Regulation of Gonadotropin Receptors and Gonadotropin Responses in a Clonal Strain of Leydig Tumor Cells by Epidermal Growth Factor*

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The MA-10 line is a clonal strain of Leydig tumor cells that has receptors for human chorionic gonadotropin (hCG) and mouse epidermal growth factor (mEGF). These cells respond to hCG, cholera toxin, and 8-Br-adenosine 3':5'-monophosphate with increased steroid production. It is reported herein that exposure of the MA-10 cells to mEGF results in a substantial (80 to 90%) reduction in the number of hCG receptors per cell. The loss of hCG receptors is accompanied by a corresponding reduction in the ability of hCG to stimulate steroidogenesis. The steroidogenic responses to cholera toxin and 8-Br-adenosine 3':5'-monophosphate, however, are not affected.

Other results presented show that mEGF is not a mitogen for these cells.

Epidermal growth factor is a small polypeptide whose main action is thought to be the stimulation of cell multiplication (see Refs. 1 and 2 for reviews). In some cell types, EGF does not act as a mitogen, but it affects the expression of other cellular functions. These include the stimulation of hCG and hCGα production in chorionicarcinoma cells (3), prostaglandin biosynthesis in canine kidney cells (4), ruffling and fluid pinocytosis in A-431 cells (5, 6), and prolactin biosynthesis in pituitary tumor cells (7).

Except for the well recognized stimulatory effect of androgens on the levels of EGF in the submaxillary glands of mice (8, 9), there is little information about a possible relationship between gonadal tissue and EGF. A possible role for EGF on gonadal function, however, is suggested by its ability to stimulate ornithine decarboxylase activity in the neonatal mouse testes (10), and by the finding that it is required for the multiplication and maintenance of cultured normal testicular cells in serum-free medium (11). Moreover, Gospodarowicz and co-workers have shown that EGF is a mitogen for granulosa cells, but not for luteal cells (12-14).

A recent report from this laboratory showed that mEGF stimulates cell multiplication and reduces 125I-hCG binding activity in mass cultures of functional Leydig tumor cells (15). In this communication, the effects of mEGF on a clonal line derived from such cultures are explored. The MA-10 is a clonal line of cultured Leydig tumor cells that have functional hCG receptors. The cells have been shown to bind hCG and to respond to the hormone with increased steroid production (15).

The results presented herein show that exposure of these cells to mEGF for 24 to 48 h results in an 80 to 90% reduction in the number of hCG receptors per cell, which can be correlated with a similar reduction in the ability of hCG to stimulate steroidogenesis. The steroidogenic response of the cells to cholera toxin and 8-Br-cAMP, however, is not affected.

The results presented also suggest that mEGF has a slight, direct stimulatory effect on the production of 20a-dihydroprogesterone. An examination of the effects of mEGF on cell multiplication revealed that it is not a mitogen for the MA-10 cells.

MATERIALS AND METHODS

Hormones and Supplies—hCG (batch CR-121) was obtained from the National Institute of Child Health and Human Development and iodinated as described elsewhere (16). The specific activities of 125I-hCG were 4 to 6 x 10^6 cpm/mg. Mouse EGF and γ-globulins against hEGF were a generous gift of Professors Stanley Cohen and Graham Carpenter of this institution. 125I-mEGF was prepared as described by Carpenter and Cohen (17) to give a specific activity of 1.9 x 10^7 cpm/mg. Prior to use, this preparation was diluted 2-fold with unlabeled mEGF. Antiserum to 20a-dihydroprogesterone was obtained from Accurate Chemical. Antiserum to progesterone was prepared in this laboratory (18). 125I-labeled steroids were purchased from New England Nuclear, and 35Na was from Amersham. All other materials were obtained as described elsewhere (15, 16, 18).

Cell Culture—The origin and properties of the MA-10 and MA-14 cells have been described recently (15). Stock cultures were maintained in medium (Waymouth MB752/1) supplemented with 15% horse serum and subcultured every 3 to 4 days (split ratio, 1:8) in 6-cm culture dishes containing 5 ml of growth medium and treated as described in the figure legends. mEGF was added as a 200-fold concentrated solution in 10 mM sodium phosphate, 0.1 M NaCl, 1 mg/ml of albumin, pH 7.4.

Hormone Binding and Steroid Production—Unless otherwise indicated, assays were carried out in Waymouth MB752/1 containing 1.12 g/liter of NaHCO3, 20 mM Hepes, 40 μg/ml of gentamicin, 1 mg/ml of albumin, pH 7.4 (referred to below as assay medium). The dishes were removed from the incubator, the medium was aspirated, and the cells were washed twice (2 ml each time) with warm (37°C) assay medium. The dishes then received 2 to 4 ml of assay medium. Hormones and other compounds were added in a total volume of 50 to 100 μl of the buffer indicated above.

The binding of 125I-hCG and 125I-mEGF was measured as described before (15). Nonspecific binding was determined in the presence of 2.5 μg/ml of unlabeled ligand, and it accounted for 1 to 2% of the total binding. All binding data are reported as specific binding. Binding to
duplicate dishes varied, in general, by 10%. Binding constants were calculated by the method of Scatchard (19) using least square analysis.

Progesterone and 20α-dihydroprogesterone were measured by radioimmunoassay in suitable aliquots of the incubation medium (15).

**Other Methods**—For growth curve determinations, cells were plated at a density of about 6 X 10⁵ cells/dish in 5 ml of medium supplemented with either 15 or 0.5% horse serum. On the following day (Day 1), the medium was changed, and half of the dishes received 10 ng/ml of mEGF. The medium (and mEGF) were replaced every other day until Day 5, and every day thereafter. On the days indicated, duplicate dishes from each group were used for cell counting. The cells were detached with trypsin (15) and counted in a Coulter counter. The number of cells per duplicate dish varied at most by 15%.

The ability of the MA-10 cells to convert progesterone to 20α-dihydroprogesterone was measured by incubating cells (in 5 ml of growth medium) with [1,2,6,7-³H]progesterone (1 ìg/ml, 0.5 ìCi/ìg) for 12 h at 37°C. At the end of the incubation, the medium was collected and extracted with 5 volumes of methylene chloride. The extracts were dried, redissolved in 50 ìl of benzene, and chromatographed on silica gel sheets using benzene/ethyl acetate (120:30) as the solvent system. The sheets were cut into 1-cm strips and counted. Under these conditions, the radioactivity reaches a maximum in 30 to 45 min, and then declines to a constant level corresponding to 20% of the initial amount bound. This is likely to be due to the internalization (and degradation) of receptor-bound EGF and subsequent receptor down-regulation (17-20). Fig. 1 also shows that the time course of mEGF binding to these cells is similar whether they are in serum-free or serum-containing medium.

In order to show that the mEGF and hCG receptors are different, two experiments were done. First, the number of hCG receptors was measured. The results presented in Fig. 2 show that the MA-10 cells, which have about 50 times more hCG receptors than do MA-14 cells (15), bind the same amount of mEGF. The maximal mEGF binding capacity for the MA-10 and MA-14 cells were 14 and 16 mol X 10⁻¹⁶/ìg of DNA, respectively. The Kᵣ values for this interaction were found to be 2.3 X 10⁻¹⁰ M (MA-10) and 2.9 X 10⁻¹⁰ M (MA-14), and are in good agreement with those reported for other cell types (7, 13, 17, 20).

Direct demonstration of the specificity of the mEGF and hCG receptors is shown in Table I. When added together, a high concentration of mEGF did not reduce the binding of ¹²⁵I-hCG to the MA-10 cells. On the other hand, when added together, a high concentration of hCG consistently reduced the binding of ¹²⁵I-mEGF by 15 to 20%.

Since hEGF was first isolated from the urine of pregnant women (11), the possibility that purified hCG was slightly contaminated with hEGF was considered. To test this hypothesis, hCG was preincubated with anti-hEGF γ-globulins for 24 h at 4°C and then tested for its ability to compete for ¹²⁵I-hCG binding. This preincubation failed to abolish the ability of hCG to inhibit ¹²⁵I-hCG binding (data not shown). Thus, this effect does not seem to be due to the presence of hEGF in purified hCG.

**Effects of mEGF on ¹²⁵I-hCG Binding**—Exposure of the MA-10 cells to mEGF for increasing periods of time resulted in the irreversible reduction of hCG binding. This is likely to be due to the internalization of hEGF receptors in two clonal lines that have widely different binding of ¹²⁵I-mEGF to the MA-10 cells is shown in Fig. 1. Similarly to results obtained with other cell types (13, 17, 20), it was found that the amount of cell-associated radioactivity reaches a maximum in 30 to 45 min, and then declines to a constant level corresponding to 20% of the initial amount bound. This is likely to be due to the internalization (and degradation) of receptor-bound mEGF and subsequent receptor down-regulation (17-20). Fig. 1 also shows that the time course of mEGF binding to these cells is similar whether they are in serum-free or serum-containing medium. In order to show that the mEGF and hCG receptors are different, two experiments were done. First, the number of hCG receptors was measured. The results presented in Fig. 2 show that the MA-10 cells, which have about 50 times more hCG receptors than do MA-14 cells (15), bind the same amount of mEGF. The maximal mEGF binding capacity for the MA-10 and MA-14 cells were 14 and 16 mol X 10⁻¹⁶/ìg of DNA, respectively. The Kᵣ values for this interaction were found to be 2.3 X 10⁻¹⁰ M (MA-10) and 2.9 X 10⁻¹⁰ M (MA-14), and are in good agreement with those reported for other cell types (7, 13, 17, 20).

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| Ligand             | Competitor | r² | p value |
|--------------------|------------|----|---------|
| ¹²⁵I-hCG (20 ng/ml)| mEGF       | 0.87|<0.01   |
|                    | hCG        | 0.91|<0.01   |
| ¹²⁵I-mEGF (8.5 ng/ml)| mEGF     | 0.86|<0.01   |
|                    | hCG        | 0.93|<0.01   |

Calculating equilibrium binding constants of EGF and hCG at 37°C is not strictly valid, because the hormone-cell interaction is not reversible. Thus, these numbers should be considered to be operational definitions only.
in a time-dependent reduction in $^{125}$I-hCG binding (Fig. 3). Half-maximal reduction occurred after 12 h of exposure, and the maximal effect (88% reduction) was observed after 24 to 36 h.

A dose-response curve for this phenomenon is shown in Fig. 4. Maximal (87%) reduction of $^{125}$I-hCG binding occurred with 2 to 5 ng/ml of mEGF. The concentration of mEGF required to produce half-maximal reduction of $^{125}$I-hCG binding was 0.5 ng/ml (0.8 x $10^{-10}$ M). This concentration is about three times lower than that required to saturate 50% of the mEGF receptors (see above), and is in good agreement with the concentrations of mEGF required to induce half-maximal stimulation of DNA synthesis in most cell types (1, 2).

Since in all the experiments described above, $^{125}$I-hCG binding was determined with a near-saturating concentration of $^{125}$I-hCG, it is likely that the decreased binding of $^{125}$I-hCG is due to a change in the number of receptors rather than in the affinity of the receptors. This was tested directly by measuring the binding constants of $^{125}$I-hCG to control and mEGF-treated cells. The results are shown in Fig. 5. In two independent experiments, the $K_r$ values for $^{125}$I-hCG binding were 3.5 and 3.9 x $10^{-10}$ M for the control cells, and 3.1 and 2.2 x $10^{-10}$ M for the mEGF-treated cells. The maximal binding capacities were 8 and 10 mol x $10^{-16}$ /µg of DNA for the control cells, and 1.2 and 1.3 mol x $10^{-16}$ /µg of DNA for the mEGF-treated cells. These results show that mEGF reduces $^{125}$I-hCG binding by reducing the number of gonadotropin receptors per cell, rather than by changing the affinity of the receptor.

Effects of mEGF on Steroidogenic Responses—The reduction in the number of hCG receptors produced by prolonged exposure of the MA-10 cells to mEGF results, as expected, in a reduction in the ability of hCG to stimulate steroid production. Since in these cells steroid production is tightly coupled to receptor occupancy (15), a reduction in the number of receptors should result in a decrease in the maximal amounts of steroid produced, rather than an increase in the concentration of hCG required to produce maximal steroid production. As shown in Fig. 6, this was found to be the case. The concentration of $^{125}$I-hCG required to produce half-maximal stimulation of steroidogenesis was 1 to 2 x $10^{-10}$ M in the

![Fig. 3. Time course of the mEGF-induced decrease in $^{125}$I-hCG binding. MA-10 cells were subcultured (split ratio, 1:8) on Day 0. On Day 1, the growth medium was replaced. mEGF (10 ng/ml) was added to the cultures at different times, in such a way that the time during which the cells had been in contact with mEGF varied as indicated in the figure. The binding of $^{125}$I-hCG was then determined at the same time in all cultures. At this time, the growth medium was removed, and the dishes were washed twice with assay medium. The cells were then incubated (37°C) in 2 ml of assay medium containing 20 ng/ml of $^{125}$I-hCG for 2 h. Cell-associated radioactivity was determined in duplicate dishes, and varied at most by 10%.

![Fig. 4. Dose-response curve for the mEGF-induced decrease in $^{125}$I-hCG binding. MA-10 cells were subcultured (split ratio, 1:8) on Day 0. On Day 1, the growth medium was replaced with growth medium containing the indicated concentrations of mEGF. Forty-eight hours latter, $^{125}$I-hCG binding was determined as described in the legend to Fig. 3.

![Fig. 5. Scatchard analysis of the binding of $^{125}$I-hCG to control (C) and mEGF (M)-treated cells. The cells were incubated in the absence or presence of 5 ng/ml of mEGF for 48 h as described in the legend to Fig. 4. After washing, 2 ml of assay medium containing increasing concentrations of $^{125}$I-hCG were added. The amount of cell-associated radioactivity was then determined in duplicate dishes after a 2-h incubation at 37°C. The different symbols represent results from two independent experiments. The correlation coefficients were 0.987 and 0.970 (controls) and 0.973 and 0.984 (EGF-treated cells).

![Fig. 6. Ability of $^{125}$I-hCG to stimulate progesterone production in control (C) and mEGF (M)-treated cells. The experimental protocol was as described in the legend to Fig. 5. Progesterone was measured in the medium of the same dishes used to determine the $^{125}$I-hCG binding constants. The medium was extracted with diethyl ether prior to the radioimmunoassay (15). The results of a single experiment are shown; each point represents the average of duplicate dishes. The bars extend to the individual values obtained.
Control cells, and 2 to $3 \times 10^{-10}$ m in the mEGF-treated cells. Maximal progesterone production occurred when about 80% of the receptors were occupied (in both groups), and it amounted to 21 to 22 ng/µg of DNA x 2 h for the control cells, and 3.8 to 4.2 ng/µg of DNA x 2 h for the EGF-treated cells. Thus, mEGF induced an 86% decrease in the number of $^{125}$I-hCG receptors, and reduced the maximal steroidogenic response to $^{125}$I-hCG by 81%.

These data suggest that the reduction in the steroidogenic ability of the cells is due entirely to the reduction in the number of hCG receptors, and not to changes induced in the "effector system." This suggestion implies that steroidogenic factors that do not act via the hCG receptor should be able to stimulate steroidogenesis to the same extent in control and mEGF-treated cells. The data presented in Table II show that this is the case. In this experiment, mEGF reduced the ability of hCG to stimulate progesterone production by 90%, but did not affect the response of the cells to cholera toxin or 8-Br-CAMP. The data also show that this is true for progesterone (the major steroid produced), but not for 20α-dihydroprogesterone. Exposure to mEGF resulted in a 2-fold increase in the amount of 20α-dihydroprogesterone production under basal conditions, or when stimulated by cholera toxin or 8-Br-CAMP. Note also that the amount of 20α-dihydroprogesterone produced in response to hCG was only slightly higher in the mEGF-treated cells than in the controls. Thus, when the amounts of these two steroids are added, mEGF reduces the steroidogenic response to hCG by about 75%.

The increased levels of 20α-dihydroprogesterone produced by mEGF-treated cells appear to be due to an effect of the growth factor on the conversion of progesterone to 20α-dihydroprogesterone. This was demonstrated by the following experiment. Cells were subcultured on Day 0 (split ratio, 1:8). On Day 1, the medium was changed, and duplicate dishes received buffer or mEGF (5 ng/ml). Thirty-six hours later, 2.5 µCi (5 µg) of $[1,2,6,7-^3H]$progesterone were added, and the medium was collected 12 h later. Thin layer chromatography of extracts of the medium (see under "Materials and Methods") revealed that in the control cells, 36% of the radioactivity co-migrated with authentic 20α-dihydroprogesterone. In the mEGF-treated cells, 60% of the radioactivity was converted to 20α-dihydroprogesterone. In a separate experiment, the levels of progesterone and 20α-dihydroprogesterone produced by cells cultured in 15% horse serum in the presence or absence of mEGF (5 ng/ml) were measured. The data (not shown) indicated that the daily production of progesterone was similar in both groups, while the daily production of 20α-dihydroprogesterone was about two times higher in the cells cultured with mEGF than in the controls.

**DISCUSSION**

The results presented in this paper show that mEGF reduces the number of hCG receptors per cell in a clonal strain of mouse Leydig tumor cells. As a consequence of this, the responsiveness of the cells to hCG is also reduced. The regulatory effect of mEGF on gonadotropin receptors is not related to its mitogenic properties, since it is expressed under conditions where cell multiplication is not affected.

These results represent the second example of negative regulation of a polypeptide hormone receptor (hCG) by a heterologous polypeptide hormone (mEGF). The first dem-
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