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Decreased Progesterone Binding and Attenuated Progesterone Action in Cultured Human Breast Carcinoma Cells Treated with Epidermal Growth Factor[1]

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ABSTRACT

Specific progesterone binding by cultured human breast carcinoma T47D, MCF-7, and ZR75-1 cells was decreased 25–40% by epidermal growth factor (EGF), with a 50% effective dose of 0.1 nM EGF. Studies with the soluble and particulate fractions prepared after homogenization of T47D cells grown in glass roller bottles revealed equivalent EGF-induced decreases in progesterone binding to receptors in both fractions. Equilibrium progesterone binding studies with these soluble and particulate fractions revealed that EGF decreased the receptor number, but had no effect on affinity. With cells grown adherent to plastic dishes, EGF treatment induced a greater decrease in binding to receptors recovered in the particulate fraction, than to receptors recovered in the soluble fraction. The decrease in progesterone binding induced by 20 nM EGF was maximal after 2 min of cellular EGF treatment for receptors recovered in the soluble fraction, but was only half-maximal after 15 min for receptors recovered in the particulate fraction. Decreased progesterone binding persisted for at least 8 days in cells cultured with 1 nM EGF.

Either insulin or EGF stimulated T47D cell proliferation by two- to threefold with a 50% effective dose of 100 nM for insulin and 0.1 nM for EGF. The progestin, R5020, decreased T47D cell growth by 30% with a 50% effective dose of 1 nM. Either EGF or insulin antagonized the inhibitory effect of R5020 on cell reproduction, but progestins did not antagonize the growth stimulatory response of cells to EGF. Progestins increased the number of EGF receptors within 12 h of their addition to T47D cells, but this response was lost after 6 days. These data show that EGF or progesterone can regulate the receptor number of the other, but for cell reproduction, the effect of EGF is dominant over that of progestins.

INTRODUCTION

Epidermal growth factor is a mitogen which stimulates many mesenchymal and epithelial cell lines to grow in culture (see reviews in References 1–4). EGF[4] induces a pleiotypic response, characterized by stimulations in transport of low molecular weight compounds, glycolytic activity, nucleic acid and protein synthesis, and phosphorylation of membrane-associated and intracellular proteins (reviewed in References 1 and 5).

Like a number of other transforming gene products, the EGF receptor is a protein tyrosine kinase (6), which phosphorylates itself as well as other substrates and the kinase activity is stimulated by EGF (reviewed in References 1, 4, 5, and 7). A role for protein phosphorylation by the EGF receptor in growth factor-induced cell division is supported by the demonstration that site-specific mutagenesis at the ATP binding site destroyed both receptor kinase and mitogenic activities (8). Purified avian oviduct progesterone receptor is a high affinity substrate for phosphorylation by purified EGF and insulin receptors (9, 10), but not for platelet-derived growth factor receptor. This observation stimulated our interest in testing for regulatory linkages between EGF or insulin actions and progesterone binding and action in cultured breast carcinoma cells.

Linkage between steroid and peptide hormone actions has an established history. Dexamethasone increases cellular EGF binding to human foreskin fibroblast cells (11) and insulin binding to 3T3-C2 Swiss mouse fibroblasts (12). Moreover, glucocorticoids potentiate mitogenic effects of EGF (11) and insulin-induced stimulation of 2-deoxyglucose transport on short term exposure (12 h), but decrease 2-deoxyglucose uptake in the presence of insulin upon longer exposure (24–48 h) (12). Progestins increase insulin (13) and EGF binding (14) to cultured human T47D breast carcinoma cells. On the other hand, androgens decrease EGF binding in rat prostate glands *in vivo* (15). Insulin down-regulates estrogen binding and antagonizes growth inhibition by antiestrogens in cultured human MCF-7 breast carcinoma cells (16).

High EGF receptor number has been associated with low estrogen receptor (17, 18) or progesterone receptor (18) contents in human breast cancer biopsies. Estrogen induces EGF- and IGF-like peptides which have been detected by competitive EGF binding and by radioimmunoassay, respectively, in medium conditioned by growth of MCF-7 cells (19). TGF-α-like activity has been detected in medium conditioned by growth of cultured MCF-7 breast carcinoma as indicated by stimulation of anchorage-independent growth of normal rat kidney cells in soft agar and inhibition of EGF binding to membranes derived from human epidermoid carcinoma A431 cells (20). IGF-I mRNAs have been detected in MCF-7 cells by Northern blot analysis, and IGF-I-like activity has been detected in medium conditioned by growth of MCF-7 cells by radioimmunoassay (21). TGF-β-like material has been detected in medium conditioned by growth of cultured human MCF-7 breast carcinoma cells in the presence of antiestrogen (22).

EGF stimulates the growth of both human neoplastic (23, 24) and normal human (25) and mouse (26, 27) mammary epithelial cells in culture. EGF induces lobuloalveolar development in estrogen and progesterone primed immature mouse mammary glands in whole organ culture (28). Studies with sialoadenectomized mice indicate that EGF is important for mammary gland development (29, 30) and milk production by the gland (29). While progestins inhibit growth of cultured MCF-7 (31) and T47D (13) human breast carcinoma cells, progesterone is essential for alveolar growth of mammary gland and it inhibits lactogenesis (reviewed in Reference 32). In normal mouse mammary epithelial cells, EGF inhibits casein synthesis (27) by differentially inhibiting the expression of casein genes (33).

Since purified progesterone receptors are substrates for purified EGF and insulin receptors (9, 10) and since EGF and progesterone have opposing effects on growth of both normal and neoplastic mammary epithelial cells, we have tested cultured human breast carcinoma cells for regulation of progesterone receptor number by EGF. In this report, we provide evi-

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[4] The abbreviations used are: EGF, epidermal growth factor; IGF-I, insulin-like growth factor-I; TGF, transforming growth factor; R5020, (17,21-dimethyl-19-norpregn-4,9-diene-3,20-dione); EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DCC-FBS, dextran-coated charcoal-treated fetal bovine serum; ACS, aqueous counting scintillation fluid; ED_{50}, 50% effective dose; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

dence that EGF affects progesterone receptor action on cultured human breast carcinoma cells by down-regulating progesterone binding and attenuating progestin-induced inhibition of cell growth.

MATERIALS AND METHODS

Materials. [1,2,6,7-^{3}H]Progesterone (91 Ci/mmol) and ACS (aqueous counting scintillation fluid) were obtained from Amersham Corporation (Arlington Heights, IL). [^{3}H]R5020 ([17α-*methyl*-^{3}H]-17,21-dimethyl-19-norpregn-4,9-diene-3,20-dione) and unlabeled R5020 were from Du Pont NEN Products (Boston, MA). Unlabeled progesterone, leupeptin, dithiothreitol, EDTA, EGTA, and phenylmethylsulfonyl fluoride, bovine serum albumin (Cohn fraction V), and bovine pancreatic insulin were purchased from Sigma Chemical Company (St. Louis, MO). Unless otherwise stated, other chemicals were of reagent grade.

Mouse EGF was isolated from male mouse submaxillary glands (Pel-Freeze Biologicals, Rogers, AR) as described by Savage and Cohen (34). Its purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (35) and its biological activity was assessed by measuring stimulation of [^{3}H]thymidine incorporation in serum-starved 3T3 cells where it exhibited an ED_{50} of 0.1 nM (36). [^{125}I]EGF was prepared using chloramine T and the specific activity was 1–2 × 10^{18} cpm/mol (37).

Cells and Cell Culture. Human breast carcinoma T47D, MCF-7, and ZR75-1 cells, obtained from Marc Lippman (National Cancer Institute, Bethesda, MD), contained estrogen, progesterone, glucocorticoid, and androgen receptors (38, 39). T47D cells contain very high levels of progesterone receptors [2.5 × 10^6 sites/cell (40); or 1221 fmol/mg cytosol protein (38)], but very low levels of glucocorticoid (19 fmol/mg cytosol protein), estrogen (3 fmol/mg cytosol protein), and androgen (31 fmol/mg cytosol protein) receptors (38). Estradiol does not induce progesterone receptors in T47D cells (40; confirmed by us) or in the MCF-7 cells used in the present study. We did not test ZR75-1 cells for induction of progesterone receptors by estradiol.

Cells were maintained routinely in 75-cm^2 polystyrene culture flasks in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) containing 5% untreated fetal bovine serum (GIBCO, Chagrin Falls, OH) and 10 μg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cell monolayers were grown to 90% confluency, treated with trypsin (0.25% trypsin in Hanks' balanced salt solution) at 37°C, suspended in 10 ml of RPMI 1640 medium containing 5% untreated fetal bovine serum, and passed (2:10, 1:10, 0.3:10 splits for ZR75-1, T47D, and MCF-7, respectively) once weekly with a change in culture medium on the third day. Cells used in all studies described here were between passages 6 and 25.

For the adherent cell hormone binding studies described in Fig. 1, cells were grown in 24-well polystyrene dishes (Corning Glass Works, Corning, NY) for 3 days to a final density of 10^5 cells/cm^2 (50% confluent). Cells were cultured only in the first and the fourth row wells of the four-row 24-well dish, since cells in the inner and outer wells sometimes grew at different rates. For the experiment described in Fig. 3, T47D cells were cultured in 840-cm^2 surface area borosilicate glass roller bottles (Wheaton, Mellville, NJ) in Dulbecco's modified Eagle's medium (Irvine Scientific, Irvine, CA) and Ham's F-12 (GIBCO) in a 1:1 ratio, containing untreated 10% calf serum (M.A. Bioproducts, Walkersville, MD) and 10 μg/ml gentamicin. Roller bottles were gassed with CO_2, and the cells were cultured at 37°C with a change in medium on the third day of culture and used when approximately 80% confluent.

Cells for the experiments described in Figs. 1–3 were grown in RPMI 1640 medium containing untreated 5% fetal bovine serum. We observed that dextran-coated charcoal treatment of fetal bovine serum by a published procedure (38) resulted in a failure of 80% of the cells to attach to the culture dish upon plating. Subsequently, we found that reduction of the temperature for dextran-coated charcoal treatment to 22°C produced serum supporting attachment of all cells plated. Cells for experiments described in Figs. 4–8 were grown in 55-cm^2 plastic Petri plates for 2–3 days in RPMI 1640 containing 5% DCC-FBS and achieved approximately 80 to 90% confluency. The cells were then plated in 1.0 ml/well (2-cm^2 24-well dish) or in 4.0 ml/well (10-cm^2 6-well dish) of RPMI 1640 medium containing DCC-FBS. There was a lag of 2 days before growth of T47D cells resumed following transfer from medium containing untreated serum to medium containing 5% DCC-FBS.

Serum-free Cell Culture. Cells plated in 55-cm^2 Petri plates in RPMI 1640 medium containing 5% DCC-FBS and grown for 2 days were transferred into 10-cm^2 6-well culture dishes at 50,000 cells/well in RPMI 1640 medium containing 5% DCC-FBS. The culture medium was replaced 18 h later with 4.0 ml of serum-free medium (RPMI 1640 containing dextran-coated charcoal treated 0.1% Cohn fraction V bovine serum albumin). After 24 h, the medium was replaced with fresh serum-free medium and at this point, hormones were added where indicated. Cells were grown with changes in serum-free culture medium, together with fresh additions of hormones, every 2 days.

Preparation of Dextran-coated Charcoal-treated Serum or Bovine Serum Albumin. Endogenous steroids were removed from sera by a modification of the procedure of Horwitz *et al.* (38), except that the temperature during charcoal treatment was 22°C. One part (100 ml) of dextran-coated charcoal suspension [0.025% (w/v) dextran and 0.5% (w/v) Norit A charcoal in 10 mM Tris-HCl (pH 7.5)] was sedimented at 30,000 × g·min. The pellet was suspended in 100 ml of fetal bovine serum or bovine serum albumin (10 mg/ml) and the suspensions were incubated at 22°C for 60 min with gentle shaking on a rotating platform. The suspensions were centrifuged at 30,000 × g·min to remove charcoal and the treated preparations were sterilized by ultrafiltration through 0.2-μm pore Nylon filters (Nalgene, Rochester, NY). When 20 nM [^{3}H]progesterone was added to sera as a tracer, >99% of the label was removed following the charcoal treatment. We observed that over 80% of the cells plated in fetal bovine serum treated with dextran-coated charcoal at 45°C as described by Horwitz *et al.* (38) did not attach to the culture dish even 2 days after plating. When the temperature of charcoal treatment of sera was changed to 22°C, this phenomenon was not observed. Addition of human plasma-derived fibronectin (Collaborative Research, Lexington, MA) at 50 ng/ml to culture medium containing the 45°C dextran-coated charcoal-treated fetal bovine serum resulted in attachment of approximately 80% of the cells plated in 24 h.

Preparation of Cytosol and Particulate Fractions. T47D cells grown in glass roller bottles were washed twice with 50 ml/roller bottle of Tris-buffered saline (150 mM NaCl and 10 mM Tris-HCl, pH 7.4). Cells (1.5 × 10^8 cells/roller bottle) were resuspended by incubating at 37°C for 15 min in 15 ml/roller bottle with Tris/saline/EDTA [25 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, and 1 mM Na_2HPO_4 (pH 7.4 at 37°C)], rolling continuously at 4 rpm. The suspended cells obtained from two roller bottles were collected by a 4,000 × g·min centrifugation, washed twice with 40 ml of homogenization buffer [10 mM Tris-HCl, 1 mM Na_2EDTA, 5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, 40 μg/ml leupeptin, 10 mM sodium molybdate, and 10% (v/v) glycerol (pH 7.4)], and homogenized with 50 strokes of a Dounce homogenizer in 4.0 ml of ice-cold homogenization buffer. The homogenate was centrifuged at 40,000 × g·min and the supernatant fraction was collected. The particulate fraction was suspended in 4.0 ml of homogenization buffer and centrifuged again at 40,000 × g/min. The supernatant fractions were combined and adjusted to 9.0 ml final volume with homogenization buffer to yield the cytosolic fraction. The cytosolic fraction was diluted 5-fold and 50 μl of this dilution was used for progesterone binding assay.

In experiments conducted with adherent T47D cells grown in 55-cm^2 Petri plates (to approximately 50% confluency), the cells were washed twice with 10 ml/dish of homogenization buffer and removed with 3.0 ml/plate of this buffer at 0°C using a rubber policeman. Cells (5 × 10^6) obtained from each Petri plate were disrupted at 0°C in 200 μl of homogenization buffer with 25 strokes of Potter-Elvhjem homogenizer. The soluble and particulate fractions were separated by a 40,000 × g/min centrifugation at 4°C. The particulate fraction was suspended in 50 μl of homogenization buffer and centrifuged as above; the supernatant fractions were then combined.

Solubilization of Particulate Fraction-associated Progesterone Recep-

tors with High Ionic Strength Buffer. Washed particulate fraction derived from 3×10^8 cells obtained from two roller bottles was suspended in 5.0 ml of homogenization buffer containing 0.5 M KCl. The particulate fraction derived from 5×10^6 cells grown on a Petri plate was suspended in 75 μl of homogenization buffer containing 0.5 M KCl. The fractions were incubated with gentle shaking on a rotating platform at 4°C for 60 min and centrifuged at 40,000 × *g*/min. The supernatant fraction containing solubilized receptors was used for progesterone binding experiments. Tests for progesterone binding to the particulate fraction showed that receptor extraction was generally >90% complete.

Dextran-coated Charcoal Assay for Progesterone Receptors. Aliquots (50 μl) of the cytosol or high ionic strength extract of the particulate fraction were combined with 50 μl of homogenization buffer containing [^{3}H]progesterone at the concentrations indicated. The samples were incubated either at 4°C for 16 h or at 23°C for 1 h. They were then chilled at 0°C for 5 min and combined with one part (v/v) of dextran-coated charcoal suspension [0.025% (w/v) dextran and 0.5% (w/v) Norit A charcoal in homogenization buffer] (41). After mixing by vortex action, the samples were incubated at 0°C for 5 min, with 2 min intermittent mixing, and centrifuged at 47,000 × *g*/min. A 50-μl aliquot of the supernatant fraction was assayed for radioactivity in 7 ml of ACS.

Progesterone Binding to Adherent Cells. Progesterone binding to adherent cells was conducted with cell monolayers grown in 2-cm^2 24-well culture dishes. The cells were washed twice with 2 ml of progesterone binding medium (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered RPMI 1640, pH 7.2) per well per wash. The cells were incubated at room temperature (22–23°C) in 0.5 ml of binding medium containing 20 nM [^{3}H]progesterone. Replicate samples were incubated with 2 μM unlabeled progesterone for determination of nonspecific binding. The cells were washed three times at 0°C in progesterone binding medium to remove unbound progesterone and solubilized in 300 μl of 1 M NaOH at 60°C for 60 min. Aliquots (150 μl) were neutralized with HCl and assayed by scintillation counting for bound [^{3}H]progesterone in 7 ml of ACS.

EGF Binding to Adherent Cells. Substratum adherent cells grown in 2-cm^2 24-well culture dishes were washed twice with EGF binding medium (RPMI 1640 medium, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 0.1% bovine serum albumin, pH 7.2) and incubated at 22°C in 300 μl of this medium containing 20 nM [^{125}I]EGF for 60 min. Replicate samples were incubated in the presence of 2 μM unlabeled EGF to determine nonspecific binding. The cells were washed rapidly at 0°C (within 30 s) three times with 2 ml of binding medium per well. Bound [^{125}I]EGF was determined by gamma counting following solubilization of the cells at 22°C for 30 min in 300 μl of 1 M NaOH.

Cell Counting. Cells were rinsed once with Tris/saline/EDTA solution [25 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, and 1 mM Na_2HPO_4 (pH 7.4)] containing 10 μg/ml phenol red and treated at 37°C for 45 min with 0.13% trypsin in Tris/saline/EDTA. Cells grown in roller bottles were suspended in Tris/saline/EDTA as described under "Preparation of cytosol and particulate fractions," and an aliquot of the suspension was treated with trypsin as described above. Cells were counted using a hemocytometer, diluting when necessary to maintain the cell count of the sum of nine squares within 50 to 300.

Protein Determination. Protein content in samples was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA), based on the method of Bradford (42), with bovine serum albumin (Cohn fraction V) as the standard.

RESULTS

EGF Decreases Progesterone Binding in Cultured Breast Carcinoma Cells. The effect of EGF on progesterone binding to substratum-adherent breast carcinoma cells was determined with cells incubated first with EGF for 60 min at 37°C, and then with [^{3}H]progesterone at 22°C for 60 min to achieve maximal binding. EGF decreased cellular specific [^{3}H]progesterone binding by 25–40% in T47D, MCF-7, and ZR75-1 cells (Fig. 1, *A–C*). The effect of EGF was dose dependent with an ED_{50} value of approximately 0.1 nM. Maximal inhibition of progesterone binding was achieved at approximately 1 nM EGF. Since T47D cells metabolize progesterone rapidly, we also studied the effect of EGF on binding of the nonmetabolizable progestin R5020. When T47D cells were treated with 3 nM EGF as described in the legend of Fig. 1 and then with 20 nM [^{3}H]R5020, cellular [^{3}H]R5020 binding was decreased by 30%. Insulin also decreased progesterone binding to cells by 25–30%. The ED_{50} for insulin was approximately 1 nM for MCF-7 and ZR75-1 cells and 10 nM for T47D cells (data not shown).

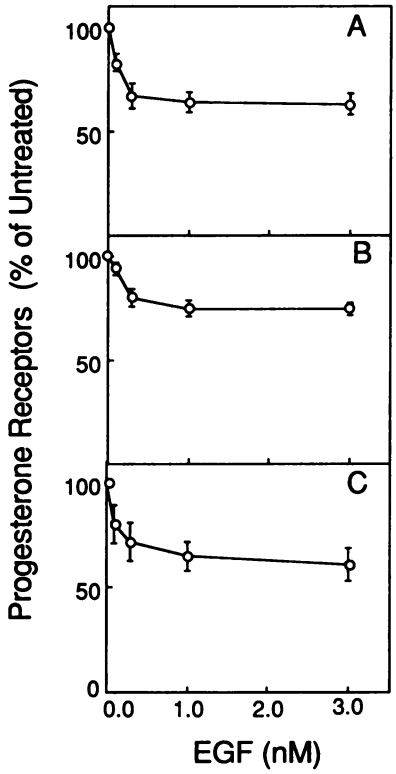

Fig. 1. EGF decreases progesterone binding to cultured human breast carcinoma cells. Adherent breast cancer T47D, MCF-7, or ZR75-1 cells grown in 2-cm^2 24-well culture dishes were washed twice with 2 ml/well of progesterone binding medium, and then incubated first at 37°C for 60 min in 0.5 ml of binding medium containing 0.1 to 3.0 nM EGF, and then at 22°C for 60 min with EGF and 20 nM [^{3}H]progesterone (see "Materials and Methods"). Progesterone binding is expressed as percentage of that detected in untreated cells ± SD ($n = 6$) and was 1020 ± 40, 481 ± 13, 370 ± 30 (× 10^3 sites/cell) for T47D, MCF-7 and ZR75-1 cells, respectively. *A*, *B*, and *C*, data for T47D, MCF-7, and ZR75-1 cells, respectively.

The influence of order of addition of EGF and progesterone was tested. An EGF-induced decrease in progesterone binding of approximately 30% was observed regardless of whether cells were incubated with EGF prior to or after progesterone addition (data not shown).

Decreased Progesterone Binding to Receptors Recovered in Resolved Particulate or Soluble Fractions as a Function of Duration of Cellular EGF Treatment. T47D cells contain two classes of progesterone binding sites that can be demonstrated in intact cells or cell free preparations (43). The high affinity sites bind progesterone with a k_d value of 2 nM and are present in the soluble fraction of a cell homogenate, whereas the lower affinity sites bind progesterone with a k_d of 20–60 nM and are present exclusively in the particulate fraction. To test for preferred inhibition of progesterone binding to receptors in the soluble or particulate fractions, cells were incubated at 37°C with 20 nM EGF for times extending from 2 to 120 min, and then processed for preparation of the particulate and soluble

fractions. The EGF-induced decrease in progesterone binding to receptors in the soluble fraction was maximal within 2 min of EGF addition, but greater than 15 min of incubation with EGF was required to achieve maximal inhibition of progesterone binding to lower affinity receptors in the particulate fraction (Fig. 2). Moreover, the magnitude of the EGF-induced decrease in progesterone binding to the particulate fraction was twice that observed in the soluble fraction.

EGF Decreases Progesterone Receptor Number without Affecting Receptor Affinity. To determine if EGF affects progesterone receptor number or affinity in T47D cells, we examined equilibrium progesterone binding to receptors in the soluble fraction and in the high ionic strength extract of the particulate fraction following EGF treatment. EGF decreased progesterone binding in either fraction by one half with no change in affinity (Fig. 3, Table 1). This effect on receptor number is different from that shown in Fig. 2, where progesterone binding to receptors in the soluble fraction of EGF-treated cells grown on plastic dishes was decreased to a lesser extent than progesterone binding to receptors in the particulate fraction.

Cells grown in glass roller bottles (Fig. 3), responded consistently to EGF with equal decreases in progesterone binding to receptors in the soluble and particulate fractions, whereas progesterone receptors in cells grown in plastic wells behaved consistently in response to EGF as described in Fig. 2. This could be due to properties induced by cell-substrate interactions on plastic *versus* glass. For example, Pourreau-Schneider *et al.* (45) showed that MCF-7 cells grown as spheroids on hydrophobic plastic coated with 1% Bactoagar have twice as many progesterone receptors as cells grown as monolayers on hydrophilic plastic. Gospodarowiz *et al.* (46) found that the response of corneal epithelial cells to growth factors depended on cell substrate: EGF was a mitogen for corneal epithelial cells cultured on a collagen gel but not on plastic, with fibroblast growth factor this response to cell substrate was reversed.

For the studies shown in Figs. 1–3 cells were grown in untreated serum. Identical studies conducted with cells grown in DCC-FBS yielded the same findings. In these, however, there was far more experimental variation resulting from a tendency of DCC-FBS grown cells to dissociate from the culture dish (see "Materials and Methods"). All other experiments (Figs. 4–8) cells were grown in DCC-FBS to facilitate comparison of our results with those of published studies (13, 16, 31, 40, 47) where DCC-FBS was utilized.

Cellular Progesterone Binding in Response to EGF as a Func-

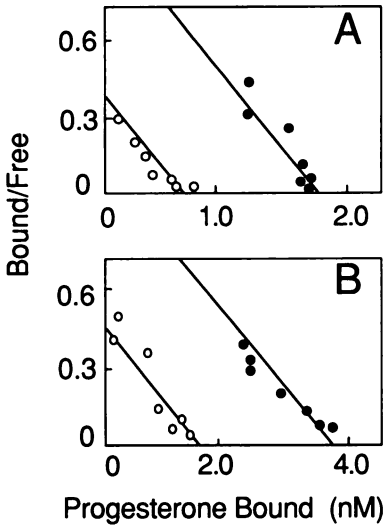

Fig. 3. Effect of cellular EGF treatment on progesterone receptor number and affinity. T47D cells, cultured in roller bottles to approximately 80% confluency, were washed once and then incubated *in situ* at 37°C for 60 min in 15 ml progesterone binding medium containing 3 nM EGF or no EGF. The cells were then washed and homogenized, and the soluble and the high ionic strength extract of the particulate fraction were prepared and incubated at 4°C for 16 h with 0.4 to 100 nM [^{3}H]progesterone prior to determining binding by the dextran-coated charcoal assay (see "Materials and Methods"). Replicate samples contained 10 μM unlabeled progesterone for determination of nonspecific binding. Nonspecific binding was also determined computationally using the LIGAND program (44) as well as total receptor number and dissociation constants. Data are shown as Scatchard plots of progesterone binding to the soluble fraction (*A*) or high ionic strength extract of the particulate fraction (*B*) from EGF-treated cells (○) or untreated (●) cells.

Table 1 *Number and dissociation constant for progesterone receptors from EGF-treated or untreated T47D cells*

Receptor number and dissociation constants ± SE were determined with the LIGAND program (44) and are from the data shown in Fig. 3.

	Cytosol		Particulate extract	
EGF (3 nM)	k_d (nM)	Receptor number (pmol/mg protein)	k_d (nM)	Receptor number (pmol/mg protein)
–	1.1 ± 0.2	6.0 ± 1.0	5.3 ± 1.0	5.2 ± 0.9
+	1.4 ± 0.2	2.7 ± 0.6	4.5 ± 0.5	1.9 ± 0.6

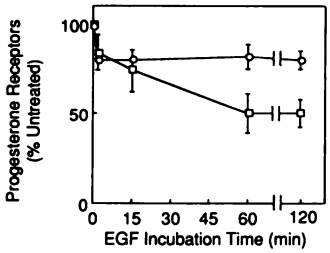

Fig. 2. Time course for EGF inhibition of progesterone binding to soluble and particulate progesterone receptors. T47D cells, grown for 3 days in 55-cm^2 Petri plates to approximately 50% confluency, were washed twice with 10 ml/plate of progesterone binding medium and then incubated at 37°C for 2 to 120 min in the absence or presence of 20 nM EGF in 4.0 ml of progesterone binding medium. Cells were washed twice with 10 ml of homogenization buffer at 22°C, and the soluble fraction and the high ionic strength extract of the particulate fraction were prepared and assayed for progesterone binding at 22°C with 20 nM [^{3}H]-progesterone using dextran-coated charcoal to remove unbound ligand (see "Materials and Methods"). Progesterone receptor content is expressed as percentage of that in samples not treated with EGF ± SD ($n = 4$) for soluble (○) or particulate extract (□) fractions. Progesterone binding to soluble and particulate extract fractions from untreated cells was 5.7 ± 0.2 and 7.9 ± 0.4 fmol progesterone bound/μg protein, respectively.

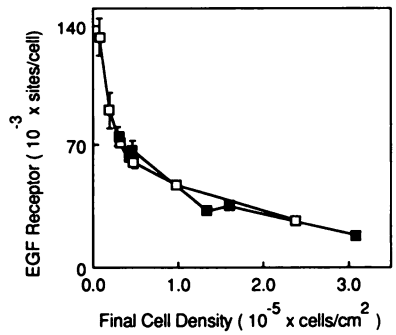

Fig. 4. Effect of cell density on EGF receptor number on T47D cells. T47D cells were seeded at different cell densities (16,000–160,000 cells/well for 3-day growth or 4,000–40,000 cells/well for 6-day growth) in 2-cm^2 24-well culture dishes to achieve the final cell densities shown after 3 or 6 days in culture. The culture medium was changed every 2 days. After 3 or 6 days in culture, cells from three independent wells were counted to determine final cell densities, and adherent cells in replicate wells were assayed for EGF receptor content (see "Materials and Methods"). EGF binding is expressed as sites per cell (mean ± SD of six independent wells) following growth for 3 (■) or 6 (□) days.

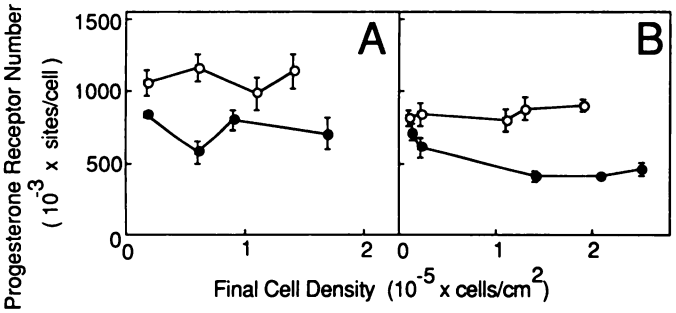

Fig. 5. Progesterone receptor content in T47D cells incubated for 2 or 8 days with EGF. T47D cells were plated in 2-cm^2 24-well dishes at varying cell densities (16,000–160,000 cells/well for 2-day growth or 1,000–10,000 cells/well (control) and 500–5,000 cells/well (EGF treated) for 8-day growth. At 24 h after plating, 1 nM EGF was added to one set of wells at each cell density and all the cells were grown for either 2 or 8 days with a change in medium and further addition of EGF every 2 days. After 2 or 8 days of culture the cells from three independent wells were counted to determine final cell densities, and progesterone binding was determined by the adherent cell progesterone binding assay (see "Materials and Methods"). Data show progesterone receptor contents as sites/cell ± standard deviation (n = 6) for 2 (A) or 8 (B) days of culture in absence (○) or presence (●) of EGF.

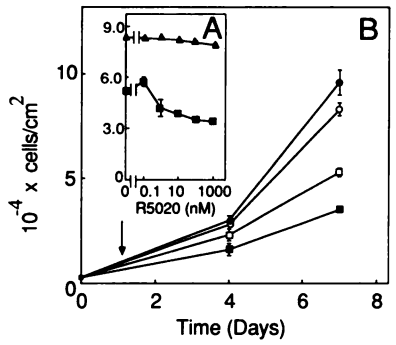

Fig. 6. Time course for EGF action as an antagonist or progestin-induced inhibition of cell growth. T47D cells were plated at 130,000 cells/10 cm^2 well (see "Materials and Methods"). At 24 h after plating, indicated by the *arrow*, R5020 (a 1,000-fold concentrated solution in ethanol) or EGF were added to the cells. Control and EGF-treated cultures received the same amount of ethanol as R5020 treated cells. Medium containing these agents was changed every 2 days. Cells were counted on the days as indicated. A, shows a dose-response experiment for the effect of R5020 on cell reproduction for cells grown 6 days without (■) or with 1 nM EGF (▲); B, shows the time courses of cell growth for untreated cells (□), and for cells treated with 100 nM R5020 (■), 1 nM EGF (●), or 1 nM EGF + 100 nM R5020 (○). Values are means ± SD (n = 3).

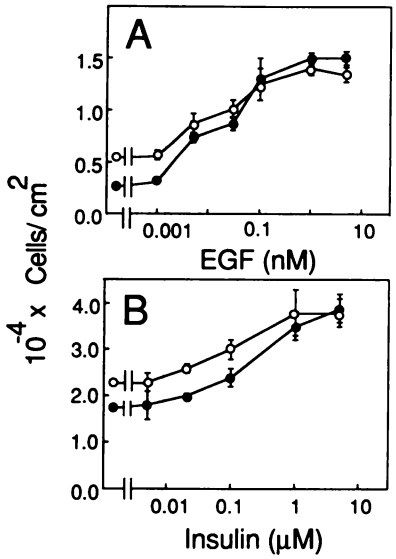

Fig. 7. Dose response of EGF or insulin for stimulation of cell growth in the absence or presence of R5020. For EGF dose-response studies (A), T47D cells were plated at 30,000 cells/well in 10 cm^2 6-well culture dishes as described in "Materials and Methods." At 24 h after plating, EGF or R5020 was added to the cells and the culture medium containing these agents was changed every 2 days. Cells were counted after 6 days of hormone treatment. Insulin dose-response studies (B) were done as above, except that 50,000 cells/well were plated and cultured under serum-free conditions (see "Materials and Methods"). Values for cell numbers are means ± SD (n = 3) for cells grown in the absence (○) or presence (●), respectively, of 100 nM R5020.

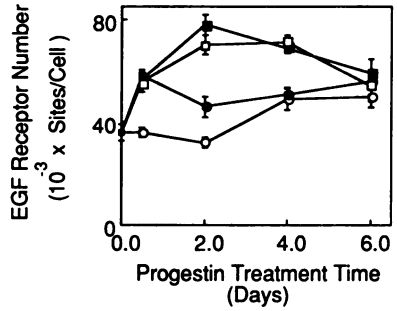

Fig. 8. Effect of progestins on EGF receptor number. T47D cells were plated in 2-cm^2 24-well dishes at varying cell densities to achieve approximately 50% confluent cultures after 2, 4, or 6 days of growth (see "Materials and Methods"). All cells were grown for at least two days after seeding prior to progestin addition and EGF binding assay. For 0 and 12 h time points cells were cultured for 2 days prior to addition of progestins. For other time periods, progestins were added to the cells 24 h after plating and the culture medium was changed every 2 days with fresh additions of progestins. The seeding densities for 0, 12 h, and 2-day progestin treatment times were the same (100,000 cells/well), for 4-day 25,000 cells/well (control) or 30,000 cells/well (progestin treated), and for 6-day 10,000 cells/well (control) or 15,000 cells/well (progestin treated). At the times indicated, cell number was counted and replicate samples were assayed for EGF receptor content (see "Materials and Methods"). Values are means ± SD (n = 6) for untreated cells (○), or cells treated with 10 nM progesterone (●), 1,000 nM progesterone (□), or 1 nM R5020 (■).

tion of Cell Density. EGF receptor density on cultured BSC-1 kidney epithelial cells decreases at high cell density (48). EGF receptor number in T47D cells also was decreased at high cell density; there were approximately 5-fold more receptors at lower than at higher cell density (Fig. 4). This inverse relationship between EGF receptor and cell density was also observed with MCF-7 cells (50,000 and 10,000 sites/cell at 0.5 × 10^5 cells/cm^2 and at 2.5 × 10^5 cells/cm^2, respectively) and ZR75-1 cells (90,000 and 15,000 sites/cell at 0.5 × 10^5 cells/cm^2 and at 2.5 × 10^5 cells/cm^2, respectively).

To study the effect of EGF on progesterone binding as a function of cell density, T47D cells were grown to high or low cell densities in the absence or presence of 1 nM EGF for 2 or 8 days. In contrast to EGF receptor behavior, progesterone receptor content of T47D cells was not affected by cell density (Fig. 5). The decrease in progesterone binding induced in these cells by EGF was as high as 30–50%, and persisted after 8 days of EGF treatment (Fig. 5). The magnitude of the decrease in progesterone binding induced by 2 days of EGF treatment was not affected by cell density (Fig. 5A). However, of cells treated for 8 days with EGF, those that had achieved higher densities were more responsive to EGF (Fig. 5B) in spite of their lower EGF receptor levels (Fig. 4).

EGF and Insulin Stimulate Cell Growth and Antagonize Progestin-induced Inhibition of Cell Growth. Since treating cells with EGF decreased progesterone binding, experiments were performed to determine if EGF antagonized progesterone-mediated inhibition of cell reproduction. Progestins, such as R5020, inhibit the growth of MCF-7 and T47D cells by 30–70% (13, 31). R5020 inhibited the growth of T47D cells by approximately 30% (Fig. 6A), with an ED_{50} value of 1 nM. This is approximately equal to the dissociation constant for R5020 binding to high affinity progesterone receptors (47; and con-

firmed by us). Inhibition of cell growth induced by R5020 persisted for at least 6 days (Fig. 6*B*).

EGF stimulated T47D cell growth (Fig. 6*B*). When cells were cultured in the presence of both EGF (1 nM) and R5020 (100 nM), the effect of EGF on cell growth dominated (Fig. 6*B*). No concentration of R5020 tested (up to 1,000 nM) overcame the growth stimulatory effect of 1 nM EGF (Fig. 6*A*). EGF stimulated cell reproduction with an ED_{50} value of <0.1 nM and a maximal response at 1 nM (Fig. 7*A*). T47D cells seeded at lower density (30,000 cells/10 cm^2 area) consistently had a greater response to EGF (2- to 3-fold) than those seeded at a higher density (130,000 cells/10 cm^2 area; 1.5- to 2-fold stimulation, compare Figs. 7*A* and 6*B*). Approximately 0.1 nM EGF completely overcame the growth inhibitory effect of 100 nM R5020 (Fig. 7*A*). The growth studies described above for EGF-treated cells were conducted in RPMI 1640 medium containing 5% DCC-FBS (see "Materials and Methods"). Identical responses to progestins and EGF were obtained in medium containing untreated serum and in serum free medium (data not shown).

Insulin did not stimulate T47D cell reproduction in medium containing serum (not shown), but in serum-free medium it increased cell number by approximately twofold (Fig. 7*B*). The ED_{50} value for this response was approximately 0.1 μM insulin, and the response was maximal at 1 μM insulin (Fig. 7*B*). Insulin also completely overcame inhibition of cell growth by 100 nM R5020, but a very high insulin concentration (1 μM or greater) was required (Fig. 7*B*).

Progestins Increase EGF Receptor Number. Progestins can increase both EGF and insulin receptor numbers in T47D cells (13, 14). The increase in insulin receptors was observed after 1 day of progestin treatment, but after 4 days of progestin treatment, insulin receptor number returned to approximately the level in untreated cells (13). An increase in EGF receptor number was evident after 1 h treatment with R5020 and persisted for 24 h, the maximal time studied (13). We performed experiments to determine if EGF receptors were maintained in an up-regulated state upon longer progestin treatment (>1 day). Progesterone or R5020 increased EGF receptor number 1.5- to 2-fold during a 12-h incubation (Fig. 8). However, when cells were incubated with progestins for 6 days, EGF receptor number returned to levels characteristic of untreated cells. At a lower progesterone dose (10 nM), EGF receptors returned to a level characteristic of untreated cells after 2 days of treatment, possibly due to progesterone metabolism (49).

DISCUSSION

EGF regulates progesterone binding activity in cultured breast cancer cells and attenuates progestin-induced inhibition of their reproduction. The ED_{50} value for both these responses was 0.1 nM EGF (Figs. 1 and 7), which is approximately the k_d value for a very high affinity class of cellular EGF binding detected in T47D cells (14) and other cell lines (50–52). This suggests the involvement of very high affinity EGF binding sites in decreasing progesterone binding. EGF also decreased dexamethasone binding to glucocorticoid receptors in HBL 100 cells (nontumorigenic human breast epithelial cells) in a dose-dependent manner with an ED_{50} value of 0.1 nM (53).

The mechanisms by which EGF decreases progesterone or glucocorticoid binding have not been established. For both progesterone (Fig. 3) and glucocorticoid (53) binding, EGF decreased total steroid binding activity without affecting affinity. These results are similar to findings of Butler *et al.* (16), who showed that insulin decreased total estradiol receptor number in MCF-7 cells without affecting affinity, and that of Roos *et al.* (54), who reported that TPA treatment decreased progesterone receptor number in T47D cells with no change in receptor affinity. EGF decreased progesterone (Fig. 2) or glucocorticoid (53) binding rapidly. We have demonstrated a rapid EGF-induced phosphorylation of glucocorticoid receptors on tyrosine and serine residues in HBL 100 cells, suggesting a possible direct action of EGF receptor kinase on glucocorticoid receptors (55). However, neither EGF nor TPA induced progesterone receptor phosphorylation in intact T47D cells (56). This shows that the EGF-induced decrease in progesterone receptor binding is not caused by a change in the phosphorylation state of progesterone receptors. EGF, insulin, and TPA must therefore decrease progesterone binding in intact cells by acting through other intracellular mediators.

Either EGF or insulin stimulated T47D cell growth in the absence of serum (Fig. 7). Although EGF stimulated T47D cell growth in the presence of serum, insulin did not, suggesting that insulin-like growth factors in serum can mask the response to insulin. Our data showing that micromolar insulin concentrations were required to stimulate T47D cell growth are in agreement with published findings of Furlanetto and DiCarlo (57). Their studies on competition between insulin and somatomedin-C (IGF-I) for binding to cultured breast cancer cells indicate that insulin stimulates cell growth by binding to IGF-I receptors. A high concentration (approximately 1 μM) of insulin also was required to stimulate cell reproduction and overcome progestin-induced inhibition of cell reproduction (Fig. 7*B*), indicating that these responses to insulin are mediated through IGF-I receptors. The findings that nanomolar insulin was half-maximal for decreasing progesterone binding, but that micromolar insulin was required for stimulating cell growth and antagonizing progestin-induced inhibition of cell growth show that the former is not causal for the latter. The finding that insulin overcame progestin-induced cell growth inhibition only in serum-free culture, but that EGF had this effect either in the presence of serum or in serum-free culture, suggest that EGF and insulin antagonize progestin action via different mechanisms.

In agreement with findings of Murphy *et al.* (14), who studied only the short-term aspects of the phenomenon, progestins increased EGF receptor number in T47D cells (Fig. 8). We discovered that after 6 days of progestin treatment, EGF receptors in T47D cells returned to levels characteristic of untreated cells (Fig. 8). This was also observed with cells subjected to frequent changes of medium containing the progestin R5020, which is relatively stable to metabolism. Therefore, the loss of progestin-induced upregulation of EGF receptors is unlikely to be caused by progestin metabolism. Progestins did not increase EGF receptor number in the experiment described in Fig. 8 by altering cell density, since that factor was controlled experimentally. Recently, Murphy *et al.* (58) showed that progestins increased EGF receptor mRNA (2- to 3-fold) in T47D cells and this increase was proportional to the progestin-induced increase in EGF binding. The mechanism by which progestins increase insulin receptor numbers is not known (13). Cell growth inhibitors such as the antiestrogen tamoxifen induce release of transforming growth factors such as TGF-β by cells (22). TGF-β has been reported to increase EGF receptor number in normal rat kidney fibroblasts (59) and rat ovarian granulosa cells (60). Recently, Fernandez-Pol *et al.* (61) demonstrated that EGF induces the synthesis of its own mRNA, and that TGF-β potentiates this response to EGF in cultured MDA-468 human

breast carcinoma cells. Since progestins increase the number of EGF (14) and insulin (13) receptors, it has been suggested that these steroids may sensitize cells to growth factor action (14). Our studies indicate that this was not the case for T47D cells (Fig. 7).

Both EGF (25–28, 30) and progesterone (28, 32), in concert with other hormones such as estrogen, glucocorticoid, thyroid hormone, insulin, and prolactin, are involved in the growth, development of and lactation by mammary glands (32). Recent studies suggest that EGF is essential for lactation and for production of milk proteins (29, 62), whereas progesterone inhibits lactation during pregnancy (32). When lactation ensues following parturition, plasma progesterone level falls (32) and lactating mammary glands have no detectable progesterone receptors (63), but possess EGF receptors. Our findings suggest that EGF could contribute to synthesis of milk proteins during lactation by down-regulating progesterone receptors.

The progestin R5020 inhibited the growth of cultured T47D cells by approximately 40% (Fig. 6). Our results are in agreement with findings that progestins inhibited the growth of cultured human breast carcinoma MCF-7 cells (31), T47D cells (13) and cultured human endometrial carcinoma cells (64) to similar extents. EGF, even at very low concentration, overcame the cell growth inhibitory effects of R5020 (Figs. 6 and 7) or dexamethasone (53) and partially overcame the growth inhibitory effect of antiestrogens (65) on steroid hormone-responsive cultured human breast epithelial cells. There are reports of other growth factors attenuating growth inhibitory responses to steroid hormones. High concentrations of insulin, apparently acting as IGF-I, overcame the growth inhibitory responses of cultured MCF-7 cells to the antiestrogen tamoxifen (16), and platelet-derived growth factor overcame glucocorticoid-induced cell cycle arrest in a cultured ductus deferens smooth muscle tumor cell line (66).

Progestins are exploited in hormone therapy of advanced breast cancer (67, 68), and progesterone receptor status is an important predictor of the response to endocrine therapy (69–72). Studies with biopsied breast tumors reveal a high EGF receptor number associated with low estrogen or progesterone receptor content (17, 18). Our studies indicate that down-regulation of steroid receptors by EGF could contribute to this condition.

We have demonstrated that EGF or insulin, the latter acting as IGF-I, stimulate proliferation of cultured breast carcinoma T47D cells and overcome progestin-induced inhibition of cell growth. The present study taken together with studies by others (16, 53, 66, 65) suggests that growth factors can competitively abolish inhibition of cell growth in response to steroids. Our findings that EGF or a high level of insulin overcame progestin-induced inhibition of cell growth in cultured human breast carcinoma cells show that secreted TGF-α-like factors or secreted IGF-I-like peptides may antagonize steroid action and lead to failures in progestin therapy for breast cancer.

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