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TRANSFORMING GROWTH FACTOR ALPHA (TGFα) INDUCTION OF C-FOS AND C-MYC EXPRESSION IN C3H 10T1/2 CELLS

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Summary: We have investigated the effects of transforming growth factor α (TGFα) in C3H10T1/2 cells, on S phase entry and early gene activation events associated with cell cycle progression. We find that EGF and TGFα, which both utilize the EGF receptor for signal generation, are able to stimulate DNA synthesis in these cells with nearly superimposable kinetics; however, the stimulation by TGFα was slightly greater at nearly all time points assayed. This report is the first showing that TGFα, like EGF, vigorously induces c-myc and c-fos gene expression in these cells. A significant stimulation of c-myc and c-fos mRNA levels is observed with both TGFα and EGF; c-myc mRNA levels show an 8-fold induction with both mitogens, while c-fos inductions were on the order of 12 to 14-fold at maximum. However, the induction of c-myc mRNA by TGFα has slower kinetics than by EGF.

Transforming growth factor α (TGFα) shares significant homology to EGF and binds to the EGF receptor (1). Alone, TGFα has only minimal effects on inducing anchorage independent growth, but is capable of inducing a mitogenic response (2). Since TGFα production has been shown to be a product of transformation, particularly K-ras transformation (3,4) we sought to look in detail at the mitogenicity of TGFα in C3H10T1/2 fibroblasts. Given the homology to EGF and the fact that TGFα uses the EGF receptor system, we have also compared the effects of TGFα to EGF in an attempt to uncover any differences in the action of these growth factors which may account for the ability of TGFα to play a significant role in malignant transformation. It has been demonstrated previously that TGFα often exerts different and more potent effects than does EGF in some systems (5-8), so the question arises whether TGFα derives its transforming ability from a more potent induction of certain parameters involved in mitogenesis. With this in mind, we have studied the effects of TGFα and EGF on early genetic events (c-myc and c-fos stimulation). EGF has previously been shown to stimulate c-myc and c-fos (9). Here, we report the

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novel observation that exogenous addition of TGFα is able to activate c-fos and c-myc in C3H 10T1/2 fibroblasts.

Materials and Methods

Cell culture.

C3H 10T1/2 fibroblasts were plated in 12-well plates and 100 mm dishes (Corning) at densities of $3x10^4$ and $5x10^5$ cells respectively, in Eagle's basal medium (BME) supplemented with 10% (v/v) fetal bovine serum (GIBCO) and .001% gentamycin as an antimicrobial agent. The medium was changed every three days, and cells reached confluency 10 days post plating. To ensure that the cells were indeed quiescent before experimentation, assays were performed four days after the last feeding.

Preparation of RNA

Quiescent cultures of 10T1/2 cells were treated with EGF (20 ng/ml) or TGFα (20ng/ml) for 15-120 minutes as indicated on the figures. At the indicated times, the media was aspirated and replaced with ice cold PBS. Cells were then scraped, collected and pelleted at 2500 rpm for five minutes. The supernatant was aspirated and the pellet was dissolved in 4M guanidinium isothiocyanate (10). RNA was purified by density gradient centrifugation through 5.8 M CsCl (11). The pellet was reprecipitated twice in 1x Robinson's buffer and cold ethanol and then dissolved in distilled water.

RNA electrophoresis, blotting and hybridization

Using the procedure of McMaster and Carmichael (12), ten ug whole cell RNA was electrophoresed through a 1.1% agarose gel and transferred to a nylon-66 membrane, Hybond-N (Amersham). RNA was cross-linked to the membrane by exposure to UV light (254 nm) for five minutes. The 1.5 kb ClaI/EcoRI fragment of c-myc DNA and the 2.8 kb XbaI/BamHI fragment of c-fos DNA were used to construct probes by the random primer method. Fragments were labelled to a specific activity of 1-2 x 10^9 cpm/ug and 3-5 x 10^6 cpm/ml of hybridization buffer was used for hybridization (13). The method of Church and Gilbert (14) was used to hybridize and wash the blots, which were then exposed to X-ray film for three to five days (Kodak, XAR). Band intensities were quantitated by densitometric scanning with a Quick Scan R & D (Helena Laboratories) and fold induction calculated relative to control levels of c-myc and c-fos bands.

DNA synthesis assays

Post-confluent 10T1/2 cells were treated with EGF or TGFα (20 ng/ml), and for cumulative ^{3}H-dThd assays, 1 uCi of 40-60 Ci/mmol specific activity isotope (ICN Radiochemicals) was added simultaneously, with the reaction being stopped 24 hours later. For pulse incorporation kinetics, the same amount of radioactive deoxythymidine was added to the treated cells at T= 12, 16, 20, 24, 28 and 32 h. Each reaction was allowed to proceed for one hour before stoppage. All reactions were stopped by aspirating the media and washing the wells twice with 1 ml cold PBS. 0.5 mls of cold 5% trichloroacetic acid (TCA) was then added to each well, and the plates were placed in a refrigerator for 30 minutes. The TCA is then aspirated and discarded, the wells are rinsed once with cold PBS, and each well is then treated with 0.5 mls of cold 0.1 N NaOH to dissolve the acid insoluble fraction. The plates are allowed to stand for 2 h at 4° C, after which the acid insoluble fraction is quantitatively transferred to a plastic counting vial, along with a 0.5 ml PBS wash, neutralized, and counted in a Beckman LS-230 liquid scintillation counter.

Growth Factors

Epidermal growth factor was a kind gift from Dr. Richard Savage, Temple University, Philadelphia, PA. Rat transforming growth factor alpha was chemically synthesized as previously described (15) and quantitated in radiolabelled EGF receptor competition assays on formaldehyde fixed human epidermoid carcinoma cells (16).

Results

Stimulation of DNA synthesis by TGFα - When post-confluent C3H 10T1/2 fibroblasts are treated with 20 ng/ml TGFα, there is a significant increase in DNA synthesis, as measured by ^{3}H-deoxythymidine incorporation into the acid insoluble fraction. We studied the pulse incorporation kinetics of ^{3}H-dThd as stimulated by EGF and TGFα. Both ligands induce thymidine incorporation with nearly superimposable kinetics (Fig. 1). The stimulation by TGFα is greater at nearly all time points assayed. However, there is a significant difference in the responses at T=12 and T=16h. At these time points, as can be seen in Figure 1, the stimulation of DNA synthesis is significantly higher with TGFα. Although the response to TGFα is greater at T=20 and 24h, the standard deviations were too large to claim a significant difference between TGFα and EGF. These results demonstrate that TGFα is an effective mitogen in these cells.

Proto-oncogene expression induced by EGF and TGFα - Given the observed increase in DNA synthesis, we undertook the study of induction of c-myc and

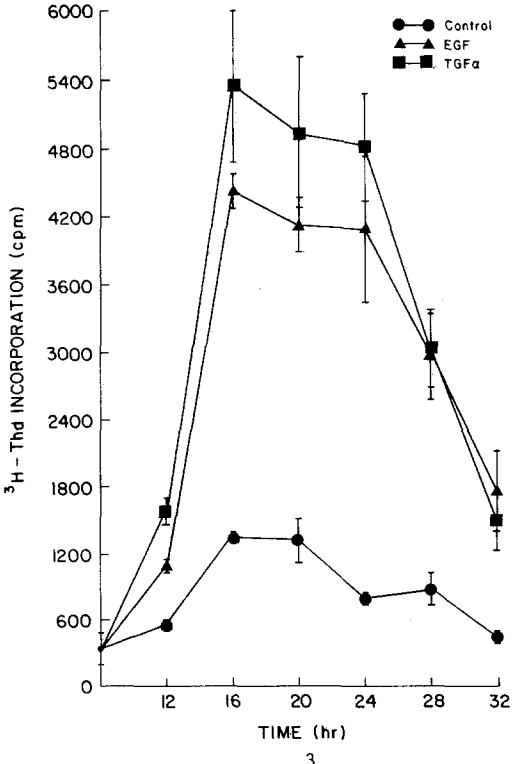

Figure 1 Pulse incorporation kinetics of ^{3}H-dThd. Density arrested quiescent 10T½ fibroblasts were stimulated with EGF (20 ng/ml) or TGFα (20 ng/ml) at T=0. At the indicated times in the figure, ^{3}H-dThd was added and the reaction was allowed to proceed for one hour, then stopped and counted as in Materials and Methods. The values for each point are the mean and standard deviation of quadruplicate samples. ●—●control; ▲—▲EGF; ■—■TGFα.

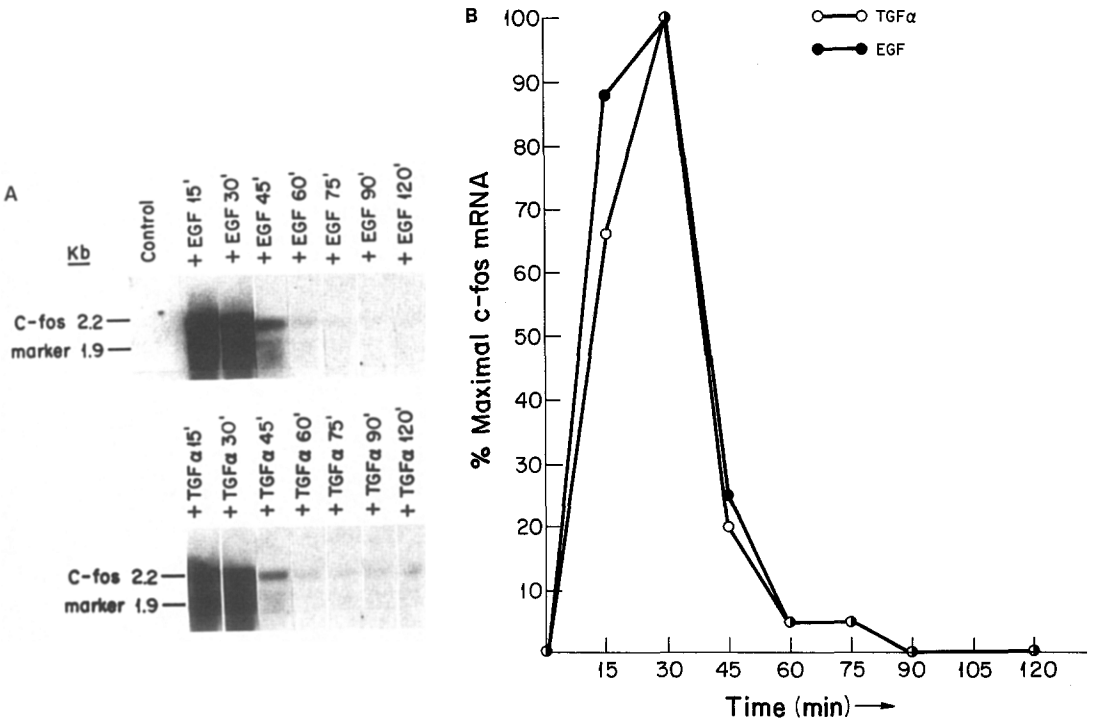

Figure 2 Stimulation of c-fos expression by EGF and TGFα. (A) Following stimulation of quiescent 10T½ cells with 20 ng/ml EGF (top panel) or 20 ng/ml TGFα (bottom panel) for the indicated times, RNA was isolated and then analyzed by Northern blotting as described in Materials and Methods. The photo shown was shot from an autoradiograph which was allowed to expose for three days. The control lane applies to both panels. The 2.2 kb and 1.9 kb bands are the position of c-fos mRNA and 18S rRNA, respectively. (B) Densitometric evaluation of relative band intensities in (A) was performed as described in Materials and Methods and plotted graphically. Increases in mRNA content are given as fold changes relative to control mRNA levels, which is assigned a value of "1". — — — EGF; ——— TGFα.

c-fos proto-oncogenes, both of which have been associated with cell-cycle progression (17,18). Initially, we stimulated quiescent cells with 20 ng/ml EGF or TGFα for only a single time point, 60 minutes, and observed significant stimulation of both c-myc and c-fos (data not shown). The time course of induction of these proto-oncogenes was examined to compare the effects of EGF and TGFα on this process. c-fos mRNA was rapidly induced by EGF and TGFα (Fig. 2A). The response to both ligands drops off rapidly by 45 minutes and returns to basal levels within 120 minutes. The levels of c-fos mRNA were quantitated by densitometric scanning of the autoradiograph. The maximal response of c-fos to both these growth hormones is similar (Fig. 2B). The maximal stimulation of c-fos by EGF was reached at 30 minutes; TGFα elicited a similar response at the same time point.

EGF and TGFα also elicit very rapid induction of c-myc mRNA that is observed as early as 15 minutes with EGF. c-myc mRNA levels reach a maximum

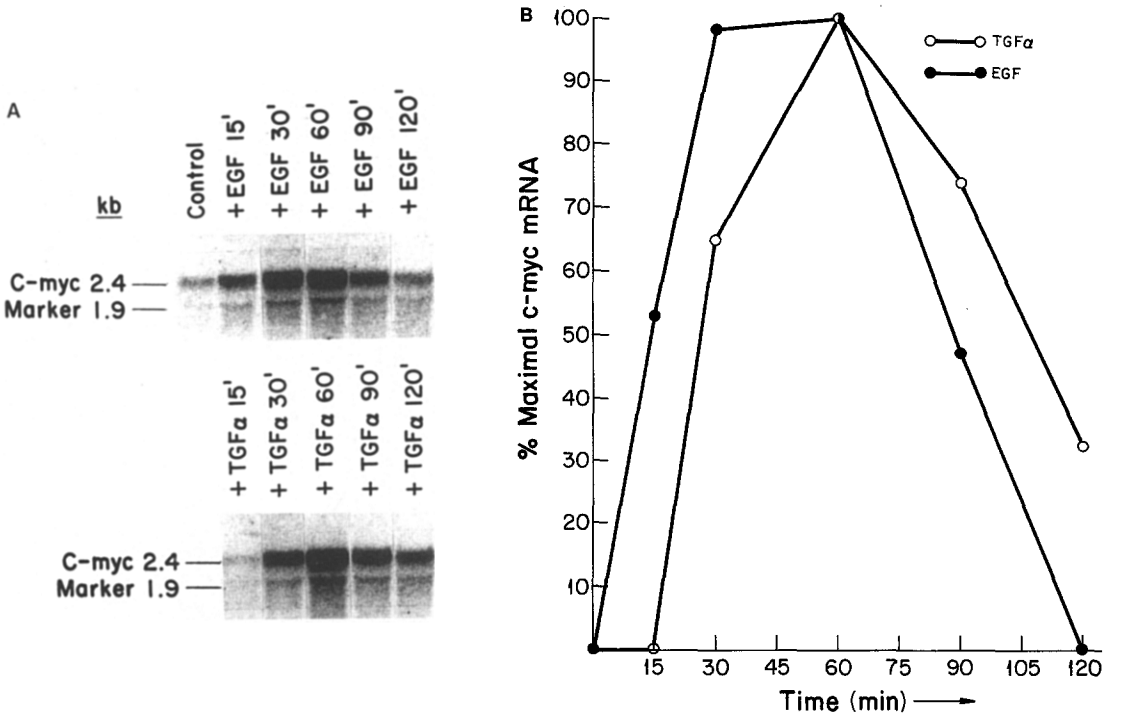

Figure 3 Stimulation of c-myc expression by EGF and TGFα. Following stimulation of quiescent 10T½ fibroblasts with 20 ng/ml EGF (top panel) or 20 ng/ml TGFα (bottom panel) for the indicated times, RNA was isolated and then analyzed by Northern blotting as described in Materials and Methods. The photo shown was shot from an autoradiograph which was allowed to expose for five days. The control lane applies to both panels. The 2.4 kb and 1.9 kb bands are the position of c-myc mRNA and 18S rRNA, resepectively. (B) Densitometric evaluation of relative band intensities in (A) was performed as described in Materials and Methods and plotted graphically. Increases in mRNA content are given as fold changes relative to control mRNA levels, which is assigned a value of "1". — — — EGF; ———— TGFα.

by 30 minutes post-stimulation with EGF. On the other hand, TGFα-mediated stimulation of c-myc lags behind EGF appreciably at the earliest time points assayed, reaching a maximum at 60 minutes (Fig. 3A). The maximal level of c-myc expression is nearly identical for either growth factor, as illustrated in the densitometric analysis shown in Figure 3B. c-myc mRNA levels remain higher at the later time points (90 and 120 minutes) in cells stimulated with TGFα, which is most likely a consequence of the delayed induction of c-myc by TGFα

Discussion

Unlike TGFβ, which is a structurally unique growth factor, TGFα shows a striking resemblance to EGF and uses the EGF receptor system for generation and transduction of the mitogenic signal (Derynck, 1986). Furthermore, several reports have cited differential responses of various cell types to

TGFα and EGF, a surprising finding for two ligands which utilize and stimulate autophosphorylation of the same cell surface receptor. For example, TGFα is a much more potent inducer of calcium release in murine calvaria and rat fetal long bones than is EGF (7,8) and is a more efficient wound healing agent than is EGF (19). Consequently, TGFα has often been thought of as a "superagonist," which may contribute to its role in neoplasia.

We have shown that TGFα stimulates c-myc and c-fos gene expression in C3H 10T1/2 cells. This is in contrast to results obtained with NIH 3T3 fibroblasts, which are relatively unresponsive to EGF and, presumably, TGFα. This indicates that the response to a ligand, in this case EGF or TGFα, can be a cell-type specific response rather than a general response common to all fibroblastic cells. Although there are subtle differences in the effects of TGFα and EGF, the results do not indicate that TGFα is a "superagonist" with respect to the parameters studied in C3H 10T1/2 fibroblasts. There is a kinetic delay in the induction of c-myc but not c-fos mRNA by TGFα. There may be a kinetic delay in TGFα stimulation of c-fos, but this may be experimentally difficult to observe because of the rapid rise and fall in c-fos mRNA levels. At present, we do not understand the mechanistic basis for TGFα's slower c-myc mRNA induction.

King and Cuatrecasas (20) were able to resolve two EGF binding sites on the cell surface: a high affinity class and a low affinity class. The high affinity class is not present in an active form on the cell surface; rather, it exists in a "cryptic" state which becomes accessible to the ligand after exposure of the cell to EGF. The appearance of this high affinity binding site depends on new protein synthesis and is temperature dependent. Furthermore, it is the high affinity class of receptor that is susceptible to protein kinase C phosphorylation, which results in decreased ligand binding and lower tyrosine kinase activity (21,22). Presently, we are developing a hypothesis to explain our results based on the existence of two different receptor affinity classes. Prelimary work shows that there may be differential usage of high and low affinity EGF receptors by EGF and TGFα, specifically that TGFα may be less dependent on the high affinity class of receptors for eliciting its mitogenic response than is EGF. A previous report has indicated that the loss of high affinity EGF binding sites abrogates the ability of EGF to induce c-myc mRNA expression (23).

Such a model may be used to explain the kinetic delay we see with c-myc. If TGFα preferentially uses the low affinity class of receptors, it would be reasonable to assume that TGFα attains receptor saturation more slowly than does EGF, which utilizes both the high and low affinity receptors. Consequently, there is a slight delay in the generation of the mitogenic signal by TGFα. Receptor binding studies are presently being conducted in an attempt to verify this hypothesis.

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