Title

Transforming Growth Factor Alpha and its Receptor in Human Mammary Epithelial Cells: Modulation of Epidermal Growth Factor Receptor Function with Oncogenic Transformation

Authors

Stampfer, MR
Valverius, E
Bates, SE
et al.

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Transforming Growth Factor α Production and Epidermal Growth Factor Receptor Expression in Normal and Oncogene Transformed Human Mammary Epithelial Cells

Eva M. Valverius, Susan E. Bates, Martha R. Stampfer, Robin Clark, Frank McCormick, David S. Salomon, Marc E. Lippman, and Robert B. Dickson

Medical Breast Cancer Section (E.M.V., S.E.B., M.E.L., R.B.D.)
and Laboratory of Tumor Immunology and Biology (D.S.S.)
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20892

Lawrence Berkeley Laboratory (M.R.S.)
Berkeley, California 94720

Cetus Corporation (R.C., F.McC.)
Emeryville, California 94710

We have characterized the expression of transforming growth factor α (TGFα) and its receptor, the epidermal growth factor receptor (EGF-R), in normal and malignantly transformed human mammary epithelial cells. Human mammary epithelial cells were derived from a reduction mammoplasty (184), immortalized by benzo-*a*-pyrene (184A1N4), and further transformed by the oncogenes simian virus 40 T (SV40 T), v-Ha-*ras*, and v-*mos* alone or in combination using retroviral vectors. 184 and 184A1N4 cells require EGF for anchorage-dependent clonal growth. In mass culture, they secrete TGFα at high concentrations and exhibit an attenuated requirement for exogenous EGF/TGFα. SV40 T transformed cells have 4-fold increased EGF-R, have acquired the ability to clone in soft agar with EGF/TGFα supplementation, but are not tumorigenic. Cells transformed by v-*mos* or v-Ha-*ras* are weakly tumorigenic and capable of both anchorage dependent and independent growth in the absence of EGF/TGFα. Cells transformed by both SV40 T and v-Ha-*ras* are highly tumorigenic, are refractory to EGF/TGFα, and clone with high efficiency in soft agar. The expression of v-Ha-*ras* is associated with a loss of the high (but not low) affinity binding component of the EGF-R. Malignant transformation and loss of TGFα/EGF responsiveness did not correlate with an increase in TGFα production. Thus, TGFα production does not appear to be a tumor specific marker for human mammary epithelial cells. Differential growth responses to EGF/TGFα, rather than enhanced production of TGFα, may determine the transition from normal to malignant human breast epithelium. (Molecular Endocrinology 3: 203–214, 1989)

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INTRODUCTION

Transforming growth factor alpha (TGFα) is a mitogenic polypeptide produced by a variety of retrovirally, chemically, or oncogene-transformed human and rodent cell lines (1). TGFα is also present in human mammary tumor tissues and cell lines (2–4), and has recently been demonstrated in normal human keratinocytes (5). TGFα is operationally defined by its ability to reversibly induce the transformed phenotype as measured by anchorage-independent cloning of normal rat kidney (NRK) cells (6). Structurally and functionally related to epidermal growth factor (EGF), TGFα competes with EGF for binding to the same receptor (7). Transformed cells *in vitro* generally synthesize and secrete TGFα, demonstrate a partial or complete relaxation of their growth factor requirements, and exhibit a down-regulation of their cell surface EGF receptors (EGF-R), as defined by reduced ligand binding capacity (1, 8). It was proposed in the autocrine hypothesis (9), that the TGFα produced by transformed cells could act on the cells' own EGF receptors to promote unrestrained cellular proliferation.

We have previously demonstrated expression of TGFα mRNA in several human breast cancer cell lines and in approximately 70% of primary infiltrating ductal

breast carcinomas, as well as evidence for a possible autocrine pathway for TGFα in hormone-dependent human breast cancer cells *in vitro* (10). Similarly, a role for EGF/TGFα in supporting the limited *in vivo* growth of xenografts of the human estrogen-responsive breast cancer cell line MCF-7 has also been described (11). Modulation of EGF-R levels may also have an important role in human breast neoplasia. In two recent studies, increased numbers of EGF-R in human breast cancer cell lines and in breast tumor biopsies have been correlated with increased rates of proliferation (12) and with clinically more aggressive mammary malignancies (13).

Protooncogene expression has also been studied in breast cancer (14–18). Overexpression of p21 *ras* protein has been found in human breast cancer biopsies (14), and increased production of TGFα and a loss of responsiveness to exogenous EGF or TGFα has been reported after transfection of MCF-7 human breast cancer cells with v-Ha-*ras* oncogene (15) or after transformation of normal mouse mammary epithelial cells with a point-mutated c-Ha-*ras* protooncogene (16). In addition, point mutations of the c-Ha-*ras* and c-Ki-*ras* genes have been observed in two TGFα refractory and hormone-independent breast cancer cell lines (Hs578T and MDA MB-231, respectively) (17, 18).

The present study was undertaken to examine the level of expression of TGFα and the EGF-R in normal human mammary epithelial cells (HMEC) and in HMEC after immortalization and after oncogene transformation. Long-term growth of nontransformed HMEC has been achieved with reduction mammoplasties of young, nonpregnant, nonlactating women (19). These cell strains are characterized by complex media requirements, contact inhibition, lack of anchorage-independent growth, lack of tumorigenicity, and senescence after 12–22 passages in culture. An immortalized clonal cell line, 184A1N4, was established after benzo-*a*-pyrene treatment of the normal HMEC 184 cells (20). Malignantly transformed sublines of the 184A1N4 cells were made by introducing the oncogenes v-*mos*, v-Ha-*ras*, and simian virus 40 T (SV40 T), through retroviral vector infection (21). The cell lines thus established displayed marked variations in expression of the transformed phenotype, ranging from lack of anchorage-independent growth and tumorigenicity, to highly anchorage-independent and fully tumorigenic (Table 1). None of the 184-derived cells were found to express the estrogen receptor, as determined by binding assays or immunocytochemistry (unpublished data). Since transformation has been reported to lead to overproduction of growth factors such as TGFα, receptor desensitization and receptor down-regulation (1), the aims of this study were to establish whether nontransformed HMEC express TGFα as well as the EGF-R, and to elucidate the influence of oncogenic transformation in human mammary epithelial cells on the TGFα/EGF-receptor system. We sought to determine whether these phenotypic variations among nonneoplastic and transformed HMEC could be correlated with alterations in the response to exogenous TGFα, production of endogenous TGFα, or EGF-R expression.

Table 1. Characteristics of the HMEC

Cell	Cloning Efficiency (in soft agar)	Tumorigenicity	Doubling Times (h)
184	≤0.1%	0	20 ± 4
184A1N4	≤0.1%	0	32 ± 6
184A1N4-T	≤0.1%	0	32 ± 5
184A1N4-M	0.6%	±	48 ± 20
184A1N4-H	0.6%	+	21 ± 5
184A1N4-MH	2.3%	+	30 ± 5
184A1N4-TH	27.5%	++	30 ± 3

Cloning efficiencies in soft agar was tested with 2×10^4 cells plated in their respective regular growth medium with 0.36% Bacto agar in 35-mm dishes. Colonies were counted after 9–11 days incubation, scoring colonies with greater than or equal to 60 μm diameter as positive. Tumorigenicity was tested as described (21) in nude athymic mice (Balb/c Nu/Nu) using 4×10^6 cells in Dulbecco's PBS after sc injections. Twenty days post inoculation, animals were killed and examined for tumors.

RESULTS

Growth Responses of the HMEC to EGF/TGFα

184 and 184A1N4 were found to be dependent upon EGF supplementation for anchorage-dependent clonal growth. In mass culture, however, the cellular requirement for exogenous EGF by the 184 cells was considerably attenuated, as shown in Fig. 1A. The 184A1N4 cells were more sensitive to EGF deprivation both in mass cell culture and under clonal growth conditions.

All oncogene transformed cells were tested for anchorage-dependent growth responses to EGF in their regular medium containing 10% fetal bovine serum (FBS). Parallel experiments performed in the low serum variant medium normally used for the 184A1N4 cells without EGF supplementation and containing 0.5% FBS as well as hydrocortisone and insulin showed no effect of serum content on the cellular responsiveness to EGF (data not shown). Figure 1B shows the results of tests in the regular growth medium for the cells, in the presence of 10% FBS for the oncogene transformants and 0.5% FBS for the 184A1N4 cells. Of the oncogene transformants, only the 184A1N4-T and 184A1N4-M cells could be additionally stimulated by EGF. When assayed for anchorage-independent growth (Fig. 2), the different cell lines displayed large variations in basal and EGF/TGFα-induced cloning. 184 and 184A1N4 cells did not clone under anchorage-independent conditions. 184A1N4-T did not clone in the absence of EGF. In the presence of 5 ng/ml EGF or TGFα, however, 184A1N4-T displayed colony forming efficiencies of up to 7% comparable to the cloning efficiencies of the established breast cancer cell lines MCF-7 and MDA-MB 231 (not shown). EGF or TGFα did not facilitate anchorage-independent cloning in the other onco-

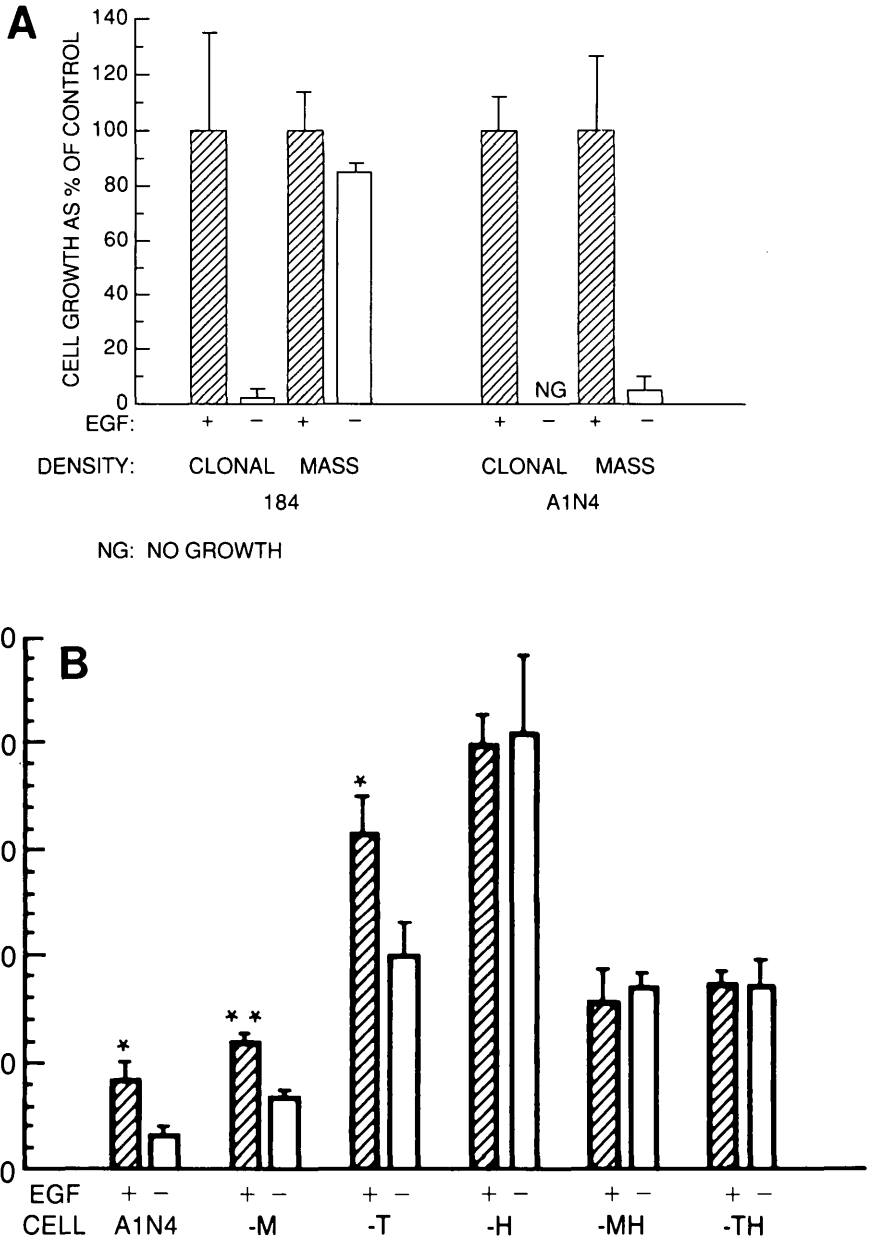

Fig. 1. HMEC Anchorage-Dependent Growth Response to EGF

A, EGF requirement of 184 and 184A1N4 cells at clonal density *vs.* mass culture. 184 and 184A1N4 (abbreviated A1N4 in figure) were tested for dependence upon EGF supplementation in MCDB 170 medium. Clonal density: 500 cells were seeded into 100-mm dishes. After 10–13 days, colonies greater than or equal to 30 cells were counted. Mass culture: 5×10^4 cells were seeded into 35-mm dishes. All cells were counted when control dishes were just confluent. □, No EGF. ▨, 5 ng/ml EGF. NG, No growth. Growth reduction in the absence of EGF was significant in all tests ($P < 0.01$) except for mass culture of 184 (not significant). B, Anchorage-dependent growth response of oncogene transformed HMEC to EGF. The immortalized and oncogene transformed HMEC were tested for anchorage-dependent growth response to EGF in their respective regular growth media containing 0.5% FBS for the 184A1N4 cells and 10% FBS for the oncogene transformants. Cells (0.5 or 1×10^4) were seeded in 12-well cluster dishes. After allowing cell attachment, medium was changed to ±5 ng/ml EGF. After 5 days incubation, cells were trypsinized and counted. *, $P < 0.05$; **, $P < 0.01$.

gene transformants. Their basal cloning efficiencies were 0.6% for both 184A1N4-M and 184A1N4-H, 2.3% for 184A1N4-MH, and 27.5% for 184A1N4-TH.

Expression of TGFα mRNA by HMEC

To ascertain whether normal HMEC express TGFα mRNA and if the level of expression increases after oncogenic transformation of these cells, total cellular mRNA was subjected to Northern blot hybridization using a synthetic TGFα riboprobe. RNA extracted from four established human breast cancer cell lines were included for comparison. As shown in Fig. 3, the phenotypically normal 184 and 172 cells and all the transformed 184A1N4 sublines displayed similar, high levels

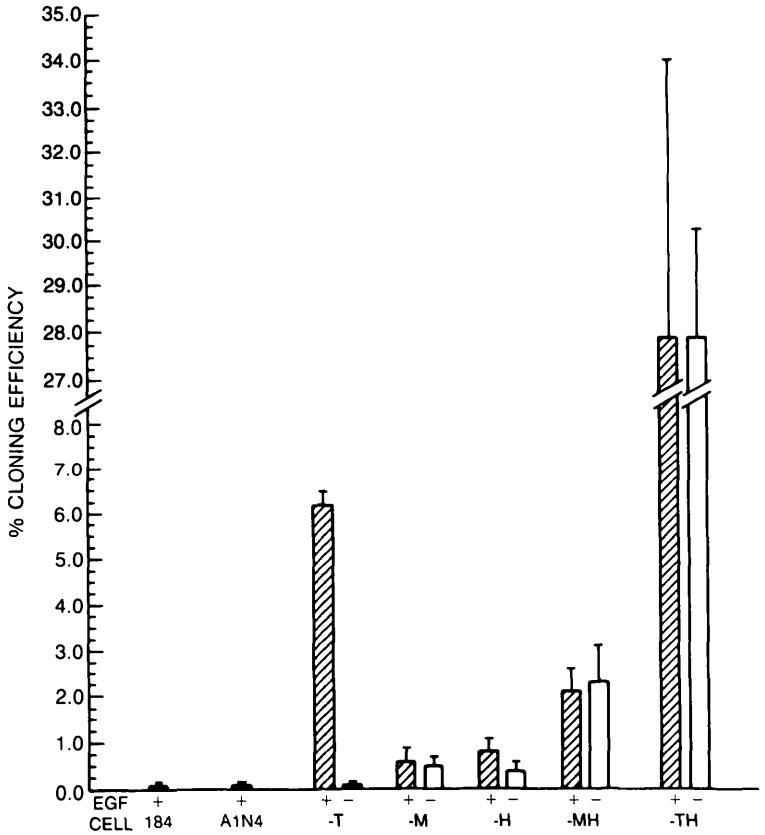

Fig. 2. Anchorage-Independent Growth Response of HMEC to EGF

Two × 10^4 cells were plated in their respective regular growth medium with 0.36% Bacto agar in 35-mm dishes, with or without 5 ng/ml EGF. 184 and 184A1N4 were tested only in the presence of EGF. After 9–11 days, colonies greater than or equal to 60 μm were counted. Cloning efficiencies shown are means ± SD of triplicates of three separate experiments. □, No EGF. ▨, 5 ng/ml EGF.

of a 4.8 kilobase (kb) TGFα mRNA (3). Gels were comparably loaded as seen in ethidium bromide staining. The TGFα signal was found by densitometric scanning to vary no more than 1.8-fold among the HMEC, and less than 2.4-fold when normalized to the 28S ribosomal RNA. A less abundant 1.6 kb mRNA species was also observed on some of the blots. This species has been previously reported in several human breast cancer cell lines (10); however, its significance remains unknown. Levels of these TGFα mRNA transcripts for all the HMEC were comparable to levels in the aggressive human breast cancer cell lines MDA MB-231 and MDA MB-468.

Production of TGFα in Conditioned Medium (CM)

In agreement with the Northern blot analysis for TGFα mRNA expression, all the oncogene transformed HMEC produced comparable levels of TGFα, with no significant differences in immunoreactive and bioactive protein production observed between the different cell lines (Table 2). Although the 184 cells displayed the same levels of TGFα mRNA, they produced higher amounts of TGFα immunoreactive protein than the oncogene transformants. 184 HMEC require a complex serum-free medium containing EGF, bovine pituitary extract, and insulin for their growth (20, 22). Deletion of the EGF, bovine pituitary extract, and insulin from the medium resulted in a 5-fold reduction in the CM levels of TGFα from the 184 cells to a level which was comparable to the levels in the 184A1N4 cells and the transformed 184A1N4 sublines. The apparently slightly higher levels of bioactive than immunoreactive TGFα in the 184 cells are probably due to trace amounts of EGF-containing medium having been left behind despite the washes before the CM collections, and are considered to be within the limits of our assay systems.

Expression of EGF-R mRNA and EGF-R Binding Characteristics

Since variations in TGFα production could not explain the observed phenotypic differences, and since variations in a possible autocrine loop may occur at the level of the receptor for this growth factor, EGF-R expression was examined in the various HMEC lines. Using an EGF-R nick-translated cDNA insert from pE7, Northern blots of total RNA from the 184 transformed series, 172, and two breast cancer cell lines were probed for expression of EGF-R mRNA. As shown in Fig. 4A, high

levels of specific hybridization to the 10 kb EGF-R mRNA species and in some cases the 5.6 kb species (23), were found in all the HMEC lines. By densitometric scanning the EGF-R signal was found to vary less than 2.0-fold among the HMEC, and less than 2.5-fold when normalized to the β-actin signal. The high levels of expression observed in the HMEC lines contrast sharply with the EGF-R mRNA expression in MCF-7 cells which in our hands is not detectable in Northern analysis of total RNA.

Southern blots of DNA digestion with *Hin*dIII were probed with the same labeled pE7 probe and showed no apparent EGF-R gene overexpression or rearrangements in the HMEC (Fig. 4B). A breast cancer cell line with known EGF-R amplification, MDA MB-468 (24), is shown for comparison. Densitometric scanning showed a maximum of 1.7-fold difference in band intensities among the HMEC.

In several other systems, either *mos* or *ras* transformation has been shown to cause loss of detectable EGF-R binding, probably due to a down regulation induced by an increase in TGFα production (16, 25, 26). EGF-R binding characteristics were therefore determined for all the 184 cell lines. In order to measure only cell surface receptor binding and to avoid obscuring determinations of ^{125}I-EGF binding characteristics due to possible receptor internalization and degradation of ^{125}I-EGF-R complexes, all ligand binding assays were performed at 4 C. As shown in Table 3, 184, 184A1N4, and 184A1N4-M all had approximately 3–4 × 10^5 total binding sites per cell. The 184A1N4, 184A1N4-M, and 184A1N4-T cell lines clearly demonstrated both high and low affinity binding sites (Fig. 5). (*P* values for two-site model fits were 0.034, 0.014, and 0.001 for 184A1N4, 184A1N4-M, and 184A1N4-T, respectively). The 184A1N4-T cells exhibited markedly elevated levels

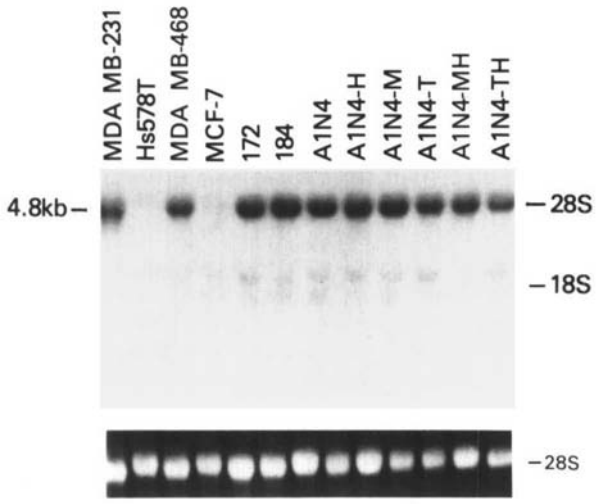

Fig. 3. Expression of TGFα mRNA in Proliferating Normal and Transformed HMEC

Northern blot analysis of 8 μg total RNA hybridized to the TGFα riboprobe is shown. RNA samples from human breast cancer cell lines MDA MB 231 and MDA 468 are included as positive controls (lanes 1 and 3), Hs578T as a negative control (lane 2), and MCF-7 as a weakly positive control (lane 4). Ethidium bromide stain of the gel (*lower panel*) showed that samples were undegraded and comparably loaded.

Table 2. Detection of TGFα-Like Activity in CM

Cell	RIA (ng/ml CM)	(pg/μg DNA)	NRK-49F (ng/ml CM)	(pg/μg DNA)
184 (complete medium)	1.04 ± 0.26	184 ± 46	ND	ND
184 (−EGF, BPE, insulin)	0.19 ± 0.05	41 ± 6	0.60 ± 0.06	159 ± 58
A1N4	0.14 ± 0.01	45 ± 2	ND	ND
A1N4-M	0.11 ± 0.04	11 ± 4	0.16 ± 0.02	10 ± 4
A1N4-T	0.17 ± 0.12	21 ± 17	0.14 ± 0.06	21 ± 7
A1N4-H	0.10 ± 0.03	9 ± 3	0.29 ± 0.09	29 ± 3
A1N4-MH	0.14 ± 0.01	17 ± 2	0.24 ± 0.01	31 ± 6
A1N4-TH	0.18 ± 0.08	22 ± 10	0.20 ± 0.03	28 ± 9
Established breast cancer lines: (RIA)				
MCF-7	1.3 ± 0.5 pg/μg DNA			
MCF-7 + E_2	10.0 ± 1.4			
MDA-MB-231	10.2 ± 0.8			

ND, Not determined. Concentrated CM from the normal and transformed HMEC lines were assayed for TGFα-like activity using the TGFα RIA kit (Biotope, Inc.) and induction of NRK 49F fibroblast cloning in soft agar. For the RIA, results shown are means ± SD of duplicate determinations from three separate experiments on two different CM collections. Values represent the TGFα activity remaining in CM after subtraction of media blanks. For 184 cells, medium blank was 26% (0.38 ± 0.18 ng/ml) of total TGFα levels in complete medium (which were 1.47 ± 0.19 ng/ml). For 184A1N4 cells, medium blank was 0.02 ng/ml, *i.e.* 11% of total TGFα levels (which were 0.16 ± 0.01 ng/ml). All medium blank values have been subtracted before calculations of averages and standard deviations shown in the table. Media blanks for the oncogene-transformants showed no immunoreactivity or biological activity. Values for breast cancer cell lines MCF-7 and MDA MB 231 are shown for comparison. For quantification in the NRK assay, three dilutions of CM were assayed in triplicates in two separate assays, using two separate CM collections. Calculations were normalized to mouse EGF titrations.

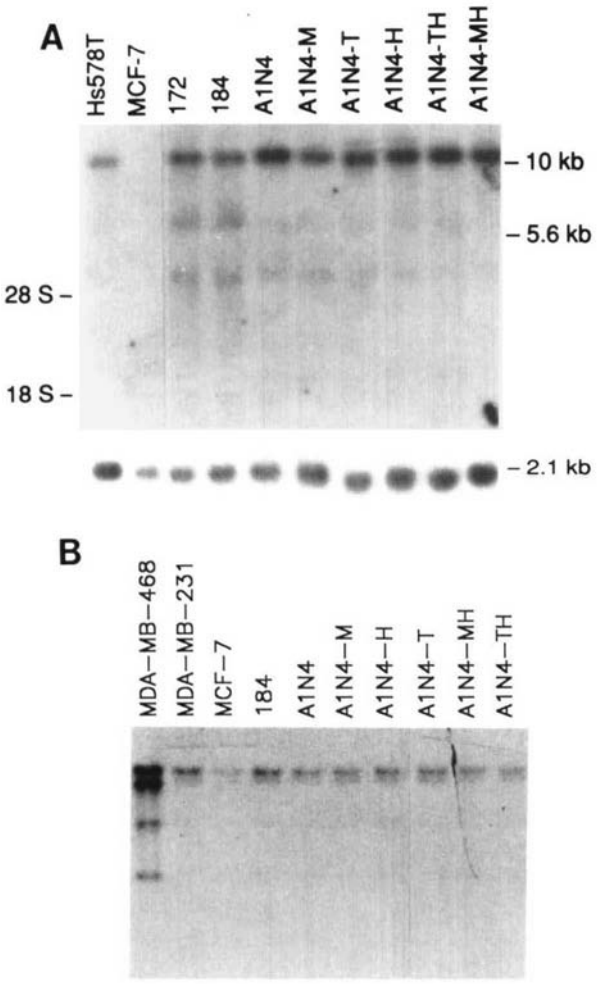

Fig. 4. EGF Receptor Gene Expression in HMEC

A, Expression of EGF receptor mRNA in proliferating normal and transformed HMEC. Northern analysis of 8 μg total RNA hybridized to the nick-translated cDNA insert from pE7, encoding the EGF-R. RNA samples from cell lines Hs578T and MCF-7 are included as controls. These lines have been shown to contain 9.0×10^4 and 2.4×10^3 binding sites per cell, respectively (53). Ethidium bromide staining showed that all samples contained comparable amounts of RNA. The *lower panel* shows hybridization of the β-actin probe to a 2.1 kb mRNA species. B, Analysis of the EGF receptor gene in HMEC. Southern analysis of 5 μg DNA was performed after digestion of the DNA with *Hin*dIII and hybridization with the nick-translated pE7 EGF-R encoding insert. The slight variations in band intensities among the oncogene transformed HMEC correspond to amounts loaded as seen in ethidium stain of the gel (not shown). The breast cancer cell lines MDA MB 231, MDA MB 468, and MCF-7 are included for comparison. The MDA MB 468 breast cancer cell line has been shown to have an amplified EGF-R gene (24).

of both EGF binding components. This was reproducible using cells either at early passage level or cells that had been propagated for 6–7 months, ruling out the potential possibility that a gradual selection had occurred during long-term serial passage of these cells. It is noteworthy that while the 184A1N4-T cells showed 5- to 10-fold greater number of EGF binding sites compared to the other cells studied, this difference was not mirrored at the mRNA level.

Although the total number of binding sites in the three cell lines containing v-Ha-*ras* were only slightly lower than in 184A1N4 cells, two-site computer fitted models gave less than 100 binding sites per cell for the high affinity component (Fig. 5).

DISCUSSION

Expression of TGFα by HMEC: Lack of Induction after Malignant Transformation

This study was undertaken to examine the expression of TGFα and the EGF/TGFα receptor in normal, proliferating, nonimmortal, reduction mammoplasty-derived HMEC and in sublines of the normal mammary epithelial cells that had been immortalized with benzo-*a*-pyrene and subsequently transformed to various degrees by single or combinations of viral oncogenes. The cells displayed marked variations in response to exogenous EGF or TGFα, ranging from an almost complete dependence upon supplementation for survival to autonomous growth and complete insensitivity. The SV40 T transformed cells required EGF or TGFα for anchorage-independent cloning. All lines carrying a v-Ha-*ras* oncogene had lost responsiveness to exogenous EGF or TGFα under anchorage-dependent and independent-growth conditions. Also, v-Ha-*ras* abrogated the SV40 T induced hypersensitivity to EGF or TGFα in soft agar and was synergistic with SV40 T in facilitating anchorage-independent cloning.

Biologically active and immunoreactive TGFα and its mRNA were produced by both the normal and malignant HMEC. Furthermore, no additional increase in TGFα expression was observed as the 184A1N4 cells progressed to an increasingly malignant phenotype after oncogene transformation. Rather, the normal parental 184 cells apparently produced more TGFα immunoreactive protein than the 184A1N4 cells or the transformed sublines. The mechanism(s) responsible for the decreased TGFα protein/mRNA ratio in the transformed HMEC 184A1N4 lines is not yet understood, and may involve altered posttranslational processing. The slightly higher levels of bioactive than immunoreactive TGFα in the 184 cells is not considered significant, since the bioassay would not discriminate between TGFα produced by the cells and traces of exogenous EGF left behind despite the washes preceding the CM collections.

Taken together, these results imply that in breast cancer, TGFα may not be a tumor specific growth factor. This is supported by several other reports demonstrating the presence of TGFα (27–29) or TGFα-like activity (30–32) in a number of other normal cells and tissues, including human milk.

The observation that TGFα production was not increased with oncogene mediated progression of HMEC to a more malignant phenotype contrasts to previous

Table 3. Binding Parameters for the EGF-Receptor on HMEC

Cell	K_D (high) (nM)	K_D (low) (nM)	B_{max} (high) Sites/Cell ($\times 10^3$)	B_{max} (low) Sites/Cell ($\times 10^3$)	B_{max} (tot) Sites/Cell ($\times 10^3$)
184	0.19 ± 0.05		403 ± 234		403 ± 24
A1N4	0.14 ± 0.14	1.12 ± 0.39	21 ± 10	290 ± 15	308 ± 17
A1N4-M	0.16 ± 0.07	2.24 ± 0.43	28 ± 11	353 ± 90	381 ± 97
A1N4-T	0.10 ± 0.03	1.75 ± 0.13	72 ± 6	1979 ± 177	2051 ± 183
A1N4-H		3.21 ± 0.45		278 ± 33	278 ± 33
A1N4-MH		0.57 ± 0.22		163 ± 47	163 ± 47
A1N4-TH		2.04 ± 0.27		184 ± 19	184 ± 19

All values are expressed as means ± SEM. Binding parameters for the EGF-R were computed from whole cell monolayer saturation experiments, as described in *Materials and Methods*. Two-site computer model fits could not be calculated for 184A1N4-H, and gave less than 100 binding sites per cell for 184A1N4-MH and 184A1N4-TH.

reports. For example, in both mouse mammary epithelial cells and rodent fibroblasts, various oncogenes induced increased expression of TGFα (1, 16, 33, 34) and transformation and desensitization to exogenous EGF were proposed to directly result from overexpression of endogenous TGFα. It should be noted that in the rodent cell lines, *ras* alone was sufficient to elicit full transformation, which was not the case with the HMEC used in the present study. For the HMEC, cooperation between SV40 T and v-Ha-*ras* (but not v-*mos* and v-Ha-*ras*) in previously immortalized cells was required for expression of the fully malignant phenotype.

Expression of EGF-R by HMEC: v-Ha-*ras* Suppression of High Affinity Sites and SV40 T Induced Receptor Expression

To determine whether transformation and variations in growth factor responses of the HMEC lines were due to alterations at the level of EGF-R expression rather than to changes in TGFα production, EGF-R binding characteristics and mRNA expression were determined in all the HMEC lines. EGF-R was expressed at high levels in all the HMEC lines, with less than a 2-fold reduction in total number of binding sites per cell in the 184A1N4 cell lines transformed by two oncogenes. However, the presence of v-Ha-*ras* oncogene in any of the cell lines was associated with a loss of the high affinity EGF-R binding component. These findings are in agreement with several other studies demonstrating a reduction in high affinity EGF-R binding in the presence of a v-Ha-*ras* oncogene or activated c-Ha-*ras* proto-oncogene (16, 35) or other viral oncogenes such as *mos* or *abl* (34, 36, 37).

The 184A1N4 cell line transformed by SV40 T alone was found to express 5- to 10-fold more EGF-R binding sites than all the other HMEC lines, without a corresponding increase in EGF-R mRNA levels. The mechanism for this is not clear but may involve regulation at a posttranscriptional level. In these cells, TGFα or EGF induced cloning in soft agar, whereas the other oncogene transformants were unresponsive to EGF/TGFα under anchorage-independent growth conditions. Our observations of the necessary interaction between a high number of EGF-R and presence of an appropriate ligand for eliciting the fully transformed phenotype in the HMEC transformed by SV40 T, are paralleled in two recent studies using the mouse fibroblast NIH 3T3 cells transfected with a functional EGF-R (38, 39). In both studies, the cells displayed soft agar growth and focus formation only in the presence of exogenous EGF. It is noteworthy that cells of such divergent origins as human epithelial cells and rodent mesenchymal cells should possess such similar characteristics. This may indicate the existence of a still unknown common pathway to transformation. In contrast, two other human epithelial cell lines with constitutive overexpression of even higher levels of EGF-R, the epidermoid carcinoma line A431 (40) and the MDA MB-468 breast cancer cell line (24), are both growth inhibited by EGF. On the other hand, enhanced responsiveness to EGF can be induced in rodent and chicken mesenchymal cells overexpressing c-*src* or c-*myc*, respectively (41–43), without alterations in cellular phenotype or EGF-R expression. All of these studies, including our present findings, indicate that elevated levels of EGF-R may be necessary in some cases but are not sufficient in all cases for eliciting the malignant phenotype, and that additional activation at some other level may be required.

The Autocrine Hypothesis in Relation to Oncogene Induced Transformation of HMEC

An autocrine feedback loop involving TGFα may be functioning for the nontransformed HMEC. In mass culture, they have reduced requirement for exogenous EGF and produce substantial amounts of TGFα activity, apparently enough to partially abrogate their requirements for exogenous EGF. It may be that the TGFα/EGF-R system is related to a common mechanism of cellular growth control in both normal and malignant breast epithelial cells and that overrexpression of either TGFα and/or the EGF-R may not be unique to tumor cells. In this respect, it has recently been shown in human keratinocytes by *in situ* hybridization with a TGFα riboprobe (5) that the highest levels of TGFα expression were observed in the basal cell layer, a putative stem cell population with the highest prolifer-

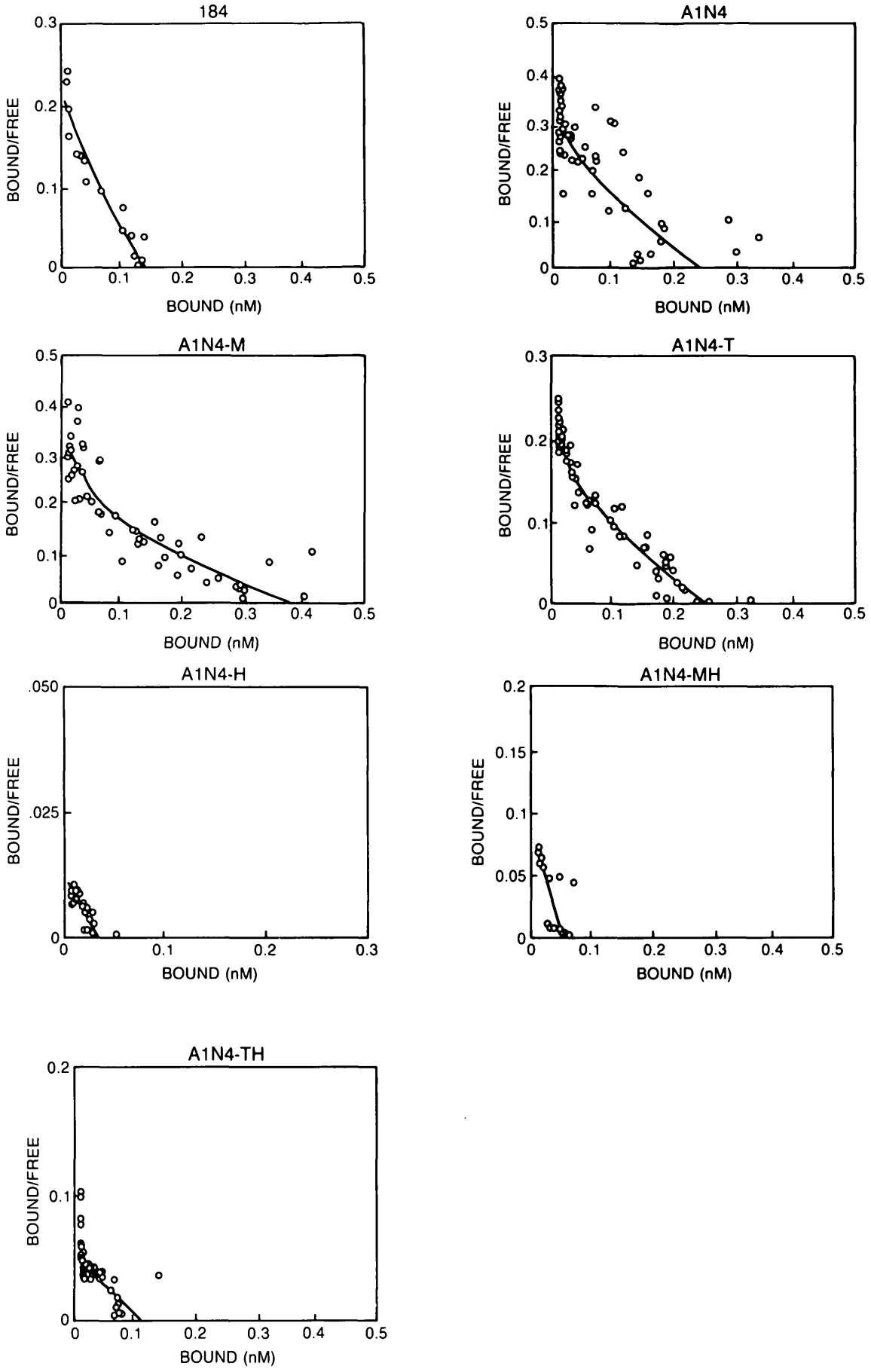

Fig. 5. Scatchard Plots for ^{125}I-EGF Binding to the HMEC Lines

Saturation binding curves of ^{125}I-EGF for EGF-R on whole cell monolayers were performed using 1 pM–25 nM ^{125}I-EGF at 4 C, with the addition of greater than or equal to 100-fold excess unlabeled EGF for nonspecific binding. Binding parameters were computed using the LIGAND program.

ation rates in the epidermis. Furthermore, whereas primary chemically-induced rat mammary tumors produce TGFα, transplantable rat mammary tumors, which were derived from these same tumors, express little or no TGFα (44). It is possible that differential responses to EGF/TGFα, rather than changes in the levels of production of endogenous TGFα, are associated with the transition from normal to malignant breast epithelium.

Taken together, these observations suggest that oncogenes may alter normal cellular growth in a neoplastic direction without obvious effects on TGFα production and/or EGF-receptor expression and function. As illustrated by the SV40 T transformed line, the presence of an oncogene can considerably increase the number of EGF-R binding sites without conferring an obviously transformed phenotype. In the presence of exogenous ligand, however, transformation is induced as measured by anchorage-independent growth. In contrast, *ras* is capable of inducing transformation in the absence of obvious changes in either TGFα production or EGF-R binding characteristics, and abrogates cellular responsiveness to exogenous EGF/TGFα. In this system, SV40 T and *ras* appear to have synergistic effects in eliciting the fully transformed and malignant phenotype. Evidently, this occurs via a mechanism other than direct alterations in the TGFα/EGF-R system. Perhaps a sustained activation of the intracellular EGF-R transduction process is caused by the G-protein-like properties of the p21 *ras* protein (35), thus bypassing activation of the EGF cell surface receptor. The SV40 T and v-Ha-*ras* oncogenes may interact only coincidentally with TGFα production or EGF-R expression, while inducing phenotypic transformation at another level. Clearly, oncogenes may have effects on growth factor pathways in addition to simply augmenting growth factor production, as initially proposed.

MATERIALS AND METHODS

Cell Lines

Normal HMEC strains 184 and 172 were routinely cultured in serum-free MCDB 170 medium (University of California at San Francisco, Tissue Culture Unit) containing EGF, bovine pituitary extract, and insulin, as previously described (45), in a less than or equal to 1.0% CO_2 atmosphere. Experiments were performed with cell cultures at passages 10-12. 184A1N4, a subclone of the established immortalized benzo-a-pyrene treated line 184A1 (20), was routinely grown in a variant medium consisting of Improved Modified Eagle's Medium (IMEM) (GIBCO, Grand Island, NY) with 0.5% FBS (GIBCO), insulin (10 μg/ml; Sigma, St. Louis, MO), hydrocortisone (0.1 μg/ml; Sigma), and EGF (5 ng/ml; Collaborative Research, Bedford, MA), in 5% CO_2 atmosphere. The oncogene-transformed (not clonally selected) lines 184A1N4-T, 184A1N4-M, 184A1N4-TH, and 184A1N4-MH (carrying SV40 T, v-*mos*, v-Ha-*ras*, or SV40 T + v-Ha-*ras*, v-*mos* + v-Ha-*ras*, respectively) (21) were maintained in IMEM supplemented with 10% FBS. 184 cells are normal diploid, 184A1N4 near triploid, 184A1N4-T near hexaploid (Clark, R., unpublished data), and two clones of 184A1N4-TH were near tetraploid.

MCF-7 cells were donated by Dr. Marvin Rich, Michigan Cancer Foundation (Detroit, MI). HT1080 fibrosarcoma cells, MDA-MB-231, MDA-M8-468, and normal rat kidney cells (NRK) clone 49F were obtained from the American Type Culture Collection (Rockville, MD); Hs578T was provided by Helene Smith, Peralta Cancer Research Institute (Oakland, CA). All of these cell lines were maintained in IMEM with 10% FBS, in 5% CO_2 atmosphere.

Effect of EGF/TGFα on Anchorage-Dependent and Anchorage-Independent Growth

Anchorage-dependent requirement for EGF by 184 and 184A1N4 cells was tested at clonal density and in mass culture. For assays at clonal densities, freshly trypsinized cells were seeded into 100-mm dishes, 500 cells per dish, in regular MCDB 170 medium with or without 10 ng/ml EGF. After 10–13 days incubation without refeeding, plates were stained and colonies were counted. For assaying EGF dependence at high cellular densities, 5×10^4 cells were seeded into 35-mm dishes in MCDB 170 medium in the absence or presence of EGF (10 ng/ml). Medium was changed every 2–3 days during the culture period, and when control dishes were just confluent, the cells were trypsinized and counted. Results represent the average of triplicate determinations from two experiments for each growth condition tested.

Growth responses of the oncogene transformed cells were evaluated on cells plated in 12-well cluster dishes, $5–10 \times 10^3$ cells per well, in IMEM containing 10% FBS. Medium was changed and EGF or TGFα were added 4 h after plating. The cells were trypsinized and counted after 5 days of culture while still less than 90% confluent. Results represent the average of triplicate determinations from three separate experiments.

Anchorage-independent growth responses to 5 ng/ml EGF or TGFα were tested by plating 0.8 ml/35-mm dish of mixture containing 2×10^4 cells in their regular growth medium in the presence or absence of EGF or TGFα and 0.36% (vol/vol) Bactoagar. After 9–11 days incubation, colonies greater than or equal to 60-mm diameter were counted on a Bausch & Lomb stem cell colony counter. Results represent the average of triplicate determinations from three separate experiments.

Preparation of CM

Cells were grown to 75–85% confluency in T-175 tissue culture flasks (Falcon Oxnard, CA) in their regular growth media. After an overnight serum-free medium wash, serum-free medium was collected after 48 h of conditioning (at which time the cells were generally less than or equal to 95% confluent). The 184 parent cells were either maintained in the MCDB 170 mixture routinely used for their growth or in MCDB 170 medium without bovine pituitary extract, EGF, and insulin, in the presence of prostaglandin E_2 (Sigma) (22). For the 184A1N4 cells, CM consisted of variant medium without FBS. For the oncogene-transformed 184A1N4 lines and for control cell lines, serum-free medium consisted of IMEM supplemented with transferrin (2 mg/liter; Sigma), fibronectin (1 mg/liter; Collaborative Research), HEPES buffer (20 mM; GIBCO), and trace elements (GIBCO). After collection of the CM, protease inhibitors aprotinin (0.2% vol/vol) (Sigma), leupeptin (2 μg/liter) Boehringer-Mannheim, Indianapolis, IN), and phenylmethylsulfonylfluoride (10^{-7} M) (Sigma) were added, media were filtered stored at −20 C. At the time of assay, CM was thawed, and concentrated and dialyzed against distilled H_2O on Centriprep centrifugal devices (Amicon Danvers, MA) as described in the manufacturer's instructions. Aliquots of 50-fold concentrated medium were assayed at various dilutions for TGFα-like activity as described below.

DNA was quantitated using a fluorescence method (46) from aliquots of pooled cells harvested from the same flasks from which CM had been collected.

Detection of TGFα-Like Activity in CM

CM was assayed both for TGFα-immunoreactivity, using a TGFα RIA kit (Biotope, Inc.), and for EGF/TGFα bioactivity by induction of anchorage-independent colony growth of NRK 49F cells. Complete MCDB 170 medium CM from 184 cells, and variant medium CM from 184A1N4 cells, which contained EGF, could only be assayed by the RIA. Several dilutions of concentrated and dialyzed CM from three separate collections were assayed by both methods.

The presence in CM of biologically active TGFα activity on NRK 49F cells in soft agar was assayed as previously described (4) using 3.2–12.5 ml equivalents of CM plated with 3 × 10^3 cells in the presence of 2.6 ng/ml TGFβ (34) in 0.36% Bacto-agar in 35-mm dishes. Different concentrations of mouse EGF were used as a reference for calibrating the amount of TGFα in the CM aliquots as determined from standard titration curves using mouse EGF and human TGFα. EGF and TGFα are equipotent in this bioassay (10, 16, and data not shown). Plates were counted after 7 days using a Bausch & Lomb stem cell colony counter. Colonies of greater than or equal to 60 μm diameter were scored as positive. Two separate experiments with triplicates of every dilution of CM were performed.

TGFα immunoreactivity was assayed in CM using a TGFα RIA kit (Biotope, Inc., Seattle, WA) with a polyclonal anti-rat TGFα antibody which recognizes reduced and denatured human and rat TGFα, but does not cross-react with up to 1 μM mouse EGF (47 and data not shown). Standard curves of unlabeled rat TGFα, against which the antibody was raised, were performed in competition with trace amounts of ^{125}I-rat TGFα. The limit of detection of the RIA for TGFα was approximately 0.08 ng/assay with 50% displacement of labeled peptide occurring at approximately 0.7 ng/assay. Duplicate or triplicate tests were performed in three separate experiments on dilutions of each individual CM sample.

EGF Receptor Binding

Whole-cell EGF-receptor analyses were performed as described (48). Briefly, oncogene transformed cells were grown to confluency in poly-D-lysine coated 24-well cluster dishes (Falcon) in IMEM with 5% FBS and washed and incubated with serum-free medium (composed as described for CM) for 3–7 h before the binding assay in order to reduce nonspecific competition by serum factors. The 184 and 184A1N4 cells were incubated overnight in their regular medium without bovine pituitary extract, EGF, or FBS, before EGF receptor analysis. Binding was carried out in Hank's Basic Salt Solution (with 0.1% BSA and 40 mM HEPES, pH 7.4) containing either varying concentrations of ^{125}I-EGF (human, ICN Radiochemical, Irvine, CA; SA, 80–160 μCi/μg), or varying concentrations of unlabeled mouse EGF with a constant amount of labeled EGF. Nonspecific binding was assayed in the presence of greater than or equal to 100-fold excess unlabeled mouse EGF. After incubation for 2.5 h at 4 C, cells were washed with ice-cold binding buffer on ice and solubilized in lysis buffer (0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% sodium dodecyl sulfate SDS). Lysates were counted in an LKB γ-counter. Three to six separate experiments were performed on each cell line, and binding sites were normalized to cell numbers determined from parallel wells which had been comparably treated. Preliminary kinetic binding experiments showed that equilibrium was attained for the high affinity binding component and approximated for the low affinity component (data not shown).

RNA Preparation and Northern Blot Analysis

Total cellular RNA was isolated by centrifugation of guanidine isothiocyanate homogenates over cushions of cesium chloride, and total RNA was electrophoretically separated on 1% agarose-6% formaldehyde gels as previously described (10). Gels were examined after staining with 2 μg/ml ethidium bromide to ensure that only comparably loaded and undegraded RNA samples were used. Subsequently, gels were subjected to alkaline hydrolysis, neutralization, and Northern transfer to nitrocellulose by capillary blot. The Northern blots were hybridized to TGFα or EGF-R synthetic riboprobes. A synthetic TGFα riboprobe was prepared by SP6 polymerase transcription of a 1.3 kb TGFα cDNA insert (49) ligated into plasmid SP65, received from Rik Derynck (Genentech, Inc., South San Francisco, CA). An EGF-R riboprobe was synthesized from a 136 basepair sequence isolated from pE7 (gift of Glen Merlino, NCI) (23) and cloned into pGEM4. Finally, a 1.7 kb β-actin cDNA (50) cloned into pGEM3 was used to construct a β-actin riboprobe with SP6 polymerase.

Nitrocellulose filters were hybridized in 50% formamide, 5× Denhardt's, 5× SSC, 0.1% SDS, and 200 μg/ml salmon sperm DNA for 18 h at 55 C for the riboprobes (2 × 10^6 cpm/ml). Filters probed with riboprobes were washed twice for 30 min at 21 C in 1× SSC + 0.1% SDS warmed to 68 C; and in 0.1× SSC + 0.1% SDS for 1 h in a 68 C water bath. Filters probed with nick-translated cDNAs were washed at 21 C in 2× SSC + 0.2% SDS for 1 h, followed by 1× SSC + 0.1% SDS warmed to 42 C. Autoradiography was performed with Kodak XAR-5 film at −70 C. Autoradiograms were subsequently quantified by densitometric scanning in a Beckman gel scanner.

DNA Preparation and Southern Blot Analysis

High molecular weight DNA was isolated by SDS-proteinase K lysis, RNAse A digestion, extraction with phenol and chloroform, and precipitation with sodium acetate in cold ethanol. DNA was digested with *Hin*dIII, electrophoresed in an 0.8% agarose gel, and transferred to nitrocellulose filters by Southern blotting. Nitrocellulose filters were baked in a vacuum oven for 2 h at 80 C. Hybridizations were performed with 1 × 10^6 cpm/ml nick-translated cDNA insert pE7 (23). Molecular size markers used were *Hin*dIII fragments of lambda DNA (Bethesda Research Laboratories, Gaithersburg, MD).

Data Analyses

Receptor binding parameters for saturation and displacement experiments were calculated using the LIGAND computer program (51). Nonspecific binding was estimated together with the other binding parameters and did not exceed 3% in any assay (51).

All parameters obtained are expressed as means ± SD (for mitogenic assays) or means ± SEM (for receptor binding assays). Means were compared using the two-tailed Student's *t* test (52). Values of $P \leq 0.05$ were taken to indicate statistical significance.

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Address requests for reprints to: Dr. Robert B. Dickson, Medical Breast Section, National Cancer Institute, Building 10, Room 12N226, Bethesda, Maryland 20892.

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