Autocrine Transforming Growth Factor α Provides a Growth Advantage to Malignant Cells by Facilitating Re-entry into the Cell Cycle from Suboptimal Growth States*

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CBS human colon carcinoma cells are poorly tumorigenic in athymic nude mice, whereas FET colon carcinomina cells are non-tumorigenic. Both cell lines have well differentiated properties in tissue culture. Transforming growth factor α (TGF-α) was ectopically expressed by stable transfection of a TGF-α cDNA under repressible tetracycline control. The TGF-α-transfected cells showed enhanced clonal initiation and shortened lag phase growth in tissue culture without an alteration in doubling time in exponential phase relative to untransfected cells. Furthermore, the TGF-α transfectants showed increased independence from exogenous growth factors in clonal growth assays and induction of DNA synthesis after release from quiescence. Growth factor independence was associated with sustained epidermal growth factor receptor activation in quiescent TGF-α-transfected cells and the requirement of exogenous insulin for stimulation of quiescent cells to re-enter the cell cycle. Higher cloning, reduced lag time in tissue, and the acquisition of growth factor independence for DNA synthesis without a change in doubling time of TGF-α-transfected cells indicate that autocrine TGF-α functions by facilitating re-entry into the cell cycle from sub-optimal growth states rather than promoting or controlling the proliferation of actively cycling cells. The modulation of growth regulation by autocrine TGF-α was associated with increased malignant properties as TGF-α transfectants showed increased tumorigenicity in athymic nude mice. The administration of tetracycline reversed the effects of TGF-α expression in these cells both in vivo and in vitro, indicating that the alterations of the biological properties were due to the expression of TGF-α. Since these cells are continuously grown in a completely chemically defined medium without serum supplementation, it was possible to assign the mechanism underlying the generation of growth factor independence to the replacement of a requirement for exogenous insulin in parental cells by autocrine TGF-α.

Transforming growth factor α (TGF-α) is a potent mitogen structurally and functionally related to the epidermal growth factor (EGF) family of proteins (1). It binds to the EGF receptor (EGFR) to produce a positive growth stimulus (2, 3). Elevated expression of TGF-α has been associated with neoplastic transformation (4, 5). Overexpression of TGF-α by stable transfection of a constitutive vector induced transformation of normal cultured fibroblasts (6). Co-infection with retroviruses encoding both TGF-α and EGFR produced NIH3T3 cell clones that grew efficiently in soft agar and formed tumors in nude mice (7). Transgenic mouse experiments revealed that the overexpression of TGF-α results in the appearance of malignant tumors in liver, mammary gland, and benign tumors in skin (8–10). Moreover, overexpression of TGF-α in vivo enhanced oncogene-induced carcinogenesis in pancreas, liver, and mammary gland (11–13) and accelerated chemically induced hepatocarcinogenesis (14, 15).

The contribution of TGF-α in the malignant progression of human carcinomas has not been well studied. Moreover, although it is clear that TGF-α promotes tumorigenicity and malignant progression in a variety of in vitro and in vivo assays, it is not apparent what specific growth advantages it imparts to cells in the various contexts of proliferative function and what specific growth functions it replaces in generating the independence from exogenous growth factors associated with malignant progression. More specifically, it is not known whether autocrine TGF-α functions by driving exponential growth or whether it is more critical in other contexts of proliferation such as re-entry into the cell cycle from growth-arrested states. These gaps in our understanding of TGF-α function in malignancy reflect a general lack of understanding as to how autocrine-positive growth factors contribute a growth advantage to malignant cells, especially in light of the well documented autocrine activity of normal cells (34). Thus, an in depth understanding of how TGF-α works in malignancy will provide direction for the investigation of other autocrine factors. Moreover, the understanding of the pathological nature of growth regulatory disruptions associated with malignant progression may lead to new approaches for the diagnosis, prevention, and treatment of cancer.

We have addressed the issue of understanding the pathological disruption of growth regulation by autocrine TGF-α using growth factor-dependent cell line models (CBS and FET cells)

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† The abbreviations used are: TGF-α, transforming growth factor α; EGF, epidermal growth factor; EGFR, EGF receptor; TTA, tetracycline controlled transactivator; NEO, neomycin; hCMV, human cytomegalo-virus; bp, base pair; IGFI, insulin-like growth factor I.
grown in completely chemically defined medium. Consequently, specific exogenous growth factor requirements have been defined for exponential proliferation as well as re-entry into the cell cycle from growth-arrested states (16, 17). These growth factor-dependent cell lines were stably transfected with a human TGF-α cDNA under repressible control by tetracycline in order to generate a strong autocrine TGF-α loop so that specific growth requirements assumed by autocrine TGF-α could be determined and evaluated in the context of malignant progression as well. Repressible tetracycline control has permitted analysis of specific growth states in tissue culture and in vivo which are dependent upon autocrine TGF-α, whereas the completely chemically defined medium in which these cells are continuously maintained and grown permits the determination of specific exogenous growth factor requirements replaced by autocrine TGF-α activity.

Previously, we contrasted the biological properties of these early stage malignant cell models with those of highly progressed colon carcinoma cell lines (18–21). In contrast to early stage models, highly malignant colon carcinoma cells are completely growth factor-independent with respect to stimulation by exogenous growth factors, are anchorage-independent, and form tumors in 100% of athymic mice with as little as 10⁶ cells. Importantly, these highly malignant cells express TGF-α and EGFR constitutively in growth-arrested states in contrast to CBS and FET cells in which TGF-α is down-regulated during the establishment of growth arrest. CBS and FET cells are well differentiated in tissue culture as indicated by retention of basolateral polarity, microvilli, and transport function (18–21). Moreover, they are weakly anchorage-independent and poorly tumorigenic in athymic mice (18, 20). These cells are dependent upon exogenous growth factors for DNA synthesis with a requirement for insulin to re-enter the cell cycle from a growth-arrested state.

TGF-α transfection using a tetracycline-repressible system permitted the generation of CBS and FET cells in which TGF-α was not down-regulated at growth arrest since the transgene was not under control of the TGF-α promoter. This created a model that could then be modulated to behave in the same manner as the highly malignant cells described above in which TGF-α was expressed in growth arrest or to behave like weakly malignant cells that down-regulate TGF-α in growth arrest when tetracycline was added. Comparison of wild type cells with TGF-α transfectants shows that autocrine TGF-α generates malignant progression in a tetracycline-repressible manner by the criteria of tumorigenicity in athymic mice and anchorage-independent growth. In addition, several lines of evidence indicate that autocrine TGF-α mitigates the requirements for exogenous insulin and EGF in this model system. Finally, we show that inappropriate TGF-α expression in transfected cells provides a growth advantage over wild type cells by enhancing the ability of growth-arrested cells to re-enter the cell cycle in association with inappropriate EGFR activation in growth arrest. Inappropriate EGFR activation resulted in the recruitment of other EGFR family members to form complexes with the activated EGFR in growth-arrested TGF-α transfected cells. The recruitment of other EGFR family members expanded the potential for additional signal transduction pathways. The advantage of growth factor independence would be of particular importance to malignant cells under environmental conditions that are limiting to growth.

Environmental restriction on growth appears to be common in solid tumors such as colon carcinoma in which labeling studies have shown that fewer than 1% of the malignant cells are cycling at any given time (41).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human colon carcinoma cell lines were originally isolated from primary tumors as described previously (18) and continuously maintained in a chemically defined serum-free medium consisting of McCoy’s 5A medium (Sigma) supplemented with pyruvate, vitamins, amino acids, antibiotics, insulin (20 μg/ml, Sigma), transferrin (4 μg/ml, Sigma), and EGF (10 ng/ml, R & D Systems, Minneapolis, MN) (20). Containing cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and routinely checked for mycoplasma contamination. Limiting dilution clones were obtained by plating the parental cells into 96-well tissue culture plates at one cell per well. Typical limiting dilution clones (designated as CBS4 and FET6) were used for transfection to ensure the uniformity of the isolated transfectants. TGF-α transfectants and control cells were routinely maintained in serum-free medium containing 650 μg/ml geneticin (Life Technologies, Inc.).

**Transfections**—A 930-bp fragment of full-length TGF-α cDNA (23) was inserted into the EcoRI site of the polycloning region in the tetracycline-repressible vector pUHD10–3 (22) in the sense orientation relative to the human cytomegalovirus (hCMV) minimal promoter to create the expression plasmid pTET-TGF-α. Orientation of the insert was confirmed by restriction digestion. The hCMV minimal promoter requires a tetracycline-controlled transactivator (tTA) that is constitutively produced by another vector. The latter vector (pUHD15–1NEO) also contains the neomycin-resistant gene which can be used for antibiotic selection (24). Because tetracycline prevents tTA from binding to tet operators placed upstream of the hCMV minimal promoter, it represses the tTA-dependent expression system. The expression vector (10 μg) and the tTA-expressing vector (1 μg) were co-transfected into cultured cells (Embyronic 293 cells) at 250 V and 960 millifarads. The control cells were transfected with the tTA-NEO selection vector and the cloning vector without any insert. Selection of stable transfectants was carried out by addition of geneticin (650 μg/ml). After 3 weeks of selection, geneticin-resistant clones were isolated and expanded to screen for TGF-α expression.

**Administration of Tetracycline**—Tetracycline (Sigma) was dissolved in 50% ethanol and stored at −20°C. Tetracycline was added to the culture medium at a final concentration of 0.1 μg/ml. Fresh tetracycline was added when changing medium (2× weekly). Tetracycline was added to the drinking water of athymic nude mice at a concentration of 3 mg/ml on day 6 and 2 mg/ml on days 7 through 21 of the in vivo study.

**RNA Isolation and Analysis**—Total RNA was isolated from confluent cell cultures by lysis in guanidine isothiocyanate and purified by ultracentrifugation through a cesium trichloroacetic acid gradient as described previously (25). TGF-α RNase protection assays were performed as described previously (21). Briefly, a 306-bp high specific riboprobe was generated with the TGF-α riboprobe template in the presence of [α-32P]UTP (NEN Life Science Products) by Sp6 RNA polymerase. The riboprobe was hybridized with 20 μg of total RNA for 12–16 h. Following RNase digestion of excess riboprobe, the protected fragment was separated on a 6% polyacrylamide, 7 M urea sequencing gel. Loading was normalized by simultaneous hybridization of the RNA with an actin probe that yields a 145-bp protected fragment (21). The protected endogenous TGF-α fragment was 283 bp, whereas the protected, transfected TGF-α fragment was 306 bp, since the expression vector contained an additional 23 bp of vector sequence. This provided the ability to distinguish the expression of the transfected TGF-α mRNA from the endogenous mRNA. Quantitative analysis of the protected mRNA was performed with an Ambis Analysis System (Ambis, San Diego, CA).

**Conditioned Medium and TGF-α Enzyme-linked Immunosorbent Assay**—Cells were plated at 120,000 cells per well in 6-well plates in serum-free medium lacking EGF in the presence or absence of tetracycline at a final concentration of 0.1 μg/ml. On day 4, 48-h conditioned medium was collected and clarified by centrifugation. Cells were enumerated with a hemocytometer after trypsinization. TGF-α levels were measured with a TGF-α enzyme-linked immunosorbent assay kit (Oncogene Science) following the manufacturer’s instructions. The TGF-α levels were normalized to cell numbers and expressed as TGF-α protein per 10⁶ cells.

**Growth Assays**—Cells were plated at a clonal density of 300 cells/well into 24-well tissue culture plates in serum-free medium in the presence or absence of EGF. Tetracycline was used at a final concentration of 0.1 μg/ml. Cells were grown for 13 days without changing the culture medium. The cells were stained by addition of 500 μl of 0.2% of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma) to each well for 2 h. Following removal of the medium and staining solution, the colonies were solubilized in 800 μl of Me₃SO. The plates were placed on
a shaker for 5 min at room temperature after which they were read immediately at 595 nm. The absorbance of dissolved colonies is directly proportional to the number of cells in culture.

**Proliferation Assays**—Cells were plated at different cell densities (30,000–360,000 cells/well) into 6-well tissue culture plates in serum-free medium lacking EGF. Every other day the medium was changed. The cells were pulse-labeled with methyl-[3H]thymidine (Amersham Pharmacia Biotech) for 1 h. DNA synthesis was measured by incorporation of methyl-[3H]thymidine into trichloroacetic acid-precipitable material.

The mitogenesis assays were performed as described previously (16). The cells were plated at a density of either 3.0 × 10^4 cells per well in 24-well tissue culture plates or 1.2 × 10^5 cells per well in 6-well plates in medium containing transferrin, insulin, and EGF. The cells were rendered quiescent by growth factor and nutrient deprivation for 6 days in supplemental McCoy's medium after reaching saturation density (7 days). Previous work (16) showed that CBS and FET cells treated by this protocol had a labeling index of 5–10% as determined by [3H]thymidine incorporation followed by autoradiography. Cells were released from quiescence with supplemental McCoy's medium (nutrients alone) or serum-free medium (nutrients and growth factors). The cells were pulse-labeled with methyl-[3H]thymidine for 1 h. DNA synthesis was measured by incorporation of methyl-[3H]thymidine into trichloroacetic acid-precipitable material at the peak of DNA synthesis 18 h after release from quiescence (16).

**Immunoprecipitation and Western Immunoblotting**—Cells was washed with cold phosphate-buffered saline and harvested in cold lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl with 0.5% Tween 20 containing 5% nonfat dried milk for 2 h at 4 °C with continuous rotation. The immunoprecipitates with anti-EGFR antibody were then detected with phosphorosine monoclonal antibody (PT99) from Santa Cruz Biotechnology. The immunocomplexes with anti-activated EGFR antibody were then immunoblotted with anti-ErbB2 and anti-ErbB3 polyclonal antibodies (Santa Cruz Biotechnology) and anti-SHC antibody (Upstate Biotechnology Inc.), respectively. The blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature and then washed twice with water, 5 min with TTBS buffer, and 5 more times with water. The membrane was imersed in ECL for 1 min and exposed to ECL film. EGFR activation was determined by overnight at 4 °C with anti-EGFR antibody or anti-activated EGFR antibody (Transduction Laboratories, Lexington, KY). Immunoprecipitates were subsequently incubated with 50% protein A-agarose suspension (Life Technologies, Inc.) for 30 min at 4 °C and then were washed twice with cold lysis buffer 3 times followed by centrifugation. Pellets were resuspended in sample buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, and 4% β-mercaptoethanol), heated for 5 min at 95 °C, and resolved by 7.5% SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose membranes. Membranes were blocked with TTBS buffer (20 mM Tris-HCl (pH 7.5), 0.5% NaCl, 0.05% Tween 20) containing 5% nonfat dried milk for 2 h at 4 °C with continuous rotation. The immunoprecipitates with anti-EGFR antibody were then detected with phosphorosine monoclonal antibody (PT99) from Santa Cruz Biotechnology. The immunocomplexes with anti-activated EGFR antibody were then immunoblotted with anti-ErbB2 and anti-ErbB3 polyclonal antibodies (Santa Cruz Biotechnology) and anti-SHC antibody (Upstate Biotechnology Inc.), respectively. The blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature and then washed twice with water, 5 min with TTBS buffer, and 5 more times with water. The membrane was imersed in ECL for 1 min and exposed to ECL film. EGFR activation was also detected with an anti-activated EGFR antibody (Transduction Laboratories, Lexington, KY). This activated EGFR antibody is unique in that it reacts only with the tyrosine-phosphorylated (activated) EGFR receptor (26). The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose membranes. Then the membrane was probed with the anti-activated EGFR antibody directly.

**Anchorage-independent Growth**—Soft agarose assays were performed as described previously (18). Briefly, 3,000 cells suspended in serum-free growth medium minus EGF containing 0.4% agarose (Sigma) were plated per well into 6-well tissue culture plates containing 0.8% agarose underlayers. Colonies were allowed to grow for 2 weeks at 37 °C in a humidified incubator at 5% CO2, stained with 1 ml of 0.8% agarose underlayers. Colonies were allowed to grow for 2 weeks at 37 °C in a humidified incubator at 5% CO2, stained with 1 ml of methyl-[3H]thymidine and photographed.

**Tumorigenicity**—NEO control and TGF-α transfectant cells were injected subcutaneously behind the anterior forelimb of 5–6-week-old BALB/c athymic mice. Mice were maintained in a genotobiotic environment before and during each experiment. Tetracycline was given to mice in drinking water at a concentration of 3 mg/ml on day 6 and 2 mg/ml on day 7 through day 21 of inoculation. Growth curves with for xenografts were determined by externally measuring tumors in two dimensions using a caliper. Volume (V) was determined by the following equation, where L is length and W is the width of the tumor: V = (L × W^2) × 0.5.

**Flow Cytometry Analysis**—Flow cytometry was performed as described previously (43). Briefly, trypsinized cells were washed with cold phosphate-buffered saline and resuspended in 0.4–0.6 ml of low salt propidium iodide stain (0.03 g/ml polyethylene glycol, 0.05 mg/ml propidium iodide (Sigma), 0.1% Triton X-100, and 4 mM sodium citrate). The suspension was incubated with 10 μl of RNase A (2 mg/ml, DNase-free) at 37 °C for 20 min. An equal volume of high salt stain (0.03 g/ml polyethylene glycol, 0.05 mg/ml propidium iodide (Sigma), 0.1% Triton X-100, and 400 mM sodium chloride) was then added. The mixture was vortexed gently and stored at 4 °C overnight. The cell cycle phase distribution was performed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA), and cell cycle parameters were obtained using a ModFit LT program (Verity Software House Inc.).

**RESULTS**

**Expression of TGF-α**—Following transfection, genicin-resistant clones were isolated and expanded for screening of TGF-α expression. Four clones (designated CBS4 α2S, α3S, α5S, and α6S) were identified. The CBS4 transfectants (Fig. 1, lanes 4, 6, 8, and 10) expressed 15–25-fold higher TGF-α mRNA levels relative to CBS4-NEO (lane 2). As described under "Experimental Procedures," the size of the protected transfected TGF-α mRNA fragment in RNase protection assays was larger than the protected endogenous TGF-α mRNA. Tetracycline (0.1 μg/ml) repressed the expression of transfected TGF-α mRNA (Fig. 1, lanes 3, 5, 7, and 9).

**TGF-α protein levels** in conditioned medium also showed a marked increase of 15–25-fold as compared with CBS4-NEO (Fig. 2 and data not shown). Similarly, the TGF-α protein levels were repressed by the presence of 0.1 μg/ml tetracycline in the growth medium (Fig. 2). Therefore, the overexpression of TGF-α mRNA is reflected by increased expression of the amount of TGF-α released into culture medium. Similar results were obtained from FET-NEO and TGF-α-transfectant cells.

**Cloning Efficiency of CBS4 TGF-α Transfectants**—The effects of expression of TGF-α on the clonal growth of CBS4 cells...
were determined. Our previous work has shown that CBS cells require exogenous growth factors for optimal growth (16, 20). We hypothesized that the overexpression of TGF-α would reduce the growth factor requirements of CBS4 cells and result in increased clonal formation in the absence of growth factors. As expected, the clonal initiation of CBS4 cells overexpressing TGF-α was markedly increased, as compared with the CBS4-NEO cells (Fig. 3). The control cells (NEO) showed an EGF-dependent phenotype in cloning efficiency experiments. The TGF-α transfectants showed an EGF-independent phenotype in the absence of tetracycline treatment (Fig. 3A). These findings suggested that overexpression of TGF-α could move more cells from the non-dividing, quiescent stage back into the cell cycle and drive more cells to initiate the clonal growth. However, in the presence of tetracycline which suppressed expression of TGF-α in the transfected cells, the clones revealed a growth factor-dependent phenotype similar to that of the NEO control cells (Fig. 3A). These data provide one line of evidence that autocrine TGF-α enhances re-entry into the cell cycle under environmental conditions that are not optimal for growth.

**Mitogenesis of TGF-α Transfectants**—Another line of evidence of increased growth factor independence of TGF-α transfectants was demonstrated by mitogenesis studies. Quiescent growth-arrested CBS cells require exogenous growth factors in addition to fresh nutrients for re-entry into the cell cycle and induction of DNA synthesis as described previously (16). CBS4 control cells required exogenous insulin for initiation of DNA synthesis (NEO in Fig. 4A). In contrast, DNA synthesis by quiescent TGF-α transfectants stimulated with fresh medium lacking any growth factors (nutrient replenishment alone) was comparable to that of cells released with insulin-containing medium or a medium containing insulin, transferrin, and EGF (Fig. 4A). Cell cycle distribution of wild type and TGF-α transfectant cells was determined by flow cytometry following the 6-day period of growth factor and nutrient deprivation to generate quiescence in order to ensure that growth arrest in TGF-α transfectants was comparable to control cells. Flow cytometry analysis of CBS NEO and CBS TGF-α transfectants indicated that 91 and 92% of cells were in G0/G1, respectively. When tetracycline was used to repress the expression of transfected TGF-α, the TGF-α transfectants regained the insulin-dependent phenotype for DNA synthesis (Fig. 4B). These data demonstrated that expression of TGF-α reduced the requirements for exogenous growth factors by these cells, thus providing growth factor independence for re-entry into the cell cycle from quiescence. These results provide a second line of evidence that autocrine TGF-α functions by enhancing growth and survival under stringent environmental conditions. Similar results were obtained for FET6 TGF-α transfectant cells (data not shown). Moreover, the acquisition of growth factor independence was reflected by the ability of TGF-α transfectants to proliferate in the absence of growth factor supplementation, whereas control cells could not sustain proliferation in the absence of exogenous growth factors added to the medium (Fig. 4C). Addition of tetracycline to the medium of TGF-α transfec-
activation of endogenous TGF-α was followed by Western analysis to probe for ErbB2 and ErbB3 proteins and visualized by sequential incubation with horseradish peroxidase-conjugated secondary antibody. Immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting with anti-EGFR antibody for normalization.

Sustained TGF-α binding and EGFR activation could lead to the recruitment of other EGFR family members to form complexes with EGFR and thus further augment the potential for enhanced signal transduction (28–30). The activated EGFR antibody was employed to immunoprecipitate activated EGFR followed by Western analysis to probe for ErbB2 and ErbB3 complex formation with the EGFR. Heterodimer formation between activated EGFR and ErbB2 or ErbB3 was observed in exponential CBS4 TGF-α cells as well as exponential CBS4 NEO cells (Fig. 5). When cells were rendered quiescent, the CBS4 TGF-α transfectants retained high levels of heterodimerization, whereas a significant reduction was seen in NEO control cells (Fig. 5D). Activated EGFR should also induce downstream components of signal transduction. The association of SHC SH2 domain proteins with activated EGFR was therefore examined. Results indicated that only the 52- and 46-kDa iso-
forms of SHC interacted with the activated EGFR in CBS cells. Quiescent TGF-α-transfected cells retained a high level of association with SHC relative to NEO cells. These results suggested that autocrine TGF-α activity in CBS4 cells not only induced higher EGFR phosphorylation and activated other EGFR family members but also stimulated the association of activated EGFR with downstream molecules.

Reduction of Lag Time in TGF-α Transfectants—Lag phase of cell culture is the period of adaptation following subculture and reseeding during which cells demonstrate reduced proliferation. If increased autocrine TGF-α activity mitigates the effects of environmental conditions retarding growth, it would be expected that the time required for entering exponential growth by TGF-α-transfected cells would be reduced relative to CBS4 NEO cells. CBS4 TGF-α-transfectant cells (αS2) exhibited a shortened lag phase as compared with CBS4-NEO cells when cultures were inoculated at low cell density (Fig. 6, A–D). The effect of low cell density on the time to reach exponential growth in tissue cultures was abrogated by increasing the number of cells plated. The growth curves in Fig. 6 indicate that increasing the number of cells plated leads to a reduction in the difference between the TGF-α-transfectants and CBS NEO cells. At high cell density the exponential growth rates of the transfectant and control cells were virtually identical. Thus, TGF-α expression appears to generate increased clonal initiation of cultures at a given inoculum resulting in shortened lag phase. The lack of effect on exponential growth indicates that autocrine TGF-α functions by allowing re-entry into the cell cycle rather than affecting cell cycle transit. The results in Fig. 6, A and E, were confirmed using flow cytometry (Table I). Flow cytometric data from day 11 at an inoculum of 10⁴ cells (Fig. 6A) were compared for TGF-α-transfectants and wild type cells. The percentages of cells from TGF-α-transfectants in S or G₂/M were approximately 1.5-fold higher than wild type cells, indicating earlier escape from lag phase by TGF-α-transfectants. This is contrasted by comparison of TGF-α-transfectants with wild type cells at day 2 and 4 after seeding at high density (3.6 × 10⁵ cells) in order to abrogate lag phase where percentages of cells in S and G₂/M were essentially equal for both cell types.

Expression of TGF-α Enhanced Malignant Properties—CBS cells have previously been shown to have low cloning efficiency in soft agarose and to be poorly tumorigenic in athymic nude mice (18, 20). Consequently, we compared the ability of CBS TGF-α-transfectants and control cells to form colonies in soft agarose because this property is considered to be reflective of malignancy (31, 32). TGF-α-transfectants showed both increased colony number and colony size, compared with CBS4-NEO cells (Fig. 7). The number of colonies from TGF-α-transfectants was increased more than 2-fold relative to NEO (Fig. 7A). Tetracycline treatment reversed these effects (Fig. 7B). These in vitro data demonstrated that expression of TGF-α increased the malignant properties of the transfectants.

We next examined the tumorigenic properties of CBS and FET transfectant cells in vivo. TGF-α-transfectants and control NEO cells (5 × 10⁶) were injected into athymic mice to determine whether increased expression of TGF-α would affect the tumorigenicity. Both CBS4 NEO cells and αS2 clone formed xenografts in 10/10 inoculations by day 6. However, the αS2 clone showed a marked increase in tumor growth, giving rise to a 4-fold increase in tumor volume as compared with CBS4-NEO (Fig. 8A). Repression of TGF-α by addition of tetracycline in the drinking water of mice from day 6 to day 21 of the inoculation resulted in reversion of the slope of the growth curve to that of the NEO control (Fig. 8A). After terminating tetracycline treatment, αS2 tumors regained a rapid growth rate similar to that of αS2 cells without tetracycline treatment. Thus, the overexpression of TGF-α in CBS4 cells is sufficient to enhance malignant properties of these cells in vivo. In contrast to CBS4 cells, FET6 cells do not form progressively growing tumors (42). FET6 cells form initial nodules of approximately 200 mm³ at a high subcutaneous inoculum (10⁷ cells) which ultimately disappear. Therefore, it was of interest to determine whether TGF-α transfection could generate a progressively growing phenotype which would not regress after initial tumor nodule formation. Fig. 8B shows that TGF-α transfection of FET6 cells resulted in a fully tumorigenic phenotype in 10/10
inoculations, whereas tetracycline treatment of the animals from day 10 to day 24 resulted in complete loss of tumorigenicity.

DISCUSSION

We have demonstrated that high levels of autocrine TGF-α contribute to the progression of malignant properties in the CBS4 and FET6 human colon carcinoma cell lines. The CBS TGF-α-transfected cells formed larger tumors than control cells in athymic nude mice, and repression of TGF-α by tetracycline treatment in vivo resulted in the reacquisition of tumor growth at the same rate as control cells in the CBS model. TGF-α transfection was permissive for progressive tumor growth in the non-tumorigenic FET6 cell line, whereas tetracycline treatment led to the complete resolution of tumors. Moreover, the TGF-α transfectants showed decreased exogenous growth factor requirements for clonal initiation in tissue culture and the initiation of DNA synthesis, as well as increased cloning efficiency in soft agarose in vitro. These results demonstrated that autocrine TGF-α plays a role in the acquisition of properties associated with malignant progression of cells such as growth factor independence and tumorigenicity.

Moreover, these results address important issues regarding the mechanism whereby autocrine TGF-α activity can impart a growth advantage to malignant cells. One issue is whether autocrine TGF-α provides independence from other growth factors as well as the expected mitigation of any exogenous EGF family ligands required for EGFR activation to enhance cell cycle entry. The utilization of model systems in which cells are grown in completely defined medium permitted the recognition that autocrine TGF-α activation can free the cells from an exogenous source of IGF1 receptor activation for re-entry into the cell cycle from quiescence. In addition, the present work provides three lines of evidence showing that autocrine TGF-α activity can enhance re-entry into the cell cycle under environmental conditions that are highly restrictive to parental CBS cells. These included increased growth factor-independent clonality, decreased lag time in tissue culture, and complete abrogation of exogenous growth factor requirements for DNA synthesis from a quiescent state. These effects were contrasted by the lack of difference of doubling times observed in cells plated at high cell density. Thus, it appears that the primary function of TGF-α does not involve actively cycling cells. Perhaps some other autocrine factors function in this growth state.

**TABLE I**

| Flow cytometry analysis | Cells in CBS4 TGF-α transfectants and control cells plated at low and high density. |
|-------------------------|--------------------------------------------------------------------------------------|
|                         | Cells                                     | CBS NEO | CBS4 aS2          |
| Day 11**                | G0/G1: 75                                | 61      |
|                         | S: 21                                    | 30      |
|                         | G2/M: 4                                  | 9       |
| Day 2*                  | G0/G1: 67                                | 69      |
|                         | S: 25                                    | 23      |
|                         | G2/M: 8                                  | 8       |
| Day 4†                  | G0/G1: 66                                | 64      |
|                         | S: 26                                    | 28      |
|                         | G2/M: 8                                  | 8       |

**a** Cells were plated at low density (1 x 10⁴/60-mm well) as described in Fig. 6A. Flow cytometry analysis was determined at day 11.

**b** Cells were plated at high density (3.6 x 10⁵/60-mm well) as described in Fig. 6E and cell cycle analysis was determined at day 2 and day 4 as described under “Experimental Procedures.”

TGF-α Enhances Malignancy

**FIG. 7.** Anchorage-independent growth. CBS4 NEO cells and TGF-α clones (aS2 and aS5) were plated at 3,000 cells per well in 6-well plates in soft agarose containing serum-free medium lacking EGF and tetracycline (A). Some cells were treated with (+) tetracycline at 0.1 µg/ml or remained untreated (−) (B). Colonies were allowed to grow for 2 weeks, stained with p-iodonitrotetrazolium violet (Sigma), and photographed. Colonies were counted, and bar graphs were drawn (C and D).
TGF-\(\alpha\) Enhances Malignancy

It is important to note that demonstration of autocrine TGF-\(\alpha\) in normal cells and nonmalignant adenoma cells has thus far been restricted to actively cycling cells (34–37). The original autocrine hypothesis was formulated to account for the growth advantage of malignant cells over their normal counterparts (33). As with most hypotheses, the addition of new information over the course of time suggests the need for fine tuning of the hypothesis. One of the most important pieces of information pertinent to the autocrine hypothesis in recent years has been the recognition that normal cells demonstrate autocrine-positive activity (frequently in the form of TGF-\(\alpha\)) as an integral element of their growth regulation (34). The observation of autocrine TGF-\(\alpha\) activity in normal cells indicates the need to re-examine how TGF-\(\alpha\) imparts a growth regulatory advantage to malignant cells. Normal cells do not, in as far as we know now, exhibit independence from exogenous growth factors for re-entry into the cell cycle under the various conditions used in this study. Normal keratinocytes, for example, have an absolute requirement for exogenous growth factor activation of the EGFR at low cell densities but do not require exogenous EGF or TGF-\(\alpha\) for clonal expansion (35, 36). Sensitivity to EGFR blockade during clonal expansion indicates the need for autocrine-mediated EGFR activation in actively cycling cells. Similarly, nonmalignant colon adenoma cells show autocrine TGF-\(\alpha\) activity during exponential growth but are incapable of clonal growth without supplementation (37). Thus, in this regard, parental CBS cells appear to show many of the growth restrictions of non-malignant cells. Taken together, these lines of evidence and the results reported herein for TGF-\(\alpha\)-transfected CBS cells imply that the principal advantage of autocrine TGF-\(\alpha\) in the malignant cells may be the enhanced ability to re-enter the cell cycle from growth arrest in environments that are not conducive to proliferation. As such labeling studies have shown that less than 1% of malignant cells are actively cycling in colon cancers (41). Similar observations have been made with other solid tumors. These observations suggest the tumor environment is not particularly conducive to proliferation. Thus, an autocrine factor enabling re-entry of cells into the cell cycle could be highly advantageous to the malignant cells. A similar advantage could easily be envisioned for cells involved in seeding metastases since the number of cells initiating a metastatic deposit is generally regarded as being quite limited.

Most intriguing was the observation that high levels of autocrine TGF-\(\alpha\) not only replaced requirements for exogenous EGF but for insulin as well. Insulin through activation of the IGFI receptor could enhance EGFR expression and/or activation in growth factor-dependent cells. Alternatively, insulin could supply a different array of signal transduction pathways from the EGFR. Independence from insulin is intriguing because it implies that signal transduction by inappropriate autocrine TGF-\(\alpha\) differs qualitatively and/or quantitatively from the autocrine TGF-\(\alpha\) demonstrated by CBS4 control cells.

It could be speculated that TGF-\(\alpha\)-transfected cells generate higher levels of EGFR activation such that the increased magnitude encompasses insulin signal transduction. However, exponentially growing TGF-\(\alpha\)-transfected and wild type cells show similar levels of EGFR activation. Thus, although large increases in TGF-\(\alpha\) resulted from transfection, autocrine activation of EGFR in growth-arrested TGF-\(\alpha\) transfectants was only about 3-fold over wild type cells. This suggests that it is the inappropriate activation of EGFR in the growth-arrested state by cells which normally down-regulate TGF-\(\alpha\) and lower EGFR activation that provides independence from insulin. Inappropriate EGFR activation in growth-arrested states may generate signal transduction consequences that might not occur in the context of exponential cells. For example, EGFR activation in growth-arrested TGF-\(\alpha\)-transfected cells could involve the modification of signal transduction pathways through the generation of new dimer interactions between EGFR and other EGFR family members that might themselves be differentially regulated in exponential and quiescent cells.

Inappropriate expression of TGF-\(\alpha\) and/or recruitment of other EGFR family members could similarly interact with cell cycle control molecules such that there is overlap with insulin func-
Finally, autocrine TGF-α expression could cause autocrine activation of IGFII receptor through induction of IGFII and/or IGFII or might abrogate dependence on insulin for EGFR activation in cell cycle re-entry. Along this line we have noted that insulin induces re-expression of down-regulated TGF-α in the wild type CBS and FET models where these cells are stimulated by exogenous growth factor treatment to re-enter the cell cycle from quiescence. Inappropriate EGFR activation as seen in TGF-α transfectants could abrogate this type of requirement for insulin. Thus, our results raise an important issue regarding the mechanistic advantage of autocrine TGF-α in malignancy which remains to be resolved.

The use of a tetracycline-repressible transfection system provided a particularly powerful control for determining the alterations in biological properties resulting from TGF-α transfection. We have reported the use of this repressible vector in reversing growth properties in tissue culture (38). Although tetracycline-repressible systems have been utilized in transgenic mice (39, 40), this is the first example that we are aware of in which this type of system has been used to control tumor growth in vivo. When mice harboring TGF-α transfectants were given tetracycline, tumor growth was minimal and was virtually identical to that of NEO control. Removal of tetracycline was effective in permitting a growth rate that was then the same as that of TGF-α transfectant cell inoculated mice that had not received tetracycline. This result is significant because it directly demonstrates that indolent tumor growth in an in vivo setting can be stimulated to undergo significant progressive changes by a single event, which in this case is aberrant TGF-α expression.

Although the levels of TGF-α expression were increased 15–25-fold in TGF-α transfectants, the level of EGFR activation in exponential transfected cells was only about 2–3-fold higher than NEO controls. A similar level was observed in quiescent TGF-α-transfected cells. Since autocrine TGF-α functions through the EGFR exclusively, this result implies that malignant progression could result from relatively small increments of TGF-α. This raises an important issue as to the role of autocrine TGF-α in the pathogenesis of growth regulation of highly progressed malignant cells. Is the level of autocrine TGF-α important or is it inappropriate continued expression during growth arrest that imparts an advantage? The results from this study cannot directly answer this question. However, they are consistent with the hypothesis that it is inappropriate expression since there is a relatively small change in endogenous EGFR activation of the TGF-α transfection as described above. The hypothesis that inappropriate expression of TGF-α at growth arrest is the important determinant of autocrine growth advantage is also consistent with a lack of effect on doubling time in TGF-α-transfected cells compared with CBS NEO controls. Along these lines, previous work has shown that highly progressed human colon carcinoma cell lines, such as HCT116, are independent of exogenous growth factors for re-entry into the cell cycle and show increased TGF-α expression in growth arrest similarly to the TGF-α CBS4 transfectants generated in this study (16, 27). Moreover, blockade of autocrine TGF-α in HCT116 cells results in the loss of growth factor independence for DNA synthesis (21).