Expression of Transforming Growth Factor α and its Messenger Ribonucleic Acid in Human Breast Cancer: Its Regulation by Estrogen and its Possible Functional Significance

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We have studied the estrogenic regulation and the potential autocrine role of transforming growth factor α (TGFα) in the human breast cancer cell line MCF-7. A biologically active apparent mol wt 30 k TGFα was identified by gel filtration chromatography in medium conditioned by MCF-7 breast cancer cells. We previously reported induction of TGFα levels in medium by 17β-estradiol. We now report correlated increases in TGFα mRNA, by Northern and slot blot analysis, after estrogen treatment of MCF-7 cells *in vitro*. *In vivo* experiments confirmed these data: estrogen withdrawal from MCF-7 tumor-bearing nude mice resulted in a decline in tumor size and TGFα mRNA levels. To explore the functional significance of TGFα in MCF-7 cells, anti-TGFα antibody was added to MCF-7 soft agar cloning assays. Inhibition of MCF-7 growth resulted, supporting an autocrine role for TGFα. Further experiments using an anti-EGF receptor antibody expanded this data, demonstrating inhibition of estrogen-stimulated monolayer MCF-7 cell growth. Examining the generality of TGFα expression, 4.8 kilobase TGFα mRNAs were seen in three other human breast cancer cell lines, MDA-MB-231, ZR 75B, and T47D. Expression of TGFα mRNA was detected in 70% of estrogen receptor positive and negative primary human breast tumors from 40 patients when examined by slot blot and Northern analysis. Thus, we have demonstrated broad expression of TGFα in human breast cancer, its hormonal regulation in an estrogen-responsive cell line, and its possible functional significance in MCF-7 cell growth. (Molecular Endocrinology 2: 543–555, 1988)

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INTRODUCTION

Breast cancer is one of a small number of malignancies in which both genesis and growth have been linked to hormonal factors. About one-third of all metastatic human breast cancers are responsive to endocrine therapies (1). Hormone withdrawal in these estrogen-responsive cancers results in regression but rarely complete resolution of the disease. Study of these estrogen-responsive tumors has been facilitated by the existence of estrogen-receptor positive human breast cancer cell lines which respond to estrogen by increased cell growth *in vitro* and are dependent upon estrogen for tumor formation in nude mice. However, the mechanism of estrogen action in these cell lines, in human primary breast tumors, and in normal breast epithelium is incom-

pletely understood. We have postulated that estrogen may act by inducing the elaboration of secreted factors which promote the growth of cells.

We have previously reported the regulation by estrogen of the mitogenic polypeptide, transforming growth factor α (TGFα), in several human breast cancer cell lines (2, 3). TGFα was originally characterized by its ability, in combination with transforming growth factor β, to induce the anchorage-independent growth of a normal fibroblast cell line, the NRK cell line (4, 5). Its potential involvement in malignant transformation was underscored by its presence in a variety of human tumors, and in cell lines transformed by both chemical carcinogens and viral oncogenes (6–12), but the precise role which TGFα plays in human neoplasia has never been established.

Both TGFα and its functional homologue, epidermal growth factor (EGF), appear to act through a common cell surface receptor, generally termed the EGF receptor (13, 14). They share many biological activities including stimulation of cell growth (9, 15, 16). Induction of epithelial cell colony formation by TGFα has been reported in a limited number of studies (17–19). EGF has been shown to stimulate the growth of three human breast cancer cell lines *in vitro*, including the MCF-7 cell line (16, 20–22). Further, EGF has been shown to stimulate MCF-7 tumor formation in nude mice in the absence of estrogen (23) and has been directly implicated in the induction of mouse mammary tumorigenesis (24).

Because human breast cancer cell lines and human breast tumors contain EGF receptors (25–28), an autocrine pathway can be proposed for TGFα in human breast cancer cells. According to this hypothesis, cells secrete mitogenic polypeptides which act through their own cell surface receptors to stimulate growth (29). Since human breast cancer cells contain the EGF/TGFα receptor and respond to EGF by increased growth, demonstration of TGFα production by these cells suggests that an autocrine loop may be operative in breast cancer cell growth. Further, regulation of TGFα production in estrogen-responsive cell lines implies that this autocrine pathway for cellular growth is hormonally regulated.

Purified human TGFα is a 50-amino acid peptide with a mol wt of 5600, which is homologous to EGF (6, 12). A 17–19 kDa precursor protein has been identified as a biologically active form of TGFα which is equipotent with the mature polypeptide (30). A cDNA clone encoding a predicted 160-amino acid precursor recognizes a 4.8 kb mRNA species in Northern blots (31–33).

Tests of the autocrine hypothesis using antibodies to interfere with TGFα action have had variable results. Antisera directed against the EGF receptor were shown to block EGF/TGFα-induced anchorage-independent colony formation by normal rat fibroblasts (34). However, in a TGFα-secreting melanoma cell line, it was shown that a monoclonal antibody which blocked EGF and TGFα binding to the EGF receptor failed to block growth or anchorage-independent colony formation (35). It was noted, though, that these cells had no detectable EGF receptors on their cell surface. In a third study, three monoclonal antibodies directed against the EGF receptor were found to block tumor formation by two of four human tumor cell lines in nude mice (36).

TGFα protein has been identified in conditioned media from a number of breast cancer cell lines, and in extracts of primary breast tumors (2, 3, 7, 8). TGFα is induced by estrogen 2- to 8-fold in MCF-7 cells. This induction may account for a portion of the mitogenic and tumor promoting effects of estrogen. We now report further studies of TGFα expression in primary human breast tumors and four human breast cancer cell lines. We sought to determine whether the observed induction of TGFα-like activity by estrogen *in vitro* in MCF-7 cells could be correlated with induction of TGFα mRNA, whether TGFα mRNA regulation could be demonstrated *in vivo* in MCF-7 tumors, whether we could provide evidence for an autocrine role for TGFα, and whether our observations of TGFα expression in human breast cancer cell lines could be extended to primary human breast tumors.

RESULTS

Production of Authentic TGFα by MCF-7 Cells

Initial experiments using gel filtration chromatography suggested the presence of a 30 k apparent mol wt TGFα activity in conditioned medium from MCF-7 cells (2). To determine whether this activity was recognized by antibodies developed against low mol wt TGFα, conditioned medium was acid treated and fractionated in 1 M acetic acid on a Bio-Gel P100 column. Two principle peaks of activity were detected, one with apparent mol wt of 30 k, and the other at 7 k (Fig. 1). Each of these peaks contained TGFα activity as detected by TGFα RIA, NRK colony formation, and EGF receptor competition. The activities measured by EGF receptor competition and TGFα RIA were comparable on a molar basis. Confirmation of the existence of a secreted high mol wt form of TGFα in MCF-7 cells was provided by the detection of multiple immunoreactive species near 30 k after immunoprecipitation of [^{35}S] cysteine labeled conditioned medium from MCF-7 cells (Fig. 2). Multiple bands of immunoprecipitated material were seen at approximately 30 k after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. These bands were absent when the immunoprecipitation was performed in the presence of excess unlabeled TGFα. These results confirm our initial observations and show that a biologically active high mol wt immunoreactive TGFα is synthesized by MCF-7 cells.

Induction of TGFα Activity and TGFα mRNA by Estrogen in MCF-7 Human Breast Cancer Cells

To determine whether the induction of TGFα by estrogen occurred in conjunction with alterations in TGFα

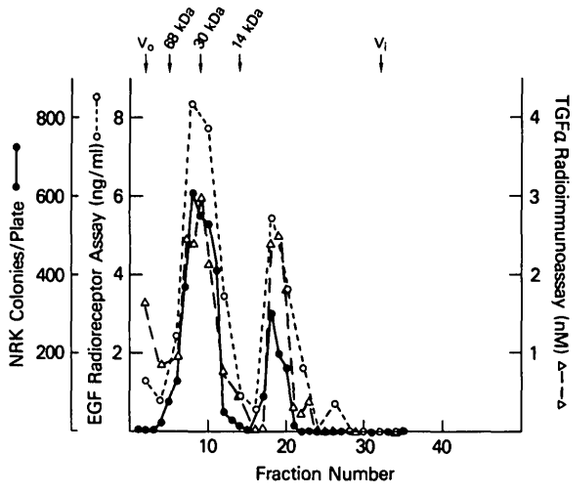

Fig. 1. Characterization of TGFα-Like Activities in Conditioned Medium from MCF-7 Cells after Bio-Gel P100 Chromatography

Four liters of serum-free conditioned medium from cells previously grown in 10% FBS were concentrated and then acidified by dialysis against 1 M acetic acid. The sample was applied to a 2.6 × 70-cm Bio-Gel P100 column equilibrated with 1 M acetic acid. Seven-milliliter fractions were collected, lyophilized, and resuspended in 1 ml 4 mM HCl for assay. Fractions were assayed by induction of NRK colony formation (●——●), EGF receptor competition (O- - -O), and TGFα RIA (Δ- - -Δ). These chromatograms are representative of three separate experiments.

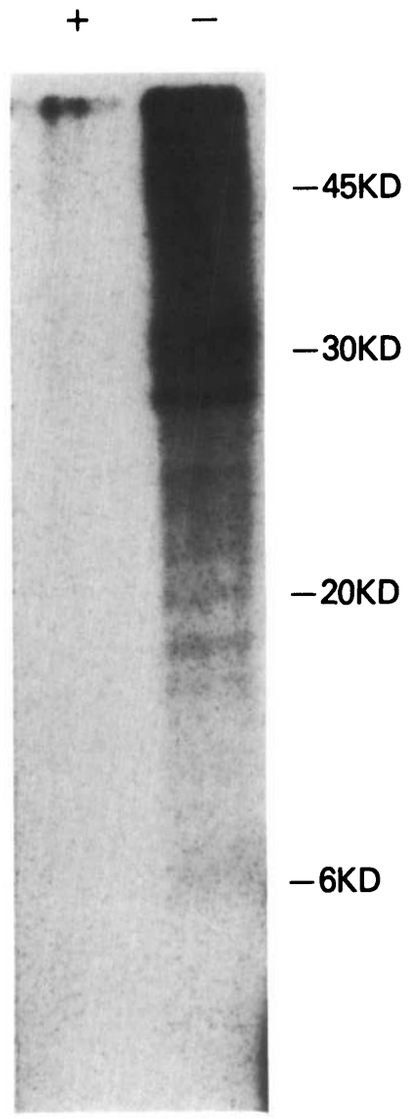

Fig. 2. SDS-Polyacrylamide Gel Electrophoresis of Immunoprecipitated Media from ^{35}S-Cysteine Labeled MCF-7 Cells

Immunoprecipitations were carried out in the presence (+) or absence of (−) of 250 ng human TGFα (54). The immunoprecipitates were washed and bound material eluted from the Sepharose by incubation with 1 ml 1 M acetic acid for 30 min at 60 C. The material was lyophilized and run on a 15% discontinuous SDS-polyacrylamide gel, incubated with En-Hance (New Nuclear Corp.) dried, and exposed to Kodak X-Omat for 4 weeks at −70 C. Similar results were obtained with a different antibody in two subsequent immunoprecipitations.

mRNA levels, MCF-7 cells were grown with and without addition of 10^{-8} M 17β-estradiol for 48 h in medium depleted of estrogen. Three paired experiments were done with cells grown in serum-containing medium used for RNA preparation, and cells grown in serum-free medium used for conditioned medium collections. We again demonstrated induction of TGFα activity by estrogen in medium conditioned by these cells, greater than 2-fold in both dose-response curves for EGF/TGFα-induced NRK colony-forming activity and in EGF radioreceptor competing activity. Conditioned media from three separate experiments contained 0.102 ± 0.014 pg/μg DNA EGF receptor competing activity after estrogen treatment for 48 h, significantly differing from the control media containing 0.044 ± 0.024 pg/μg DNA ($P < 0.005$). Total RNA from estrogen-treated and control cells was purified and directly applied to nitrocellulose filters using a slot blot apparatus. As shown in Fig. 3A, there was 2.6-fold induction of the TGFα message by estrogen in MCF-7 cells. Additionally, both total and poly(A)$^+$ selected RNA were examined by Northern analysis (Fig. 3B). The previously reported 4.8 kb message was easily identified in Northern transfers containing both total RNA (data not shown) and poly(A)$^+$ selected RNA, and was increased 2- to 3-fold by estrogen treatment. Additionally, a 1.6 kb message 10-fold less abundant and not visible on photographs of MCF-7 lanes was faintly detected in poly(A)$^+$ selected RNA. Control hybridizations with β-actin revealed no differences in RNA loading in these experiments.

Dose-response and time course studies of estrogen induction of TGFα mRNA were performed. A 2-fold estrogen stimulation of TGFα mRNA was seen as early as 6 h after estrogen treatment, and remained stable over 6 days of estrogen treatment (data not shown).

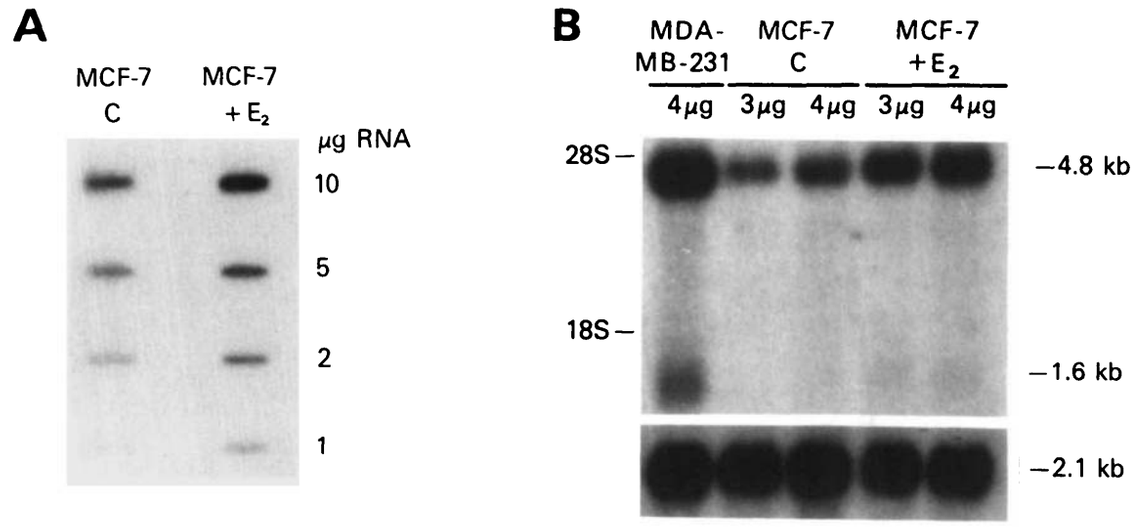

Fig. 3. Estrogen Induction of TGFα mRNA by Northern and Slot Blot Analyses

Filters were hybridized to the TGFα riboprobe and subsequently to the β-actin riboprobe. A, Total RNA from cells treated 48 h with 10^{-8} M 17 β-estradiol (E_2) or its vehicle, ethanol (C) was serially diluted and applied to nitrocellulose using a slot blot apparatus. There was a 2.6-fold induction of the message measured by densitometry in this experiment. B, Three or four μg poly(A)$^+$ selected RNA were applied to 1% agarose-6% formaldehyde gels and transferred to nitrocellulose after electrophoresis. MDA-MB-231 mRNA was included in the first lane as a positive control. The estrogen induction of TGFα mRNA was independently confirmed in over six experiments. Hybridization to β-actin confirmed that RNA loading was comparable in all cases. Densitometric scanning showed a 2- to 3 fold induction by estrogen in this experiment.

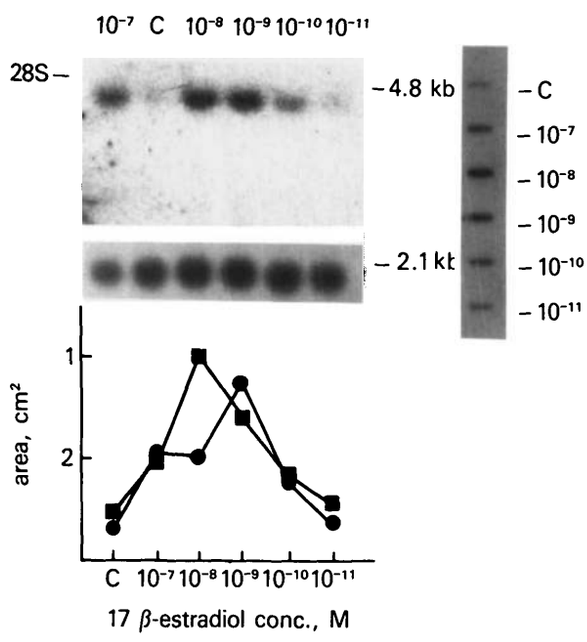

Fig. 4. Dose-Response Study of Estrogen Induction of TGFα mRNA

Total RNA from cells treated with 10^{-7}–10^{-11} M 17 β-estradiol for 48 h or with its vehicle, ethanol (C), was analyzed for TGFα mRNA three times and the results of two experiments are shown here. Eight micrograms of total RNA were subjected to Northern analysis, hybridizing with the TGFα riboprobe. Ten micrograms of total RNA were applied to the accompanying slot blot. Quantification of the TGFα signals observed by densitometric scanning of the slot blot (■) and Northern blot (●) is also shown.

Figure 4 shows the dose-response curve for TGFα mRNA induction by estrogen. Total RNA prepared from MCF-7 cells treated with varying concentrations of estrogen revealed levels of TGFα mRNA exceeding control levels with as little as 10^{-10} M 17β-estradiol. The highest level of induction of TGFα mRNA was observed between 10^{-9} and 10^{-8} M 17β-estradiol. In this experiment 10^{-8} M 17β-estradiol induced TGFα mRNA 4-fold over control TGFα mRNA levels after 48 h of treatment.

In vivo Regulation of TGFα mRNA by Estrogen in MCF-7 Xenografts

Growth of MCF-7 tumors in nude mice is dependent upon the presence of estrogen. To determine whether induction of TGFα mRNA by estrogen may occur during tumor formation *in vivo*, TGFα mRNA levels were examined in MCF-7 tumor xenografts grown in female nude mice implanted with estrogen pellets. No tumors formed in animals without estrogen pellets. Tumor-bearing animals were killed, or the estrogen pellets were removed, 3 weeks after injection of tumor cells. Tumors were subsequently removed at varying time intervals, and the RNA purified, poly(A)$^+$ enriched and analyzed by Northern analysis with a human TGFα cDNA probe. The levels of TGFα mRNA declined with increasing time after estrogen withdrawal (Fig. 5). After 10 days of estrogen withdrawal the level of 4.8 kb TGFα mRNA was 2.5-fold lower (by densitometric scanning of the Northern blots) than the level in tumors from control estrogenized animals. The tumors at this time were not actively growing, and were regressing slightly. This

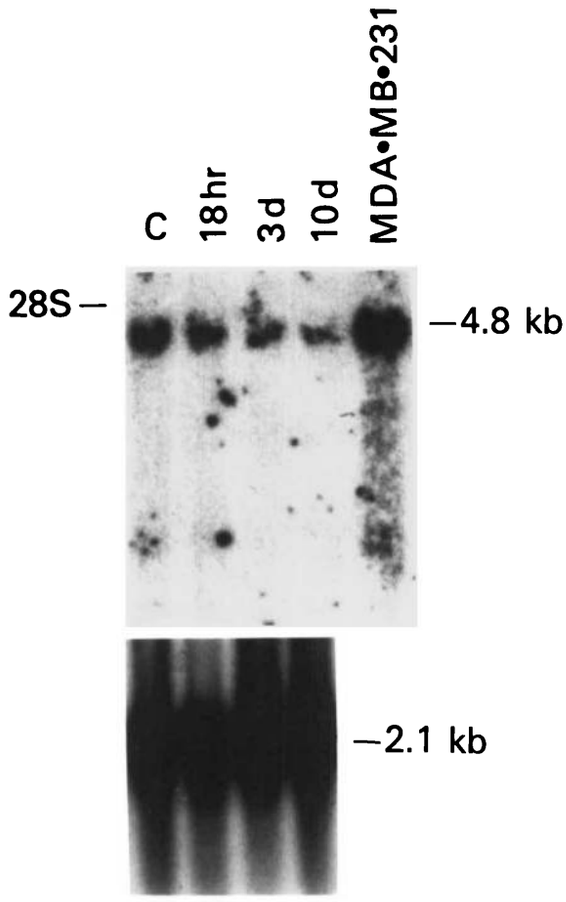

Fig. 5. Regulation of TGFα mRNA by Estrogen in MCF-7 Xenografts

MCF-7 tumors growing sc in nude Balb/C mice with an implanted 17β-estradiol pellet were harvested before; and 18 h, 3 days, and 10 days after removal of the estrogen pellet. Northern analysis of 10 μg poly(A)$^+$ enriched RNA from these tumors is shown. Nick-translated cDNA probes for human TGFα (*top*) and β-actin (*bottom*) were used for the hybridizations. By densitometric scanning there was a 2.5-fold decrease in TGFα mRNA levels 10 days after removal of the estrogen pellet. These results represent pooled mRNA samples from 20 mice.

effect was specific for the 4.8 kb TGFα mRNA since there was no decrease in the 2.1 kb β-actin mRNA levels after estrogen withdrawal. These results confirm the regulation by estrogen of TGFα and extend that observation to an *in vivo* setting.

Inhibition of MCF-7 Anchorage-Independent Growth by Antihuman TGFα Antibody

The effect of a polyclonal anti-TGFα antibody on the anchorage-independent growth of MCF-7 cells was studied to determine whether TGFα could be functioning as an autocrine growth factor for MCF-7 cells. As shown in Table 1, NRK colony formation, which is dependent upon exogenous TGFα or EGF, was reversed by the antibody at low concentrations of TGFα (0.3 ng/ml). MCF-7 colony formation in the presence of fetal calf serum (containing endogenous estrogens) was inhibited 50–90% by a 1:50 dilution of 60 or 125 μg/ml purified immunoglobulin G (IgG) from a polyclonal rabbit antihuman TGFα antibody. Increasing the dilution of anti-TGFα antibody or addition of 1 ng/ml recombinant TGFα resulted in loss of growth inhibition. These studies suggest that TGFα produced by MCF-7 cells is acting to promote the clonal growth of the cells in soft agar.

Inhibition of Estrogen-stimulated MCF-7 Anchorage-Dependent Growth by Anti-EGF Receptor Antibody

We also sought to determine whether interference with a TGFα autocrine growth loop could reverse the estrogen stimulation of MCF-7 cell growth after anchorage-dependent culture. A mouse anti-EGF receptor monoclonal antibody previously shown to block binding of EGF to its receptor (35) was added to cells grown in the presence or absence of estrogen. These experiments were performed on cells grown in monolayer cultures because the effects of estrogen on MCF-7 cell growth are most easily demonstrable under these culture conditions. As shown in Table 2, addition of 10 μg/ml/day anti-EGF receptor antibody inhibited estrogen-stimulated cell growth after 5 days of treatment but did not inhibit MCF-7 cells growing without estrogen. Control mouse IgG inhibited neither estrogen-treated or control MCF-7 cell growth. The effect of the anti-EGF receptor antibody was significant during the first 5 days of treatment, but not after the longer 10-day interval. To determine whether the failure of the EGF receptor antibody to significantly inhibit growth after longer treatment intervals was simply due to effects of cell density, MCF-7 cells were plated in Exp 3 to achieve a comparable density at day 5 to that at day 10 in Exps 1 and 2. Anti-EGF receptor antibody again inhibited estrogen-induced proliferation, suggesting that TGFα mediated growth as occurring at the higher cell density. The results in Table 2 are consistent with the hypothesis that estrogen stimulation of MCF-7 cell growth over a 5-day treatment interval depends upon induction of a TGFα-mediated autocrine pathway. After longer estrogen treatment intervals, and in the absence of estrogen, additional growth factors or other mechanisms may perform roles in regulating MCF-7 cell growth.

Expression of TGFα mRNA in Human Breast Cancer Cell Lines

Four additional human breast cancer cell lines in which we had previously studied TGFα activities (2, 3) were also examined for TGFα mRNA expression. As shown in Fig. 6, poly(A)$^+$ enriched mRNA analyzed on formaldehyde-agarose gels demonstrated a 4.8 kb mRNA for TGFα in the T47D, ZR-75-1, and MDA-MB-231 cell lines. Levels of TGFα mRNA expressed by T47D and

Table 1. Effects of Antihuman TGFα Antibody on MCF-7 and NRK 49F Colony Formation

	Control (No IgG)	Rabbit Antihuman TGFα IgG				Rabbit IgG
		(1:50)	(1:100)	(1:500)	(1:1000)	
Exp 1						
NRK 49F						
No added TGFα	17 ± 5					
+1 ng/ml TGFα	260 ± 16	147 ± 13[a]	321 ± 45			
+0.3 ng/ml TGFα	345 ± 15	52 ± 12[a]	44 ± 1[a]			
MCF-7						
No added TGFα	677 ± 3	23 ± 5[a]	25 ± 2[a]			
Exp 2						
NRK 49F						
No added TGFα	50 ± 7					
+1 ng/ml TGFα	614 ± 76	350 ± 20[b]	463 ± 50	631 ± 52	690 ± 41	555 ± 38
MCF-7						
No added TGFα	148 ± 41	50 ± 6[b]	58 ± 10[b]	160 ± 50	175 ± 23	113 ± 5
+1 ng/ml TGFα		117 ± 6				

Two × 10^4 MCF-7 cells or 5 × 10^3 NRK 49F cells were plated in soft agar for 14 days and numbers represent colonies per dish after nitrobluetetrazolium staining. Purified rabbit antihuman TGFα IgG was obtained from rabbit antiserum by protein-A sepharose chromatography. Serial dilutions of 125 μg/ml and 60 μg/ml aliquots of IgG were used in Exp 1 and 2, respectively. Recombinant human TGFα, 0.3 or 1 ng/ml, was added to test for neutralization of antibody effects. Rabbit IgG, 125 μg/ml, was used as a control. All tests were done in duplicate. Values shown are the mean ± SD from each of two of the three separate experiments performed with this antibody.

[a] $P < 0.05$ by Student's t test comparing antibody treated to untreated controls.

[b] $P < 0.05$ by Student's t test comparing anti-TGFα antibody treated to nonspecific antibody-treated controls.

Table 2. Effects of Anti-EGF Receptor Antibody on MCF-7 Cell Number after Estrogen Treatment

	No IgG		Mouse IgG		Anti-EGF Receptor IgG	
	Control	E_2	Control	E_2	Control	E_2
Exp 1						
Day 5	667 ± 30	1570 ± 310[a]	699 ± 84	1799 ± 135[b]	603 ± 76	948 ± 63[c]
Day 10	1482 ± 72	12538 ± 632[a]	1862 ± 112	11402 ± 1057[b]	1989 ± 498	9739 ± 1029
Exp 2						
Day 5	468 ± 82	2152 ± 142[a]	1105 ± 116	2487 ± 163[b]	1067 ± 109	1428 ± 192[c]
Day 10	1976 ± 106	18064 ± 478[a]	2217 ± 124	17781 ± 521[b]	2317 ± 216	11765 ± 391
Exp 3						
Day 5	7950 ± 526	21368 ± 23[a]			7892 ± 154	7582 ± 63[d]

One × 10^5 MCF-7 cells per well (Exps 1 and 2) or 5 × 10^5 cells per well (Exp 3) were plated in six-well CoStar dishes in phenol red-free IMEM containing 5% CCS. Experiments were performed in triplicate and cells were harvested with PBS-0.1% EDTA and counted at the end of 5 or 10 days. Cell counts represent 0.2% of the total cells per well in each case. Anti-EGF receptor monoclonal antibody (35) was added daily at 10 μg/ml/day, without concomitant media changes. Control nonspecific antibody (mouse IgG, Jackson Immuno Research Labs, Westgrove, PA) was added in the same fashion. One × 10^{-8} M 17β-estradiol (E_2) was added for estrogen treatment while the remainder of the wells received vehicle, ethanol 0.1% (vol/vol) alone. Values represent mean ± SD from three of the four separate experiments performed with this antibody.

[a] $P < 0.05$ using Student's t test to compare estrogen treated to untreated controls.

[b] $P < 0.05$ using Student's t test to compare estrogen treated to untreated nonspecific mouse IgG.

[c] $P < 0.05$ using Student's t test to compare combined estrogen plus nonspecific IgG treatments to combined estrogen plus anti-EGFR treatment.

[d] $P < 0.05$ using Student's t test to compare estrogen treated without antibody to estrogen treated combined with EGFR treatment.

ZR-75-1 cells were lower than those observed in the MDA-MB-231 cells, an estrogen unresponsive cell line. In the MDA-MB-231 cell line, the 4.8 kb mRNA was readily identified and the 1.6 kb message faintly detected in MCF-7 cells was also observed. Expression of TGFα mRNA by the Hs578T cell line was not detectable after Northern analysis with the TGFα riboprobe. Thus, levels of TGFα mRNA correlated well with levels of secreted biologically active TGFα previously reported. It is of interest that the estrogen-receptor negative Hs578T cell line is a carcinosarcoma, and thus has a histology (and possibly origin) distinct from the other epithelial breast cancer cell lines studied (37).

Range of Expression of TGFα mRNA in Primary Human Breast Tumors

Since TGFα mRNA is produced in varying levels by several breast cancer cell lines, we sought to learn whether similar variations could be found in primary

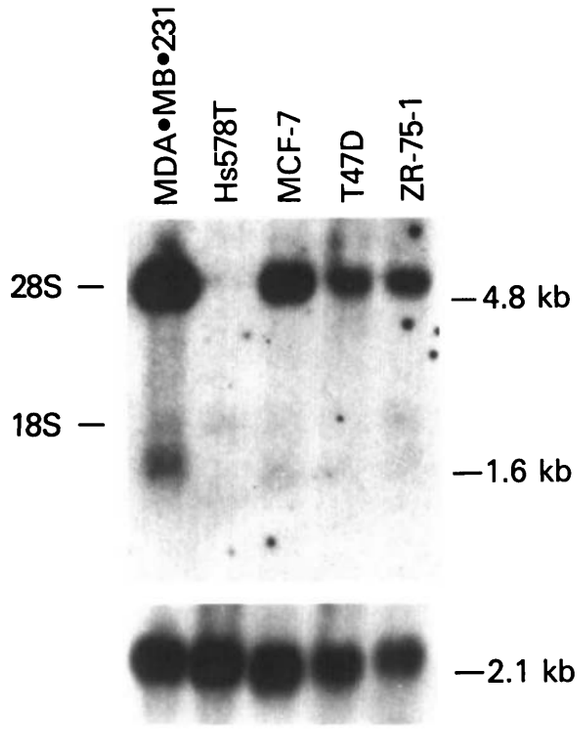

Fig. 6. Expression of TGFα mRNA in Human Breast Cancer Cell Lines

Four micrograms of poly(A)$^+$ selected RNA from five breast cancer cell lines, MDA-MB-231, Hs578T, MCF-7, T47D, and ZR-75-1 are shown. RNA from the three estrogen-responsive cell lines, MCF-7, T47D, and ZR-75-1 was made from cells grown in the presence of the estrogen contained in fetal calf serum. The TGFα riboprobe was used for hybridization. The striking differences in TGFα expression were specific, as confirmed in three experiments and by control hybridization to β-actin.

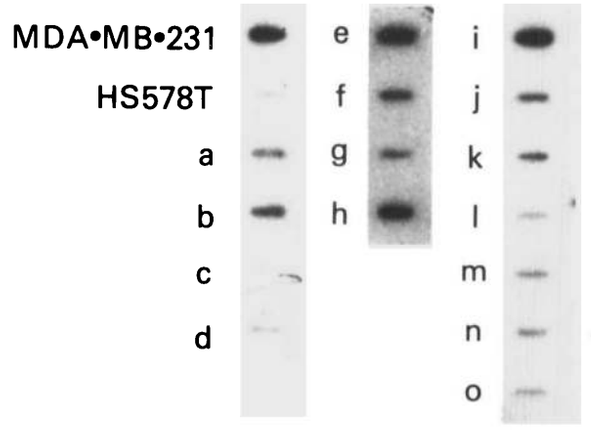

Fig. 7. Expression of TGFα mRNA in Human Breast Tumors

All breast tumors were analyzed twice by both slot blot and Northern analysis. Slot blot analysis of 10 μg total RNA hybridized to the TGFα riboprobe from a representative group of the human breast tumors is shown here. Patients a–h are estrogen-receptor positive, while i–o are estrogen-receptor negative. The Hs578T cell line is used as a negative control in this experiment, since poly(A) enriched mRNA reveals no hybridization signal by Northern analysis.

human breast tumors and whether such variations could be correlated with estrogen receptor status. Both total and poly(A)$^+$ selected mRNA from 40 human breast tumors were examined. RNA from the Hs578T cell line was included as a negative control. Fifteen representative samples of the 40 examined on slot blots are seen in Fig. 7. The first two columns (samples a–h) include estrogen-receptor positive tumors, while samples from estrogen receptor negative tumors are shown in the third column (samples i–o). For comparison, a Northern blot including nine of these is shown in Fig. 8. Total RNA contained detectable levels of TGFα mRNA in 70% of the 40 samples, and a few tumors showed very high TGFα mRNA levels. As shown in Table 3, there was no correlation between the estrogen or progesterone receptor status of the tumor and TGFα mRNA expression.

Northern analysis of poly(A)$^+$ selected mRNA from 10 human breast tumors demonstrates the expression of the expected 4.8 kb TGFα mRNA in seven of these tumors (Fig. 9). The 1.6 kb TGFα mRNA species observed in the breast cancer cell lines is also seen in the two tumors expressing the most TGFα mRNA. By densitometric scanning, the 1.6 kb TGFα mRNA was 4- to 10-fold less intense than the 4.8 kb TGFα mRNA. These results confirm broad expression of TGFα mRNA in human tumors, show that expression is not directly related to hormone-receptor content, and demonstrate that the previously unreported 1.6 kb TGFα mRNA is present in human tumor tissue.

DISCUSSION

We have studied the expression of TGFα mRNA in human breast cancer cell lines and in primary human breast tumors. These studies demonstrate that TGFα mRNA is commonly found in human breast cancer. Although its role as a mitogenic growth factor capable of inducing cellular proliferation in monolayer culture is generally accepted, its role in generating or maintaining the malignant phenotype in human cancer is entirely unknown.

That TGFα may play an important role in the generation or maintenance of the malignant phenotype of breast cancer is suggested by its regulation by estrogen. While estrogen promotes the growth of MCF-7 cells *in vitro* and is required for tumor formation *in vivo*, the mechanism by which estrogen acts has never been fully understood. We and others have shown that estrogen regulates the level of a number of mRNA species and proteins, including the pS2 mRNA (38), the mitogenic 52 kDa protein (39), a 24 kDa protein (40), an IGF-I-related protein (41), a 39 kDa protein (42), and TGFα (43). For none of these has a physiological role in breast cancer *in vivo* been clearly elucidated, but all

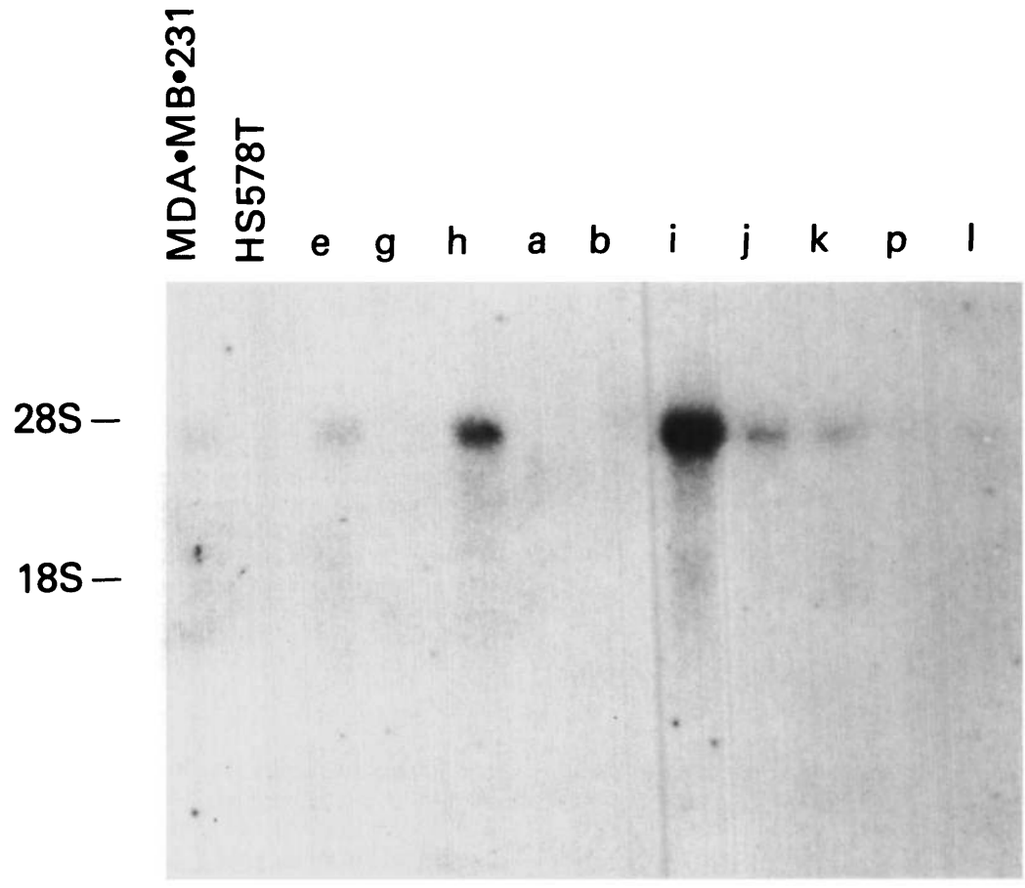

Fig. 8. Northern Analysis of 8 μg Total RNA Hybridized to the TGFα Riboprobe from Human Breast Tumors

Tumors from patients e, g, h, a, b, i, j, k, and l, shown in Fig. 8, are also seen here. The five patients on the *left*, e, g, h, a, and b, had estrogen-receptor positive tumors, and the five on the right, i, j, k, p, and l, estrogen-receptor negative tumors. Control β-actin hybridization confirmed comparable loading.

have been proposed as intermediaries of estrogen action.

Estrogen regulates the secretion of TGFα in human breast cancer cells. This regulation occurs, at least in part, by regulation of mRNA levels. An increased level of 4.8 kb TGFα mRNA is seen shortly after addition of physiological concentrations of estrogen to breast cancer cell lines deprived of estrogen. Evidence that this estrogen regulation of TGFα occurring *in vitro* may occur *in vivo* is provided by demonstration that withdrawal of estrogen from MCF-7 xenografts results in a decline in TGFα mRNA levels. Similar *in vitro* and *in vivo* demonstrations of TGFα regulation have been previously reported in primary estrogen-dependent carcinogen-induced rat mammary tumors (44).

Because breast cancer cells secrete TGFα, respond to TGFα or EGF, and contain EGF/TGFα receptors, an autocrine hypothesis can be proposed in which the cell secretes TGFα, which acts through its own cell surface receptor to stimulate growth (29). Partial confirmation of this postulated mechanism has been achieved by the inhibition of anchorage-independent MCF-7 colony formation with anti-TGFα antibodies, and the reversal of estrogen-stimulated anchorage-dependent MCF-7 cell growth by anti-EGF receptor antibodies. Thus, inhibition of cell growth can be achieved by addition of antibodies against either the ligand or its receptor. Such inhibition suggests interruption of an autocrine loop important for the growth of these cells. Because the growth inhibition is incomplete, involvement of other growth factors in the proliferation of these cells is implied. Estrogen regulation of TGFα production in estrogen-responsive breast cancer suggests that hormonal control can be exerted on the autocrine regulation of cellular growth.

In Northern blots, the previously described 4.8 kb TGFα mRNA is seen (31–33) as well as hybridization with a novel 1.6 kb species. The significance of the 1.6 kb mRNA is unknown. Both species are also seen in poly(A)$^+$ enriched RNA from human primary breast cancers. The expression of these 4.8 and 1.6 kb mRNAs are associated with the presence of a high mol wt form of secreted TGFα in the MCF-7 cell line. Although multiple intermediate-sized TGFα polypeptide species have been described as processing products derived from a common glycosylated and palmitoylated high mol wt precursor of 18 k encoded by the cloned human TGFα gene (45), none of these species approximates the multiple 30 k apparent mol wt TGFα species de-

scribed in these studies. The structures of both this high mol wt form of TGFα and the 1.6 kb mRNA are subjects requiring further study.

TGFα mRNA is detectable in approximately 70% of primary human breast cancers, and is present in exceedingly high levels in some of these. Our studies with several human breast cancer cell lines suggest that a correlation exists between the levels of TGFα mRNA and the concentration of TGFα released into the conditioned medium. Similarly, in the estrogen induction studies the degree of enhancement of TGFα mRNA is well correlated with observed alterations in TGFα protein levels. By inference, a majority of human primary tumors can be expected to synthesize TGFα protein. A previous study using detergent extracts of 22 primary breast carcinomas for RIA found detectable levels of TGFα in all samples assayed (3). Highest levels were found in estrogen receptor positive tumors, although the number analyzed was too few to be statistically significant. Expression of TGFα mRNA did not show any correlation with the presence of estrogen or progesterone receptor in the 40 tumors examined in the present study. However, a previous analysis of a more limited sample of 15 human breast tumors had noted that 75% of estrogen receptor and progesterone receptor positive tumors contained TGFα mRNA while only 29% of the TGFα mRNA negative tumors were of this phenotype (46). In contrast to these findings in human tumors, carcinogen-induced estrogen-independent transplantable rat mammary tumors are reported to contain no detectable TGFα mRNA (44). Whether this difference is due to the human *vs.* rodent system or to some other selection bias, is unknown. The variability of TGFα expression implies that there can be differences in responsiveness or sensitivity to autocrine reg-

Table 3. Correlation of the Expression of TGFα mRNA in Primary Human Breast Tumors with Estrogen and Progesterone Receptor Status

	No. of Samples	TGFα mRNA + (%)	TGFα mRNA − (%)
ER+ PR+	15	10 (67)	5 (33)
ER+ PR−	1	1	0
ER− PR+	3	2	1
ER− PR−	15	11 (73)	4 (27)
ER+/− PR+	1	1	0
ER+/− PR−	1	1	0
	36	26 (72)	10 (28)

Estrogen receptor (ER) and progesterone receptor (PR) data were available in 36 of the 40 primary human breast tumors analyzed for TGFα mRNA expression. Samples were scored as positive if they contained levels of TGFα mRNA by slot blot analysis above that of the negative control, Hs578T. This determination was made using the area under the peak seen by densitometric scanning ER and PR values obtained from patient medical records were defined as positive if greater than 10 fm/mg cytosolic protein.

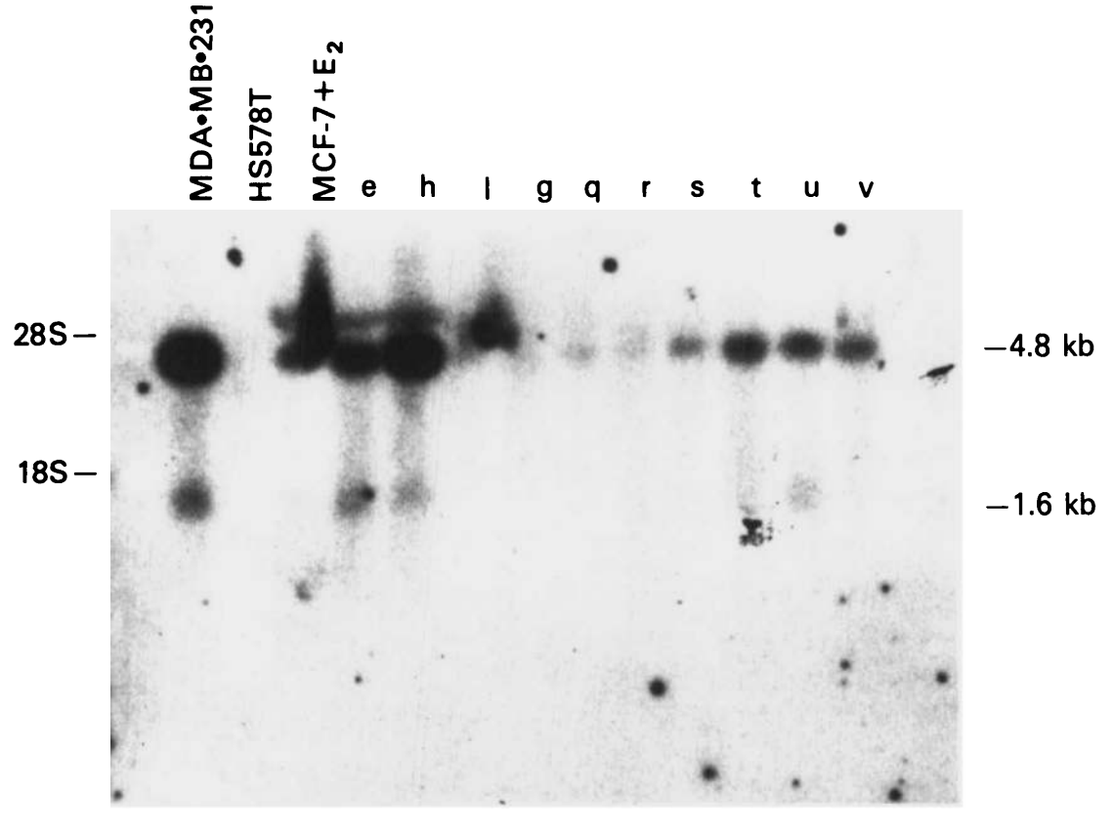

Fig. 9. Northern Analysis of 4 μg Poly(A)$^+$ Enriched RNA from Human Breast Tumors, Hybridized to the TGFα Riboprobe Tumors from patients e, h, l, and g, in Figs. 8 and 9, are seen here.

ulation and involvement of other pathways of growth regulation among human breast cancers. However, the presence of TGFα as a mitogenic polypeptide, its regulation by estrogen in hormone-dependent systems, and its frequent expression in malignant breast tissue suggest that TGFα is playing a role in either the genesis or maintenance of human breast cancer.

MATERIALS AND METHODS

Cell Lines

Cell lines from the following sources were used: MCF-7 (80th passage), originally obtained from the Michigan Cancer Foundation (47); T47D, MDA-MB-231, and NRK clone 49F fibroblasts, obtained from the American Type Culture Collection (Rockville, MD); ZR-75-1 (48); Hs578T, kindly provided by Helene Smith (37); and A431 cells, kindly provided by Ira Pastan. All cell lines were propagated in T-175 flasks in improved modified Eagle's medium (IMEM) (GIBCO, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS) (GIBCO). For experiments involving estrogen induction, cells were passaged for 1 week in IMEM supplemented with 5% calf serum (GIBCO), which had been treated with dextran-coated charcoal and sulfatase (Sigma, St. Louis, MO) to remove endogenous estrogens (CCS) (49). Subsequently cells were plated in 5% CCS in IMEM lacking the pH indicator phenol red, which is a weak estrogen (50). For determination of estrogen effects, pairs of flasks were treated with 10^{-8} M 17β-estradiol or the vehicle, 0.1% ethanol.

Tumors

Primary human breast tumors from 40 female patients were dissected from surgical specimens, frozen in dry ice, and stored at −70 C until use.

Conditioned Medium Collections

Conditioned medium collections were carried out as previously described (2). The medium was concentrated 100-fold in an Amicon ultrafiltration cell (YM 5 membrane) (Amicon, Danvers, MA) and used directly in the NRK colony forming assay which was performed as previously described (2). For radioreceptor studies, the medium was acidified with 4% (vol/vol) acetic acid after addition of 10^{-7} M pepstatin, passed through a Waters SEP-PAK C-18 reverse phase cartridge, and washed with 4% acetic acid. The EGF-receptor competing material was quantitively eluted with 50% acetonitrile (Fisher Scientific, Pittsburgh, PA) (3).

EGF RRAs

A431 membranes were prepared according to the method of Kimball and Warren (51). The cells were disrupted under nitrogen, and the nuclei and organelles pelleted by low speed centrifugation. The membranes were then pelleted by centrifugation at 35,000 rpm for 1 h and resuspended in 20 mM HEPES buffer. Membranes were plated into 96-well plates and allowed to dry overnight at 37 C before use. Standard binding competition studies were performed using [^{125}I]EGF (SA, 100 μCi/μg; 50,000 cpm/assay). A standard curve of 0.2–50 ng unlabeled mouse (receptor grade, Collaborative Research, Waltham, MA) EGF was constructed. The SEP-PAK acetonitrile fractions were lyophilized and reconstituted in 4 mM HCl (0.5 ml/50 ml conditioned media). After incubation of the labeled EGF and 100-μl samples for 2 h at 21 C in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) containing 50 mM HEPES, 0.1% BSA, the wells were washed, cut from the plate, and counted in a γ-spectrometer. EGF competing activity was computed using a Hewlett-Packard RIA program (52).

RIA

RIAs were performed using polyclonal rabbit antirat TGFα antibody and ^{125}I-rat TGFα (Biotope, Inc., Seattle, WA), according to manufacturer's instructions. The antibody used was raised against a 17-amino acid synthetic peptide from the COOH-terminus of mature rat TGFα (53).

Metabolic Labeling and Immunoprecipitation of ^{35}S-Labeled Conditioned Medium

Confluent 100-mm culture dishes of MCF-7 cells were washed with serum-free DMEM and incubated for 2 hours in cysteine-free DMEM containing 10 μg/ml each of bovine pancreatic insulin and human transferrin with 1% dialyzed FBS. Cells were labeled 18 h with 100 μCi/ml ^{35}S-cysteine (Amersham Corp., Arlington Heights, IL; 1250 Ci/mmol). The labeled medium was then collected, centrifuged at 10,000 rpm, and chromatographed over a Sephadex G-25 column in distilled water to remove unincorporated ^{35}S-cysteine. Fractions containing radioactive protein were lyophilized and reconstituted in 1 ml radioimmunoprecipitation (RIPA) buffer containing 1% Triton X-100, 0.5% deoxycholate, 100 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsufonyl fluoride (Sigma), 50 μg/ml leupeptin, and 50 μg/ml aprotinin in 10 mM Na(P04), pH 8.0. After reduction with 40 mM dithiothreitol, 400 μl reconstituted labeled conditioned medium was incubated in the absence or presence of 250 ng human TGFα with 50 μl polyclonal goat antihuman TGFα IgG (Biotope Inc.) covalently coupled to Sepharose 4B at a concentration of 5 mg IgG/ml gel for 48 h at 4 C. The immunoprecipitates were washed five times in RIPA buffer, and the bound material was eluted from the antibody-Sepharose matrix by incubation with 1 ml 1 N acetic acid for 30 min at 60 C. The eluted radioactivity was lyophilized, reconstituted in sample buffer containing SDS, and run on a 15% discontinuous SDS-polyacrylamide gel. The synthetic TGFα used in competition was prepared as previously described (54).

MCF-7 Tumor Formation in Nude Mice

Two × 10^7 MCF-7 cells were injected sc into twenty 5-week-old nude Balb/C mice with implanted 10 mg 17β-estradiol pellets. After 3 weeks, tumors were harvested from control animals; and then 18 h, 3 days, and 10 days after removal of the estrogen pellets from the experimental animals. Tumors were stored at −70 C before RNA extraction.

MCF-7 Cloning and Monolayer Growth Experiments

Two × 10^4 MCF-7 cells were plated in DMEM containing 0.4% Bacto-agar (Difco, Detroit, MI) and 10% FBS over a bottom layer containing 0.8% agar in 35-mm tissue culture dishes (Costar, Cambridge, MA). Purified polyclonal rabbit antihuman TGFα IgG which was isolated by protein A-sepharose chromatography of rabbit antiserum raised against synthetic human TGFα (54) was used in varying dilutions to test for inhibition of MCF-7 colony formation in agar. Colonies were incubated for 14 days, then stained with nitrobluetetrazolium (NBT), and counted. The antibody is specific for human TGFα, and does not recognize mouse or human EGF (Tam, J., and C. K. Chang, unpublished data). Purified rabbit IgG (Cappel Laboratory) was used as a control. Recombinant human TGFα (31) used for antibody neutralization was a gift from Rik Derynck (Genentech, Inc.).

For monolayer growth studies of estrogen-induced MCF-7 proliferation, purified mouse monoclonal antihuman EGF re-

ceptor antibody was used (35). This antibody has been previously characterized to be a pure antagonist of the EGF receptor; it blocks EGF-stimulated proliferation of human fibroblasts in monolayer. Details of the procedure used in blockade of estrogen-induced MCF-7 growth with this antibody are in the footnote of Table 2. Data representative of four separate experiments are shown.

TGFα Hybridization Probes

A 1.3 kb cDNA probe encoding the precursor for mature human TGFα (31) ligated into the plasmid SP65, a gift from Rik Derynck, was used to make a synthetic riboprobe with SP6 polymerase (Promega Biotech, Madison, WI). Alternatively, the human TGFα cDNA insert was obtained by restriction digest of plasmid pTGF-CI with *Eco*RI, agarose gel purified, and used for nick translation. A 1.7 kb β-actin cDNA cloned into pGEM3 was used to make a riboprobe with SP6 polymerase (55).

RNA Extraction, Electrophoresis, and Filter Preparation

Total cellular RNA was extracted from cells by homogenizing in guanidine isothiocyanate followed by centrifugation over a cesium chloride cushion. RNA from tumor samples was extracted after pulverization of frozen samples and homogenization in guanidine isothiocyanate. Poly(A)$^+$ mRNA was eluted in 10 mM Tris after passing total cellular RNA over an oligo(DT) cellulose (Pharmacia, Piscataway, NJ) column equilibrated with 10 mM Tris-0.5 M NaCl, pH 8.0. After precipitation in ethanol (66% vol/vol) and 0.1 M NaAc, both total and poly(A)$^+$ selected RNA were resuspended in 10 mM Tris-1 mM EDTA buffer, and separated on 1% agarose, 6% formaldehyde gels. Electrophoresis was carried out at 20 V over 14–16 h in 5 mM NaAc, 1 mM EDTA, 20 mM 3-[N-Morpholino]propanesulfonic acid (MOPS) (Sigma), pH 7.0. Gels were stained with 2 μg/ml ethidium bromide to allow inspection of the quantity and quality of RNA. Only gels containing comparably loaded RNA samples of the highest quality were used for Northern transfer. RNA gels were subjected to partial alkaline hydrolysis in 50 mM NaOH, 10 mM NaCl, followed by neutralization with 0.1 M Tris HCl (pH 7.5) and equilibration with 10× SSC (1.5 M NaCl, 0.15 M trisodium citrate). Northern transfer of RNA to nitrocellulose was by capillary blot. Nitrocellulose filters used for slot blot preparation were presoaked in 10× SSC. All filters were then baked for 2 h at 80 C under vacuum.

Hybridizations

Synthetic riboprobe was prepared by SP6 polymerase transcription using uridine 5′-triphosphate, tetra (triethylammonium) salt [α-^{32}P] (SA, 3000 Ci/mmol) (New England Nuclear, Boston, MA). The DNA template was linearized with *Hind*III. Filters were prehybridized for 4 h at 55 C in 50% formamide (Fluka, Buchs, Switzerland) 5× Denhardt's, 5× SSC, 0.1% SDS, and 200 μg/ml salmon sperm DNA (Sigma), and hybridized for 16–18 h in the same buffer with 2 × 10^6 cpm/ml labeled riboprobe. Filters were washed twice for 30 min with 1× SSC, 0.1% SDS heated to 68 C, and twice for 30 min with 0.1× SSC, 0.1% SDS in a 70 C water bath. After hybridization, autoradiography was performed using Kodak XAR-5 film exposed for 2–4 days at −70 C.

For cDNA insert probes, deoxycytidine 5′ triphosphate, tetra (triethylammonium) salt, [α^{32}P] (SA, 3000 Ci/mmol) (New England Nuclear) was incorporated into the human TGFα cDNA probe pTGF-CI using the nick translation kit from New England Nuclear. Prehybridization and hybridization were performed for 24 h at 42 C in the same buffers as for the riboprobe labeling with 1 × 10^6 cpm/ml nick translated DNA.

In addition to ethidium bromide staining of gels to ensure comparable loading, filters were routinely hybridized to a β-actin probe to demonstrate equivalent levels of mRNA. In some of the estrogen-induction experiments where ethidium bromide staining clearly demonstrated comparability of loading, β-actin hybridization demonstrated variability. Alterations were felt to be due to limited regulation of β-actin message by estrogen and/or cell growth. Densitometric analysis was performed to quantify differences in levels of TGFα mRNA.

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Order of first two authors is to be considered arbitrary.

REFERENCES

1. Lippman ME 1985 Endocrine responsive cancers of man. In: Williams RH (ed) Textbook of Endocrinology. WB Saunders Co, Philadelphia, pp 355–411
2. Bates S, McManaway ME, Lippman ME, Dickson RB 1986 Characterization of estrogen responsive transforming activity in human breast cancer cell lines. Cancer Res 46:1707–1713
3. Perroteau I, Salomon D, DeBortoli M, Kidwell W, Hazarika P, Pardue R, Dedman J, Tam J 1986 Immunological detection and quantitation of alpha transforming growth factors in human breast carcinoma cells. Breast Cancer Res Treat 7:201–210
4. DeLarco JE, Todaro GJ 1978 Growth factors from murine sarcoma virus-transformed cells. Proc Natl Acad Sci USA 75:4001–4005
5. Anzano MA, Roberts AB, Smith JM, Sporn MB, DeLarco JE 1983 Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type α and type β transforming growth factors. Proc Natl Acad Sci USA 80:6264–6268
6. Marquardt H, Todaro GJ 1982 Human transforming growth factor. Production by a melanoma cell line, purification, and initial characterization. J Biol Chem 257:5220–5225
7. Nickell KA, Halper J, Moses HL 1983 Transforming growth factors in solid human malignant neoplasms. Cancer Res 43:1966–1971
8. Salomon DS, Zwiebel JA, Bano M, Losonczy I, Fehnel P, Kidwell WR 1984 Presence of transforming growth factors in human breast cancer cells. Cancer Res 44:4069–4077
9. Luetteke NC, Michalopoulos GK 1985 Partial purification and characterization of a hepatocyte growth factor produced by rat hepatocellular carcinoma cells. Cancer Res 45:6331–6337
10. Coffey RJ Jr, Shipley GD, Moses HL 1986 Production of transforming growth factors by human colon cancer lines. Cancer Res 46:1164–1169
11. Roberts AB, Lamb LC, Newton DL, Sporn MB, DeLarco JE, Todaro GJ 1980 Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. Proc Natl Acad Sci USA 77:3494–3498
12. Marquardt H, Hunkapiller MW, Hood LE, Twardzik DR, DeLarco JE, Stephenson JR, Todaro GJ 1983 Transforming growth factors produced by retrovirus-transformed rodent fibroblasts and human melanoma cells: amino acid

sequence homology with epidermal growth factor. Proc Natl Acad Sci USA 80:4684–4688
13. Marquardt H, Hunkapiller MW, Hood LE, Todaro GJ 1984 Rat transforming growth factor type I: structure and relation to epidermal growth factor. Science 223:1079–1083
14. DeLarco JE, Reynolds R, Carlberg K, Engle C, Todaro GJ 1980 Sarcoma growth factor from mouse sarcoma virus-transformed cells. Purification by binding and elution from epidermal growth factor receptor-rich cells. J Biol Chem 255:3685–3690
15. Schreiber AB, Winkler ME, Derynck R 1986 Transforming growth factor-α: a more potent angiogenic mediator than epidermal growth factor. Science 232:1250–1253
16. Imai Y, Leung CKH, Friesen HG, Shiu RP 1982 Epidermal growth factor receptors and effect of epidermal growth factor on growth of human breast cancer cells in long-term tissue culture. Cancer Res 42:4394–4398
17. Todaro GJ, Fryling C, DeLarco JE 1980 Transforming growth factors produced by certain human tumor cells: polypeptides that interact with epidermal growth factor receptors. Proc Natl Acad Sci USA 77:5258–5262
18. Hanauske AR, Pardue R, Von Hoff DD 1986 Assessment of biological activity of linear α-transforming growth factor and monospecific antibodies to α-transforming growth factor and linear α-transforming growth factor. Cancer Res 46:5567–5570
19. Dickson RB, Kasid A, Huff KK, Bates SE, Knabbe C, Bronzert D, Gelmann EP, Lippman ME 1987 Activation of growth factor secretion in tumorigenic states of breast cancer induced by 17 β-estradiol or v-ras^H oncogene. Proc Natl Acad Sci USA 84:837–841
20. Osborne CK, Hamilton B, Titus G, Livingston RB 1980 Epidermal growth factor stimulation of human breast cancer cells in culture. Cancer Res 40:2361–2366
21. Fitzpatrick SL, LaChance MP, Schultz GS 1984 Characterization of epidermal growth factor receptor and action on human breast cancer cells in culture. Cancer Res 44:3442–3447
22. Davidson NE, Gelmann EP, Lippman ME, Dickson RB 1987 EGF receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. Mol Endocrinol 1:216–223
23. Dickson RB, McManaway ME, Lippman ME 1986 Estrogen-induced factors of breast cancer cells partially replace estrogen to promote tumor growth. Science 232:1540–1543
24. Kurachi H, Okamoto S, Oka T 1985 Evidence for the involvement of the submandibular gland epidermal growth factor in mouse mammary tumorigenesis. Proc Natl Acad Sci USA 82:5940–5943
25. Osborne CK, Hamilton B, Nover M 1982 Receptor binding and processing of epidermal growth factor by human breast cancer cells. J Clin Endocrinol Metab 55:86–93
26. Perez R, Pascual M, Macias A, Lage A 1984 Epidermal growth factor receptors in human breast cancer. Breast Cancer Res Treat 4:189–193
27. Fitzpatrick SL, Brightwell J, Wittliff JL, Barrows GH, Schultz GS 1984 Epidermal growth factor binding by breast tumor biopsies and relationship to estrogen receptor and progestin receptor levels. Cancer Res 44:3448–3453
28. Sainsbury JRC, Sherbert GV, Farndon JR, Harris AL 1985 Epidermal-growth-factor receptors and oestrogen receptors in human breast cancer. Lancet i:364–366
29. Sporn MB, Todaro GJ 1980 Autocrine secretion and malignant transformation of cells. N Engl J Med 303:878–880
30. Ignotz RA, Kelly B, Davis RJ, Massague J 1986 Biologically active precursor for transforming growth factor type α released by retrovirally transformed cells. Proc Natl Acad Sci USA 83:6307–6311
31. Derynck R, Roberts AB, Winkler ME, Chen EY, Goeddel DV 1984 Human transforming growth factor-α: precursor structure and expression in *E. coli.* Cell 38:287–297
32. Derynck R, Roberts AB, Eaton DH, Winkler ME, Goeddel DV 1985 Human transforming growth factor-α: precursor sequence, gene structure, and heterologous expression. In: Growth Factors and Transformation. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 79–86
33. Derynck R, Goeddel DV, Ullrich A, Gutterman JU, Williams RD, Bringman TS, Berger WH 1987 Synthesis of messenger RNAs for transforming growth factors and the epidermal growth factor receptor by human tumors. Cancer Res 47:707–712
34. Carpenter G, Stoscheck CM, Preston YA, DeLarco JE 1983 Antibodies to the epidermal growth factor receptor block the biological activities of sarcoma growth factor. Proc Natl Acad Sci USA 80:5627–5630
35. Kudlow JE, Khoravi MJ, Kobrin MS, Mak 1984 Inability of anti-epidermal growth factor receptor monoclonal antibody to block "autocrine" growth stimulation in transforming growth factor-secreting melanoma cells. J Biol Chem 259:11895–11900
36. Masui H, Kawamoto T, Sato JD, Wolf B, Sato G, Mendelsohn J 1984 Growth inhibition of human tumor cells in athymic mice by anti-epidermal growth factor receptor monoclonal antibodies. Cancer Res 44:1002–1007
37. Hackett AJ, Smith HS, Springer EL, Owens RB, Nelson-Rees WA, Riggs JL, Gardner MB 1977 Two syngeneic cell lines from human breast tissue: the aneuploid mammary epithelial (Hs578T) and the diploid myoepithelial (Hs578Bst) cell lines. J Natl Cancer Inst 58:1795–1806
38. Jakowlew SB, Breathnach R, Jeltsch JM, Masiakowski P, Chambon P 1984 Sequence of the pS2 mRNA induced by estrogen in the human breast cancer cell line MCF-7. Nucleic Acids Res 12:2861–2878
39. Vignon F, Capony F, Chambon M, Freiss G, Garcia M, Rochefort H 1986 Autocrine growth stimulation of the MCF-7 breast cancer cells by the estrogen-regulated 52K protein. Endocrinology 118:1537–1545
40. Edwards DP, Adams DJ, Savage N, McGuire WL 1980 Estrogen induced synthesis of specific proteins in human breast cancer cells. Biochem Biophys Res Commun 93:804–812
41. Huff KK, Knabbe C, Linsey R, Kaufman D, Bronzert D, Lippman ME, Dickson RB, Multihormonal regulation of Insulin-like growth factor-I-related protein in MCF-7 human breast cancer cells. Mol Endocrinol, in press
42. Bronzert D, Silverman S, Lippman M 1987 Estrogen inhibition and antiestrogen induction of a 39,000 MW glycoprotein secreted by human breast cancer cells. Cancer Res 47:1234–1238
43. Knabbe C, Lippman ME, Wakefield LM, Flanders KC, Kasid A, Derynck R, Dickson RB 1987 Evidence that transforming growth factor β is a hormonally regulated negative growth factor in human breast cancer cells. Cell 48:417–428
44. Liu SC, Sanfilippo B, Perroteau I, Derynck R, Salomon DS, Kidwell WR 1987 Expression of transforming growth factor α (TGFα) in differentiated rat mammary tumors: estrogen induction of TGFα production. Mol Endocrinol 1:683–692
45. Bringman TS, Lindquist PB, Derynck R 1987 Different transforming growth factor α-species are derived from a glycosylated and palmitoylated transmembrane precursor. Cell 48:429–440
46. Salomon DS, Kidwell WR, Liu H, Kim N, Callahan R, Theillet C, Lidereau R, Derynck R 1986 Breast Cancer Res Treat 8:106
47. Soule HD, Vazquez J, Long A, Albert S, Brennan M 1973 A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 51:1409–1416
48. Allegra JC, Lippman ME 1980 The effects of 17β-estradiol and tamoxifen on the ZR-75-1 human breast cancer cell line in defined medium. Eur J Cancer Clin Oncol 16:1007–1015
49. Darbre P, Yates J, Curtis S, King RJB 1983. Effect of

estradiol on human breast cancer cells in culture. Cancer Res 43:349–354
50. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS 1986 Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci USA 83:2496–2500
51. Kimball ES, Warren TC 1984 Epidermal growth factor (EGF) binding to membranes immobilized in microtiter wells and estimation of EGF-related transforming growth factor activity. Biochim Biophys Acta 771:82–88
52. Aitken SC, Lippman ME 1977 A simple computer program for quantitation and scatchard analysis of steroid receptor proteins. J Steroid Biochem 8:77–94
53. Linsley PS, Hargreaves WR, Twardzik DR, Todaro GJ 1985 Detection of larger polypeptides structurally and functionally related to type I transforming growth factor. Proc Natl Acad Sci 82:356–360
54. Tam JP, Sheikh MA, Salomon DS, Ossowski L 1986 Efficient synthesis of human type α transforming growth factor: its physical and biological characterization. Proc Natl Acad Sci USA 83:8082–8086
55. Ponte P, Ng SY, Engel J, Gunning P, Kedes L 1984 Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA. Nucleic Acids Res 12:1687–1696