Journal of Cellular Biochemistry 41:159–170 (1989)

Modulation of Transforming Growth Factor Alpha-Dependent Expression of Epidermal Growth Factor Receptor Gene by Transforming Growth Factor Beta, Triiodothyronine, and Retinoic Acid

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We have investigated the actions of transforming growth factor (TGF) type α on epidermal growth factor (EGF) receptor mRNA expression in MDA-468 human mammary carcinoma cells in serum-free media. We found that exposure of MDA-468 cells to TGFα results in elevated levels of EGF receptor mRNA. This increase in mRNA accumulation showed time and dose dependence. Addition of TGFβ1 enhanced the accumulation of EGF receptor mRNA induced by TGFα in a time- and dose-dependent manner. We also found that triiodothyronine at physiological concentrations exerts synergistic control on the action of TGFα alone, or in association with TGFβ1, on EGF receptor mRNA expression. Similarly, retinoic acid treatment also enhanced in a time- and dose-dependent manner the TGFα-dependent response of EGF receptor mRNA and acted synergistically with TGFβ1. The results described here suggest that optimum regulation of EGF receptor gene expression by TGFα is a complex process involving synergistic interactions with heterologous growth factors and hormones.

Key words: oncogenes, vitamin A, thyroid hormones, mammary cells, cancer, epithelial cells

EGF has potent effects on mammalian cell growth and acts via a specific transmembrane receptor to stimulate or inhibit growth [1,2]. The EGFR transmembrane and kinase domains are homologous to the viral oncogene erb B [1]. These observations and others [3] indicate that the EGF receptor can function as an oncogene. Thus, the level of EGFR gene expression must be accurately regulated in normal cells and is different in a number of transformed cells [1]. It has been reported that EGF controls the expression of the EGFR gene in various cell types [4–6]. However,

Abbreviations used: DME, Dulbecco-Vogt modified Eagle's medium; EGF, epidermal growth factor; EGFR, EGF receptor; kb, kilobase; RA, retinoic acid; TGF, transforming growth factor; T_3, triiodothyronine.

Received March 17, 1989; accepted June 27, 1989.

modulation of the expression of the EGFR gene by heterologous growth factors and hormones and their relationship to cell proliferation remains obscure.

It has been reported that many normal and tumor-derived human mammary epithelial cells produce and secrete two critical growth factors, TGFα and TGFβ [7,8]. The first factor, TGFα, is structurally and functionally related to EGF and binds to and operates through the EGFR [9]. The production of TGFα may be functionally important for the autocrine growth of a subset of normal and cancerous human breast cells [8,10,11]. The second factor, TGFβ, is a multifunctional agent that has a negative growth modulatory role in various epithelial cell systems [7,12,13]. One salient feature of TGFβ is that it affects the expression of a series of genes to control their expression [12,14], including the EGFR proto-oncogene [6,15]. However, little is known on the actions of TGFβ on EGFR proto-oncogene expression in human mammary cells.

Thyroid hormones are essential in the control of cell growth and differentiation [16]. Several studies have shown that T3 can modulate cellular replication [17–19] and that it plays an essential role in chemical-, radiation-, viral-, and oncogene-induced carcinogenesis [20]. Other reports imply that thyroid hormones may have a specific role in regulating the production of both EGF and EGFR [15,21,22]. It is tantalizing that the viral oncogene erb A is homologous to cellular genes that encode nuclear receptors for T3 [23,24] and that the latter genes belong to the same family as the receptors for steroid hormones and retinoic acid [25–27].

RA, an analogue of vitamin A, is of great interest since it exerts profound effects on development and differentiation and is a potent anti-carcinogenic agent [28,29]. Many studies have indicated that RA can inhibit growth, suppress the transformed phenotype, and induce differentiation of cultured carcinoma cells [28,29]. The compound is of particular interest since it has been shown that it can modulate growth factor and oncogene expression in several cell types [29,30]. Although RA has been shown to modulate EGFR concentration [29,31], activity [32] and mRNA synthesis [29], the molecular mechanisms by which the effects on replication are mediated and interaction with other polypeptide growth factors and hormones are not well understood.

In this study, we have characterized the actions of TGFα on EGFR gene expression in MDA-468 human mammary carcinoma cells. We have also investigated the actions of TGFβ1, T3, and/or RA on EGFR gene expression in TGFα-stimulated cells. The results suggest that optimum regulation of EGFR gene expression by TGFα is a complex process involving the coordinate interaction of a discrete number of heterologous growth factors and hormones.

MATERIALS AND METHODS

Cells and Culture Conditions

MDA-468 human mammary carcinoma cells [6] were routinely cultured in a humidified atmosphere (5% CO_2, 95% air) at 37°C in the presence of DME/F12 medium containing 10% calf serum [13]. For experiments, the cells were maintained in synthetic serum-free DME/F12+H+F medium [13]. The formulation of DME/F12+H+F medium was DME and Ham's F12 mixed 1:1 and supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone (50 nM), prostaglandin E_1 (25 ng/ml), selenous acid (10 nM), glutamine (4 mM), gentamicin (40 μg/ml), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mM), and bovine serum albumin (1 mg/ml).

RNA Extraction and Northern Blot Analysis

Total cellular RNA was isolated by the guanidinium isothiocyanate/cesium chloride procedure as described [6]. RNA samples were quantified by measurement of A_{260}. For RNA blot analysis, samples containing 20 μg of total RNA were fractionated on 1% agarose/formaldehyde gels [6]. Before transfer, the gels were stained with ethidium bromide (10 μg/ml), destained, and photographed under ultraviolet light in order to visualize the positions of ribosomal RNAs (28S and 18S). The RNAs were transferred to a nylon membrane (Biotrans™), essentially following the procedure of Thomas [33]. Membranes were prehybridized and hybridized with $\simeq 3.5 \times 10^7$ count/min of purified ^{32}P-labeled cDNA probes as described [6,15]. The membranes were washed [6] and autoradiographed for 1 to 4 days by using Kodak XAR-5 film with Dupont Lightening Plus intensifying screen at −70°C. The filters were hybridized to ^{32}P-labeled cDNA clones pE7 or pHER-A-64-1 [6,15]. The EGFR cDNA inserts were excised from both plasmids by appropriate restriction endonuclease digestion [6,15] and purified by electrophoresis on low melting point 1.0% agarose gels [6]. To take into account experimental variations in the amount of RNA transferred to the filters, ribosomal RNA levels were measured on the same blots as a control for total bound RNA. For estimation of ribosomal RNA content, the filters were hybridized to a nick-translated 28S ribosomal DNA probe [6]. DNA probes were radiolabeled with [^{32}P]dCTP to a specific activity greater than 1.8×10^8 cpm per μg of DNA by using a nick translation kit (New England Nuclear).

Densitometric Analysis and Other Procedures

Densitometry was performed with a microprocessor-controlled Beckman DU-70 spectrophotometer with automatic background subtraction for each lane. Subsequently, the areas of interest were digitized and the average of triplicate measurements, percent of maximum, and fold-increase were automatically calculated by utilizing SigmaScan (Jandel). Plotting and statistical analysis were done by using SigmaPlot (Jandel). Other methods utilized in this investigation were performed as described in refs. 6, 13, and 15. The results presented here were repeated with similar results in at least three independent experiments each.

Growth Factors, Hormones, Chemicals, and cDNA Probes

EGF was purchased from Biomedical Technologies (Stoughton, MA). Human TGFα was purchased from ICN Biochemicals (Cleveland, OH). Platelet-derived human TGFβ1 was obtained from R & D Systems (Minneapolis, MN); 3,5,3′-triiodo-L-thyronine and retinoic acid were purchased from Sigma (St. Louis, MO). Sources of other growth factors, hormones, chemicals, and cDNA probes were as described elsewhere [6,13,15]. All reagents were of the highest purity commercially available.

RESULTS

Time- and Concentration-Dependent Effects of TGFα on EGF Receptor mRNA Accumulation

To determine the time-dependent effect of TGFα on the expression of EGFR gene, MDA-468 cells were exposed to TGFα at 10 ng/ml for set time intervals. Samples of

RNA from each of the time points were transferred onto nylon membranes, the blot was hybridized to the pHER probe, and the signal strengths from the resulting autoradiogram were quantitated by densitometry. Figure 1A shows that no induction of EGFR mRNA was observed after 3 h of incubation with 10 ng/ml TGFα, while at 4 h, a significant increase was detected. The maximum level of accumulation (≈12-fold greater than control at time 0) was reached at ≈5–6 h (Fig. 1A). The level of EGFR mRNA declined from 8 to 16 h (data not shown). RNA blot analysis (Fig. 1B, lane 3)

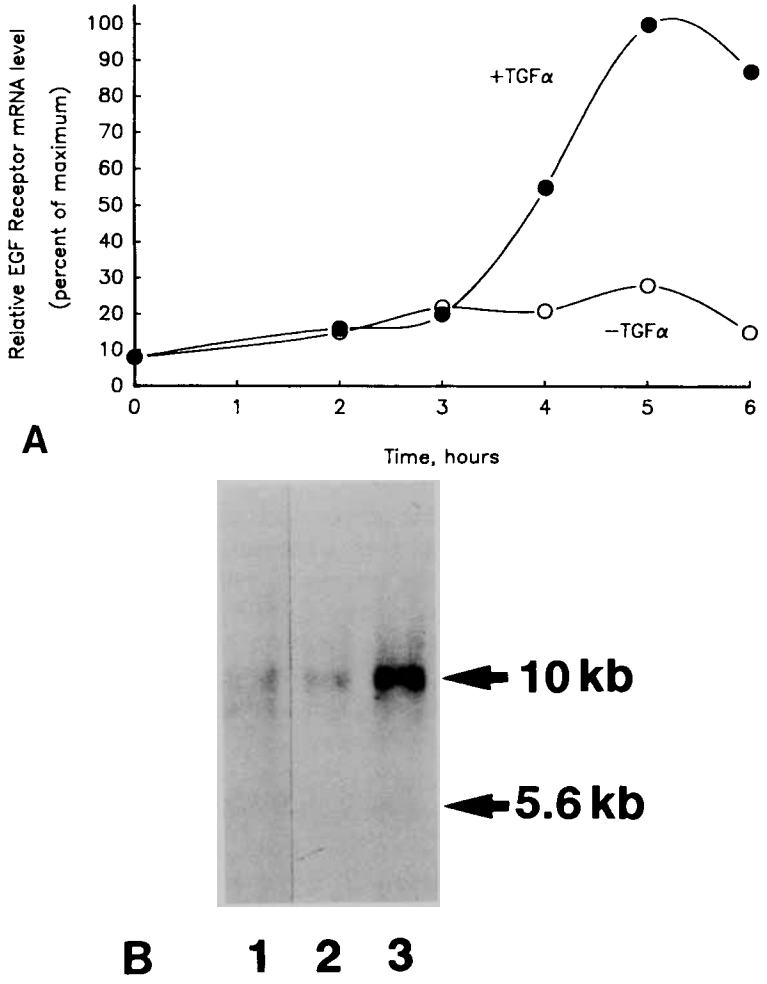

Fig. 1. **A:** Time course showing the change in EGF receptor mRNA levels after addition of TGFα. Subconfluent MDA-468 cells were maintained for 23.5 h in synthetic DME/F12+H medium. Then, the medium was replaced by the same fresh medium and 1 h later TGFα (10 ng/ml) was added. Aliquots of total RNA (20 μg per lane) were fractionated on 1% agarose gels and transferred to a nylon membrane. The transcripts were detected by hybridization with ^{32}P-labeled pHER probe and subsequent autoradiography. The resulting autoradiogram was quantified by densitometric scanning (only signals in the linear range of film sensitivity were quantified) and the relative intensity of the hybridization signal for each time point was plotted. (○), control, no additions; (●), TGFα. **B:** Autoradiograph (88-h exposure) showing the change in the levels of the 10- and 5.6-kb mRNAs encoding the EGF receptor after treatment with TGFα (10 ng/ml) for 6 h. Lane 1: No additions, time 0. Lane 2: No additions, 6 h. Lane 3: TGFα, 6 h.

showed that the rise in mRNA level is the result of accumulation of both a major 10-kb and a minor ≃5.6-kb species of mRNA.

We have also analyzed RNA obtained from MDA-468 cells treated for 5 h with increasing concentrations of TGFα. Figure 2A and B shows that the level of EGFR mRNA increased progressively with increasing concentrations of TGFα. As little as 2.5 ng/ml TGFα induced a significant increase in EGFR mRNA (Fig. 2A, lane 3, and 2B). Maximal levels of EGFR mRNA were reached at 20 ng/ml TGFα (Fig. 2A, lane 6, and 2B). A concentration of 100 ng/ml TGFα produced a significant decrease in EGFR mRNA accumulation after 5 h of treatment (data not shown).

Effect of TGFβ1 on TGFα-Stimulated EGF Receptor Gene Expression

To determine whether the TGFα-dependent expression of the EGFR gene may be modulated by TGFβ1, MDA-468 cells were treated for 5 h with increasing concentrations of TGFβ1 in the absence or presence of 10 ng/ml TGFα and the RNA was analyzed. TGFβ1 alone (5 to 100 pM) had no significant stimulatory effect on EGFR mRNA expression in the absence of TGFα. The increase in EGFR mRNA level produced by 10 ng/ml TGFα was enhanced in a dose-dependent fashion by increasing concentrations of TGFβ1 (5–50 pM). A significant enhancement of the TGFα-mediated increase in EGFR mRNA was observed with as little as 5 pM TGFβ1 in the presence of 10 ng/ml TGFα. Maximal enhancement of EGFR mRNA by TGFβ1 was observed with 25–50 pM TGFβ1 in the presence of 10 ng/ml TGFα. In the presence of 10 ng/ml TGFα, a concentration of 100 pM TGFβ1 produced a significant decrease in EGFR mRNA accumulation. These results are consistent with previous work that demonstrated that TGFβ1 enhances the EGF-mediated increase in EGFR mRNA in a time- and dose-dependent fashion in MDA-468 cells [6,15].

Modulation of EGFR mRNA Levels by the Combined Action of TGFα, TGFβ1, and T3

Because previous work from this laboratory showed that T3 regulates the expression of the EGFR gene [15], it was of interest to test whether treatment of MDA-468 cells with T3 is followed by a change in their responsiveness to TGFα and/or TGFβ1. For this purpose, MDA-468 cells were treated with or without 1 nM T_3 for 23 h. Then, the cells were stimulated with TGF α (2.5 or 20 ng/ml) and/or TGFβ1 (50 pM) for 5 h in the absence or presence of T3. The presence of 1 nM T3 enhanced the TGFα-dependent induction process in comparison to control, TGFα alone. This enhancement induced by T3 was most evident in cells treated with 20 ng/ml TGFα. Densitometry revealed that the enhancement by T3 of the TGFα (20 ng/ml)-generated increase in EGFR mRNA was ≃sixfold in comparison to control, 20 ng/ml TGFα alone. Additional experiments also showed that T3 considerably increases (≃ threefold) the TGFβ1-enhanced TGFα-dependent induction of EGFR mRNA, in comparison to control cells treated with TGFα (20 ng/ml) plus TGFβ1 (50 pM). These results are in agreement with previous results [15] and suggest that T_3 acts synergistically with TGFα and/or TGFβ1 in the EGFR mRNA induction process.

Modulation of EGFR mRNA Levels by the Combined Action of EGF(or TGFα), TGFβ1, and/or Retinoic Acid

In an attempt to determine whether the level of EGFR mRNA may be modulated by RA, MDA-468 cells were pretreated with 1 nM RA for 20 h. Then, the cells were

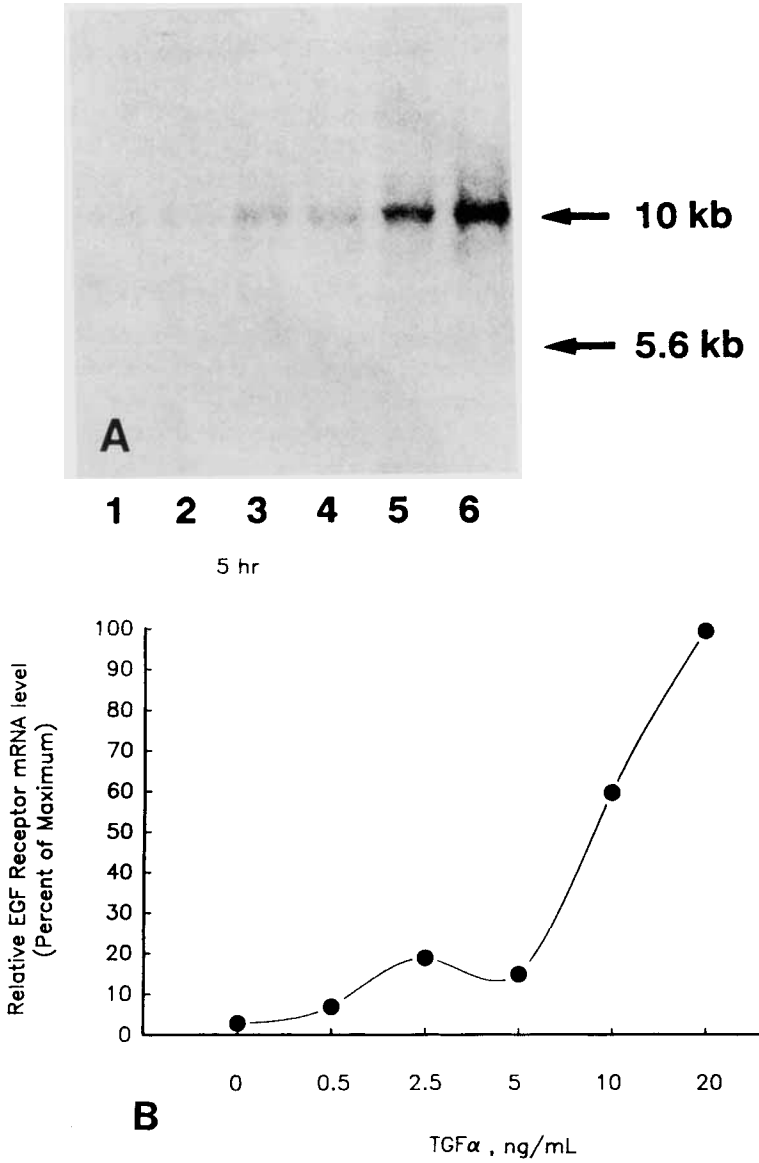

Fig. 2. Enhancement of EGF receptor mRNA levels by different concentrations of TGFα. Subconfluent MDA-468 cells were maintained for 24 h in serum-free DME/F12 medium. Then, the medium was replaced by the same fresh medium and 1 h later the indicated concentrations of TGFα were added. RNA was isolated from the cells 5 h after the addition of TGFα. The RNAs (20 μg per lane) were fractionated on 1% agarose gels and transferred to a nylon membrane. The transcripts were detected by hybridization with ^{32}P-labeled pE7 probe and subsequent autoradiography. The resulting autoradiogram was quantified by densitometry and the relative intensities of the EGF receptor mRNA signal were plotted as a function of TGFα concentrations in the culture media. **A:** Autoradiograph (90-h exposure) of an RNA blot analysis showing the changes in the levels of the 10- and 5.6-kb mRNAs encoding the EGF receptor after treatment with TGFα. The sample numbers correspond in sequence with each of the conditions (left to right) shown in the graph (**B**).

stimulated with EGF and/or TGFβ1 for 5 h in the presence of RA. The results presented in Figure 3 show that the basal level of EGFR mRNA significantly increase in response to RA. As shown in Figure 3A, lanes 2 and 5, the presence of 1 nM RA strongly enhances the EGF-dependent induction process in comparison to control EGF alone. Densitometry revealed that the enhancement by 1 nM RA of the EGF-mediated increase in EGFR mRNA at 5 h is ≃20-fold, in comparison to control EGF alone (Fig. 3B). Figure 3A, lane 6, also shows that the addition of RA markedly increases the EGF-dependent induction of EGFR mRNA in the presence of TGFβ1. However, the increase induced by RA in cells exposed to both EGF and TGFβ1 was of lower extent than that observed in cells treated with EGF plus RA in the absence of TGFβ1 (Fig. 3). Similar results were obtained when EGF was replaced by TGFα under identical experimental conditions (data not shown).

We also examined the effects of different concentrations of RA (1 and 5 nM) on the EGF (or TGFα)-dependent induction of EGF-R mRNA in the absence and presence of TGFβ1. For this purpose, the cells were simultaneously treated with RA, EGF (or TGFα), and/or TGFβ1 for 24 h. As expected [6], EGF (or TGFα) and/or TGFβ1 did not have any effect on EGFR mRNA at 24 h in the absence of RA (Fig. 4). Figure 4 shows that RA increases significantly and in a dose-dependent manner the EGFR mRNA levels under all conditions tested. Interestingly, while TGFβ1 alone was unable to modify EGFR expression, in the presence of RA it was capable of enhancing EGFR mRNA accumulation (Fig. 4). As shown in Figure 4, the effects of RA on EGFR mRNA accumulation were of greater magnitude in cells treated with both EGF (or TGFα) and TGFβ1.

The results presented here suggest that under certain conditions RA acts synergistically with EGF (or TGFα) and/or TGFβ1 in the EGFR mRNA induction process.

DISCUSSION

The hormonal and polypeptide growth factor control of cellular proliferation in human breast carcinoma cells is extremely complex and poorly understood [34]. Synergistic and antagonistic interactions between different hormones and polypeptide growth factors are known to occur in human breast cancer cells [7,11,13,15,34]. The molecular mechanisms mediating the influence of specific hormones on the expression of the EGFR gene have not been investigated extensively in these cell families. In an effort to study this problem, we have investigated the expression of proto-oncogene EGFR mRNA in MDA-468 cells as a possible mediator of growth-modulatory signals transmitted by the autocrine peptide growth factors TGFα and TGFβ1 and two hormones critically involved in growth and differentiation, T3 and RA.

The studies reported here demonstrate that physiological concentrations of TGFα (2.5–20 ng/ml) increase EGFR mRNA severalfold above control levels in a time- and concentration-dependent manner (Figs. 1, 2). The finding that TGFα dramatically increases EGFR mRNA is not unexpected, since EGF has been shown to increase EGFR mRNA in MDA-468 [6] and other cell lines [4,5]. These observations are of interest because 1) TGFα plays an important role in human mammary normal and abnormal cell proliferation by acting as an autocrine growth factor [8,10,11], and 2) they serve as the basis for subsequent studies reported here with heterologous growth factor and hormones. The molecular mechanism of action of TGFα on EGFR mRNA induction in MDA-468 cells is not known. It is conceivable that the effect of TGFα on

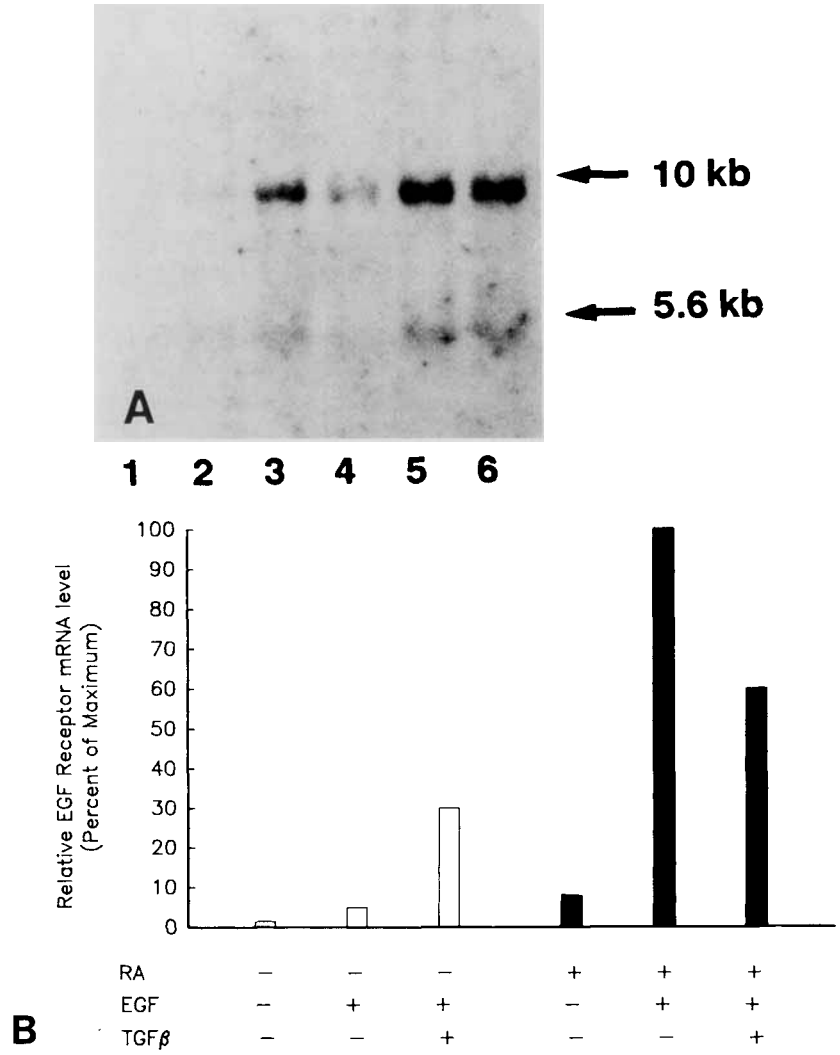

Fig. 3. Modulation of EGF receptor mRNA levels by the combined action of EGF, TGFβ1, and retinoic acid. Subconfluent MDA-468 cells were maintained for 20 h in serum-free medium with or without 1 nM retinoic acid (RA). Then, the media were replaced by identical fresh media and 1 h later EGF (25 ng/ml) and/or TGFβ1 (100 pM) was added. RNA was isolated from the cells 5 h after the addition of EGF and/or TGFβ1. Aliquots of total cellular RNAs (20 μg per lane) were fractionated on 1% agarose gels and transferred to a nylon membrane. The EGF receptor transcripts were detected by hybridization with ^{32}P-labeled pHER probe and subsequent autoradiography. **A:** Autoradiograph (64-h exposure) of the RNA blot analysis showing the change in the levels of the 10- and 5.6-kb mRNAs encoding the EGF receptor after incubation with RA, EGF, and/or TGFβ1. The lane numbers correspond in sequence with each of the conditions (left to right) shown in **B.** The autoradiogram was quantitated by densitometry (only signals in the linear range of film sensitivity were quantified) and the relative intensities of the EGF receptor mRNA signals were plotted (B).

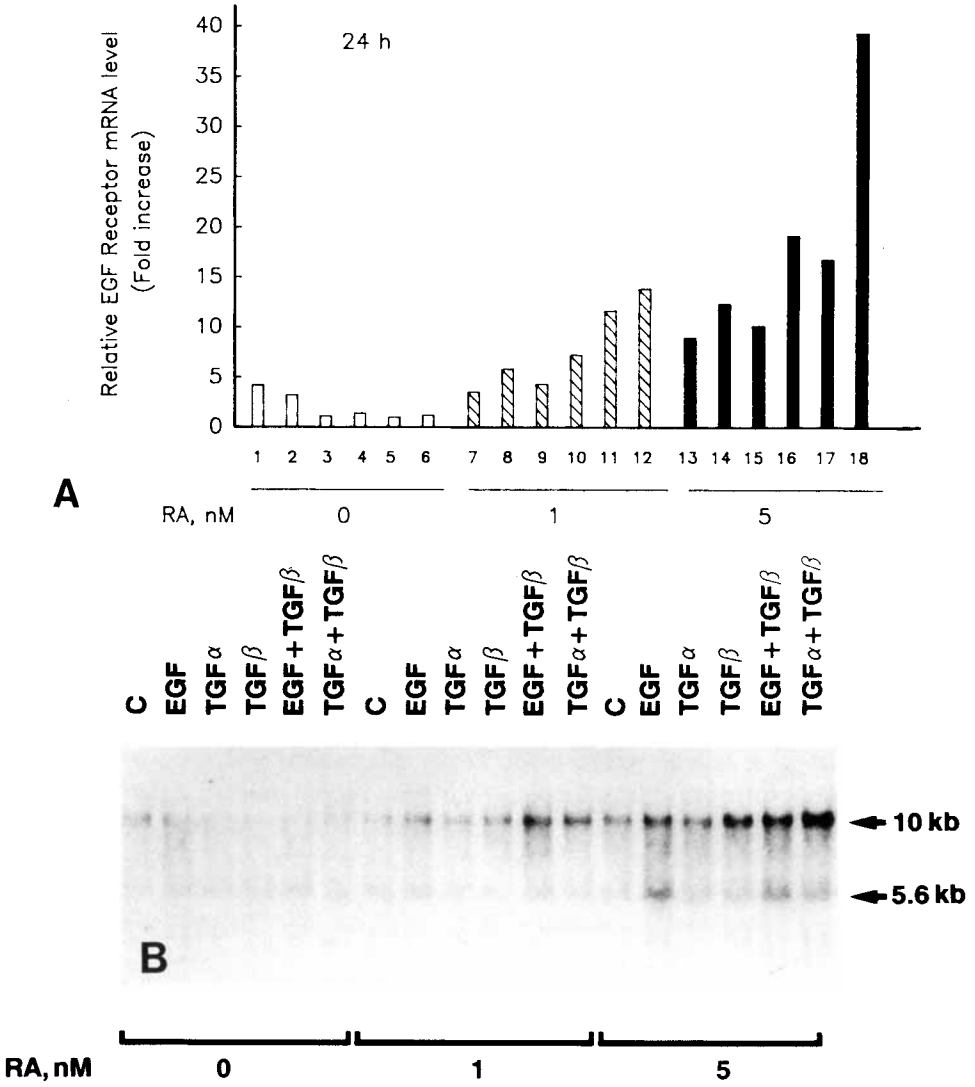

Fig. 4. Enhancement of growth factor-induced EGF receptor mRNA levels by different concentrations of retinoic acid. **A:** Total cellular RNA was isolated from MDA-468 cells cultured in serum-free DME/F12+H medium 24 h after the simultaneous addition of RA, EGF, TGFα, and/or TGFβ1. The RNAs were fractionated by electrophoresis, transferred to a nylon membrane, and hybridized to ^{32}P-labeled pHER probe. The resulting autoradiogram was quantitated by densitometry and the relative intensities of the EGF receptor mRNA signal (10-kb band) were plotted as a function of RA concentrations (1 and 5 nM) in the culture media. The sample numbers (**1–18**) correspond in sequence with each of the conditions (left to right) shown in B. **B:** Autoradiographs (88-h exposure) of representative lanes of the RNA blot analysis utilized to construct the graph presented in A are shown. Lanes, 1, 7, 13—no growth factor additions, control, C; lanes 2, 8, 14—25 ng/ml EGF; lanes 3, 9, 15—10 ng/ml TGFα; lanes 4, 10, 16—50 pM TGFβ1; lanes 5, 11, 17—25 ng/ml EGF plus 50 pM TGFβ1; lanes 6, 16, 18—10 ng/ml TGFα plus 50 pM TGFβ1.

expression of EGFR gene is very complex and is regulated at a variety of levels, including transcription [15,35,36] and mRNA stability [37].

The demonstration that TGFα-dependent expression of the EGFR gene can be experimentally regulated by TGFβ1 implicates EGFR in the system that controls growth inhibition induced by TGFβ1 in MDA-468 cells and possibly other cell lines. Previous experiments performed in our laboratory have demonstrated that TGFβ1 has analogous effects on EGF-stimulated EGFR mRNA in MDA-468 cells [6,15]. Thus, the observations presented here confirm our original contention that TGFβ1 is able to modulate the EGF (TGFα)-dependent expression of EGFR gene [6]. Based on our earlier results with EGF- and TGFβ1-stimulated MDA-468 cells, we believe that the effects of TGFβ1 are mediated by transcriptional activation of EGFR proto-oncogene [15]. However, we emphasize that posttranscriptional mechanisms such as changes in mRNA stability [37] have not been ruled out.

The data in this report identify T3 as one of the serum hormones that can modulate TGFα-dependent expression of the EGFR gene in breast cancer cells. Furthermore, the observation that T3 and TGFβ1 synergistically modulate TGFα-dependent induction of EGFR mRNA indicates a role for thyroid hormones in regulating cell responsiveness to growth-inhibitory factors. Our data are consistent with a recent report from this laboratory implying that thyroid hormones may have a specific role in regulating cell proliferation by modulating the action of EGF and/or TGFβ1 on EGFR gene expression [15]. Of particular relevance to the present studies are the findings of Mukku, who showed that thyroid hormones increase the level of cell surface EGFR in rat liver cells in vivo [22]. Although the mechanism of action of T3 on EGFR gene expression is not known, it is attractive to speculate that it is mediated by erb A–related nuclear receptors for T3 [23,24] which activate EGFR gene transcription [15]. However, we should emphasize that other cellular receptors for T3 are also possible mediators of T3 actions on EGFR gene expression. For example, it has been recently reported that a distinct authentic thyroid hormone receptor protein present in endoplasmic reticulum and nuclear envelope is homologous to protein disulfide isomerase and the β-subunit of prolyl-4-hydroxylase [38,39]. These observations suggest an alternative or more complex mechanism for T3-mediated stimulation of TGFα-dependent induction of EGFR mRNA in breast cancer cells.

Our data clearly demonstrate that treatment of MDA-468 cells with RA results in an early rise in mRNA for the EGFR (Fig. 3). These results are consistent with previous work that showed that RA can increase cell surface EGFR in various fibroblastic and epithelial cell lines [29,31]. Experiments presented here also show that long-term treatment with RA resulted in a dose-dependent elevation of basal EGFR mRNA level and potentiation of the effects of other growth factors tested, including TGFβ1 alone (Fig. 4). This finding with TGFβ1 is unanticipated, since TGFβ1 alone did not have any effect on EGFR mRNA at early or late time points in the absence of RA. One possible explanation is that RA induces autocrine production of TGFα which in turn synergizes with exogenously added TGFβ1 in inducing EGFR mRNA accumulation. The molecular mechanism of action of RA on EGFR mRNA accumulation in MDA-468 cells can only be a subject for speculation. The receptor protein for RA is homologous to the receptors for steroid hormones, thyroid hormones, and vitamin D3 [16,23–27]. Thus, RA could function by activating a specific nuclear trans-acting RA receptor protein which may enhance EGFR gene transcription.

In summary, the physiological significance of the effects of the modulatory factors tested here on EGFR gene expression is not known. However, our studies offer a potential pathway involving EGFR gene expression whereby TGFβ1, T3, and/or RA could distally modulate the effects of TGFα (EGF) on proliferation of breast cancer cells. Experiments for testing the hypothesis that the modulatory effects of TGFβ1, T3, and/or RA are predominantly mediated by transcriptional activation of EGFR gene are underway.

ACKNOWLEDGMENTS

This work was supported by Grant No. 001 from the Medical Research Service of the Veterans Administration. We thank Vera M. Schuette for expert technical assistance.

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