Identification of a Specific Receptor for Erythroid Differentiation Factor on Follicular Granulosa Cell*

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A cellular receptor for erythroid differentiation factor (EDF) was demonstrated by incubation of 125I-labeled EDF with rat follicular granulosa cell cultures. The specific binding of labeled EDF to the cells showed saturation; Scatchard analysis of binding data indicated a single class of receptors having Kd = 3.4 × 10^-10 M. A large excess of unlabeled EDF reduced labeled EDF binding almost completely, whereas similar doses of inhibin and transforming growth factor type β, which are quite similar to EDF in protein structure and subunit organization, had no effect; EDF did not share receptors with those factors. EDF receptor (activin A receptor) expression was enhanced in granulosa cells cultured in the presence of follicle-stimulating hormone; follicle-stimulating hormone raised the number of EDF binding sites/cell from 13,000 to 96,000 without altering the binding affinity.

A variety of hypophysiotropic proteins or peptides including inhibin and activin has been reported to be present in mammalian gonads. Inhibin is a gonadal hormone that specifically suppresses the secretion of follicle-stimulating hormone (FSH) but not luteinizing hormone. Two forms of inhibin (A and B) obtained from the gonadal fluid of several species are characterized as a heterodimeric protein consisting of one α subunit and one of two β subunits held together by disulfide bond(s) (1–7). On the other hand, activin A (or FSH-releasing protein) and activin AB also obtained from porcine follicular fluid are selective stimulators of pituitary FSH release in vitro; the former is a homodimer composed of two β subunits of inhibin, and the latter is a heterodimer composed of one β subunit and one β subunits of inhibin (8, 9). Additionally, the recent DNA cloning data indicate the striking homology between the β dimers and transforming growth factor type β (TGF-β); the marked homology is found especially in the COOH-terminal half of the molecules (10–16).

Recently, a protein factor termed erythroid differentiation factor (EDF), which can differentiate mouse Friend cells (lacking in ability of hemoglobin accumulation) into hemoglobin-positive cells, has been isolated from the conditioned medium of human leukemia cell line THP-1 (17). The protein chemical and DNA sequence analyses of the factor revealed that EDF is a homodimer composed of two inhibin β subunits linked by disulfide bond(s); in other words, EDF is the same molecular species as activin A (17, 18).

Although EDF was initially characterized by its ability to cause differentiation of erythroid progenitor cells, it has now been shown to possess multifunctional properties. It was confirmed that EDF can stimulate FSH secretion by cultured anterior pituitary cells with the same potency as activin A (19); this proved the molecular identity between both protein factors not only by the structure but also by the biological activity.

We recently have found that EDF has various modulatory actions on follicular granulosa cell functions; EDF significantly augments the FSH-induced luteinizing hormone receptor expression and progesterone synthesis in cultured granulosa cells, and enhances an ability of the cells to produce inhibin (20). Moreover, an activity of EDF to induce the formation of FSH receptor in granulosa cells has also been demonstrated. These findings strongly suggest that EDF plays an important role in regulation of the granulosa cell functions and differentiation, and thus, that the specific binding of EDF to its granulosa cell surface receptor is the initial event of a process which ultimately elicits cellular responses. Although it is well established that FSH is the primary regulator of granulosa cell maturation, there is increasing evidence that its actions are modulated by gonadal peptides or peptide growth factors; for instance, inhibin, activin (EDF), and TGF-β exert local actions on granulosa cell functions (20–24). However, cellular receptors for these peptide factors on granulosa cells have not been identified yet. In this paper, we describe a specific binding of 125I-labeled EDF to cultured granulosa cells.

**EXPERIMENTAL PROCEDURES**

Materials—EDF was purified from the culture fluid of human leukemia cell line THP-1 (17). Bovine 32-kDa inhibin A was obtained by immunoadfinity chromatography using the monoclonal anti-bovine 32-kDa inhibin A antibody (α-subunit specific) (25, 26). Porcine TGF-β was purchased from R & D System Inc. (Minneapolis, MN). Rat FSH (NIH-FSH-I-5) was provided by the National Pituitary Agency, National Institute of Arthritis, Metabolism, and Digestive Diseases. Diethylstilbestrol (DES) and gentamycin sulfate were purchased from Sigma. Dulbecco’s modified Eagle’s medium 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). Bovine serum albumin (Fraction V) was from Armour.

Binding of 125I-Labeled EDF to Rat Granulosa Cells—Cellular binding of 125I-labeled EDF was studied by using the primary culture of rat granulosa cells. Granulosa cells were obtained from immature female Wistar rats (21–23 days old) which were administered subcutaneous injection of DES, diethylstilbestrol.

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The abbreviations used are: FSH, follicle-stimulating hormone; EDF, erythroid differentiation factor; TGF-β, transforming growth factor type β; DES, diethylstilbestrol.
taneously at a dose of 2 mg in 0.1 ml of sesame oil per head daily for 4 days. The cells were cultured in Immulon 2 Removawell (Dynatech Laboratories, Inc., Alexandria, VA) at 37 °C in 7.5% CO₂, 92.5% air for 2 days. Each well contained 1 × 10⁶ viable cells in 0.2 ml of Ham's F-12/Dulbecco's modified Eagle's medium (1:1, v/v), medium supplemented with 14.3 mM sodium bicarbonate, gentamycin sulfate (40 μg/ml), and Fungizone (1 μg/ml). After removing the medium, labeled EDF dissolved in 0.05 ml of binding medium (culture medium plus 2 mg/ml bovine serum albumin) with or without unlabeled EDF dissolved in 0.05 ml of binding medium was added. Binding was allowed to stand at 37 °C for 1 h in 7.5% CO₂, 92.5% air. Experiments were terminated by twice washing with 0.2 ml of ice-cold binding medium. Then, each well was torn off the Removawell strips, and the amount of radioactivity remaining on the wells (cell-bound EDF) was quantitated by γ spectrometry.

Iodination of EDF—EDF was iodinated by the chloramine-T method (27). EDF (3.77 μg in 15 μl of 0.2 M phosphate buffer, pH 7.5) was incubated with 250 μg of Na[^131]I (Amersham Japan, Tokyo, Japan) for 1 min at room temperature. ^131]Labeled EDF was separated from unlabeled radioactivity on a Bio-Gel P-60 (Bio-Rad) column (1 × 10 cm) eluting with 0.06 M phosphate-buffered saline, pH 7.4. The specific activity of about 6000 cpm/ng was obtained.

RESULTS

Demonstration of ^131]Labeled EDF Binding to Rat Granulosa Cells—Rat granulosa cells cultured in serum-free medium for 2 days were incubated with various concentrations of ^131]labeled EDF for 1 h at 37 °C. Fig. 1A demonstrates a ligand saturation curve; half-maximal binding was at 200 pM, and saturation was at around 1 nM. A Scatchard plot of the binding data gave a straight line (Fig. 1B), indicating that a single class of receptors for EDF is present on granulosa cells. The apparent Kₘ was 3.4 × 10⁻¹⁰ M, and the average number of receptors was approximately 13,000/cell.

![Fig. 1. Saturation curve of ^131]labeled EDF binding to rat granulosa cells. A, granulosa cells from DES-treated immature female rats (1 × 10⁶ cells/well) were cultured for 48 h at 37 °C. After removing the medium, the resulting cells were incubated at 37 °C for 1 h with various amounts of ^131]labeled EDF. After washing with the binding buffer, the cell-associated ^131]labeled EDF was then determined. B, binding data were transformed and plotted by Scatchard analysis. E, bound; F, free.](image-url)

![Fig. 2. Specificity of ^131]labeled EDF binding to rat granulosa cells. Granulosa cells from DES-treated immature female rats (1 × 10⁶ cells/well) were cultured in the presence of FSH (30 ng/ml) for 48 h at 37 °C. After removing the medium, the resulting cells were incubated at 37 °C for 1 h with 1.25 ng (7500 cpm) of labeled EDF/well and various amounts of unlabeled EDF (O), inhibin (A), or TGF-β (B). Binding was terminated with the binding buffer and the cell-bound ^131]labeled EDF was determined.](image-url)

![Fig. 3. Induction of EDF receptor in rat granulosa cells by FSH. Rat granulosa cells from DES-treated immature female rats (1 × 10⁶ cells/well) were cultured for 48 h at 37 °C in the presence of various amounts of FSH. After removing the medium, the resulting cells were incubated at 37 °C for 2 h with 5 ng of ^131]labeled EDF in the binding medium. Binding was terminated by washing with the binding medium, and the cell-bound ^131]labeled EDF was determined.](image-url)

Lack of Cross-reactivity of ^131]Labeled EDF Receptors for Inhibin and TGF-β—Incubation of granulosa cells with a fixed amount (25 ng/ml) of ^131]labeled EDF and increasing amounts of unlabeled EDF resulted in progressive inhibition of the tracer binding; at the high doses (>100 ng/ml), the displacement was about 90% (Fig. 2). In contrast, the presence of similar amounts of bovine 32-kDa inhibin or TGF-β had no effect on the binding of labeled EDF to the cells.

Induction of EDF Receptors by FSH—The addition of FSH to rat granulosa cells in culture caused a rise in EDF receptors in a dose-dependent manner; the induction of the receptors by FSH was elevated linearly by incubation for 48 h with increasing levels of the gonadotropin (1–30 ng/ml); at higher FSH concentrations the receptor levels no longer increased (Fig. 3). Fig. 4 demonstrates the concentration-dependent binding of ^131]labeled EDF to the receptors on granulosa cells treated with FSH; when compared with the binding capacity of the nontreated cells, the binding to the FSH-treated cells was 7–8-fold greater (Fig. 4A). Scatchard analyses showed that the incubation of granulosa cells with 30 ng/ml FSH which induced maximal expression of EDF receptors augmented the number of EDF binding sites/cell from 13,000 to
The receptor is responsible for the biological activity of the molecule. Inhibin production was determined in several ways. More than 90% of the labeled cule.

Addition of FSH induced a 7-8-fold rise in the number of EDF binding sites without alteration of the binding affinity. The EDF receptor was found to be distinct from inhibin receptor and TGF-β receptor. The EDF receptor also does not bind inhibin; a large excess of inhibin had no significant effect on the binding of EDF at a concentration 1000 times that needed to give a half-maximal response in the inhibin biological assay (pituitary cell culture). This is also in good accord with the finding that a large amount of inhibin has no effect on the modulatory actions of EDF on granulosa cell functions.

For comparison, TGF-β binds to a variety of cultured cells of both epithelial and mesenchymal origin with a $K_d$ of 20-50 pM, and there are 10,000-20,000 binding sites/cell (30). Receptors for platelet-derived growth factor exist on Swiss/3T3 cells with a $K_d$ of 10 pM, and 200,000 binding sites are on the cell surface (31). Epidermal growth factor binds to human fibroblasts with a $K_d$ of 300-500 pM and with 100,000 binding sites/cell (32) and to AKR-2B cells with a $K_d$ of 1 nM and 100,000 binding sites/cell (33). Thus, like TGF-β, there appear to be significantly fewer EDF binding sites on granulosa cells than platelet-derived or epidermal growth factor binding sites. On the other hand, the binding affinity of EDF compares favorably with other factors.

The EDF binding sites on granulosa cells were augmented by treating the cells with FSH; in cultured granulosa cells, addition of 30 ng/ml FSH induced a 7-8-fold rise in the EDF receptor (approximately 100,000 binding sites/cell). Therefore, FSH should increase the responsiveness of the cell to EDF. In fact, as we reported previously (20), in the presence of FSH that induces luteinizing hormone receptor formation and progesterone synthesis during culture of granulosa cells, EDF can enhance both responses while EDF itself does not have such an inducing effect in the absence of FSH. These observations may be explained by the EDF receptor-inducible effect of FSH. Although further characterization of the EDF receptor is required to evaluate the role of EDF in the process of granulosa cell differentiation, the information derived from the present study should serve as an aid to address the question.

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REFERENCES