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ERYTHROID DIFFERENTIATION FACTOR CAN MODULATE FOLLICULAR GRANULOSA CELL FUNCTIONS

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SUMMARY: The action of human erythroid differentiation factor (EDF) on the functions of rat granulosa cells cultured in a chemically defined medium was investigated. In the presence of FSH that induced LH receptor expression and progesterone synthesis during culture of the cells, EDF augmented both responses in a dose- and time-dependent manner. Unlike FSH, EDF itself did not have such an inducing effect at all. Furthermore, in the absence of FSH, EDF was found to strongly enhance the ability of granulosa cells to produce inhibin. Thus, EDF may play an important role in the regulation of granulosa cell function and differentiation during follicle development.

During the maturation of ovarian follicles and their transformation into corpora lutea, follicular granulosa cells undergo a continuing and distinct differentiation process. There is increasing evidence that granulosa cells respond to several polypeptide factors including gonadal hormones, inhibin and activin, which have recently been purified from the mammalian follicular fluids(1-7). Inhibin, which is synthesized in granulosa cells (8), is capable of suppressing of follicle-stimulating hormone (FSH) secretion from the pituitary, and activin, whose cellular origin is uncertain, has effects opposite to those of inhibin at the pituitary level. Based on DNA cloning results with these polypeptides, inhibin is a heterodimer consisting of an α subunit and one of two β subunits (β_A or β_B) encoded by separate genes, whereas activin is a dimer of two inhibin β subunits (9-12). Furthermore, the genes for the inhibin β subunits are highly conserved in all the mammalian studies and are homologous to the genes for transforming growth factor type β(TGF-β) (13) and Müllerian duct inhibiting substance (14). Recent papers indicate that inhibin, activin and TGF-βcan exert local actions on granulosa cell steroidogenesis and LH receptor formation(15-18), but the

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exact physiological significance of the findings so far reported remains to be examined.

On the other hand, a polypeptide factor which can induce the differentiation of mouse Friend erythroleukemia cells has recently been isolated from the conditioned medium of a human leukemia cell line (19). The polypeptide, named erythroid differentiation factor (EDF), has been found to be a homodimer of two β_A subunits, that is, the same polypeptide molecular species as activin A(20), and also to stimulate the secretion of pituitary FSH (21). These facts raise the possibility that EDF is versatile enough to affect the differentiation of follicular granulosa cells. In this paper, we report the FSH dependent action of EDF on LH receptor expression and progesterone production and its direct action on inhibin production by rat granulosa cells in culture, compared with that of inhibin A.

MATERIALS AND METHODS

Hormones and reagents: Erythroid differentiation factor (EDF), was obtained from the culture medium of human leukemia cell THP-1 (19). Bovine 32 kDa inhibin A was purified by immunoaffinity chromatography using the monoclonal anti-bovine 32 kDa inhibin A antibody (α-chain specific) (22) as described previously (23). Rat FSH (NIH-FSH-I-5) was provided by the National Pituitary Agency, National Institute of Arthritis, Metabolism and Digestive Diseases. Human chorionic gonadotropin (hCG) was kindly supplied by Dr. R. E. Canfield (Columbia University, New York, NY). Diethylstilbestrol (DES) and gentamycin sulfate were purchased from Sigma Chemical Co. (St.Louis, Mo). Dulbecco's modified Eagle medium (DMEM) was a product of Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Ham's F-12 and fungizone were purchased from Irvine Scientific (Santa Ana, CA). Chloramine T was obtained from Wako Pure Chemical Industries Inc. (Osaka, Japan).

Rat granulosa cell culture: Granulosa cells were obtained from immature female Wistar rats (21-23 days old) which were pretreated subcutaneously with 1 mg of DES in 0.1 ml sesame oil daily for 4 days. Granulosa cells were cultured in Immulon 2 Removawell (Dynatech Laboratories, Inc., Alexandria, VA) at 37°C in a 7.5% CO_2-92.5% air atmosphere. Each well contained 1 x 10^5 viable cells (determined by trypan blue exclusion) in 200 µl Ham's F-12/DMEM (1:1, v/v) medium supplemented with 14.3 mM sodium bicarbonate, gentamycin sulfate (40 µg/ml) and fungizone (1 µg/ml). At various times during culture, the medium was removed and saved for assays of progesterone and inhibin. The cells were analysed for LH receptor content.

Determination of LH receptor: The LH receptor content of the cells was measured by quantitating the levels of ^{125}I-hCG bound to the intact cells. Briefly, the granulosa cell monolayers were incubated in a 1:1 (v/v) mixture solution of DMEM-Ham's F-12 containing 20 mM Hepes (pH 7.4) at 37°C with a saturating concentration of ^{125}I-hCG (1ng, ≃18,000cpm/ng). The incubation medium was removed after 2 hr of incubation and the cells were washed twice with 200 µl of the mixture solution. Then, each well was torn off from the Removawell strips and the amount of radioactivity remaining on the wells (cell-bound hormone) was quantitated by γ-spectrometry. Nonspecific binding was determined by adding excess unlabeled hCG. Receptor content was expressed as counts per min of cell-bound hormone/1 x 10^5cells.

Determination of progesterone and inhibin: The concentration of progesterone in the medium was determined by direct radioimmunoassay (RIA) (24). Inhibin

production by granulosa cells was monitored by RIA as described previously (25).

Iodination of proteins: Proteins were iodinated according to the lactoperoxidase or chloramine T method (26).

RESULTS

In our primary cultrue of granulosa cells from immature DES-treated female rats, LH receptor expression and progesterone production were induced by FSH in a time- and dose-dependent manner. The expression reached the maximum level during 65-75 hr culture (data not shown). We attempted to demonstrate the ability of EDF and bovine 32 kDa inhibin A to induce the LH receptor formation and progesterone synthesis in granulosa cells, but unlike FSH they themselves did not have such an inducing function. Then, the modulatory effects of EDF and bovine inhibin A on FSH actions were examined in a 72-hr culture of granulosa cells. LH receptor induction by FSH was remarkably enhanced by concomitant EDF (Fig.1A). A negligible level of LH receptor was detected in the presence of 10 ng/ml of FSH alone, but the receptor expression was amplified 10-fold by cotreatment with 30 ng/ml of EDF, and to more than 25-fold with 100 ng/ml of the factor. The EDF action was increased in a dose-dependent manner up to an FSH concentration of 30

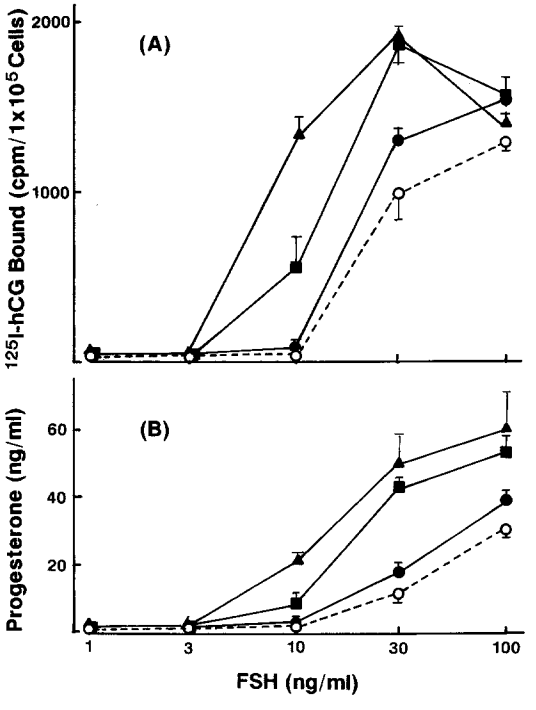


Figure 1. Effect of EDF on FSH-induced LH receptor expression (A) and progesterone production (B) by rat granulosa cells. Granulosa cells (1 x 10^5 cells/well) obtained from DES-treated rats were cultured for 72 hr with increasing concentrations of FSH plus varying amounts of EDF (0 ng/ml,--○--; 10 ng/ml, —●— ; 30 ng/ml, —■— ; 100 ng/ml, —▲—). The results are the mean ± SD for four wells.

ng/ml. Although in the presence of the higher EDF concentrations (30-100 ng/ml) a fall in LH receptor induced by 100 ng/ml of FSH was observed, EDF still increased the number of LH-binding sites more than to that in the absence of EDF. Similarly, the treatment of granulosa cells with FSH resulted in a dose-dependent increase in progesterone production (Fig.1B). Likewise, EDF significantly augmented the FSH action at higher doses than 10 ng/ml of the gonadotropin. Both progesterone and LH receptor syntheses remained unaffected in the presence of a lower concentration than 3 ng/ml of FSH, even when treated with a high concentration of EDF (100 ng/ml). These results showed that a higher FSH concentration (> 10 ng/ml) was necessary in leading up to the EDF action.

We tested the modulatory effect of bovine 32 kDa inhibin A on FSH-induced LH receptor formation and progesterone accumulation. The addition of inhibin to the 3-day culture of granulosa cells at concentrations up to 5,000 ng/ml (160 nM) did not significantly alter either the basal or FSH-induced level of LH receptor (Table I). On the other hand, inhibin A slightly suppressed the action of 30 ng FSH on progesterone production ($P < 0.01$ at an inhibin concentration of 1,000 ng/ml), although it had no effect on basal progesterone synthesis (Table I).

The cellular mechanisms of inhibin production by granulosa cells have been taken up as a new topic in the discussion of ovarian follicle development, but still there are many problems to be solved. Since it is accepted that FSH stimulates inhibin production by granulosa cells (26), we attempted to determine the effects of EDF on the biosynthesis of inhibin in cultured granulosa cells. As shown in Fig.2, EDF itself was found to have a potent ability to potentiate the biosynthesis of inhibin in granulosa cell in a dose-dependent manner: its augmenting ability was similar to that of FSH.

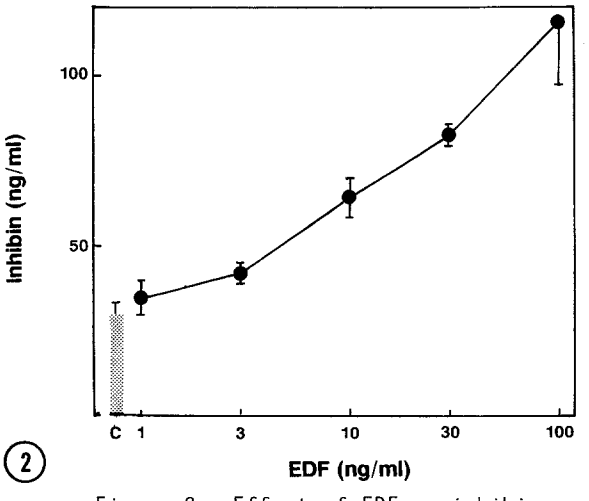
Table I. Effect of bovine 32K inhibin A on FSH-induced LH receptor formation and progesterone production by rat granulosa cells

FSH (ng/ml)	Inhibin (ng/ml)	^{125}I-hCG bound (cpm/1x10^5 cells)	Progesterone produced (ng/ml)
0	0	95 ± 57	< 0.1
0	200	51 ± 16	< 0.1
0	1000	72 ± 31	< 0.1
0	5000	60 ± 23	< 0.1
30	0	826 ± 174	15.4 ± 5.0
30	200	782 ± 58	11.3 ± 3.7
30	1000	767 ± 85	9.3 ± 2.0*
30	5000	918 ± 118	10.9 ± 3.1

Granulosa cells (1 x 10^5 cells/well) obtained from DES-treated rats were cultured for 72 hr with increasing concentrations of inhibin (0-5000 ng/ml) in the presence (30 ng/ml) or absence of FSH. The results are the mean ± SD for four wells. *, $p < 0.01$ vs. cultures treated with FSH alone.

The combined effect of FSH and EDF appeared to be simply additive, and not synergistic (data not shown).

To find how to explain the regulatory mechanisms of diverse EDF actions on granulosa cells, the time-dependent effects of EDF were tested. The FSH-induced LH receptor content was dramatically increased during 36 to 60 hr culture and a rapid decrease was observed at 96 hr (Fig.3A). EDF did not affect the timing of the peak (around 60 hr) of receptor formation. EDF also caused a time-dependent increase in FSH-stimulated progesterone production (Fig.3B). The response reached its maximum at around 48 hr. In both cases, an obligatory lag phase of 24-36 hr was required before EDF-activated increases were detectable, which suggests that important biochemical events are involved in the activation process.



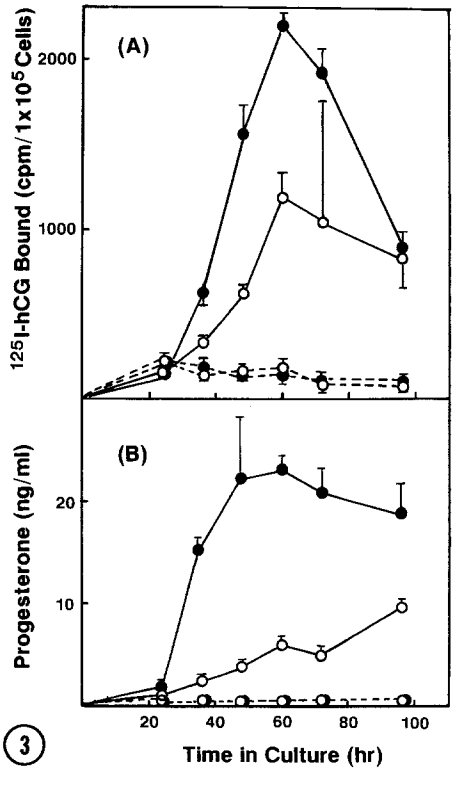


Figure 2. Effect of EDF on inhibin production by rat granulosa cells . Granulosa cells (1 x 10^5 cells/well) obtained from DES-treated rats were cultured for 72 hr with increasing concentrations of EDF (1-100 ng/ml). Histogram represents the basal level in the absence of EDF. The results are the mean ± SD for four wells.

Figure 3. Time course for LH receptor expression (A) and progesterone producation (B) by rat granulosa cells. Granulosa cells (1 x 10^5 cells/well) obtained from DES-treated rats were cultured for 72 hr with (●) or without (○) EDF (30 ng/ml) and in the presence (—) or absence (---) of FSH (30 ng/ml). The results are the mean ± SD for four wells.

DISCUSSION

The present study has shown a unique action of EDF on rat ovarian granulosa cell development. EDF can enhance the FSH induction of LH receptor expression and progesterone production by granulosa cells, while EDF itself has no effect on either basal level in the absence or lower concentrations (< 3ng/ml) of FSH. In addition to the FSH-dependent actions, EDF potentiates the ability of granulosa cells to produce inhibin in a manner comparable to that of FSH. Bovine 32 kDa inhibin A, on the other hand, exerts no effect on the increase in LH receptor and slightly attenuates the progestetone production induced by FSH.

EDF has been obtained from conditioned medium of human monocytic leukemia cell THP-1 and its primary structure has been shown to be identical to that of activin A that was isolated from porcine follicular fluid (6). Moreover, EDF has been reported to have the same biological activity as activin A (21), but some difference was found in their effect on steroidogenesis in granulosa cells. Hutchinson et al (16) reported that bovine activin A arrested FSH-induced progesterone production with an ED_{50} of 0.42 pM (10.5 pg/ml) and proposed an anti-luteinization effect. Under our experimental conditions, however, such a low concentrations of EDF as 5-50 pg/ml did not show any effect on production in the presence of 30-100 ng/ml FSH (data not shown). Althogh it is possible that this discrepancy may be due to the difference in experimental conditions, additional studies should clarify its biological significance.

Opinion is divided on the action of inhibin on FSH-stimulated granulosa cell steroidogenesis: Ying et al (15) claimed that inhibin prevented FSH-stimulated estrogen production whereas Hutchinson et al (16) observed no effect on either basal or FSH-stimulated aromatase activity and progesterone synthesis. There exist some questions about their claims, because the doses of inhibin used in their experiments were very low (up to 10 ng/ml) compared to the levels (50 ng/ml) produced by granulosa cells. Therefore, we used the higher doses of inhibin ($\geq$200ng/ml), and a slight suppressive effect on progesterone production was observed (Table I). Anyway, the specific local function of inhibin in the differentiation of follicular granulosa cells is still controversial.

The potential importance of the monocytic cell origin EDF is underscored by its homology with TGF-β that is produced by many normal and malignant tissues and has many effects. Recently, many papers have dealt with the modulatory actions of TGF-β on differentiation and proliferation of granulosa cells. The actions of EDF observed in this study are quite similar to those of TGF-β, which can augment LH receptor formation and progesterone accumulation in the developing granulosa cells, but there are

some differences in the actions of these regulatory proteins. TGF-β has a biphasic effect that enhances the stimulatory actions of low levels of FSH and inhibits the induction of LH receptor by higher levels of FSH (18), Regarding EDF, no such dual effect was observed in our study. In any case, our study and others strongly support the possibility that EDF (activin A) and TGF-β may be local endogenous ovarian regulators.

Extensive studies on regulatory mechanisms of inhibin production by cultured granulosa cells are now going on (25,27) and FSH, cAMP analog, choleratoxin and testosterone etc. are known to directly or indirectly stimulate production. Our study added one more -the direct stimulator EDF- to them. This, in other words, indicates that biosynthesis of inhibin is regulated locally at the granulosa cell level by EDF(activin A), suggesting that the physiologically important and complex control mechanism for FSH secretion exists in the pituitary-gonadal axis.

As shown in Fig.3, a lag period of 24-30 hr was required to detect noticeable EDF actions, indicating that the effects of EDF are not direct but mediated by the induction or suppression of some cellular processes. In order to solve the puzzle of EDF actions, we performed a preliminary experiment and obtained promising results. When rat granulosa cells were pretreated with EDF for 24 hr and, after removing EDF by washing the cells, the culture was continued for another 48 hr in the presence of FSH, a significant amount of LH receptor was found to be expressed. In contrast, when FSH treatment was followed by EDF treatment, no LH-receptor formation was observed at all (data not shown). Although it is known that initial events in granulosa cell differentiation require only the inductor substance, FSH, this preliminary result and the present study suggest that EDF may affect the earlier differentiation stage of granulosa cell, and trigger the differentiation process.

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