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Glucocorticoid-Mediated Alteration in Growth Factor Binding and Action: Analysis of the Binding Change

Joffre B. Baker and Dennis D. Cunningham

Department of Medical Microbiology, College of Medicine, University of California at Irvine, Irvine, California 92717

The addition of the glucocorticoid analog dexamethasone (DX) to serum-free cultures of human fibroblasts caused a twofold enhancement of the mitogenic response to epidermal growth factor (EGF), although DX by itself was not mitogenic. A basis for this effect was suggested by studies showing that DX also increased the cellular binding of ^{125}I-EGF. DX increased the ability of the cells to bind ^{125}I-EGF only at low physiological concentrations of this polypeptide. Thus, data from ^{125}I-EGF binding to cells incubated without DX produced a linear Scatchard plot, whereas the data from ^{125}I-EGF binding to DX-treated cells led to an upwardly curvilinear Scatchard plot. Measurements of ^{125}I-EGF association with the cell surface and cytoplasm indicated that this binding change involved an alteration of cell surface EGF receptors. The binding change appeared not to involve negatively cooperative interactions between EGF receptors, nor a change in the number of receptors. The binding alteration could be explained by a model in which DX converted 25–30% of the cell surface EGF receptors to a form having a fourfold increased affinity.

Key words: dexamethasone, epidermal growth factor, human diploid fibroblasts, cell proliferation, permissive effect

Glucocorticoids exert a large number of diverse effects on a variety of different tissues [1]. Many of these influences appear to be caused indirectly ("permissively") by glucocorticoids modulating the responsiveness of cells to other hormones. Among the permissive actions of glucocorticoids are growth-promoting effects. Glucocorticoids by themselves are not mitogenic for cultured cells, but they markedly enhance cell proliferation in the presence of growth promoting polypeptide hormones (growth factors) [2, 3] or serum [4]. Although hypotheses have been advanced to explain these growth effects and other permissive actions of glucocorticoids [2, 5], in no case is the mechanism of a permissive effect understood.

Recently we reported that the synthetic glucocorticoid dexamethasone (DX), which

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by itself had no growth-promoting effect on nonproliferating human diploid foreskin (HF) cells in serum-free medium, was able to sensitize these cells to the polypeptide mitogen epidermal growth factor (EGF) [6]. A possible basis for this permissive effect was suggested by our finding that concomitant with this sensitization, DX increased the ability of the HF cells to bind ^{125}I-EGF. This finding indicated that DX might enhance the response of cells to EGF by altering the EGF receptors. In the present report we probed other aspects of this DX-mediated growth effect and analyzed the nature of the DX alteration in ^{125}I-EGF binding. The present experiments indicate that DX alters ^{125}I-EGF binding by acting on cell surface receptors for EGF. DX does not increase the number of receptors per cell. Instead, DX increases the affinity of the cells for low to physiologic levels of ^{125}I-EGF through an increase in the binding association rate constant. Based on these results and Scatchard analysis of ^{125}I-EGF binding data, we propose that DX converts about 25% of the cell surface EGF receptors to a new form with a fourfold to fivefold increased affinity. The possible relevance of this binding change to the DX-mediated enhancement of EGF action is considered.

MATERIALS AND METHODS

We purchased Dulbecco-Vogt-modified Eagle's medium (DV medium) from Gibco, serum and other medium products from Irvine Scientific, and tissue culture dishes from Falcon Plastics. EGF was purified by the procedure of Savage and Cohen [7]. Rabbit antibody to EGF was prepared by the procedure of Cohen [8].

Stock cultures of human fibroblasts prepared from neonatal foreskin explants (HF cells) were grown at 37° in DV medium containing 10% serum and equilibrated with 5% CO_2 in air as previously described [6].

Quiescent, serum-free cultures of HF cells were prepared from confluent HF cultures described previously [6], by incubating confluent cells for 24 h with two parts DV medium and one part Waymouth's medium containing no serum.

^{125}I-EGF binding to cultures of HF cells was measured as previously described [6], using ^{125}I-EGF labeled by the procedure of Carpenter and Cohen [9]. Briefly, the medium on cultures was changed to DV medium containing ^{125}I-EGF and 0.1% (w/v) bovine serum albumin. Binding incubations at 37° were carried out in 5% CO_2/95% air. Binding incubations at 4° (on ice) were carried out in air and the medium was buffered with 15 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes). To terminate binding incubations, the cultures were rinsed on ice. The cells were dissolved in 0.3 N NaOH, and the amount of cell-associated ^{125}I and cell protein were quantitated with a γ counter and the Lowry protein assay [10]. Nonspecific ^{125}I-EGF binding, measured as the amount of ^{125}I-EGF binding which still occurred in the presence of nonlabeled EGF at 2 μg/ml, was subtracted from all binding determinations.

RESULTS

Unlike certain other cell types that strongly require serum for survival, confluent HF cells maintained constant cell number in serum-free, chemically defined medium for over one week (Fig 1). The addition of EGF to these nonproliferating cells stimulated cell division as previously reported [6]. In the presence of physiologic levels [11] of EGF (1.5 ng/ml) cell number increased by about 35% over five days (Fig 1A). In the presence of EGF at a high maximally effective concentration (20 ng/ml), cell number doubled during a nine-day period (Fig 1B).

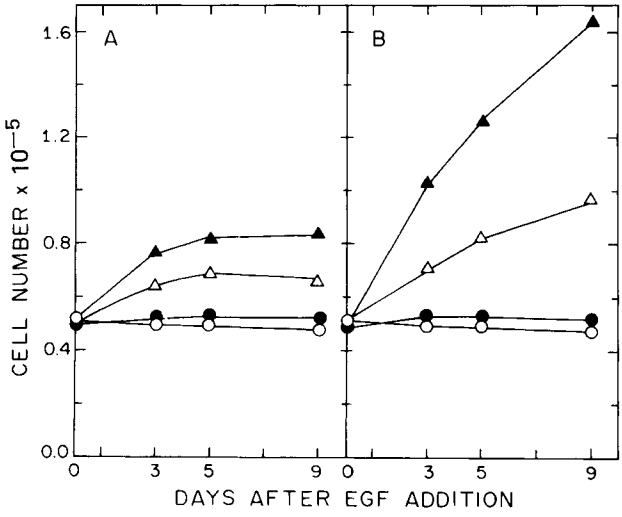

Fig 1. Growth of HF cells in serum-free cultures in the presence of EGF (△), DX (●), EGF plus DX (▲), or no additions (○). DX at 250 ng/ml was added to the indicated quiescent serum-free HF cell cultures. After three days the cultures were given a further 2 ml of medium (2 parts of DV medium and 1 part Waymouth's medium) containing 0.1% bovine serum albumin and either no EGF, or enough EGF to yield a final concentration of 1.5 ng/ml (1A) or 20 ng/ml (1B). Number of cells per dish was counted on the indicated days. Points represent averages of triplicate measurements.

In the course of these experiments with EGF, we discovered that the glucocorticoid analog DX also had a growth-promoting activity in these cultures, but only if EGF was present. Thus, Figure 1 shows that when EGF was added to cultures exposed to DX (250 ng/ml), the mitogenic response to EGF was two times greater than in cultures without DX. Addition of DX by itself had no effect on cell number (Fig 1). In two other experiments identical to the one described in Figure 1, DX permissively increased the response to EGF by 40% and 70%. This amount of variability was less than observed in our earlier experiments [6]. This may have been a result of longer DX treatment before addition of EGF in the present experiments (three days in Fig 1). It is noteworthy that in all the experiments the proportion by which DX enhanced the mitogenic response to EGF was about the same whether EGF was present at relatively low concentrations or at high maximally effective concentrations (compare Fig 1A and 1B).

In studies probing the mechanism of this "permissive" effect, we recently discovered that DX treatment enhanced the ability of the HF cells to bind ^{125}I-EGF [6]. The following experiments were designed to determine the nature of this DX-mediated binding change. Figure 2 shows the time courses of ^{125}I-EGF binding to quiescent cells in serum free medium at 37°. At a nearly physiologic concentration of ^{125}I-EGF (0.25 ng/ml), ^{125}I-EGF binding to untreated cells reached a steady-state level after about 1 h (Fig. 2A). As indicated, DX treatment increased the ability of the cells to bind ^{125}I-EGF twofold at every time examined, including the time when the maximum level of binding was attained.

In contrast, experiments in which much higher concentrations of ^{125}I-EGF were used in the binding incubation did not reveal an effect of DX on ^{125}I-EGF binding. When 25 ng/ml ^{125}I-EGF was allowed to bind to quiescent HF cells at 37°, binding attained a maximum level within an hour, and then decreased markedly over the next several hours (Fig 2B). (The decrease in binding, which occurred after several hours (Fig 2B), has been shown to involve a "down-regulation" or loss of the cellular capacity to bind ^{125}I-EGF,

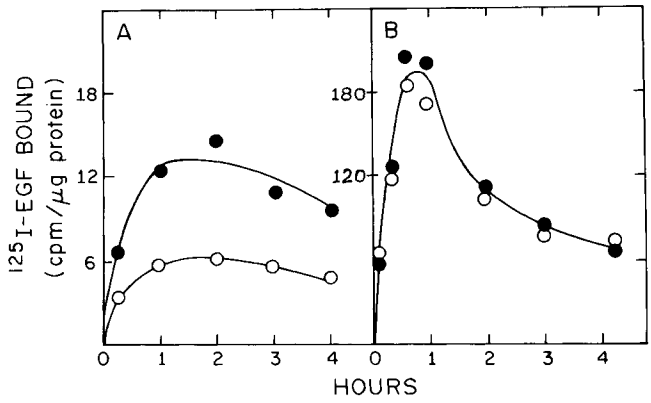

Fig 2. Time course of ^{125}I-EGF binding at 37° to HF cells incubated with (•) and without (○) DX. DX (250 ng/ml) was added to the indicated quiescent HF cultures. After three days the cells were allowed to bind ^{125}I-EGF at 0.25 ng/ml (2A) or 25 ng/ml (2B) for the indicated times at 37°, and were processed for radioactivity and protein measurements. Points represent averages of duplicate measurements. DX did not significantly change the amount of protein per cell (data not shown).

and is a characteristic response of HF cells to prolonged exposure to EGF [12].) As shown in Figure 2B, DX treatment of the HF cells did not influence their ability to bind ^{125}I-EGF at this high concentration.

These results prompted us to investigate the influence of DX on EGF receptor number and affinity. This kind of information is frequently obtained in hormone binding studies by measuring the concentration dependence of hormone binding at equilibrium and analyzing the results on a Scatchard plot. A Scatchard analysis of ligand binding assumes that the binding reaction involves only association and dissociation reactions between the ligand and its receptor [13]. However, EGF binding to cells at 37° also involves rapid internalization of cell surface-bound ^{125}I-EGF [9]. Therefore, measurements of ^{125}I-EGF binding to HF cells were carried out at 4° to minimize internalization of ^{125}I-EGF.

Measurements of steady-state ^{125}I-EGF binding to HF cells over a thousand-fold range of ^{125}I-EGF concentrations generated a linear Scatchard plot (Fig 3), suggesting one affinity class of receptors. From the slope of this line we calculated a $K_{diss} = 11 \times 10^{-10}$M. The X axis intercept of this line indicated that there were approximately 60,000 EGF receptors per cell. In contrast, Figure 3 shows that the binding data obtained from the DX-treated HF cells resulted in a curvilinear Scatchard plot. The data extrapolated to about the same point on the "bound" axis as the data from the nontreated cells, suggesting that DX did not influence the total number of EGF receptors. However, at decreasing levels of bound ^{125}I-EGF, corresponding to lower concentrations of ^{125}I-EGF in the binding medium, the Scatchard plot curved upward. Similar results were obtained when the binding study was conducted at 37° (data not shown).

Two types of hypothesis frequently have been used to explain curvilinear Scatchard plots of hormone binding data. Curvature could be produced by the presence of two or more affinity classes of receptors [15]. Alternatively, curvature could result from negatively cooperative interactions occurring between hormone binding sites, resulting in a continuously decreasing binding affinity as increasing amounts of hormone are bound [16].

Evidence of negative cooperativity has come from observations that the rate of dis-

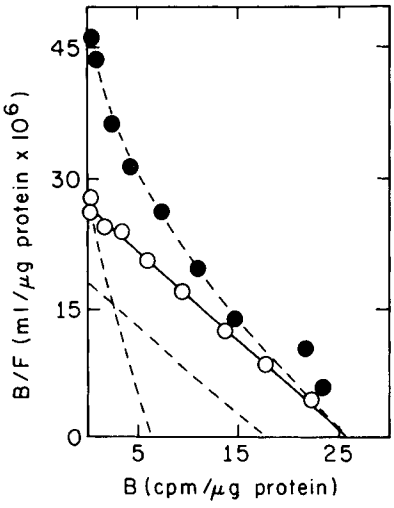

Fig 3. Scatchard plot of ^{125}I-EGF binding at 4° to DX-treated (•) and untreated (○) control HF cells. DX at 100 ng/ml was added to the indicated quiescent cultures. Three days later the cultures were allowed to bind ^{125}I-EGF at various concentrations (ranging from 0.075 ng/ml to 50 ng/ml) on ice. After 4 h, at which time binding had reached steady state (data not shown), the cultures were processed for radioactivity and protein measurements. Points represent the averages of triplicate measurements. The curved line drawn through the data from DX-treated cells was resolved by a graphical method [14] as a sum of the two straight dashed lines shown.

sociation of certain hormones from their receptors occurs more rapidly as increasing levels of hormone become bound [17]. This modulation of the dissociation rate constant has formed the basis for postulating negatively cooperative hormone binding. If DX modulated EGF binding by this mechanism, the DX-mediated binding enhancement would be least apparent at very early times in the binding reaction, when the influence of dissociation rate on binding is negligible. We examined this possibility by following the time course of ^{125}I-EGF binding to DX-treated and control HF cells at 4°. As indicated in Figure 4, DX-treated cells bound about two times more ^{125}I-EGF at 0.25 ng/ml than control cells at all times examined. It should be noted that a similar result had been obtained in a study done at 37° (Fig 2A). It is of particular interest that the DX-mediated enhancement of binding was apparent even in the initial phase of binding (0–5 min in Fig 4) during which the amount of binding was approximately proportional to the length of the binding incubation. During the initial linear phase of binding, the rate of binding, X, is given by the equation:

$$X = ka \cdot H_0 \cdot R_0,$$

where ka is the association rate constant, H_0 is ^{125}I-EGF concentration, and R_0 is receptor number. Since DX-treated and control cells appeared to have about the same number of EGF receptors (as suggested by the Scatchard analysis in Fig 3), the greater initial rate of ^{125}I-EGF binding to the DX-treated cells shown in Figure 4 indicated that DX elevated EGF binding by increasing the binding association rate constant. Thus, the increased binding of EGF to DX-treated cells appeared unrelated to the kind of negative cooperativity proposed to occur in the binding of other hormones.

The binding data are consistent with a model in which DX causes some of the EGF receptors to increase in affinity. The two dashed lines in Figure 3 demonstrate how the

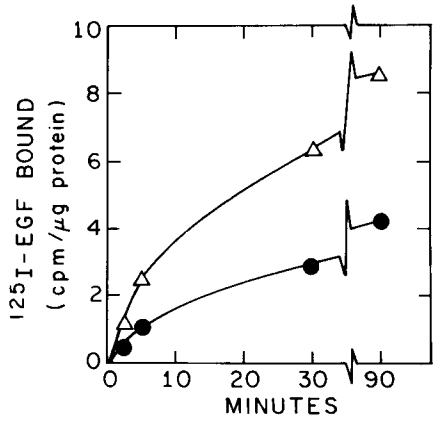

Fig 4. Time course of ^{125}I-EGF binding at 4° to HF cells incubated with (△) and without (•) DX. HF cultures incubated as described in Figure 2 were allowed to bind ^{125}I-EGF on ice for the indicated times, and were processed for radioactivity and protein measurements. Points represent the averages of duplicate measurements.

presence of two affinity classes of EGF receptors on DX-treated cells could generate the observed curvilinear Scatchard plot of EGF binding. The "low affinity" class of receptors would have the same affinity as the EGF receptors of untreated cells. A new "high affinity" class of receptors would have about a fourfold greater EGF binding affinity. These receptors would be only about one-quarter to one-third as abundant as the lower-affinity receptors. The fact that the total number of EGF receptors was apparently not altered by DX suggests that the high-affinity receptors would arise by a modification of some of the low-affinity receptors.

We considered an alternative explanation for the curvilinear Scatchard plot. If significant internalization of ^{125}I-EGF could occur even in our binding measurements at 4°, the DX-mediated alteration in EGF binding shown in Figure 3 might be caused by an effect on internalization of ^{125}I-EGF, rather than by an effect on the EGF receptors. In fact, published evidence suggested that substantial internalization of EGF could occur at 4° [9]. Therefore, we attempted to determine 1) how cell-associated ^{125}I-EGF was distributed between the cell surface and cytoplasm under the binding conditions used to generate the Scatchard plot in Figure 3, and 2) how DX affected this distribution between cell surface-bound and internalized ^{125}I-EGF.

Carpenter and Cohen reported that only about half of the ^{125}I-EGF bound to HF cells during a 1-h incubation on ice could be removed during a subsequent 0.5-h incubation with trypsin on ice [9]. We found that an alternative procedure for determining the cell-surface-to-cytoplasm distribution demonstrated that a greater fraction of cell-associated ^{125}I-EGF was external. In this method, developed by Holley et al [18], cells with bound ^{125}I-EGF were incubated with anti-EGF antibody, and the amount of ^{125}I-associated with the cells and the medium was then measured. Table I shows that approximately 90% of the ^{125}I which was bound to the cells after a 4-h 4° incubation with ^{125}I-EGF was released from the cells during the incubation with anti-EGF antibody. Most (75–90%) of the ^{125}I released from either the DX-treated or control cells was precipitable by trichloroacetic acid. This ^{125}I was very likely ^{125}I-EGF that had been associated with the cell surface, because internalized ^{125}I-EGF is released as ^{125}I-monoiodotyrosine [12]. The small fraction (10–20%) of medium-associated ^{125}I that was acid-soluble (Table I, column 1)

TABLE I. Cellular Location of ^{125}I-EGF Bound at 0°

^{125}I-EGF concentration (ng/ml)	CPM released into medium					
	TCA-soluble		TCA-insoluble (cell surface-associated)		Cell-associated CPM	
	+DX	−DX	+DX	−DX	+DX	−DX
0.4	121 ± 20	66 ± 3	954 ± 3	678 ± 38	80 ± 6	63 ± 4
20.0	3,490 ± 198	3,310 ± 53	12,962 ± 217	13,189 ± 109	1,676 ± 192	1,916 ± 70

Quiescent HF cells in serum-free medium were incubated with or without DX (150 ng/ml) and allowed to bind ^{125}I-EGF (0.4 ng/ml or 20 ng/ml) at 0° for 4 h as described in Figure 4. After the unbound ^{125}I-EGF was washed away, the cultures were incubated at 37° for 2 h in 1 ml Dulbecco-Vogt medium containing 0.1% bovine serum albumin and 20 μl/ml anti-EGF antibody. Microscopic examination showed that this treatment removed an insignificant fraction of the cells from the dishes. The medium was removed and the cells were dissolved in 0.3 N NaOH for measurement of cell-associated ^{125}I. The medium samples were refrigerated with 0.3 ml 15% trichloroacetic acid (TCA) for 12 hours, and centrifuged in a Beckman microfuge for 3 min to obtain TCA-soluble (supernatant) and TCA-insoluble (pellet) released material. The CPM shown have been corrected for CPM due to nonspecific binding.

probably represented predominately ^{125}I that had been internalized during the 4° binding incubation and was later degraded and exocytosed. However, an unknown fraction of the acid-soluble ^{125}I in Table I, as well as the ^{125}I associated with the cells in Table I, could have come from ^{125}I-EGF that was associated with the cell surface during the binding incubation but was not dissociable by anti-EGF antibody, and was subsequently internalized. Together, the data in Table I show that more than 75% of the ^{125}I-EGF bound to the cells at steady state at 4° was associated with the cell surface.

DX altered the amount of ^{125}I-EGF bound to the cell surface. This alteration in surface binding, shown in Table I, appeared similar to the modification by DX of total cell binding that has already been shown in Figure 2 and Figure 3. Thus, Table I shows that DX had no influence on the cell surface binding of ^{125}I-EGF at a high concentration (20 ng/ml), but DX increased cell surface binding of ^{125}I-EGF at a low physiologic concentration (0.4 ng/ml). DX affected the amount of ^{125}I-EGF internalized only insofar as increased cell surface binding was accompanied by a proportional increase in ^{125}I-EGF internalization. Together, these findings indicate that DX primarily affects ^{125}I-EGF binding by altering the cell surface receptors for EGF. These results are consistent with the model suggested in Figure 3 in which DX increases the affinity of a fraction of the cell surface EGF receptors.

DISCUSSION

Our previous results with HF cell cultures have shown that DX increased the binding and mitogenic action of EGF [6]. In the present studies we have analyzed the nature of the DX-mediated binding change. DX-treated HF cells had a substantially increased ability to bind ^{125}I-EGF at low concentrations (0–1 ng/ml), but this binding enhancement disappeared when binding was measured with higher concentrations of ^{125}I-EGF. Several lines of evidence suggest that DX altered EGF binding primarily by altering cell surface EGF receptors. First, the DX enhancement of ^{125}I-EGF binding was fully apparent early in the binding reaction (2–5 min), when the ^{125}I-EGF was probably binding only to the

cell surface. Second, the effect of DX on ^{125}I-EGF binding at steady state was about the same whether the binding study was conducted at 4° or at 37°, even though the amount of EGF internalized is markedly inhibited at the lower temperature [9, 18]. Third, direct measurements of the amount of ^{125}I-EGF bound to the cell surface, as determined by the amount released by anti-EGF antibody, revealed the same effect of DX on ^{125}I-EGF binding as was found with total cell binding.

Scatchard analysis of EGF binding either at 37° or 4° revealed an intriguing distinction between the ^{125}I-EGF binding properties of untreated and DX-treated cells. EGF binding to untreated cells generated a linear Scatchard plot, indicating that the cells had a single affinity class of EGF receptors. The Scatchard plot of ^{125}I-EGF binding data from DX-treated cells extrapolated to the same number of EGF receptors as demonstrated for the untreated cells, but this Scatchard plot became upwardly curvilinear at the lower levels of bound ^{125}I-EGF.

Although there are a number of ways curvilinear Scatchard plots may be caused by experimental artifacts [19] or ligand-ligand interactions [20], these mechanisms could hardly have been involved in the present case. Here, the curvilinearity undoubtedly was caused by a modification of the cell receptors, because when cells were not pretreated with DX a linear Scatchard plot obtained.

The DX-mediated binding change reflected by the curvilinear Scatchard plot did not involve the negative cooperativity phenomenon proposed to occur in certain other hormone-binding systems where curvilinear Scatchard plots have been obtained. The negative cooperativity concept is based on observations that in some systems the dissociation of bound hormone is accelerated as more receptors become occupied [17]. However, the DX enhancement of ^{125}I-EGF binding seemed to involve predominately a change in the binding association rate constant, rather than a change in the dissociation rate constant. Other considerations also argue against an explanation for the increase in ^{125}I-EGF binding based on negative cooperativity. In such a model DX would have to act by reducing negatively cooperative interactions among EGF receptors. In the untreated cells these negatively cooperative interactions would have to be at their *maximal* effectiveness at the lowest levels of receptor occupancy we observed – only 0.5% of the receptors occupied (Fig 3). Otherwise the binding data from untreated cells would have generated a curvilinear rather than a straight Scatchard plot. It is unlikely that negatively cooperative effects would be important when only 0.5% of the receptors are occupied. Thus, it is unlikely that negatively cooperative interactions play a role in ^{125}I-EGF binding to untreated cells. DX could, therefore, not reduce negative cooperativity.

The effect of DX on ^{125}I-EGF binding could be explained fairly simply by a model in which DX converted about one-quarter of the EGF receptors to a form having a fourfold to fivefold increased affinity for ^{125}I-EGF. Of course, other models are also possible. For example, DX-modified receptors might have a heterogeneous distribution of affinities.

Future studies need to be directed toward the question of whether the DX-mediated alteration of EGF receptors is causally related to the enhancement by DX of the cellular response to EGF. It is clear from the present data that DX does not increase cellular sensitivity to EGF simply by increasing the amount of EGF cells can bind. The DX-treated cells expressed enhanced responsiveness to EGF when cells were incubated with high concentrations of EGF, at which DX-treated and untreated cells bound similar amounts of ^{125}I-EGF (compare Fig 1B and Fig 2B). Therefore, if the DX-mediated alteration of EGF receptors is responsible for the enhanced sensitivity of DX-treated cells to EGF, this must be brought about by the DX-altered receptors having an increased ability to

transmit the mitogenic signal from bound EGF. (It is not unreasonable that the receptors for a hormone could be heterogeneous in their signal-transmitting capability; 90% of the glucogen receptors on liver plasma membranes appear to be incapable of transmitting the signal from glucogen to activate adenylate cyclase [21].)

In view of the present results, it is tempting to speculate that the high-affinity receptors have this property. A basic prediction of this model can be tested. DX should most enhance the cellular response to EGF when EGF is added in those low concentrations at which DX-treated cells bind substantially more EGF than untreated cells (< 0.5 ng/ml). In order to accurately measure the growth stimulation caused by EGF at these low concentrations, it will be necessary to use a very sensitive assay for proliferation such as ^{3}H-thymidine autoradiography.

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