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Altered Glucocorticoid Binding and Action in Response to Epidermal Growth Factor in HBL100 Cells[1]

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ABSTRACT

Incubation of adherent human breast epithelial HBL100 cells with epidermal growth factor (EGF) decreased [^{3}H]dexamethasone binding by 35% with no effect on affinity. Maximal inhibition was obtained at 3 nM EGF and the 50% effective dose was 0.2 nM EGF. Decreased dexamethasone binding induced by 3 nM EGF was maximal by 5 min of treatment and, in the continuous presence of EGF, persisted at a constant level over 4 days. The action of EGF was antagonized by 12-*O*-tetradecanoylphorbol-13-acetate, which did not inhibit dexamethasone binding significantly, and by concanavalin A. In homogenates of EGF-treated cells, decreased dexamethasone binding was observed only in the cytosolic fraction. Saturation dexamethasone binding inhibited the growth rate of HBL100 cells by approximately 50%, but concurrent treatment with EGF overcame this inhibition. The effect of EGF on dexamethasone-inhibited cell growth also was antagonized by 12-*O*-tetradecanoylphorbol-13-acetate.

INTRODUCTION

Glucocorticoids elicit an array of physiological responses in target tissue and cultured cells. These effects include modulation of gene expression and regulation of cell growth (for a review see Refs. 1 and 2). The first step in glucocorticoid hormone action is a reversible, noncovalent, high affinity binding to its intracellular receptor. Subsequent to this, the receptor undergoes "activation," in which it acquires the capacity to interact with chromatin (1).

There is evidence for cross-talks between steroid hormones and growth factors, presumably via action of their respective receptors. Progestins have been reported to stimulate ^{125}I-EGF[3] (3) and ^{125}I-insulin (4) binding in cultured human breast cancer (T47D) cells. Incubation of adherent cells with progestins for 10 h resulted in a 2-fold increase in EGF binding with no change in affinity for this binding (3). Glucocorticoids have also been reported to enhance EGF binding in G_1-S phase synchronized HeLa S_3 cells (5) and in quiescent human diploid foreskin fibroblasts (6). Dexamethasone-treated human fibroblasts (HF cells) exhibited a 50 to 100% increased ability to bind physiological concentrations of ^{125}I-EGF, with maximal enhancement after 24 h of dexamethasone treatment (6). This increase was specific for glucocorticoids and required protein synthesis. Dexamethasone treatment of HF cells also enhanced the mitogenic response of these cells to EGF by 50% (6). Increased insulin binding was observed when Swiss mouse 3T3-C2 cells were treated with glucocorticoids (7). Maximal stimulation was obtained 12 h after glucocorticoid administration (3–5-fold). Based on heavy isotope density shift experiments, it was suggested that this process involved decreased receptor degradation rather than fresh insulin receptor synthesis (7). Glucocorticoids have also been reported to either potentiate (8) or antagonize (9) insulin-stimulated macromolecular synthesis in different human breast carcinoma cell lines and to inhibit growth of breast (9, 10), prostate (11), and smooth muscle (12) tumor cell lines. Attenuation of that growth inhibition has been observed upon growth factor addition (11–13).

Our interest in steroid receptors is derived from tests of these substrates for the kinase action of EGF receptor in cultured cells. Previous studies from our laboratory have shown that the purified chick oviduct progesterone receptor is a high affinity substrate for tyrosine phosphorylation by purified EGF (14, 15) and insulin (15) receptor kinases. More recently, we demonstrated that EGF treatment of $^{32}P_i$ steady state-labeled human breast epithelial HBL100 cells resulted in enhanced phosphorylation of subsequently immunoprecipitated glucocorticoid receptors (16). Increased phosphorylation in response to EGF was detected on both serine and tyrosine residues. To test for possible biological relevance of EGF-stimulated glucocorticoid receptor phosphorylation, we examined the effects of EGF on glucocorticoid binding and action in HBL100 cells, a nontumorigenic epithelial cell line derived from human milk (17). HBL100 cells are rich in glucocorticoid receptors but exhibit essentially no binding capacity for other steroids (18). In this report we describe studies showing that EGF decreases glucocorticoid binding and antagonizes its action in these cells.

MATERIALS AND METHODS

Materials. [1,2,4,6,7-^{3}H]Dexamethasone (70 Ci/mmol) was obtained from Amersham Corporation (Arlington Heights, IL). Unlabeled dexamethasone, TPA, leupeptin, dithiothreitol, EDTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Company (St. Louis, MO). Unless otherwise stated, all other chemicals were of reagent grade.

Cells and Cell Culture. HBL100 cells were obtained from W. L. McGuire (University of Texas Health Science Center, San Antonio, TX). The cells were maintained routinely in 55-cm^2 polystyrene Petri plates in McCoy's 5A (modified) culture medium (GIBCO, Chagrin Falls, OH) supplemented with L-glutamine (2 mM), 10% fetal bovine serum (GIBCO), and 10 μg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were grown to near confluency and passed once a week with a change in the culture medium on the third day after passage.

For adherent cell binding studies, unless otherwise stated, cells were plated in 24-well polystyrene dishes (Corning Glass Works, Corning, NY). The medium was removed from the wells 24 h later and replaced with fresh medium containing 5% fetal bovine serum that had been stripped of steroids by treatment with dextran-coated charcoal (18). The cells were grown 4 days longer to about 0.7×10^5 cells/cm^2 (60% confluency).

In binding experiments involving supernatant and particulate fractions of cell homogenates, HBL100 cells were grown in glass roller bottles. The roller bottles were gassed with CO_2 and the cells were cultured to approximately 80% confluency with a change in medium on the third day of culture.

Preparation of Cell Suspensions. HBL100 cell suspensions were obtained from cells grown in roller bottles. The cells were washed twice with 50 ml/roller bottle of Tris-buffered saline (150 mM NaCl and 10 mM Tris-HCl, pH 7.4). The cells were then incubated in 15 ml/roller

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[3] The abbreviations used are: EGF, epidermal growth factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; dexamethasone, 9α-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione.

bottle with Tris-EDTA [25 mM Tris-HCl, 1 mM NaCl, and 1 mM Na_2HPO_4 (pH 7.4 at 37°C)] at 37°C for 15 min to detach the cells. During this incubation period the roller bottles were rolled continuously at 4 rpm.

Preparation of High Speed Cytosol and Particulate Fractions following Homogenization. Suspended cells were collected by a 4000 × g_{min} centrifugation and washed twice with 5 cell pellet volumes of homogenization buffer [10 mM Tris-HCl, 1 mM disodium EDTA, 5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, 40 μg/ml leupeptin, 10 mM sodium molybdate, and 10% (v/v) glycerol (pH 7.4)]. Cells were then homogenized in 2 pellet volumes of homogenization buffer using 50 strokes of a Dounce homogenizer in an ice bath. The cell homogenate was centrifuged at 40,000 × g_{min} and the supernatant fraction was collected. The particulate fraction was washed with 1 volume of the homogenization buffer and centrifuged at 40,000 × g_{min}. The supernatant fractions were combined and centrifuged at $6 \times 10^6 \times g_{min}$ to obtain the high speed cytosol. The 40,000 × g_{min} pellet was suspended in 3 volumes of homogenization buffer to determine dexamethasone binding to the particulate-associated receptors.

Extraction of Particulate Fraction-associated Glucocorticoid Receptors at High Ionic Strength. Washed pellets were suspended in 2 volumes of homogenization buffer containing 0.5 M KCl. The suspension was incubated with gentle shaking on a rotating platform at 4°C for 60 min and then centrifuged at 40,000 × g_{min}. The supernatant fraction containing solubilized particulate-associated receptors was used for dexamethasone-binding experiments.

Dexamethasone Binding to Receptors Present in the Cytosol and Salt-extracted Particulate Fractions. Aliquots of 50 μl from the high speed cytosol or the particulate extract were incubated at 4°C for 16 h with 50 μl of homogenization buffer containing 0.2 to 100 nM [^{3}H]dexamethasone. Three independent determinations were performed at each dexamethasone concentration and nonspecific binding was that occurring in the presence of 10 μM unlabeled dexamethasone. At the end of the incubations, bound radioactivity was determined by the dextran-coated charcoal assay (19). One part of dextran-coated charcoal suspension was added per sample and, after mixing by vortex action, the samples were incubated at 0°C for 5 min and centrifuged at 47,000 × g_{min}, and 50-μl aliquots of the supernatant fraction were used for determination of radioactivity in 7 ml of aqueous counting scintillation fluid (Amersham).

Dexamethasone Binding to Receptors Present in the Particulate Fraction. Dexamethasone binding to the particulate fraction was assayed following suspension of the 40,000 × g_{min} pellet in 2 volumes of the homogenization buffer. Aliquots (50 μl) of the pellet suspension were incubated with varying concentrations of [^{3}H]dexamethasone as described for the cytosol fraction. Bindings were terminated by centrifuging the samples at 47,000 × g_{min}; the supernatant was aspirated off and the pellets were washed twice by suspending the pellet in 500 μl of homogenization buffer followed by a 47,000 × g_{min} spin. The washed pellets were extracted with 50 μl of homogenization buffer containing 0.5 M KCl for 60 min at 4°C with gentle shaking on a rotating platform. Suspensions were then centrifuged at 47,000 × g_{min} and 250-μl aliquots of the supernatant fraction were quantified for radioactivity in 7 ml of aqueous counting scintillation fluid.

Dexamethasone Binding to Adherent Cells. Unless stated otherwise, dexamethasone binding to adherent cells was performed routinely on cells grown in 24-well dishes. Prior to ligand binding, cells were washed twice with 2 ml of binding medium [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered McCoy's Medium 5A (pH 7.4 at 37°C)] per well per wash and incubated in 0.5 ml of the binding medium containing 0.2 to 75 nM [^{3}H]dexamethasone either with or without 3 nM EGF at 37°C for 60 min. Stock solutions of dexamethasone were made up in 95% ethanol and the final concentration of ethanol in the binding medium was 0.5%. Replicate assays contained 10 μM unlabeled dexamethasone for determination of nonspecific binding. At the end of incubation, the 24-well dishes were placed on ice and within 5 min the cells were washed three times with binding medium chilled to 4°C using 2 ml per wash per well to remove free ligand. Cells were then incubated with 200 μl of 1 M NaOH for 30 min at 60°C and

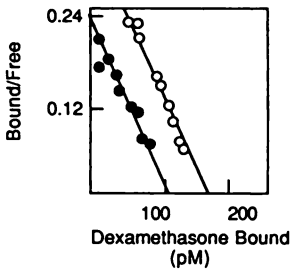

Fig. 1. Lack of effect of EGF on the affinity for specific dexamethasone binding. Replicate sets of adherent HBL100 cells, grown under identical conditions, were incubated at 37°C for 60 min in 0.5 ml of binding medium containing 0.2 to 25 nM [^{3}H]dexamethasone and 3 nM EGF (●) or no EGF (○). Specific binding was determined as described in "Materials and Methods." The resulting data were subjected to a Scatchard analysis using the LIGAND program (20). In this experiment cells that were not treated with EGF had 9.6 ± 0.5 × 10^5 dexamethasone-binding sites per cell as opposed to 6.4 ± 0.1 × 10^5 sites per cell for EGF-treated cells.

100 μl of the NaOH-solubilized material were neutralized with HCl and quantified for whole cell-associated radioactivity in 7 ml of aqueous counting scintillation fluid. For binding studies to cells grown in 6-well dishes, the volume of binding medium used was 1 ml/well. After completion of binding the cells were chilled and washed with 5 ml/well of binding medium and solubilized in 1 ml of 1 N NaOH.

Determination of Cell Count. Cells were rinsed with 0.5 ml Tris/EDTA and incubated in 1–5 ml of 0.25% trypsin in Hanks' salt solution at 37°C for 20 min. Cell counts were determined using a hemocytometer.

Protein Determination. Protein content in samples was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) and Cohn fraction V bovine serum albumin (Sigma) as the standard.

RESULTS

Dexamethasone Binding in HBL100 Cells. Adherent HBL100 cells were incubated at 37°C with 0.25 to 75 nM [^{3}H]dexamethasone to achieve equilibrium binding ("Materials and Methods"). Scatchard analysis of the data using the LIGAND program of Munson and Rodbard (20) revealed a single class of high affinity binding sites (K_d 2 nM) with about 1 × 10^5 sites/cell (not shown, but see Fig. 1).

In the course of our studies on progesterone receptor distribution in cultured breast carcinoma (T47D) cells we found that progestin binding to adherent T47D cells revealed two classes of binding affinity (21).[4] These two classes of binding sites were resolved by cell homogenization and subcellular fractionation to yield a soluble high affinity component and a particulate-associated low affinity component. While [^{3}H]dexamethasone binding to HBL100 cells revealed only a single class of high affinity binding sites, a minor population of low affinity ligand binding sites might have escaped detection. We therefore examined dexamethasone binding in the resolved cytosolic, particulate, and salt-extracted particulate fractions from HBL100 cells ("Materials and Methods"). Both the cytosolic and particulate fractions displayed a single class of dexamethasone-binding sites with identical affinities (Table 1). The major portion of ligand binding activity was associated with the cytosolic fraction; about 20% was retained in the pellet fraction. While there was no evidence for a second, lower affinity component, high ionic strength extraction of the particulate fraction increased ligand binding activity by 2-fold with no change in affinity (Table 1).

In a separate experiment, no enhanced ligand binding activity

[4] J. C. Sarup, K. V. S. Rao, R. E. Williams, and C. F. Fox, submitted for publication.

Table 1 *Scatchard analysis of specific dexamathasone binding to the cytosolic, particulate, and salt-extracted particulate fractions from HBL100 cell homogenates*

HBL100 cells grown in the glass roller bottles to about 80% confluency were suspended, homogenized, and subjected to subcellular fractionation ("Materials and Methods"). A portion ot the particulate fraction was extracted in 2 volumes of homogenization buffer containing 0.5 M KCl at 4°C for 60 min. Aliquots of all the three fractions were incubated at 4°C for 16 h with 0.2 to 100 nM [^{3}H]-dexamethasone. Nonspecific binding was that occurring in the presence of 10 μM unlabeled dexamethasone. The resulting data were subjected to Scatchard analysis using the LIGAND program of Munson and Rodbard (20). Values represent the mean ± SE of triplicate determinations.

Sample	Dexamethasone bound (fmol/2 × 10^7 cells)	K_d (nM)
Cytosol	2560 ± 333	1.0 ± 0.2
Particulate fraction	656 ± 92	1.1 ± 0.2
Salt extract of particulate fraction	1320 ± 264	1.6 ± 0.3

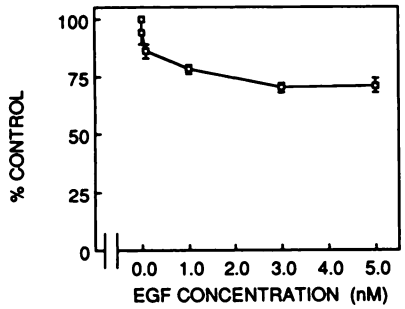

Fig. 2. Effect of EGF concentrations on specific dexamethasone binding. Cells in 24-well dishes were washed and incubated for 60 min at 37°C in 0.5 ml of binding medium containing 10 nM [^{3}H]dexamethasone and EGF at the concentrations shown. Nonspecific binding was determined as that occurring in the presence of 1 μM unlabeled dexamethasone. Values represent the average ± SD (*bars*) of three separate experiments.

was observed when the cytosolic fraction was treated at high ionic strength (not shown). High ionic strength is reported to inactivate ligand binding activity of the rat liver glucocorticoid receptor, but this inactivation can be overcome by including molybdate in the extraction buffer (22), as was the case in our experiment. Thus a significant population of dexamethasone-binding sites in the particulate fraction of HBL100 cells is not accessible to ligand. The increase in binding capacity noted for the salt extract of particulate fraction could be due either to hitherto inaccessible receptors becoming accessible on solubilization or by salt-induced activation of a cryptic population of particulate-associated receptors. This behavior does not appear to be a general characteristic of glucocorticoid receptors; extraction of receptors from the particulate fraction of cultured rat hepatoma cells at high ionic strength did not enhance glucocorticoid binding (23).

Effect of EGF on Dexamethasone Binding. Adherent HBL100 cells grown to about 60% confluency were incubated with 0.01 to 20 nM ^{125}I-EGF at 4°C for 4 h to characterize EGF binding. Scatchard analysis of the resulting data revealed 41,000 ± 4,000 (SE) EGF-binding sites per cell. Over 95% were low affinity sites (K_d 8 nM), and the remainder were high affinity sites (K_d 0.1 nM) (not shown).

Incubation of adherent cells with 10 nM [^{3}H]dexamethasone and 0 to 5 nM EGF caused a dose-dependent decrease in dexamethasone binding (Fig. 2). Maximal inhibition was achieved at 3 nM EGF with a 50% effective dose of about 0.2 nM EGF. This suggests that the high affinity component of EGF receptors (K_d 0.1 nM) may facilitate the process resulting in inhibition.

To test this, experiments were conducted in which the tumor promoter TPA was also added to the incubation medium along with EGF. TPA is known to inhibit formation of the high affinity EGF binding component (24, 25), apparently by stimulating protein kinase C-mediated phosphorylation of EGF receptor on Thr 654 (26). TPA inhibited dexamethasone binding slightly (~15%), but simultaneous incubation with EGF and TPA produced no additional inhibition (Table 2). On the other hand, EGF induced a 36% decrease in dexamethasone binding in this experiment in the absence of TPA.

Wakshull and Wharton (27) have reported that concanavalin A prevents endocytosis of the EGF-EGF receptor complex by mouse fibroblasts (27). The effect of concanavalin A on EGF-induced inhibition of dexamethasone binding was studied under conditions used in that study. HBL100 cells were first incubated with 3 nM EGF at 4°C for 4 h and then with 200 μg/ml concanavalin A for 20 min at 4°C. The cells were then warmed to 37°C and incubated with 10 nM [^{3}H]dexamethasone for 60 min. EGF had no effect on dexamethasone binding in concanavalin A-treated cells but inhibited dexamethasone binding by 36% in cells not treated with concanavalin A (Table 3). Concanavalin A alone had no significant effect on dexamethasone binding. In agreement with the findings of Wakshull and Wharton (27), ^{125}I-EGF uptake and degradation by HBL100 cells were inhibited by greater than 50% under the conditions of Table 3 (not shown).

Failure of EGF to Alter the Binding Affinity of Occupied Receptors. To determine if EGF inhibits dexamethasone binding by altering glucocorticoid receptor affinity as opposed to inactivating ligand binding activity, adherent cells were incubated at 37°C to achieve equilibrium binding with 0.3 to 25 nM [^{3}H]dexamethasone in either the presence or the absence of 3 nM EGF. Scatchard analysis revealed 33% lowered dexamethasone binding in response to EGF but no significant change in dexamethasone binding affinity (Fig. 1). In four separate experiments, the K_d for dexamethasone binding was 2.3 ± 1.4 for untreated cells and 2.1 ± 1.2 for EGF treated cells, and the average decrease in receptor number due to EGF was 36 ± 2%.

Inhibitory Effect of EGF on Dexamethasone Binding is Detected only in the Cytosolic Fraction of Cell Homogenates. To

Table 2 *Influence of TPA on the EGF-induced decrease in dexamethasone binding*

HBL100 cells in 6-well dishes were incubated for 60 min at 37°C in 1 ml/well of medium containing 10 nM [^{3}H]dexamethasone and where indicated either EGF (3 nM), TPA (100 nM), or EGF plus TPA. Nonspecific binding was determined as that occurring in the presence of 1 μM nonradioactive dexamethasone. Values represent the mean ± SE of four determinations.

Additions	Dexamethasone bound (10^{-3} × sites/cell)
None	99 ± 5
EGF	63 ± 5
TPA	84 ± 7
EGF + TPA	84 ± 11

Table 3 *Influence of concanavalin A on the EGF-mediated decrease in specific dexamethasone binding*

HBL100 cells grown in 6-well dishes were washed, cooled to 4°C, and incubated in 1 ml of chilled (4°C) binding medium where indicated with 3 nM EGF at 4°C for 4 h. Concanavalin A was then added in 20 μl of medium to the relevant wells where indicated at a final concentration of 200 μg/ml; 20 μl of medium were added to the remaining wells. All cells were incubated further for 20 min and the temperature was then raised to 37°C. [^{3}H]Dexamethasone was added at a final concentration of 10 nM was added to all wells and the cells were incubated at 37°C for 60 min. Values represent the mean ± SE of five determinations.

Additions	Dexamethasone bound (10^{-3} × sites/cell)
None	96 ± 9
EGF	62 ± 10
Concanavalin A	92 ± 10
EGF + concanavalin A	108 ± 8

determine if EGF decreased dexamethasone binding in all subcellular fractions, dexamethasone binding was studied in the cytosolic, particulate, and salt extract of the particulate fractions of EGF-treated and untreated cells (Table 4). Significantly decreased dexamethasone binding was observed only in the cytosolic fraction in response to EGF.

Rapid Initiation and Persistence of Decreased Dexamethasone Binding by EGF. The inhibitory effect of EGF on [^{3}H]dexamethasone binding was studied as a function of time at 37°C. Cells were incubated throughout the course of the experiment with 10 nM [^{3}H]dexamethasone in the presence or absence of 3 nM EGF. The ratio of dexamethasone binding in untreated *versus* EGF treated cells is presented in Fig. 3. Inhibition of dexamethasone binding was evident as early as 2 min of EGF treatment and was maximal within 5 min (Fig. 3*A*). This decrease in dexamethasone binding persisted over 4 days (Fig. 3*B*).

Table 4 *EGF decreases specific dexamethasone binding in the cytosolic fraction of cell homogenates*

HBL100 cells grown in glass roller bottles were incubated in 15 ml/bottle of binding medium in the presence or absence of 3 nM EGF at 37°C for 60 min. The cells were suspended and homogenized and the cytosolic, particulate, and salt extract of particulate fractions were obtained as described in "Materials and Methods", except that the 4×10^4 g_{min} supernatant was used as cytosol without the additional 6×10^6 g_{min} centrifugation. Dexamethasone binding in all fractions was determined with 10 nM [^{3}H]dexamethasone in the presence or absence of 1 μM nonradioactive ligand ("Materials and Methods"). Values for the two independent experiments shown represent mean ± SE of triplicate determinations. Addition of 0.5 M KCl to the cytosolic fraction from EGF-treated and untreated cells and incubating at 4°C for 60 min (*i.e.*, under conditions of particulate pellet salt extraction) had no effect on the EGF-induced decrease in dexamethasone binding, indicating that the effect of EGF is stable to salt treatment.

Sample	EGF	Dexamethasone bound (fmol/mg protein) Experiment 1	Experiment 2
Cytosol	–	580 ± 81	665 ± 18
	+	348 ± 49	454 ± 26
Particulate fraction	–	114 ± 46	154 ± 54
	+	93 ± 9	150 ± 37
Salt extract of particulate fraction	–	500 ± 75	639 ± 58
	+	458 ± 69	613 ± 84

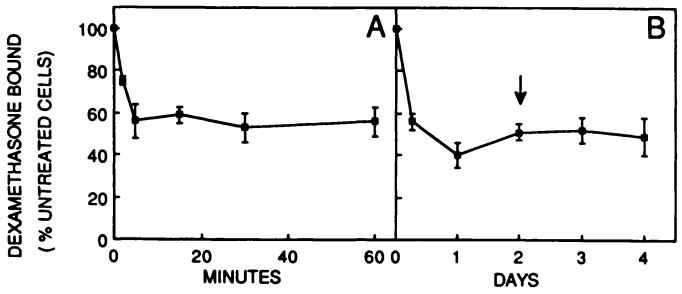

Fig. 3. Effect of EGF on specific dexamethasone binding as a function of time. *A*, effect over 60 min of EGF treatment. Cells in 24-well dishes were incubated at 37°C for the times shown in 0.5 ml medium containing 10 nM [^{3}H]-dexamethasone either with or without 3 nM EGF. To determine nonspecific binding 1 μM nonradioactive dexamethasone binding was also added to some wells. Cells were processed at the times indicated to determine specifically bound dexamethasone ("Materials and Methods"). *B*, effect of long term incubation with EGF. HBL100 cells were plated in 24-well dishes at a density of 0.3×10^4 cells/cm^2. At 24 h (day 0) the medium was replaced with fresh medium containing [^{3}H]dexamethasone either with or without EGF as described for *A*. The cells were incubated at 37°C for the times shown with a change in medium at day 2 (*arrow*). At the times indicated cells were processed to determine specifically bound dexamethasone ("Materials and Methods"). Cell counts were determined with a hemocytometer and dexamethasone bound was normalized for cell number. Values represent mean values for dexamethasone bound by EGF-treated cells expressed as a percentage of that bound by untreated cells ± SE (*bars*) of quadruplicate determinations. The down-regulation of glucocorticoid receptors in response to glucocorticoids that has been reported for several different cell lines (28–30) was also characteristic of HBL100 cells in this experiment. In the experiment in *B*, dexamethasone binding in the presence or absence of EGF was decreased by similar extents as a function of time.

Insensitivity of Occupied Receptors to Short Term EGF Action. When cells were first incubated with 10 nM [^{3}H]dexamethasone at 37°C for 30 min and with 3 nM EGF and [^{3}H]-dexamethasone for an additional 60 min, no EGF-induced decrease in dexamethasone binding was observed (Table 5). EGF inhibited dexamethasone binding by approximately 40% in the control experiment where both ligands were added together (Table 5). In independent experiments we determined that the dissociation rate of [^{3}H]dexamethasone at 37°C was not significantly different in EGF-treated cells which had been incubated for 30 min at 37°C with 10 nM dexamethasone prior to EGF addition ($t_{½}$ = 26 ± 1.5 min) or with no dexamethasone added ($t_{½}$ = 24 ± 1 min). Neither were these rates significantly different to dexamethasone dissociation from cells not treated with EGF ($t_{½}$ = 26 ± 2 min). Taken together with the earlier noted observation that EGF does not alter the equilibrium binding affinity of occupied receptors (Fig. 2), EGF appears to act by determining the number of receptors capable of binding dexamethasone but acts in the short term to decrease binding only to those receptors which are not saturated with ligand (Table 5).

Antagonism of Inhibitory Action of Dexamethasone on Growth of HBL100 Cells by EGF. Dexamethasone inhibits thymidine incorporation in several human breast cancer cell lines, and the extent of this inhibition correlates well with their glucocorticoid-binding capacity. For example, 100 nM dexamethasone inhibited the growth of MCF-7 cells by over 50% (10). Glucocorticoids have been shown to inhibit proliferation of several cultured cell lines by arresting them either in the G_0 or the G_1 phase of the cell cycle. Growth factor addition can overcome this inhibition (11–13). Attenuation of glucocorticoid-induced inhibition of cell growth has been observed in prostate-derived R3327H-G8-A1 tumor cells with EGF (11) and in a smooth muscle tumor cell line, DDT$_1$ MF-2, with platelet-derived growth factor (12, 13).

Dexamethasone also inhibited the growth of HBL100 cells (Fig. 4). Maximal inhibition was achieved at 100 nM dexamethasone with a 50% effective dose of less than 5 nM (Fig. 4), in close agreement with the observed dissociation constant for dexamethasone binding (2 nM). Dexamethasone-induced inhibition of HBL100 cell proliferation was blocked by 3 nM EGF (Fig. 5), although EGF alone had no significant stimulatory action on HBL100 cell growth (Fig. 5). Our results are thus consistent with observations of others (11–13).

Table 5 *Insensitivity of occupied glucocorticoid receptors to short term EGF action*

HBL100 cells were preincubated at 37°C for 30 min in 0.5 ml of binding medium with 10 nM [^{3}H]dexamethasone where indicated. The medium was then removed and dexamethasone-treated and untreated cells were divided into two sets. To one set 0.5 ml medium containing 10 nM [^{3}H]dexamethasone was added and to the other set 0.5 ml medium containing 10 nM [^{3}H]dexamethasone and 3 nM EGF was added. All cells were incubated for a further 60 min at 37°C prior to determination of specific whole cell dexamethasone binding ("Materials and Methods"). Nonspecific binding was determined as that occurring in the presence of 1 μM unlabeled ligand. Values are mean ± SE of five determinations.

Experiment	Dexamethasone preincubation	Dexamethasone bound (10^{-3} × sites/cell) Control	+EGF
1	–	112 ± 13	69 ± 5
	+	87 ± 13	85 ± 15
2	–	91 ± 3	70 ± 5
	+	76 ± 6	81 ± 11

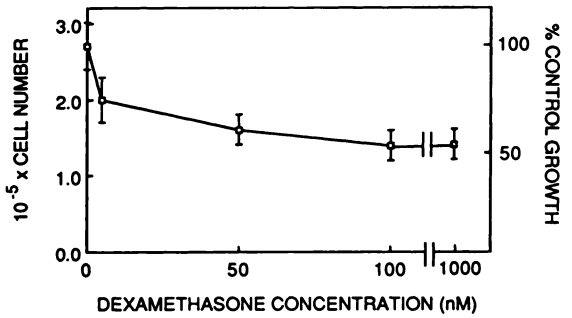

Fig. 4. Effect of dexamethasone concentrations on the growth of HBL100 cells. HBL100 cells were plated in 6-well dishes at approximately 10^4 cells/cm^2 and grown for 24 h. The medium was removed and replaced with 5 ml of fresh medium containing dexamethasone as indicated. The cells were grown for 48 h further when cell counts were determined. The values represent mean ± SE (*bars*) of four determinations.

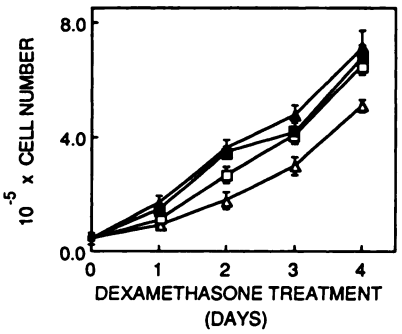

Fig. 5. EGF overcomes the growth inhibition induced by dexamethasone. HBL100 cells were plated in 6-well dishes at approximately 10^4 cells/well. At day 0 (48 h later), the medium was replaced with 5 ml/well of fresh medium alone (□) or with 100 nM dexamethasone (△), 3 nM EGF (■), or 100 nM dexamethasone plus 3 nM EGF (▲). The cells were grown with a replenishment of medium every 48 h. At the designated times, cell counts were determined in 1–5 ml of 0.25% trypsin solution as described in "Materials and Methods." Each *point* represents the mean of four determinations. *Bars*, SE. Similar results were obtained in two additional, independent experiments. The experiment in Table 6 also demonstrates the dominance of EGF over dexamethasone-induced growth inhibition.

Table 6 *TPA antagonism of the stimulatory effect of EGF on growth of dexamethasone-inhibited HBL100 cells*

Cells were plated in 24-well dishes at a density of 10^4 cells/cm^2. At day 0 (24 h later), the medium was replaced with 1 ml/well of medium containing the various agents as shown. The concentrations used were: Dex,[a] 100 nM; EGF, 1 nM, and TPA, 20 nM. Cells were incubated at 37°C with a change in medium after a further 48 h (day 2). After an additional 48 h (day 4), cell counts were determined ("Materials and Methods"). Values are mean ± SE of four determinations.

Additions	10^{-5} × cell no.	% of control
None	7.4 ± 0.68	(100)
Dex	4.4 ± 0.14	59
EGF	8.6 ± 0.67	116
TPA	8.2 ± 0.24	111
Dex + EGF	8.5 ± 0.77	115
Dex + TPA	5.5 ± 0.46	74
EGF + TPA	7.9 ± 0.26	107
Dex + EGF + TPA	6.0 ± 0.44	81

[a] Dex, dexamethasone.

TPA Blockage of Antagonistic Action of EGF on Dexamethasone-induced Inhibition of HBL100 Cell Growth. Since the EGF-induced decrease in dexamethasone binding was antagonized by TPA (Table 2), we determined if TPA could also block the EGF action on dexamethasone-induced inhibition of HBL100 cell growth. The growth-inhibitory effect of dexamethasone was not influenced by TPA (Table 6), and the growth rates of cells incubated in the presence of EGF, TPA, or EGF plus TPA were similar. However, TPA addition effectively blocked the antagonistic effect of EGF on dexamethasone-induced growth inhibition (Table 6).

DISCUSSION

In responsive cells, epidermal growth factor can stimulate protein phosphorylation on tyrosine, serine, and threonine residues (31–36). The protein tyrosine kinase activity is a property of the EGF receptor protein (31). The protein threonine kinase activity can be attributed to protein kinase C (32) which acts in turn to phosphorylate EGF receptor at Thr 654 (26, 37), attenuating its protein tyrosine kinase activity (25, 38, 39). The source of increased serine phosphorylation has not been established, although there are several candidate activities (33–36).

Glucocorticoid receptors are phosphorylated on serine residues in a variety of tissues and cultured cells (16, 40, 41), and more recent evidence has indicated a significant but smaller phosphorylation complement on tyrosine residues (16). Increased phosphorylation at both serine and tyrosine residues has been noted in EGF-stimulated cells (16). Phosphorylation at serine residues has been proposed to play an obligatory role in maintaining ligand-binding activity (42, 43). No role has been proposed for phosphorylation at tyrosine residues.

Equilibration of cells with dexamethasone at a concentration sufficient to saturate ligand-binding sites on glucocorticoid receptors in HBL100 cells inhibited alkali-stable phosphorylation of the glucocorticoid receptor occurring in response to EGF (16). Preequilibration with dexamethasone also blocked the ability of EGF to induce a short term decrease in dexamethasone-specific binding activity (Table 5). These experiments suggest a causal relationship between EGF receptor-mediated phosphorylation of glucocorticoid receptor and EGF-induced decreased glucocorticoid-binding activity but are not conclusive (16). The question of whether EGF receptor inactivates cell-free system glucocorticoid binding by phosphorylating glucocorticoid receptors directly on tyrosine residues is a subject currently under investigation in our laboratory.

The properties of TPA and concanavalin A in antagonizing the ability of EGF to decrease glucocorticoid-binding activity (Tables 2 and 3) support a causal role for EGF-dependent protein tyrosine kinase action in decreasing glucocorticoid binding but do not exclude the possibility of EGF receptor acting through activation of other catalytic activities. The cytosolic confinement of the EGF effect on glucocorticoid binding (Table 4) is consistent with the localization and thus the direct action of EGF receptor kinase and indicates that the cellular functions of glucocorticoid receptors remaining associated with the particulate fraction upon homogenization are unlikely to be affected by direct EGF receptor action.

While the magnitude of the effect of EGF on dexamethasone binding is not substantial, nevertheless this interaction could be important in regulating at least the short term effects of glucocorticoids. Amplified responses to small changes in stimuli are common regulatory events in biological systems (44) and the condition has been referred to as a "threshold" phenomenon.

The mechanism by which EGF acts to attenuate HBL100 cell growth inhibition occurring in response to dexamethasone is not obvious from our study. It is unlikely that EGF acts to uncouple glucocorticoid growth-inhibitory action by decreasing glucocorticoid binding. This would require that EGF (*a*) act to decrease glucocorticoid binding nearly completely in a subpopulation of cells which is unique in its growth inhibition sensitivity to glucocorticoids or (*b*) induce preferential phosphorylation of a unique subpopulation of glucocorticoid receptors which are responsible for the growth-inhibitory action. While we consider these possibilities extremely unlikely, we are pre-

paring to test for differences in responses to glucocorticoids or EGF in subclones of the HBL100 cell line.

The action of EGF to attenuate glucocorticoid-induced inhibition of HBL100 cell growth reinforces the observations of Smith *et al.* (11) and Syms *et al.* (12) with cultured R3327H-G8-A1 and DDT$_1$MF-2 cells. Glucocorticoid-induced growth inhibition of R3327H-G8-A1 and DDT$_1$MF-2 cell growth can be reversed nearly completely by EGF in the former case and by platelet-derived growth factor in the latter (11, 12). This indicates that growth factors can act through pathways independent of and dominant over those through which glucocorticoids act to inhibit cell growth. This underscores the value of determining EGF receptor levels (and responsiveness) and possible autocrine or paracrine EGF or transforming growth factor-α secretion (45) in tumors in assessing the prognosis of glucocorticoids as tumor growth inhibitors.

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