Regulation of Rat Ovarian Cell Growth and Steroid Secretion

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ABSTRACT A cultured rat ovarian cell line (31A-F2) was used to study the effect of growth factors (epidermal growth factor [EGF] and fibroblast growth factor [FGF]), a survival factor (ovarian growth factor [OGF]), a hormone (insulin), and an iron-binding protein (transferrin) on cell proliferation and steroid production under defined culture conditions. EGF and insulin were shown to be mitogenic (half-maximal response at 0.12 nM and 0.11 μM, respectively) for 31A-F2 cells incubated in serum-free medium. EGF induced up to three doublings in the cell population, whereas insulin induced an average of one cell population doubling. FGF, OGF, and transferrin were found not to have any prominent effect on cell division when incubated individually with 31A-F2 cells in serum-free medium. However, a combination of EGF, OGF, insulin, and transferrin stimulated cell division to the same approximate extent as cells incubated in the presence of 5% fetal calf serum.

EGF or insulin did not significantly affect total cell cholesterol levels (relative to cells incubated in serum-free medium) when incubated individually with 31A-F2 cells. However, cell cholesterol levels were increased by the addition of OGF (250%), FGF (370%), or a combination of insulin and EGF (320%). Progesterone secretion from 31A-F2 cells was enhanced by EGF (25%), FGF (80%), and insulin (115%). However, the addition of a mitogenic mixture of EGF, OGF, insulin, and transferrin suppressed progesterone secretion 150% below that of control cultures. These studies have permitted us to determine that EGF and insulin are mitogenic factors that are required for the growth of 31A-F2 cells and that OGF and transferrin are positive cofactors that enhance growth. Also, additional data suggest that cholesterol and progesterone production in 31A-F2 cells can be regulated by peptide growth factors and the hormone insulin.

Mitogenic activity of low molecular weight proteins on cultured cells has been reported for several cell lines (8). A major drawback has been the requirement of a minimal amount of serum to maintain the viability of the cells and yet not so much as to initiate multiple cell division. The question arises as to whether the addition of a growth factor in an assay system is alone responsible for growth. Hayashi et al. (9) have reported that a mixture of hormones can stimulate the growth of GH3 rat pituitary cells in the absence of serum. Wyche and Noteboom (15) found that rat ovarian cells (31A-F2) maintained in serum-free medium required the addition of a factor isolated from fetal calf serum (FCS) that could initiate and maintain continuous cell division. We have also reported that a growth factor derived from serum-free conditioned medium in which human pituitary cells have been cultured also stimulates 31A-F2 cells in an analogous manner (16). We have suggested that complex interactions may occur between isolated growth factors under defined culture conditions (15). Recently, we have demonstrated that 31A-F2 cells synthesize and secrete sex steroids into the culture medium. This communication presents further findings on the growth-promoting activities of isolated growth factors and the hormone insulin on 31A-F2 cell growth and steroid production.

1 Abbreviations used in this paper: EGF, epidermal growth factor; FCS, fetal calf serum; FGF, fibroblast growth factor; OGF, ovarian growth factor.

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MATERIALS AND METHODS

Cell Culture

Experiments were performed with a rat ovarian epithelial subline (31A-F2) which has been previously described (15). These cells have been grown continuously for over three years in Coon’s Modified Ham’s F-12 Medium (Coon’s medium, obtained from Pacific Biological Co., Richmond, Calif.) plus a factor (15) isolated from FCS (obtained from Grand Island Biological Co., Grand Island, N. Y.) and ovarian growth factor (OGF) (8) which was generously supplied by Dr. Denis Gospodarowicz at the University of California, San Francisco. The growth and handling of stock cells and the composition of the medium have been described elsewhere (15). These cells have been periodically checked for mycoplasma contamination (12) by autoradiography and found to be mycoplasm-negative.

Growth Assays

Stock 31A-F2 cells were detached from plastic dishes by trypsinization with 0.5 ml of a 0.01% sterile trypsin (American Biochemicals Co., Cleveland, Ohio) solution within 5 min at 37°C. The activity of trypsin was inhibited with 0.5 ml of a 5 mg/ml sterile solution of ovomucoid trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.). The cells were spun down at 1,000 rpm for 5 min, and the cell pellet was washed once with 5–10 ml of fresh serum-free Coon’s medium.

The cell pellet was resuspended in fresh serum-free Coon’s medium and plated at a density of about 5 x 10^4 cells/60 x 15 mm culture dish (Corning Glass Works, Science Products Div., Corning, N. Y.), each dish containing a total volume of 5 ml of serum-free Coon’s medium with or without additions. The protein factors (insulin and transferrin, Sigma Chemical Co., St. Louis, Mo.; OGF, epidermal growth factor [EGF], and fibroblast growth factor [FGF], Collaborative Research Inc., Waltham, Mass.) assayed for growth were added in I ml of methanol. Both the cell and medium methanol mixtures were extracted with 10 times the volume of chloroform, evaporated to dryness at 45°C, and then extracted with 30% potassium hydroxide at 90°C for 4 h. The solution was then made 50% with respect to ethanol. The alcoholic mixture was then evaporated to dryness, and then extracted with anhydrous ether and the extracted residue was reconstituted in methanol as recently described. The cells were washed once with cold serum-free medium, obtained from Sigma (Chemical Co.) for 8 d in a humidified CO_2 incubator. At the end of the incubation period the medium was removed and extracted separately in I ml of a phosphate-buffered physiological saline solution and counted with a Coulter counter. Each point was done in triplicate, and standard deviations did not exceed 10% of the mean value.

Steroid Analyses

Stock 31A-F2 cells were prepared as outlined above and plated at a density of about 1.5 x 10^4/75-cm<sup>2</sup> culture flask (Corning Glass Works) containing 10 ml of serum-free medium. Cells were incubated without and with proteins (insulin, EGF, FGF, insulin, transferrin) or other chemicals (epinephrine, N<sub>2</sub>-2'-0-dibutyryl cyclic AMP (Bu<sub>2</sub>cAMP), N<sub>2</sub>-2'-0-dibutyryl cGMP (Bu<sub>2</sub>cGMP), obtained from Sigma (Chemical Co.) for 8 d in a humidified CO_2 incubator. At the end of the incubation period the medium was removed and extracted separately with anhydrous ether and the extracted residue was reconstituted in methanol as recently described. The cells were washed once with cold serum-free medium and then extracted with 30% potassium hydroxide at 90°C for 4 h. The solution was then made 50% with respect to ethanol. The alcoholic mixture was then evaporated to dryness, and the residue was reconstituted in various volumes of chloroform, evaporated to dryness at 45°C, and the residue was washed five times with 10 ml of distilled water. The remaining residue was extracted with anhydrous ether, evaporated to dryness, and reconstituted in 1 ml of methanol. Both the cell and medium methanol mixtures were stored at -40°C for total cholesterol and progesterone analyses, respectively.

The medium extracts were assayed by a modified specific radioimmunoassay for progesterone (2, 14, and footnote 2) using [1,2,6,7-3H(N)]progesterone (New England Nuclear, Boston, Mass.) as tracer at 97.9 Ci/mmol stock specific activity. Progesterone antiserum was obtained from Dr. R. E. Short at the USDA Range Station, Miles City, Mont. The cell extracts were assayed for total cell cholesterol by the method of Rudell and Morris (13) as modified from the Zlatkis and Zak (18) procedure. The standard curve used re-crystallized cholesterol (generously supplied by Dr. J. L. Gaylor, University of Missouri) as the standard and was linear from 2.5 to 20 µg. Data from progesterone and cholesterol assays were analyzed by the Student’s t-test for significance.

RESULTS

Effect of EGF, FGF, and OGF with and without Serum on 31A-F2 Cell Growth

 Cultured 31A-F2 rat ovarian cells were found to be very sensitive to factors present in low concentrations in FCS. When the cells were incubated with serum concentrations ranging from 0.2 to 3.0% FCS the growth response was linear (Fig. 1) during an 8-d incubation period. We investigated the ability of the growth factors EGF, FGF, and OGF to stimulate cell growth in the presence and absence of FCS. Cells were separately incubated with various concentrations of the growth factors in serum-free medium for 6 d. EGF consistently gave a 100–200% increase in cell numbers vs. cells incubated in serum-free medium alone (Fig. 2). A peak response was observed at ~2 ng/ml EGF with the half-maximal response occurring at 0.8 ng/ml (1.2 x 10^-8 M). The response of 31A-F2 cells to insulin was also determined and it was found that cell growth was stimulated 100–150% above the serum-free control at 1.0 µg/ml (Fig. 2). The half-maximal growth response was achieved with 0.6 µg/ml (1.1 x 10^-7 M) insulin addition. The stimulation of 31A-F2 cell growth by EGF or insulin addition alone in serum-free medium was roughly comparable to that obtained on the addition of 0.05–0.1% FCS to serum-free medium (Fig. 1).

As previously reported, OGF alone did not enhance the growth of 31A-F2 cells in serum-free medium. It did, however, enhance cell survival and has been classified as a survival factor for these cells (15). An examination as to whether OGF could interact with components present in FCS to further enhance FCS stimulation of growth was also studied. The addition of OGF at 25 ng/ml to an FCS gradient of 0.025–0.30% resulted in an increase in cell numbers relative to cells incubated in FCS alone. OGF stimulated a 68–100% increase in cell numbers when added to 0.025–0.1% FCS (Fig. 3), indicating that OGF can play some secondary role as a growth cofactor. The interaction of other growth factors with components present in FCS was also studied (Fig. 4). FGF behaved much like OGF, but was somewhat less consistent in enhancing 31A-F2 cell survival in serum-free medium. When FGF was incubated with a limiting amount (0.2%) of FCS, the increase in cell numbers obtained (60%) was nearly identical to that obtained in the presence of similar concentrations of OGF and FCS. EGF addition alone, on the other hand, increased cell numbers 150–200% over those of the serum-free control. In the presence of 0.2% FCS, EGF stimulated a 120% enhancement...
of cell numbers over that of the FCS control. Thus, evaluating the effect of growth factors on the growth of 31A-F₂ cells is dependent on the test medium. When incubated individually in serum-free medium with 31A-F₂ cells, EGF is a mitogen. OGF behaves as a survival factor, and FGF seems to have little effect but is not easily differentiated from OGF. When incubated with a limiting amount (0.2%) of FCS, all three

Effect of Insulin in Defined Medium on 31A-F₂ Cell Growth in the Presence and Absence of Growth Factors

The effect of insulin alone and in combination with EGF, FGF, and OGF on the growth of 31A-F₂ cells was studied in serum-free medium. The addition of insulin at 1.0 µg/ml (1.75 x 10⁻⁷ M) resulted in a 100% increase in cell numbers relative to the serum-free control after a 7-d incubation (Fig. 5). The addition of FGF and OGF at 2.5 ng/ml increased cell numbers 35 and 65%, respectively. When the same concentrations of either FGF or OGF and insulin were added together, the net increase in cell numbers (140%) was about the same as that obtained with a single addition of EGF (150%) at 2.5 ng/ml in serum-free medium. The co-incubation of insulin plus EGF with the cells stimulated a slight increase in cell numbers (170%) above that obtained with EGF alone (150%). The co-incubation of EGF, FGF, OGF and insulin with 31A-F₂ cells resulted in a 246% increase in cell numbers above that of the serum-free control. The same effect on growth (~250% increase) was obtained with EGF, OGF, and insulin (data not shown). Therefore, the addition of FGF was omitted in all subsequent experiments.

Modulatory Effect of Transferrin on 31A-F₂ Cell Growth

Various factors (e.g., prostaglandin F₂δ, and E₁, dexamethasone, triiodothyronine, gonadotropin release hormone, thyro-
tropin release hormone, follicle-stimulating hormone, transferrin) were assayed for their ability to further augment the mitogenic effect of EGF, OGF, and insulin on 31A-F2 cells in serum-free medium. It was found that, of those assayed, transferrin mimicked a growth cofactor in its effect. While the addition of transferrin alone to serum-free medium had no effect on cell growth or cell survival (Fig. 6, closed circles), it did enhance cell numbers when added with the mitogenic mixture (EGF, OGF, and insulin). To 2.5 ng/ml each of EGF and OGF and 1 µg/ml insulin were added various concentrations (0.01-10 µg/ml) of transferrin. After 6 d, the maximal response to transferrin was observed at 1 µg/ml (Fig. 7). Transferrin added at 1 µg/ml along with EGF, OGF, and insulin stimulated cell numbers about twofold above those of cells grown in the absence of transferrin (Fig. 7). The effect of the combined factors (EGF, OGF, insulin, and transferrin) was approximately equal to that of ~5% FCS (Fig. 1). After one medium change (arrow, Fig. 6), the cells incubated in the presence of EGF, OGF, insulin, and transferrin reached confluence on day 12.

Effect of Growth Factors and Insulin on 31A-F2 Cell Steroid Levels

In a recent communication it has been shown that 31A-F2 cells synthesize and secrete low levels of the sex steroids...
(progesterone, testosterone, and estradiol-17β) while maintained in serum-free medium. We evaluated the effects of EGF, OGF, and insulin on total cell cholesterol and progesterone secretion under conditions similar to those in which growth were defined.

Neither of the mitogens (EGF and insulin) active on 31A-F2 cells has any significant effect on total cell cholesterol levels. On the other hand, the addition of 2.5 ng/ml of OGF stimulated an increase of ~250% over that of controls (Fig. 8). FGF had virtually no effect on the growth or survival of 31A-F2 cells (Figs. 4 and 5) but stimulated an increase in cell cholesterol of 365% over that of control cultures. While EGF or insulin alone had no effect, when incubated together they increased the total cell cholesterol 320% over that of controls. The addition of OGF and/or transferrin did not further increase cell cholesterol levels. Neither the catecholamine epinephrine or the cyclic nucleotides (Bu)2cAMP and (Bu)2cGMP had any significant effect on cell cholesterol levels.

The effect of the same factors on progesterone secretion from 31A-F2 cells into the culture medium was somewhat different. Progesterone levels in the medium of cells incubated in the presence of OGF were not significantly different from those of control cultures (Fig. 9). The addition of EGF to the cultures produced a small (25%) but significant increase in progesterone secretion (Fig. 9). Insulin addition had the most dramatic effect in increasing the progesterone secretion 116% over that of control cultures. Thus, both of the mitogens stimulated progesterone secretion but not net cholesterol production. The co-incubation of insulin with other factors (EGF, OGF, and transferrin) seemingly reversed insulin's effect some 80–300% below the progesterone values obtained with insulin alone. In contrast, OGF alone had no effect on progesterone secretion, whereas it stimulated cell cholesterol levels 250% (Fig. 8). Thus, the mitogens (EGF and insulin) were effective in stimulating progesterone secretion from 31A-F2 cells, but had no significant effect on cell cholesterol levels. The growth cofactor OGF acted in an opposite manner. OGF had no effect on progesterone secretion, but was highly effective in stimulating cell cholesterol concentrations. FGF was clearly different in that it stimulated a 79% increase in progesterone secretion (Fig. 9) and also maximally stimulated cell cholesterol levels relative to those of controls (Fig. 8). The effect of other nonpeptide factors (Bu)2cAMP and epinephrine) on progesterone secretion was also tested. Epinephrine and (Bu)2cAMP increased the concentration of progesterone in the medium 72 and 46%, respectively, while (Bu)2cGMP had no significant effect. In contrast, neither of the three chemicals had any effect on cell cholesterol levels (Fig. 8).

DISCUSSION

Control of ovarian cell functions represents a very complex and intriguing regulatory pattern of cellular growth and steroid production. The development of tissue culture has permitted investigators to use primary cultures of granulosa and luteal cells (6, 7) to study the factors that regulate ovarian growth, while primary (4) and hybrid (17) granulosa cells have been used to study the luteinization process (3). In recent studies from our laboratory, it has been shown that a cultured rat ovarian subline (31A-F2) has been isolated that can be maintained in the absence of total serum addition to the medium (15, 16). These cells have been shown to be capable of synthesizing and secreting the sex steroids progesterone, testosterone, and estradiol-17β when maintained in serum-free medium. Thus, the 31A-F2 system is the first permanently established ovarian cultured cell system that is steroidogenic. The present study summarizes data that demonstrate that growth and steroid production in 31A-F2 cells are regulated by specific growth factors and hormones under defined culture conditions.

The growth factors OGF and FGF did not behave like mitogens for 31A-F2 cells. Instead, OGF was a survival and growth cofactor (Figs. 5 and 6 and reference 15). However, EGF and insulin, singly, did stimulate cell growth (Fig. 2) and were, therefore, considered to be mitogenic. A combination of insulin plus EGF and other factors further increased cell growth. The inclusion of OGF and transferrin with EGF and insulin (Fig. 6) increased cell numbers by 1.570% over those of the serum-free control. In this system, transferrin is not a primary mitogen (such as EGF and insulin) but acts as a cofactor, i.e., it enhances the effect of EGF and insulin but has

FIGURE 8 Effect of growth factors on 31A-F2 cell cholesterol levels. Cells were plated at about 4 × 10⁵/75-cm² flask each containing 20 ml of serum-free (SF) medium plus or minus 2.5 ng/ml EGF, 2.5 ng/ml FGF, 1 µg/ml insulin (INS), 2.5 ng/ml OGF, 1 µg/ml transferrin (TFN), 5.46 µM epinephrine (EPI), 2 µM (Bu)2cAMP (DBcAMP) or 2.4 µM (Bu)2cGMP (DBcGMP). The cells were incubated for 8 days and the cells and medium were removed and separately extracted. Each treatment was done in triplicate, and samples were assayed in triplicate and values are expressed as the mean ± SE.
no effect when incubated with 31A-F₂ cells by itself. The combination of EGF, OGF, insulin, and transferrin was approximately equal to ~5% FCS addition when assayed in 7- to 8-d growth experiments.

It has been reported that the growth of primary cultures of granulosa cells is stimulated by EGF and FGF, while luteinized granulosa cells (luteal cells) respond only to FGF (6, 7). The 31A-F₂ cells respond to EGF but not to FGF. It would be of interest to see if primary cultures of thecal cells respond only to EGF. No augmentative effect of dexamethasone on growth was observed when 31A-F₂ cells were incubated alone or in combination with EGF, OGF, insulin, and/or transferrin (J. H. Wyche, unpublished data). It had been previously reported for the 31A parent cell line that dexamethasone stimulated cell growth (5). While the iron-binding protein transferrin augmented EGF-mediated growth 2- to 2.5-fold, it does not alter ¹²⁵I-EGF binding to 31A-F₂ cells in the presence or absence of OGF and insulin. Dexamethasone, on the other hand, has been shown to modulate EGF binding to cultured human fibroblast (1).

Insulin has been implicated in playing a role in granulosa cell survival and augmenting progestin secretion when these cells were maintained for a few days in serum-free medium (4). In addition to affecting growth, insulin was found to have a stimulatory effect on the secretion of progesterone from 31A-F₂ cells (Fig. 9). Insulin did not have a generalized effect because cellular cholesterol (Fig. 8) levels were not significantly affected. Thus, it may be postulated that insulin may also have a specific role in regulating progestin secretion and possibly its synthesis in 31A-F₂ rat ovarian cells. The effect of a combination of hormones and growth factors on cholesterol biosynthesis in 31A-F₂ cells represents a generalized induction of one or more rate-limiting steps (e.g., 20a-hydroxycholesterol) in the sterol biosynthetic conversion of cholesterol to the sex steroids. While insulin seems to increase progesterone secretion from 31A-F₂ cells, its effect is lost when EGF is added. The data suggest that EGF and insulin act synergistically on 31A-F₂ cell growth but not on progesterone production. The addition of OGF and transferrin to EGF and insulin further inhibited progesterone secretion below control levels (Fig. 9). This could represent a shunting of the important cell cholesterol pool into membrane components that are under heavier demand when cell division becomes more important than cell differentiation (i.e., progestosterone production). Such may be the case when 31A-F₂ cell division is maximally stimulated in serum-free medium by the mitogenic mixture of EGF, insulin, OGF, and transferrin (Fig. 6). It is not possible to explain at present the significant stimulation in cell cholesterol by OGF and FGF (Fig. 8) and in progesterone secretion by FGF (Fig. 9). It may be that the site(s) of action for OGF and FGF on 31A-F₂ cells may differ from that of EGF, insulin, and possibly transferrin. We postulated that the predominant or primary metabolic effect of FGF and OGF may be at some other site (e.g., mitochondria) involving cholesterol conversion perhaps analogous to the effect that adrenocorticotropic hormone has on cholesterol conversion in adrenal cells (11). Whatever the mode of action, (Bu)cAMP and (Bu)cGMP were incapable of mimicking the stimulatory effect of growth factors on cell cholesterol, which suggests that the effect may not be mediated through a membrane-associated adenylate or guanylate cyclase.

It has been reported that catecholamines (e.g., epinephrine) activate adenylate cyclase which is involved in mediating the response of hormones on progesterone production in luteal cells (10). While epinephrine and (Bu)cAMP had no effect on 31A-F₂ cell growth (J. Wyche, unpublished data), both did significantly enhance progesterone secretion (Fig. 10). This seems to be a selective event because cell cholesterol levels were not significantly affected. Also, (Bu)cGMP had no effect on either cell cholesterol or progesterone production in 31A-F₂ cells, suggesting that the adenylate cyclase system may be specifically involved in progesterone secretion. Thus, there may be two modes of hormone action involved in steroid formation in 31A-F₂ cells. The synthesis and conversion of cholesterol takes place presumably at the mitochondria. It is at this site that the nonmitogenic growth cofactors (FGF and OGF) may have their primary regulatory effect (cAMP-independent action). Substances whose primary effect is elicited by some association with cell membrane components (e.g., adenylate cyclase) may have little effect on mitochondrial cholesterol metabolism in 31A-F₂ cells. Therefore, it is proposed that EGF, insulin, epinephrine and (Bu)cAMP may directly stimulate the adenylate cyclase activity (cAMP-dependent action) associated with the cell membrane, resulting in enhanced progesterone secretion in 31A-F₂ cells.

In summary, the data presented in this communication and elsewhere (footnote 2 and reference 15) have shown that 31A-F₂ rat cultured cells are the first permanently established ovarian system. These cells can be maintained in completely defined medium and their growth can be regulated by the addition of a protein growth factor (EGF), a peptide hormone (insulin), and a serum-binding protein (transferrin). Under specific culture conditions the differentiated status of the cells can be regulated. Progesterone secretion can be regulated by insulin, (Bu)cAMP, epinephrine, FGF, and possibly EGF. While 31A-F₂ is a hormonally responsive ovarian system, it does not possess all the functional characteristics (e.g., loss of gonadotropin receptors) of normal ovarian cells. However, it does maintain some properties relevant to the problems of ovarian growth and steroid function that can be studied in vitro under defined culture conditions.

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