Autocrine TGFα Expression in the Regulation of Initiation of Human Colon Carcinoma Growth

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Previously, we reported that unaggressive, growth factor-dependent FET human colon carcinoma cells downregulated their transforming growth factor alpha (TGFα) expression in a quiescent state (G0/G1) induced by growth factor and nutrient deprivation (Mulder, 1991, Cancer Res., 51:2256–2262). In contrast, highly aggressive, growth factor-independent HCT116 human colon carcinoma cells aberrantly upregulated this autocrine activity in the quiescent state (Mulder, 1991, Cancer Res., 51:2256–2262; Howell et al., 1998, Mol. Cell. Biol., 18:303–313). In this report, the role of autocrine TGFα and the mechanism of its regulation of expression during reentry into the cell cycle from a noncycling growth state were determined in FET cells. Optimal induction of DNA synthesis from a quiescent state in FET cells is dependent upon autocrine TGFα as well as exogenous transferrin and insulin. Reentry into the cell cycle resulting from treatment with exogenous transferrin and insulin resulted in ~3-fold induction of TGFα expression within 1 hr. TGFα induction was controlled at the transcription level, and the cis-controlling element was localized to the region between bp −370 to −201 relative to the translation start codon within the TGFα promoter. Thus neutralization of autocrine TGFα protein revealed that the induced TGFα autocrine activity was necessary for DNA synthesis and acted only in the early G1 phase of the cell cycle. Blockade of autocrine TGFα expression early in the cell cycle resulted in the reduction of DNA synthesis, whereas treatment with neutralization antibody at later times had no effect. This suggested that autocrine TGFα functions to initiate cell growth from noncycling states. This was further confirmed by the dependence of FET cells upon autocrine TGFα for colony formation in experiments where the plating density was sufficiently low to generate a lag phase in tissue culture. In contrast, TGFα autocrine activity was not required for exponential phase cells, as evidenced by the failure of TGFα neutralizing antibody to inhibit proliferation in this growth state. Taken together, these results suggest that autocrine TGFα acts primarily in the process of growth initiation by moving cells from a noncycling state back into the cell cycle, rather than supporting cell growth already initiated. J. Cell. Physiol. 177:387–395, 1998. © 1998 Wiley-Liss, Inc.

The autocrine hypothesis was proposed to explain the loss of dependence upon exogenous growth factors associated with the transformed phenotype in tumor cells. This hypothesis stated that polypeptide growth factors secreted by tumor cells stimulate their cognate receptors on the same cells, thus imparting a growth advantage to the transformed cells (Sporn and Roberts, 1985). Transforming growth factor alpha (TGFα), a member of the epidermal growth factor (EGF)-like family of mitogens, was the first positive growth factor described to behave in this manner. It elicits its biological functions through binding to the EGF receptor (EGFr) (Cantty et al., 1991; Ullrich and Schlessinger, 1990); Lee et al., 1995). Ligand binding triggers receptor dimerization and activation of the intracellular kinase domain, resulting in receptor autophosphorylation on tyrosine residues and signal transduction (Ullrich and Schlessinger, 1990).

Subsequently, TGFα and the EGFr were found to be widely expressed in developing and adult tissues, and have been suggested to play a role in embryonic development and adult physiology (Kudlow and Bjorge,

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1990; Derynck, 1992; Lee et al., 1995). However, TGFα levels in tumor tissues are generally significantly higher than in their normal counterparts (Derynck et al., 1987). Overexpression of TGFα is characteristic of gastrointestinal tract tumors (Lee et al., 1995), and TGFα content in colon tumors is 4-fold higher than in normal mucosa (Liu et al., 1990). Increased production of TGFα, and thus elevated EGFβ activation, in colon cancer cell lines have been associated with autonomous proliferation and tumorigenicity (Baldwin and Zhang, 1992; Karnes et al., 1992; Modjtahedi et al., 1992; Watkins et al., 1988; Hirsch et al., 1996). TGFα transgenic mice showed epithelial hyperplasia in the colon (Sandgren et al., 1990; Vassar and Fuchs, 1991). More directly, overexpression of a TGFα cDNA in a human colon carcinoma cell line significantly stimulated its malignant progression (Ziobet al., 1993). Thus, TGFα appears to have an important role in the progression of colon cancer.

Despite its importance as a positive growth factor in colon as well as other types of carcinoma cells, the mechanism by which autocrine TGFα confers a growth advantage to tumor cells, or for that matter to normal cells (Bates et al., 1990; Pittelkow et al., 1993), and how this autocrine activity is controlled, are still largely unclear. We previously reported that manipulation of TGFα autocrine activity in human colon carcinoma cell lines by TGFα sense (Ziobet al., 1993) or antisense (Howell et al., 1995) transfections significantly altered their lag phase in tissue culture, while their growth rate in exponential phase remained unchanged. These observations suggest that autocrine TGFα acts primarily in the process of growth initiation. Therefore, the work reported herein was based on the hypothesis that the major role of autocrine TGFα in tumor progression is to move cells from a noncycling, growth arrested state back into the cell cycle.

A previous report indicated that unaggressive, growth factor-dependent FET human colon carcinoma cells downregulated their TGFα expression at a quiescent state (G0/G1) induced by growth factor and nutrient deprivation (Mulder, 1991), but that highly aggressive, growth factor-independent HCT116 human colon carcinoma cells aberrantly upregulated TGFα expression, perhaps contributing to their malignant progression (Mulder, 1991). In this report, we utilized FET cells as a model to study the role of autocrine TGFα and the mechanism of its regulation of expression during reentry into the cell cycle from a noncycling growth state. We found autocrine TGFα to be critical for reentry into the cell cycle from two growth states in which FET cells exhibit high levels of noncycling cells. One of these states was the dependence of FET cells upon autocrine TGFα for colony formation at low cell density (lag phase of tissue culture), while the other was the stimulation of DNA syntheses in cells which had been rendered quiescent (G0/G1) by growth factor and nutrient deprivation. Optimal induction of DNA synthesis in quiescent FET cells required exogenous growth factors. However, stimulation of autocrine TGFα expression at the transcriptional level in early G0 phase was necessary for induction of DNA synthesis. In contrast, exponential phase cells were not dependent upon autocrine TGFα, as indicated by the failure of TGFα-neutralizing antibody to inhibit proliferation in this growth state.

**MATERIALS AND METHODS**

**Cell culture**

The FET cell line was developed from an early-stage human colon cancer and has been extensively characterized (Brattain et al., 1981, 1994; Chantret et al., 1988; Schlechte et al., 1990). It has been continuously maintained in a chemically defined serum-free medium as previously described (Brattain et al., 1984; Howell et al., 1993). The medium consists of McCoy’s 5A medium (Gibco BRL, Gaithersburg, MD) supplemented with amino acids, pyruvate, antibiotics, insulin (20 μg/ml, Sigma, St. Louis, MO), and transferrin (4 μg/ml, Sigma). Working cultures were maintained at 37°C in the presence of 5% CO2. Cells were routinely subcultured with 0.25% trypsin in Joklik’s medium containing 0.1% EDTA. The medium used in this study was designated as “IT” medium, while the growth factor-free medium was designated as supplemental McCoy’s medium (“SM” medium). The TGFα- and EGFβ-neutralizing antibodies were purchased from Oncogene Science (Cambridge, MA).

**Cloning efficiency assay**

FET cells were plated at 400 cells/well (200 cells/cm2) in 1 ml of the indicated medium into 16-mm wells and incubated until colonies of suitable size for staining formed (~12 days). The colonies were then stained with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) and photographed.

**3H-thymidine incorporation assay**

The protocol was carried out as previously described, with some minor modifications (Mulder and Brattain, 1989). Briefly, FET cells were grown in IT medium to about 70% confluency. They were then rendered quiescent by growth factor and nutrient deprivation for 6 days in SM medium and subsequently released from quiescence by providing fresh nutrients (changing into SM medium) or nutrients plus growth factors (IT medium). Cells were pulsed for 1 hr with 3H-thymidine (25 μCi, 50 Ci/mM, Amersham, Arlington Heights, IL) at the indicated times. The free 3H-thymidine was removed and the cells were washed three times with ice-cold PBS. TCA was added and precipitated radioactivity was solubilized in 0.2 M NaOH solution. Incorporation of radioactivity into DNA was then determined.

**RNase protection assay**

High-specific-activity TGFα riboprobe was synthesized in vitro from a 306-bp 5’-fragment of TGFα cDNA cloned into the pGEM3Z(−) vector (Promega) in the presence of [32P]-UTP (3,000 Ci/mM, NEN) as previously described (Howell et al., 1995). Synthesis of the

**Abbreviations:** CAT, chloramphenicol transferase; cDNA, complementary DNA; CsTFA, cesium trifluoroacetate; DNA, deoxyribonucleic acid; EGF, epidermal growth factor; EDTA, ethylenediaminetetraacetic acid; EGFβ, epidermal growth factor receptor; mRNA, messenger RNA; PBS, phosphate-buffered saline; RNA, ribonucleic acid; RNase, ribonuclease; SM, McCoy’s 5A medium supplemented with amino acids, pyruvate, and antibiotics; TCA, trichloroacetic acid; TGFα, transforming growth factor alpha; TTI, SM medium supplemented with insulin (20 mg/ml) and transferrin (4 mg/ml).
Fig. 1. Repression of TGFα transcription during establishment of quiescence. A: FET cells were grown to ~70% confluence and then rendered quiescent, as described in Materials and Methods. Cells were harvested for RNA extraction at the indicated times and TGFα RNase protection analysis was performed. Control, 40 μg untransfected FET yeast tRNA. B: FET-2 cells were rendered quiescent and harvested for CAT assay of TGFα promoter-reporter chimeras at the indicated times, as described in Materials and Methods. Control, lysate of cells.

Fig. 2. Location of the cis-controlling element in the TGFα promoter. FET-370 and FET-201 cells at 80% confluence (L) or quiescence (Q) were harvested for CAT assays, as described in Materials and Methods.

EGFα and γ-actin riboprobes was the same, except that the DNA templates were 404- and 145-bp 5’-fragments of their cDNAs, respectively. Following DNase I digestion of the template, the riboprobe was isolated with an NACS-52 column (Gibco BRL).

Total cellular RNA was isolated in 5 M guanidine isothiocyanate and purified using a CsTFA (cesium trifluoroacetate) gradient (Pharmacia, Piscataway, NJ) (Chirgwin et al., 1979). Total RNA (40 μg) was hybridized with the indicated riboprobes overnight. After RNase digestion of excess riboprobe, protected fragments were resolved on a 6% urea-polyacrylamide sequencing gel followed by autoradiography. Actin was used to normalize the sample loading (Enoch et al., 1986).

Stable transfection

Eighty percent confluent FET cells were harvested by trypsinization. Cells (2.0 x 10⁷) were then transferred to a 0.4-cm electro gap cuvette (BioRad, Richmond, CA) with 5 μg SV-40 Neomycin (Neo) plasmid and 30 μg TGFα promoter-CAT construct. After electroporation at 250 v and 960 μF in a BioRad gene pulser, the cells were plated at a 1:20–1:30 dilution and changed to medium containing 600 μg/ml Geneticin (G418, Gibco BRL) 48 hr later. Neo-resistant clones were expanded and tested for CAT expression. FET cells transfected with the full-length TGFα promoter-CAT construct were designated FET-2 cells. Other transfectants were named by the length of the deletion fragments in the chimeric constructs used in the transfections. For example, FET-370 indicates FET cells stably transfected with promoter-CAT chimeras con-
Fig. 3. Induction of TGFα mRNA in quiescent FET cells. A: Quiescent FET cells were changed to fresh TI or SM medium. 3H-thymidine incorporation assays were performed at indicated hours. Fold of induction over quiescent 3H-thymidine incorporation level was plotted (mean ± SD (bar), n = 3). B: Quiescent FET cells were stimulated to reenter the cell cycle with fresh TI medium or SM medium and harvested for RNA extraction at 0 (Qui.), 1, 4, and 8 hr. The RNase protection assays were then performed, as described in Materials and Methods.

**CAT assay**

After three PBS washes, cells were harvested in 1 ml TEN buffer (40 mM Tris-HCl, 1 mM EDTA, and 150 mM NaCl, pH 7.4) and pelleted by centrifugation. The pellet was resuspended in buffer (250 mM Tris-HCl, pH 7.8) and lysed by three freeze-thaw cycles. Cellular debris was pelleted by centrifugation, and the protein concentration of the lysate was determined using the BioRad protein assay reagent. Protein (20 μg) from FET-2 and FET-370 cells, and 50 μg protein from FET-201 cells, were used for CAT assays.

The CAT reaction was performed in 250 mM Tris-HCl (pH 7.8), 0.2 μCi [14C]-chloroamphenicol (55 mCi/mM, Amersham), and 3.6 mM acetyl-CoA (Pharmacia) for 8 hr at 37°C. Following ethyl acetate extraction, samples were resolved by thin-layer chromatography and visualized by autoradiography (Maniatis et al., 1982).

**RESULTS**

**Repression of TGFα autocrine activity during induction of quiescence**

Initiation of DNA synthesis from quiescent cells was studied to test the hypothesis that autocrine TGFα is critical for reentry into the cell cycle by noncycling cells. Quiescence was induced by nutrient and growth factor deprivation for 6 days, during which DNA synthesis was monitored by 3H-thymidine incorporation assays. The acquisition of a steady low basal level of 3H-thymidine incorporation is operationally defined as quiescence in this model system (Mulder and Brattain, 1989). Flow cytometry assays showed that ~85% of the cells were arrested in G0/G1 at this time (data not shown).

A TGFα-specific RNase protection assay, as described in Materials and Methods, was utilized to monitor TGFα expression at the mRNA level during the establishment of and subsequent release from quiescence. At quiescence, TGFα mRNA levels were significantly lower, as decreased TGFα mRNA was observed by day 4 of starvation, with continued diminution through day 6 (Fig. 1A).

**Repression of autocrine TGFα was controlled at the transcription level**

To determine whether TGFα repression was controlled at the transcription level, a series of previously described deletion fragments of the TGFα promoter (Saeki et al., 1991; Lynch et al., 1993; Howell et al., 1998) was inserted upstream of the bacterial CAT gene.
in the pGCAT-C vector. FET cells were then stably transfected with these promoter-reporter chimeras, as described in Materials and Methods. A 3-fold decrease of CAT activity in FET cells transfected with the 2.8-Kb TGFα promoter-CAT construct (FET-2) was observed by day 4 of starvation (Fig. 1B), indicating that the repression of TGFα mRNA expression was controlled at the transcription level. FET-370 cells showed a similar reduction of CAT activity to that in FET-2 cells at quiescence, but FET-201 cells showed the same amount of CAT activity at quiescence as in the exponential phase (Fig. 2). This indicated that the controlling element was located in the region between −370--1 bp (relative to the translation start codon) within the TGFα promoter. Interestingly, other investigators found an EGF/TGFα response element of the TGFα promoter within the same region (Raja et al., 1991; McClain et al., 1992). This raised the possibility that the element controlling the loss of TGFα transcription during induction of quiescence could be the same as that controlled by EGFr activation. Consequently, the loss of EGFr activation would then lead to reduction of TGFα expression in a circular fashion.

**Stimulation of autocrine TGFα expression preceded induction of DNA synthesis**

When quiescent cells were supplied with fresh TI medium, 3H-thymidine incorporation activity was stimulated by −24-fold over the quiescent level at 22 hr. However, nutrient replenishment alone (SM medium) induced only a small fraction of this DNA synthesis (20%), indicating that exogenous transferrin and insulin were required for optimal reentry of quiescent FET cells into the cell cycle (Fig. 3A).

We next examined the kinetics of induction of TGFα expression after release from quiescence by growth factors. If autocrine TGFα was required for reentry of the cells into the cell cycle, induction of TGFα expression and activation of EGFr should occur prior to DNA synthesis. Significant induction of DNA synthesis in quiescent cells began at 12 hr after release from quiescence and peaked at 24 hr, while stimulation of TGFα expression at the mRNA level was already apparent by 1 hr (Fig. 3B). TI medium stimulated TGFα expression by −3-fold. Consistent with its inability to fully stimulate DNA synthesis, SM medium elicited a significantly weaker induction of TGFα expression (Fig. 3B). Similarly, TGFα promoter activity was stimulated −3-fold by the TI medium and to a lesser extent by the SM medium within 1 hr in quiescent FET-370 cells (Fig. 4A). However, promoter activity of the 201-bp TGFα promoter fragment lacking the EGF/TGFα response element was not affected by TI treatment in quiescent FET-201 cells (Fig. 4B), therefore implying the importance of EGFr activation in the induction of TGFα expression.
insulin. Despite these advances, how autocrine growth factor stimulated to reenter the cell cycle with transferrin and signal transduction has seen a precipitous expansion.

When the cells were plated at clonal density, transferrin and insulin added at time of plating were sufficient to initiate growth of FET colonies. Removal of either transferrin or insulin abolished clonal growth (Fig. 6A), suggesting that transferrin and insulin are the minimal requirement for FET cells to grow in serum-free medium. To examine whether autocrine TGFα acts in concert with these two factors to support clonal initiation and expansion, neutralizing antibodies against TGFα or EGF were utilized. If autocrine TGFα were necessary for colony formation, addition of either antibody should inhibit the clonal growth. Addition of the TGFα-neutralizing antibody inhibited clonal growth in a dose-dependent manner (Fig. 6B). A neutralizing antibody against EGF, which blocks ligand binding (Sunada et al., 1990), also inhibited clonal growth (Fig. 6C).

**Fig. 5.** Effect of TGFα-neutralizing antibody treatment at different times after stimulation of DNA synthesis. Quiescent FET cells were stimulated with fresh SM, TI, or TI medium with 15 μg/ml TGFα-neutralizing antibody added to the tissue culture medium at time of release from quiescence. T3H-thymidine incorporation assays were then performed at 22 hr. Fold of induction over quiescent 3H-thymidine incorporation level was plotted (mean ± SD (bar), n = 3).

**Autocrine TGFα was required in early G1 for DNA synthesis after release from quiescence**

TGFα antibody was added to the medium at various times after release from quiescence in order to determine at which point autocrine TGFα acted to support reentry into the cell cycle. When TGFα-neutralizing antibody (20 μg/ml) was added at time of release from quiescence along with TI medium, DNA synthesis was inhibited by ~50% (Fig. 5). In a separate experiment, TI medium-induced DNA synthesis at 22 hr was inhibited by ~65% by the addition of 30 μg/ml TGFα antibody to the TI medium at time of release (data not shown). However, if the antibody addition was delayed until 4 hr after release from quiescence, inhibition was drastically reduced, while delay until 8 hr after release from quiescence resulted in no effect on peak 3H-thymidine incorporation into cellular DNA (Fig. 5). Thus, TI-induced TGFα autocrine activity acted in early G1 to support DNA synthesis after quiescent FET cells were stimulated to reenter the cell cycle with transferrin and insulin.

**Autocrine TGFα was required for initiation of cell growth in FET cells**

Like normal cells, FET cells show a substantial lag period after plating in tissue culture before acquiring exponential growth. The lower the seeding density, the longer the lag phase. Therefore, clonal growth assays in which the cells were plated at a density of 200 cells/cm² represented an extreme of the lag phase in which FET cells exhibited high levels of noncycling cells. When the cells were plated at clonal density, transferrin and insulin added at time of plating were sufficient to initiate growth of FET colonies. Removal of either transferrin or insulin abolished clonal growth (Fig. 6A), suggesting that transferrin and insulin are the minimal requirement for FET cells to grow in serum-free medium. To examine whether autocrine TGFα acts in concert with these two factors to support clonal initiation and expansion, neutralizing antibodies against TGFα or EGF were utilized. If autocrine TGFα were necessary for colony formation, addition of either antibody should inhibit the clonal growth. Addition of the TGFα-neutralizing antibody inhibited clonal growth in a dose-dependent manner (Fig. 6B). A neutralizing antibody against EGF, which blocks ligand binding (Sunada et al., 1990), also inhibited clonal growth (Fig. 6C).

**Autocrine TGFα was not necessary for cell growth in the exponential phase**

We then examined the effect of TGFα antibody treatment on exponentially growing FET cells at this growth state. If our hypothesis concerning a role for autocrine TGFα for reentry into the cell cycle from a growth arrested, noncycling state were correct, antibody treatment should have no effect on exponential cells. FET cells were plated at a density of 5,000 cells/cm². TGFα antibody (20 μg/ml) was added at the time of plating and cells were counted 4 days later. The addition of TGFα antibody at initial plating inhibited cell growth by ~50%. However, after the cells were grown for 3 days to allow them to enter exponential phase, addition of the antibody had no effect on cell growth (Fig. 7). The TGFα mAb is a high-affinity antibody which neutralizes TGFα when used at ratio of 1 μg antibody to 0.5 ng TGFα protein (Sorvillo et al., 1990; Oncogene data sheet for TGFα Ab-3, Oncogene Science, Cambridge, MA). The concentration of TGFα protein in tissue culture medium conditioned by exponentially growing FET cells ~70 μg/ml, as analyzed by TGFα ELISA assay (data not shown). Therefore, the antibody concentration used in our experiment was well in excess of the amount needed. These results suggest that autocrine TGFα is necessary to support clonal initiation but not cell growth at the exponential phase. This is in contrast to normal cells, which require exogenous TGFα/EGF for clonal initiation (Pittelkow et al., 1993).

**DISCUSSION**

Since the introduction of the autocrine hypothesis to account for the growth advantage of malignant cells over their normal counterparts, our understanding of polypeptide growth factors and their mechanisms of signal transduction has seen a precipitous expansion. Despite these advances, how autocrine growth factor action is controlled and when it is activated in the cell cycle remain largely unknown. The work presented here shows that prolonged growth arrest leads to down-regulation of TGFα transcription. This autocrine activity must be reactivated for reentry into the cell cycle by the bulk of growth-arrested cells. Submaximal reactivation of TGFα expression occurs as a result of nutrient replenishment of quiescent FET cells and might reflect the recognized role of glucose conversion to glucosamine in the stimulation of TGFα transcription.
Fig. 6. Autocrine TGFα is required for clonal growth of FET cells. A: Cloning efficiency assay of FET cells grown in transferrin and insulin (TI), SM + I, SM + T, and SM medium. B: Cloning efficiency assay of FET cells grown in TI medium with 0, 6, and 15 μg/ml TGFα-neutralizing antibody, respectively. C: Cloning efficiency assay of FET cells grown in TI medium with or without 200 ng/ml EGFα-blocking antibody.

(Sayeski and Kudlow, 1996). Importantly, the low level of TGFα stimulation achieved by nutrient replenishment alone is not sufficient to lead to stimulation of DNA synthesis or reentry of cells into the cell cycle at substantial levels. Our results indicate that in order to obtain substantial reentry into the cell cycle, exogenous insulin must be present in addition to nutrients. The induction of autocrine TGFα-mediated EGFα activation in early G1 is an essential event for DNA synthesis, as shown by TGFα-neutralizing antibody blockade of DNA synthesis and the failure of TGFα-neutralizing antibody to block DNA synthesis when its addition is delayed until 4–8 hr after growth factor and nutrient treatment.

Our results show for the first time the interdependence and crosstalk between insulin and autocrine TGFα in stimulating DNA synthesis. It is not clear whether insulin receptor or insulin-like growth factor receptor (IGF-IR) activation by insulin is responsible for this observed crosstalk. However, activation of IGF-IR has been identified as the target for the mitogenic effect of insulin, and the concentration of insulin used in this study was sufficient to activate IGF-IR (Stewart et al., 1990). Thus, activation of IGF-IR by exogenous ligand appears to be essential for TGFα induction.

The most obvious growth advantage imparted by autocrine TGFα to malignant FET cells is abrogation of the requirement for an exogenous source of EGFα activation for reentry into the cell cycle. This advantage does not appear to be shared by nonmalignant cells, since, in contrast to FET cells, normal keratinocytes (Pittelkow et al., 1993) and a nonmalignant colon adenoma cell line (Markowitz et al., 1990) showed an absolute requirement for exogenous growth factor to activate EGFα for initiation of clonal growth at low cell densities. Significantly, subsequent colony expansion of keratinocytes was independent of endogenous or exogenous TGFα (Pittelkow et al., 1993). This suggested that EGFα activation was required for initiation of clonal growth, but these normal cells were unable to produce sufficient autocrine TGFα or any other autocrine EGF-like growth factor to support this process at
Fig. 7. Autocrine TGFα is not necessary for cell growth in exponential phase. FET cells (TI medium) were plated into two sets of 35-mm wells (6 × 10^6 cells/cm²). TGFα antibody (20 μg/ml) was added to one set of wells at time of plating (Lag Phase), and cells were counted 4 days later. The other set of cells was grown for 3 days to allow entry into the exponential phase (Exponential Phase). The TGFα antibody was then added, and cells were counted 4 days later. Cell counting was performed with a hemocytometer, as previously described (Zieber et al., 1993). Percentages of respective controls were plotted with each control cell number set as 100% (mean ± SD, n = 3).

Clonal densities. The lack of a TGFα requirement for colony expansion of keratinocytes parallels the lack of an autocrine TGFα requirement for exponential FET cell growth. These observations support the hypothesis that autocrine TGFα acts primarily by facilitating the movement of noncycling cells back into the cell cycle, and that it imparts a growth advantage to malignant cells through its inappropriate expression in noncycling states, such as lag phase and quiescence in tissue culture, or inappropriate induction of expression early in the cell cycle by other signaling pathways, as observed in FET cells.

TGFα promoter-reporter (CAT) assays showed that the growth-related expression of TGFα was regulated at the transcription level during the establishment of and subsequent release from quiescence in the FET cells. The controlling element resided in the region between −370 to −201 bp (relative to the translation start codon) in the TGFα promoter. Work by other investigators suggests the involvement of two potential mechanisms for controlling TGFα transcription under these conditions. The first involves the metabolism of glucose to glucosamine through the L-glutamine: D-fructose-6-phosphate amidotransferase (GFAT) enzyme. Transcription of the TGFα gene was increased when arterial smooth muscle cells were exposed to supraphysiological concentrations of glucose (McClain et al., 1992). Glucose metabolism to glucosamine through the GFAT enzyme activity, as observed in human skeletal muscle cells (Daniels et al., 1996). The glucose/glucosamine response element in the TGFα promoter was located in the same region we identified in this study (McClain et al., 1992). Therefore, it is feasible that glucose in SM nutrient replenishment medium stimulates TGFα expression in quiescent FET cells, and that insulin in the TI medium further amplifies this stimulation by increasing the expression of the GFAT enzyme and/or allosteric activation of the enzyme. The second mechanism controlling TGFα transcription involves EGFr activation (Coffey et al., 1992; Mulder, 1991; Zipfel et al., 1993). Raja et al. (1991) identified an EGF/TGFα response element which resides between −373 to −201 bp of TGFα promoter. Both the downregulation and upregulation of TGFα expression noted in our study were accompanied by parallel changes in levels of EGFr activation (data not shown). EGF/TGFα stimulation of TGFα transcription was independent of GFAT enzyme activity (Roos et al., 1996; Paterson and Kudlow, 1995), suggesting that glucose and EGF/TGFα utilize different signaling pathways to stimulate TGFα transcription. Moreover, the divergence of GFAT and EGFr controls of TGFα activation implies that the transcription factor(s) binding to this region of TGFα promoter might be a convergence point for multiple signaling pathways.

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LITERATURE CITED


