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Correlation between hormone dependency and the regulation of epidermal growth factor receptor by tumor promoters in human mammary carcinoma cells

(breast cancer cells/phorbol ester/proliferation/protein kinase C/steroid receptor)

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ABSTRACT **The effects of the tumor promoter phorbol 12-tetradecanoate 13-acetate (TPA) on the epidermal growth factor (EGF) receptor levels were investigated in hormone-dependent (MCF-7, T-47-D, and ZR-75-1) and hormone-independent (MDA-MB-231, HBL-100, and BT-20) human mammary carcinoma cell lines. In the absence of TPA, hormone-independent cell lines contained high concentrations of low-affinity EGF receptors (apparent $K_d = 8 \times 10^{-10}$ M), whereas hormone-dependent cell lines exhibited low concentrations of high-affinity receptors (apparent $K_d = 1 \times 10^{-10}$ M). TPA causes a change of the receptor from a high- to the low-affinity state in hormone-dependent cell lines (MCF-7, T-47-D, and ZR-75-1), as well as in the hormone-independent HBL-100, whereas the affinity remained unchanged in MDA-MB-231 and BT-20 cells. In addition, progesterone receptor levels are decreased after TPA treatment in the hormone-dependent cell lines MCF-7, T-47-D, and ZR-75-1, whereas the estrogen receptor levels remained unchanged. Tumor promoters such as TPA or teleocidin inhibited the proliferation of these cell lines at concentrations above 10 μM with the exception of the T-47-D cells. The most sensitive cell line towards growth inhibition by tumor promoter was the hormone-dependent MCF-7 cell line. Evaluation of different TPA analogs indicated a positive correlation between the growth-inhibitory effects and their ability to stimulate the subcellular redistribution of protein kinase C activity in MCF-7 cells. These data suggest a protein kinase C-mediated down-regulation of the progesterone receptor concentration and of the EGF receptor affinity, which is supposed to mediate the mitogenic response. Furthermore, these results support the hypothesis that the tumor-derived growth factors induced by estradiol act via the EGF receptor in hormone-dependent mammary carcinoma cells.**

The mechanism of estrogen-dependent growth of breast cancer cells is poorly understood. Preliminary evidence suggests that in the mammary carcinoma cell line MCF-7, the action of estradiol is mediated by growth factors that are released into the cell culture medium (1). Recently Salomon *et al.* (2) reported the presence of transforming growth factors (TGFs) in conditioned media of MCF-7 cells. By their ability to compete with epidermal growth factor (EGF) for its receptor, these factors were characterized as α-TGFs (3). If hormone dependency is mediated by estradiol-induced release of anti-TGF, interaction of anti-TGF with the EGF receptor could represent a crucial mechanism for growth regulation of hormone-dependent carcinomas and may represent a mechanism to discriminate between hormone-dependent and -independent breast cancer cells.

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It has been established that binding of EGF to its receptor activates a tyrosine kinase that is part of the intracellular domain of the receptor (4, 5). Additionally, EGF causes an increased turnover of phosphatidylinositol (6, 7), giving rise to the production of diacylglycerol, which is an activator of protein kinase C (8). This enzyme is able to inactivate the EGF receptor by phosphorylating it at a threonine site (9), possibly causing a feedback inhibition of the EGF-signal input.

A simple way to block this system is the application of tumor promoters like phorbol 12-tetradecanoate 13-acetate (TPA) (10), which are agonists of diacylglycerol, causing phosphorylation and down-regulation of the EGF receptor by enhancing the activity of protein kinase C (11–13). TPA and its analogues have been shown to exert numerous effects on a variety of tissues and cell lines (14, 15) long before protein kinase C was identified as their intracellular target (8). Only part of these effects seems to be related to tumor promotion.

In the study reported here, we analyzed the influence of tumor promoters on the proliferation of hormone-dependent and -independent cell lines, as well as their effect on the EGF receptor affinity and the steroid receptor levels in these cells.

MATERIALS AND METHODS

Cell Culture Conditions. The mammary carcinoma cell lines MCF-7, T-47-D, ZR-75-1, MDA-MB-231, HBL-100, and BT-20 were obtained from the Mason Research Institute (Rockville, MD). Estrogen and progesterone receptor concentrations were in agreement with the originally published values (16–18). All cell lines were grown in IMEM-ZO (improved minimal essential medium, zinc option) as described by Richter *et al.* (19), supplemented with L-glutamine (2 mM), insulin (5 μg/ml), tylocin (45 μg/ml), gentamycin (0.4 μg/ml), minocyclin (3 μg/ml), Hepes at pH 7.3 (10 mM), and 5% (vol/vol) fetal calf serum (Boehringer Mannheim) at 37°C in a humidified atmosphere containing 5% CO_2.

Growth Experiments. Cells were plated at densities of 1–8 × 10^4 cells per well into 16-mm multiwell dishes (Falcon no. 3047). After 2–4 days, incubation was started by addition of the experimental media, which were changed after 2 days. Proliferation was evaluated 4 days later by trypsinizing and counting the cells in a Sysmex CC-108 microcell counter or by measuring DNA by the method of Burton (20) as modified by Taylor *et al.* (21). All determinations were done in triplicate. TPA and its analogues 4β-phorbol 13α,20-diace-

Abbreviations: EGF, epidermal growth factor; TGF, transforming growth factor; TPA, phorbol 12-tetradecanoate 13-acetate; HBSS, Hanks' balanced salt solution.

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tate, 4β-phorbol 12β,13α-didecanoate, 4β-phorbol 12β,13α-dibenzoate, 4β-phorbol 12β,13α-dibutyrate, and 4β-phorbol were obtained from Sigma. Teleocidin was kindly provided by T. Sugimura (National Cancer Center Research Institute, Tokyo, Japan). All compounds were dissolved in dimethyl sulfoxide at 1–10 mM and were diluted into the media to final dimethyl sulfoxide concentrations of <0.1%, a concentration that does not affect the growth of the cell lines.

EGF-Receptor Assay. ^{125}I-labeled EGF (^{125}I-EGF; specific activity, 175 μCi/μg; 1 Ci = 37 GBq) was obtained from New England Nuclear, and unlabeled mouse maxillary EGF was from Collaborative Research (Waltham, MA). Cells were grown in 16-mm multiwell dishes to a cell density of 1–5 × 10^5 cells per well. Before the assay they were washed once with 1 ml of Hanks' balanced salt solution containing 1 mg of bovine serum albumin (HBSS/albumin). For the binding assay, the cells were incubated for 3 hr at 25°C with 0.5 ml of HBSS/albumin containing 0.2 ng of ^{125}I-EGF per ml and between 0.4 and 30 ng of unlabeled EGF per ml. Nonspecific binding was detected by adding a 50-fold excess of unlabeled EGF at four concentrations covering the whole range of the binding curve. The curve for unspecific binding was determined by linear regression of the four points. Kinetic studies showed that equilibrium was reached within 3 hr. After the incubation, the medium was removed and the wells were washed three times with 1 ml of ice-cold HBSS/albumin. Cells were dissolved in 0.1 M NaOH/0.5% Triton X-100, and radioactivity was measured. Parallel wells were trypsinized to determine the cell number. Saturation-binding curves were analyzed by using ONESITE, a computer program performing a one-site-weighted least-squares fit of the binding data.

Steroid Receptor Determination. Estrogen- and progesterone-receptor determinations were done by using whole-cell monolayer cultures grown in 16-mm multiwell dishes. When the cells were confluent (about 6 days after plated), medium was removed and replaced by 0.25 ml of IMEM-ZO containing 5% of dextran/charcoal-extracted fetal calf serum (22) and the following receptor ligands: 2.5 nM [^{3}H]estradiol (134 Ci/mmol; NEN) in the presence or absence of 250 nM diethylstilbestrol to determine nonspecific binding for the estrogen receptor and 5 nM [^{3}H]R 5020 (87 Ci/mmol, NEN) in the presence or absence of 500 nM progesterone for the progesterone receptor. After 1 hr of incubation at 37°C, the cell layer was washed four times in ice-cold phosphate-buffered saline (137 mM NaCl/2.5 mM KCl/6 mM Na_2HPO_4/1.5 mM KH_2PO_4/0.7 mM $CaCl_2$/0.4 mM $MgCl_2$) containing bovine serum albumin (1 g/liter). Finally, the cells were trypsinized and transferred to counting vials containing 8 ml of Instagel (Packard). Cell numbers were determined in parallel-treated wells as described above. Saturation-binding studies, which were done under the same conditions, resulted in linear Scatchard plots. The method is reliable to measure the receptor content in cultured cells.

Determination of Protein Kinase C. MDA-MB-231 cells, grown in 150-mm dishes, were washed three times in 20 mM Tris·HCl (pH 7.4) containing 150 mM NaCl, 5 mM glucose, 20 μg of leupeptin per ml, and 2 μg of aprotinin per ml. The cells were harvested and resuspended in homogenization buffer [20 mM Tris·HCl (pH 7.4) containing 2 mM EGTA, 2 mM EDTA, 6 mM mercaptoethanol, 20 μg of leupeptin per ml, and 2 μg of aprotinin per ml] at a density of 1–4 × 10^7 cells per ml and then were disrupted by sonication (3 sec with a Branson model B-12 sonifier at 70 W). The homogenate was centrifuged at 2000 × *g*, and the supernatant was centrifuged at 100,000 × *g* for 1 hr. The resulting supernatant was used to measure protein kinase C with the polyacrylamide gel electrophoresis technique as described (23).

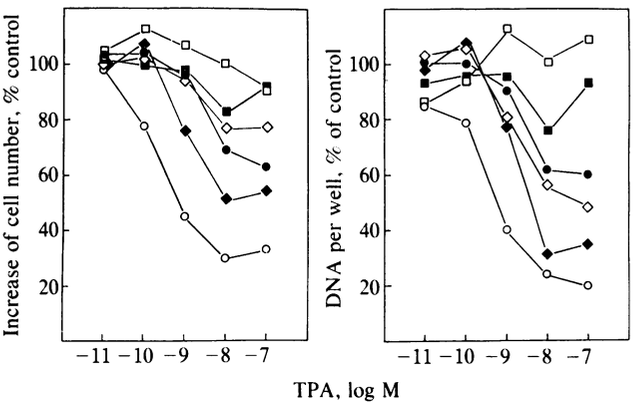

FIG. 1. Dose–response curve for the effect of TPA on the growth of human mammary carcinoma cell lines. Cells were incubated for 4 days in the presence of TPA as described. Data were normalized to the value measured in the control and presented as the increase in cell numbers (*Left*) or concentration of DNA after 4 days (*Right*). The data points are means of triplicates; SEMs were generally lower than 10%. Cell lines: ○, MCF-7; □, T-47-D; ◇, ZR-75-1; ●, MDA-MB-231; ■, HBL-100; ◆, BT-20.

RESULTS

With the exception of the cell line T-47-D, TPA inhibited growth of all cell lines (Fig. 1). The maximal effect was reached at a concentration of 10 μM of TPA. No correlation was evident with the hormone dependency of the cells. Virtually the same growth-inhibiting effects were observed with Teleocidine, a tumor-promoting compound of different structure belonging to the indolalkaloid class of tumor promoters (24). The strongest inhibition was seen in the cell line MCF-7, which was investigated in detailed growth experiments (Fig. 2). The cells were not killed by TPA; however, the inhibition was not readily reversible. Removal of TPA from the medium did not lead to the resumption of growth during the period of measurement, whereas replating the cells at lower densities restored the ability to proliferate.

The possibility that TPA-resistant variants were responsible for this result has been ruled out by the following experiment: MCF-7 cells were kept in 0.1 μM TPA for 3 months. At the end of that period the cells were cloned. Evaluation of 33 clones showed that the growth of all of them was still inhibited by TPA.

The ability of different TPA analogues to inhibit the growth of MCF-7 cells (Fig. 3 *Upper*) correlated well with their effect on protein kinase C (Fig. 3 *Lower*). There was a dose-dependent decrease of cytosolic protein kinase C (Fig. 3 *Lower*) that matched well with the growth-inhibiting action of

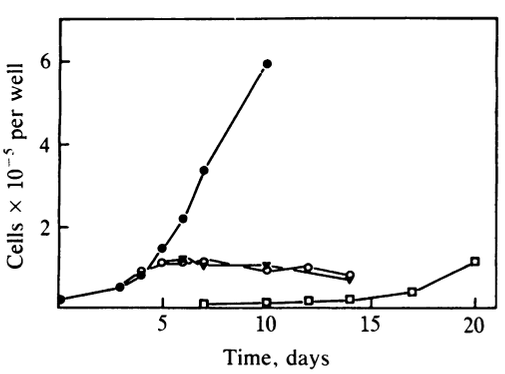

FIG. 2. Effect of TPA on the growth on MCF-7 cells. ●, Control growth curve in the absence of TPA; ○, 10 μM TPA starting at day 3; ▼, 10 μM TPA, rescue at day 5 by change to TPA-free medium; □, 10 μM TPA, replated without TPA at day 7 under 1:6 dilution.

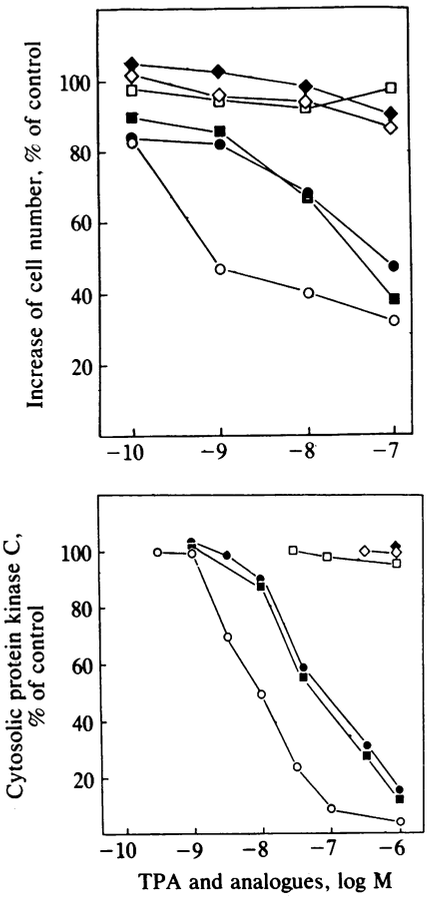

FIG. 3. (*Upper*) Dose–response curve of the effect of TPA and its analogues on the proliferation of MCF-7 cells. Cells were incubated for 4 days in the presence of TPA and its analogues as described. Data were normalized to the value measured in the control. ○, TPA; □, 4β-phorbol 13α,20-diacetate; ◇, 4α-phorbol 12β,13α-didecanoate; ●, 4β-phorbol 12β,13α-dibenzoate; ■, 4β-phorbol 12β,13α-dibutyrate; ◆, 4β-phorbol. (*Lower*) Effect of increasing concentrations of phorbol esters on cytosolic protein kinase C activity. MDA-MB-231 cells were treated for 30 min with the same phorbol ester analogs used in *Upper*. After the incubation, cytosols were prepared to measure protein kinase C activity as described. Determinations were done in triplicate; the symbols are as in *Upper*.

the respective compounds in the growth experiment (Fig. 3 *Upper*). Such phorbol ester-mediated decline of cytosolic protein kinase C, which has been shown by several authors (25, 26), reflects the translocation of the enzyme to the plasma membrane and is equivalent to its activation. These findings strongly suggest that TPA effects on proliferation are also mediated by protein kinase C.

The influence of TPA on the steroid hormone receptor concentrations in the receptor-positive cell lines was investigated in intact cells by using an *in vivo* receptor assay (Fig. 4). Whereas estrogen receptors were not affected, progesterone receptor levels were significantly reduced after 24 hr in the presence of 10 μM TPA. In additional saturation-binding experiments comparing the cytosolic progesterone receptor in TPA-treated and untreated T-47-D cells, we were able to demonstrate that the decline of progesterone receptor levels is not due to changes of the receptor affinity (data not shown).

A possible target for the protein kinase-mediated action of TPA is the receptor for EGF. EGF receptor has been shown to be present in most human mammary carcinoma cell lines (27, 28). Our results (Table 1) are in good agreement with the presented reports. In addition, however, our experiments yielded a highly significant difference in the EGF-binding

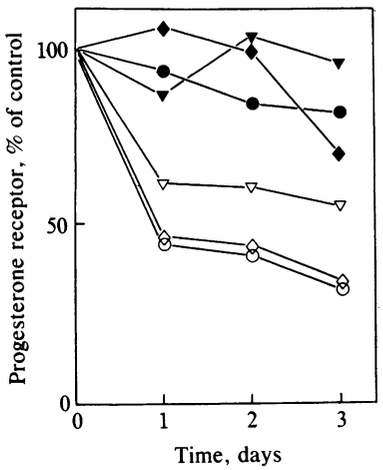

FIG. 4. Effect of TPA on progesterone receptor levels in hormone-dependent mammary carcinoma cell lines. Cells were incubated in the presence (○, ◇, ▽) or absence (●, ◆, ▼) of 10 μM TPA. The values calculated as fmol per 10^6 cells were normalized to the receptor concentration at day 0. ○ and ●, MCF-7; ▽ and ▼, T-47-D; ◇ and ◆, ZR-75-1.

capacity between estrogen-dependent and -independent cell lines ($P < 0.001$; Wilcoxon U-test). Hormone-dependent cell lines (MCF-7, T-47-D, and ZR-75-1) had very low concentrations of EGF receptor ($<10^4$ sites per cell), whereas high levels (up to 5×10^5 sites per cell in the BT-20 cell line) were found in the hormone-independent cell lines (Table 1). Recent reports on the production of tumor growth factors by breast cancer cells (1, 2) raise the question of whether the low EGF-receptor levels in hormone-dependent cell lines are caused by the autocrine production of anti-TGF, which is known to compete for EGF-binding sites. Therefore, extracts prepared from conditioned media of all cell lines were checked for their ability to inhibit EGF binding. Competing activity could be demonstrated in all cell lines; however, it was negligibly small and only evident in extensively concentrated extracts (10-fold). In contrast to other reports, our binding data were reasonably well-fitted by a one-site binding model resulting in linear Scatchard plots. Occasionally observed indications for curvilinear Scatchard plots, arguing for multiple binding sites—especially in the hormone-dependent cell lines—could not be substantiated on a significant level. It has been shown in several cell lines of different origin that the addition of TPA to the growth medium reduces the binding of EGF (10, 29). Preliminary evidence for the same effect in breast cancer cells was presented by Imai *et al.* (27). We did the same experiment, comparing the cell lines MCF-7 and T-47-D, which differ widely in their response to TPA with respect to growth inhibition (Fig. 5). Measured at nonsaturating concentrations of EGF, both cell lines showed a strong decrease of EGF binding with increasing concentrations of TPA. Saturation-binding curves and Scatchard plots demon-

Table 1. EGF receptor in hormone-dependent and -independent mammary carcinoma cell lines

Cells	ER	Sites/cell	K_d, M × 10^9
MCF-7	+	2,940 ± 1,440	190 ± 67
T-47-D	+	7,440 ± 1,920	32 ± 9
ZR-75-1	+	3,660 ± 1,440	31 ± 9
MDA-MB-231	−	102,000 ± 36,000	964 ± 206
HBL-100	−	24,600 ± 8,400	295 ± 102
BT-20	−	462,000 ± 114,000	878 ± 362

The values are means ± SD of five experiments. ER, estrogen receptor.

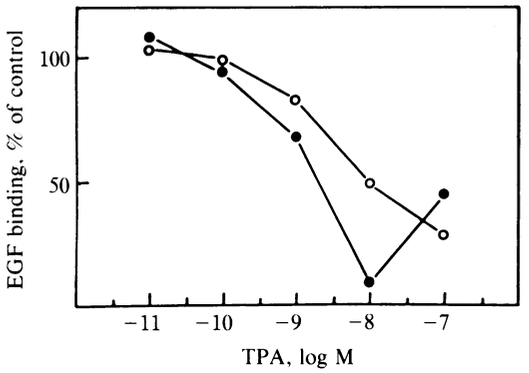

FIG. 5. Dose–response curve for the effect of TPA on EGF binding in the cell lines MCF-7 (●) and T-47-D (○). Cells were incubated for 2 hr in the presence of 10 μM TPA. The values were normalized to control levels measured in the absence of TPA. EGF concentration was 1 ng/ml.

strated that the apparent reduction of binding capacity is actually caused by an altered affinity (Fig. 6). In the hormone-dependent cell lines (MCF-7, T-47-D, and ZR-75-1) as well as in the hormone-independent cell line HBL-100, TPA induced a transformation of the EGF receptor to a low-affinity state with an apparent dissociation constant similar to that of MDA-MB-231 and BT-20 cells (Fig. 7). In contrast, variations in binding capacity were not significant or did not occur consistently in either direction. In the hormone-independent cell lines MDA-MB-231 and BT-20, TPA did not affect the affinity of the EGF receptor.

DISCUSSION

In most cell lines, TPA has proved to be a mitogen; only a few cases are reported in the literature (30, 31) in which the

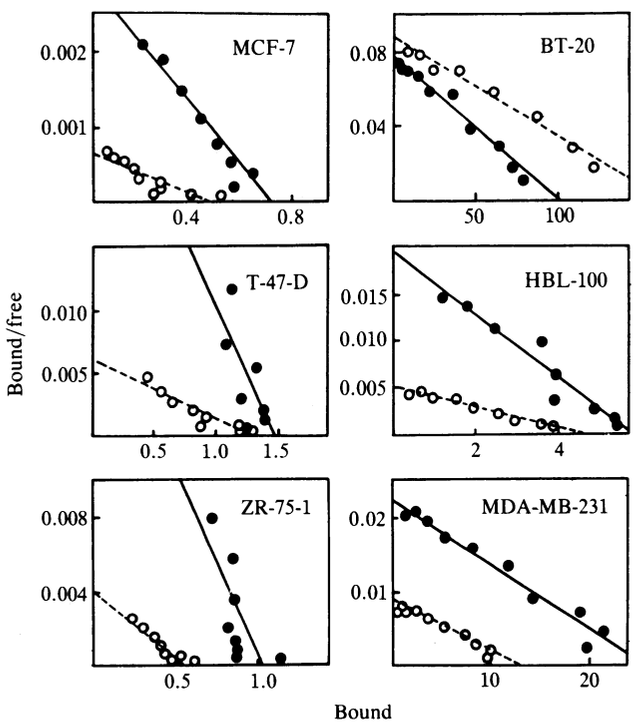

FIG. 6. Scatchard plots of EGF binding to human mammary carcinoma cell lines. Before the saturation-binding experiment, cells were incubated for 2 hr at 37°C with (○) or without (●) 10 μM TPA. To allow comparison of the slopes, the ratio between the axes is the same in all six plots.

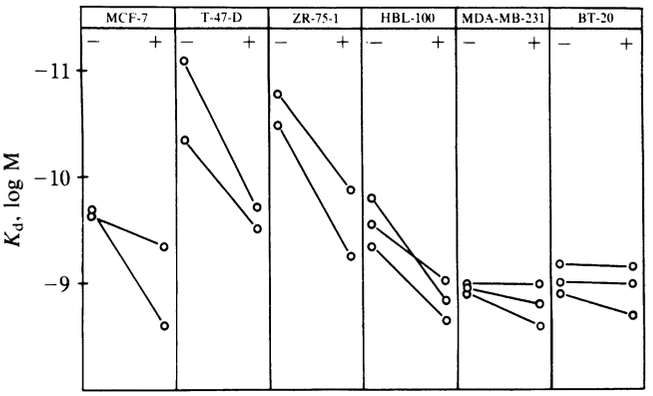

FIG. 7. Effect of TPA on the apparent dissociation constant of EGF binding in human mammary carcinoma cell lines.

growth of cells was inhibited. In agreement with results of Osborne *et al.* (32), the growth of several breast cancer cell lines examined by us was inhibited by TPA. In the present study, only one of six mammary carcinoma cell lines (T-47-D) did not show significant growth inhibition by TPA. The absence of a correlation between hormone dependency and TPA effect does not support a possible involvement of TGFs and their receptors in the estrogen-dependent growth regulation of breast cancer cells, as proposed by the work of Lippman *et al.* (1). The fact that subculturing in the absence of TPA was necessary to reverse the effects may not be of biological significance. Because of its well-known lipophilic nature, TPA is likely to partition rapidly into cellular membranes (33, 34), and repeated washing is not sufficient to deplete TPA from the cell membrane.

In the hormone-dependent cell lines MCF-7, T-47-D, and ZR-75-1, the levels of progesterone receptor were shown to be significantly reduced by TPA. The recently discovered phenomenon (35) that progesterone receptor can serve as a substrate for EGF receptor kinase provides a mechanism by which TPA could control the levels of the steroid receptor. Protein kinase C-mediated down-regulation of the EGF receptor could change the state of phosphorylation of the progesterone receptor, finally resulting in reduced receptor concentrations. However, these results were obtained in a system of purified receptor proteins, and their biological significance has still to be established.

Treatment of cultured cells with tumor promoters decreases the binding affinity for EGF (10, 29) and reduces the activity of EGF receptor protein kinase (12, 36). Therefore, we compared the EGF receptor and its regulation by TPA in several breast cancer cell lines with respect to hormone dependency of proliferation. Our results demonstrate clear differences in the EGF-binding capacity between hormone-dependent and -independent cell lines, very low levels of binding sites being present in the hormone-dependent cell lines relative to the hormone-independent cell lines. The same holds true for the binding affinity if we do not take into consideration the HBL-100 cell line. (There is some justification to do so, because HBL-100 are nontransformed cells of nonmalignant origin in contrast to the other cell lines.) Apparent dissociation constants measured in hormone-dependent cell lines were about 1 order of magnitude lower than in the hormone-independent cell lines MDA-MB-231 and BT-20.

Effects of TPA on the apparent K_d of the EGF receptor could only be demonstrated in cells with high-affinity EGF receptor. In the cell lines MDA-MB-231 and BT-20, EGF receptor affinity was not noticeably affected by the treatment with TPA in spite of the fact that the growth of both cell lines was inhibited by TPA. A possible explanation for this

contradiction would be the presence of a small subset of high-affinity EGF receptors obscured by a large excess of low-affinity receptor. In the human epidermoid carcinoma cell line A-431, which contains high amounts of low-affinity EGF receptor, a small subfraction of high-affinity receptor could be demonstrated that is supposed to be responsible for the mitogenic response of these cells to EGF (37) and has been shown to be sensitive to TPA (36).

It remains to be determined if the differences in level and affinity of the EGF receptor are responsible for the growth response of these cell lines to EGF. Osborne *et al.* (38) observed stimulation of growth in MCF-7 cells and no response in the cell line MDA-MB-231. Fitzpatrick *et al.* (28) could enhance proliferation of the cell lines MCF-7 and T-47-D and found no effect in the cell lines ZR-75-1 and MDA-MB-231, whereas Imai *et al.* (27), working at low concentrations of fetal calf serum, could only stimulate T-47-D cells. Thus, there is no sufficient evidence yet to differentiate between hormone-dependent and -independent cell lines on the basis of their growth response to EGF. However, these results have to be regarded cautiously because, in all cases, cells that were normally grown in the presence of fetal calf serum were submitted to suboptimal medium conditions that do not allow their permanent cultivation.

There is increasing evidence that EGF is an important regulator for mammary epithelium growth and development *in vivo* (39, 40). Mammary carcinoma cell lines seem to have retained to some extent this mechanism of growth control. Preliminary results of Fitzpatrick *et al.* (41) on the presence of EGF receptors in human breast cancer biopsies were impressively confirmed by Sainsbury *et al.* (42), demonstrating a highly significant inverse correlation between EGF-receptor levels and the presence of estrogen receptors. Thus, there is some evidence that the basic differences between hormone-dependent and -independent human mammary carcinoma cell lines with respect to EGF-receptor binding hold true at the tissue level.

In conclusion, our data support the role of the EGF receptor in mediating mitogenic signals in mammary cancer cells. It may be possible to use the EGF receptor as a target for pharmacological intervention in human breast cancer—especially in estrogen receptor-negative cases, not amenable to endocrine treatment.

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