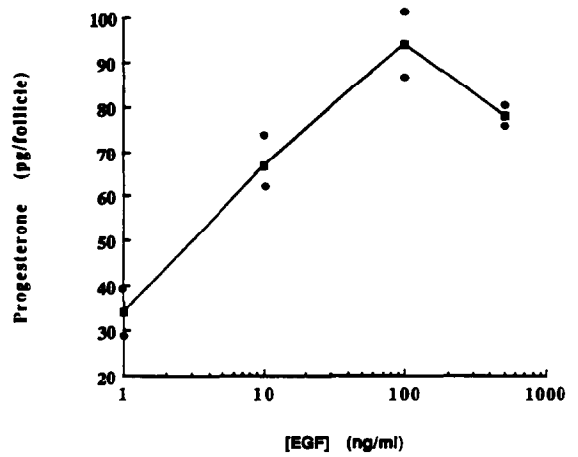

Fig. 3. Concentration dependence of EGF-induced progesterone production. Isolated follicles were incubated for 6 h with the indicated concentrations of EGF. Culture medium was collected at the end of the incubation period and progesterone levels were determined by radioimmunoassay. Experiments were repeated 3 times in duplicates. The data of duplicates of one representative experiment is presented.

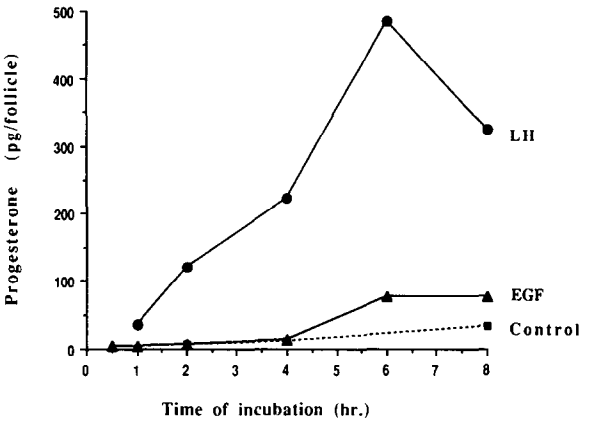

Fig. 4. Time course of LH- and EGF-induced progesterone production. Follicles were incubated in the presence of 5 μg/ml LH or 100 ng/ml EGF for the indicated times. Progesterone levels were determined by radioimmunoassay, in samples of culture medium collected at the end of the indicated incubation period. The results are presented as means, $n = 2$–4.

reached its maximal level. It is of interest to note that at this time point (6 h) the response to FSH was similarly intense as that to LH (FSH, 100 ng/ml, 458 pg progesterone/follicle).

Discussion

In the present study we demonstrate that oocytes which underwent maturation in response to EGF were successfully fertilized, although at a lower rate than oocytes matured under the influence of LH (EGF: 61%, LH: 79%). The term 'oocyte maturation' comprises a series of events which take place in different compartments of the cumulus-oocyte complex. These changes are manifested by nuclear maturation, expansion and mucification of the cumulus oophorus, an increase in zona pellucida penetrability to sperm and maturational changes in the ooplasma (Clarke and Masui, 1986, 1987; Fowler, 1988; Tesarik et al., 1988; Flood et al., 1990; Rufas and Shalgi, 1990; Cuasicu and Bedford, 1991). The difference in fertilizability between the EGF and the LH group of oocytes could possibly result from a response that was limited to some of the follicular compartments. In the case that maturation of the oocyte is not accompanied by an appropriate maturation of the cumulus cells and the zona

pellucida, the rate of fertilization will probably be affected. This idea is supported by our later findings that once fertilized the potential of the zygote to further develop and cleave in the EGF and LH group was almost identical (48% and 45% respectively).

In previous studies we have demonstrated that oocytes induced to mature by a GnRH analog (GnRHa) in vivo or in vitro, are fertilizable and have the potential to develop into normal embryos (Dekel and Shalgi, 1987; Shalgi and Dekel, 1990; Ben-Yosef et al., unpublished). In the present study, using a similar in vitro maturation system, we found that the fertilization rate of oocytes exposed to EGF was somewhat lower than that obtained by GnRHa. In spite of these differences in fertilization rate, it is interesting to note that the developmental potential of fertilized eggs in both groups (EGF and GnRHa) was similar, and not different from that of the control (Ben-Yosef et al., unpublished).

Although EGF acts as a potent inducer of oocyte maturation (Dekel and Sherizly, 1985), its effect on ovarian steroidogenesis was very poor (77 pg progesterone/follicle). In our study maximal progesterone production induced by EGF was 6-fold lower than that induced by both LH and FSH. The same proportion between EGF and FSH stimulation is reported in another study in which rat granulosa cells have been used (3 ng/ml and 17 ng/ml progesterone respectively, Jones et al., 1982); similarly, in hamster follicles EGF induced the production of 2000 pg progesterone/follicle with 10,000 pg progesterone/follicle induced by FSH (Roy and Greenwald, 1991). Both FSH and LH are known to induce progesterone production via the cAMP second messenger system (Lindner and Bauminger, 1974). Taken together these findings suggest that biosynthesis of progesterone is effectively induced by hormones that act via cAMP, rather than by hormones that activate other biochemical pathways, such as EGF. This idea seem to coincide with the effect of GnRH on ovarian steroidogenesis which is not significantly different from that of EGF (GnRHa 10^{-7} M; 24.5 pg/follicle at 4 h of incubation, 98 pg/follicle at 8 h of incubation; unpublished results). Eckstein et al. (1986) showed that progesterone production induced by GnRHa in rat granulosa cells in vitro was low. It is interesting to note that both EGF and GnRHa effectively induce oocyte maturation, yet showing a very limited effect on progesterone production. These findings may suggest that both these agonists activate similar biochemical pathways.

EGF is known as a mitogen, affecting cellular proliferation in a variety of somatic cells. It also causes proliferation of granulosa cells (Skinner et al., 1987; Hill, 1989). This growth factor may possibly serve as a physiological intraovarian regulator of granulosa cell proliferation, but probably does not play a role in the luteinization process. Nevertheless, our demonstration of a high fertilization and cleavage rate of oocytes induced to mature by EGF supports the idea that EGF may be involved in the control of meiotic maturation in vivo. It is possible that EGF acts in the follicle in an endocrine manner since this growth factor has been reported to reach serum concentrations that are probably high enough to affect meiosis, as suggested by Byyny et al. (1974). Alternatively, local production of EGF as well as other growth factors could possibly function in an autocrine or paracrine manner within the developing follicle. This hypothesis is strongly supported by the existence of an EGF-like protein in granulosa, theca, interstitial cells as well as in the follicular fluid of several species (Skinner et al., 1987; Roy and Greenwald, 1990), and by the presence of specific and high affinity binding sites for EGF within the ovary (St-Arnaud et al., 1983; Feng et al., 1987; Rose et al., 1991).

Other mitogenic agents such as lectins were found to stimulate oocyte maturation in the mouse (Fagbohun and Downs, 1990). These and our findings strongly support the idea that both mitogenesis and the induction of meiosis are mediated by common signals. These signals may involve activation of the maturation-promoting factor (MPF), which is a ubiquitous cytoplasmic protein that plays a key role in regulation of both meiosis and mitosis (Dunphy and Newport, 1988; Kishimoto, 1988; Murray and Kirschner, 1991).

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