TGF-β_1 Is an Autocrine-negative Growth Regulator of Human Colon Carcinoma FET Cells In Vivo as Revealed by Transfection of an Antisense Expression Vector

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Abstract. Transforming growth factor-β_1 (TGF-β_1) has previously been implicated as a potential negative autocrine or paracrine growth regulator of certain cell types (Arteaga, C. L., R. J. Coffey, Jr., T. C. Dugger, C. M. McCutchen, H. L. Moses, and R. M. Lyons. 1990. *Cell Growth & Differ.* 1:367–374; Hafez, M. M., D. Infante, S. Winawer, and E. Friedman. 1990. *Cell Growth & Differ.* 1:617–626; Glick, A. B., K. C. Flanders, D. Danielpour, S. H. Yuspa, and M. B. Sporn. 1989. *Cell Regulation.* 1:87–97). This is based mainly on experiments assessing the effects of exogenous TGF-β_1 or neutralizing antibodies to TGF-β_1 on normal or tumor cell proliferation in vitro. However, direct evidence demonstrating such a negative regulation of tumor cell growth in vivo is still lacking. To overcome this problem we have constructed and used an antisense expression vector for TGF-β_1 as a means of regulating endogenous TGF-β_1 expression in tumor cells. Antisense-transfected FET human colon carcinoma cells showed a fivefold reduction in TGF-β_1 mRNA and 15-fold reduction in TGF-β_1 secretion. Antisense mRNA was detected in transfected cells by an RNase protection assay. Compared to control cells, cultured antisense-transfected cells showed a reduction in lag phase time rather than a change in doubling time. Cloning efficiencies of transfected cells were four times greater than control cells in anchorage-independent assays. Control cells did not form tumors at 5×10^5 in athymic nude mice. Antisense-transfected cells formed tumors in 40% of animals injected. At higher inocula (1×10^6 cells) antisense-transfected cells formed tumors in 100% of animals injected, but control cells still failed to form tumors. These results show that TGF-β_1 acts as a negative growth regulator of human colon carcinoma cells in vivo as well as in vitro. Acquisition of partial or full resistance to such inhibitory effects may therefore contribute to tumor development and progression.

THE transforming growth factor-βs (TGF-β)[1] are a family of hormonal-like polypeptide growth factors with multifunctional effects on a variety of target cells (Massagué, 1990; Sporn et al., 1987). The type of effect elicited by the TGF-βs is a function of the target cell exposed to the polypeptide and its microenvironment. Reported TGF-β effects have included induction of differentiation, inhibition of differentiation, inhibition of proliferation, stimulation of proliferation, and immunosuppression. There are currently three known human TGF-β (β_1, β_2, and β_3) genes. Although the three forms of TGF-β show some differences in potency (Graycar et al., 1989) and are differentially regulated (Bascom et al., 1989), they have similar biological effects.

Almost all cell types and tissues express one or more of the TGF-β mRNAs (Derynck et al., 1988). Although TGF-βs are released from cells via a constitutive secretion pathway, most of the secreted material has been shown to be released in a latent, inactive form (Lawrence et al., 1985). Despite the predominance of latency in conditioned media containing secreted TGF-βs from cultured cells, there is evidence that a sufficient portion to act in an autocrine manner is present in the medium of at least some cell lines (Arteaga et al., 1990; Hafez et al., 1990; Glick et al., 1990; Singh et al., 1990).

Most studies of TGF-β_1 autocrine-negative effects have depended on indirect stimulation of target cells by neutralizing antibodies to TGF-β_1. There are some distinct limitations in using neutralizing antibodies to demonstrate auto-

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Shaoping Wu and Dan Theodorescu contributed equally to this manuscript.

1. *Abbreviation used in this paper:* TGF-β, transforming growth factor-β.

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crine TGF-β inhibitory activity in transformed cells. One of these is the low level of induction of proliferation or enhancement of anchorage-independent growth seen in these studies as a result of neutralization (Arteaga et al., 1990; Hafez et al., 1990). This may be due to relatively weak effects of autocrine TGF-β on proliferation or to the loss of neutralizing antibody activity during the course of an experiment. A second and perhaps more important limitation is the difficulty of extending neutralizing antibody experiments from tissue culture to animals to determine autocrine effects of TGF-β on the biological behavior of tumors. Consequently, we have constructed an expression vector for an antisense mRNA to the human TGF-β_1 cDNA to study the autocrine effects of TGF-β_1 in a human colon carcinoma cell line (designated FET) which expresses TGF-β_1 mRNA, but has no detectable TGF-β_2 or TGF-β_3 mRNA. Antisense approaches have been successfully used to study the functions of a number of oncogenes (Inouye, 1989) and products associated with extracellular matrix degradation (Khokha et al., 1989).

The FET cell line was isolated from a well-differentiated primary human colon carcinoma and has been extensively characterized. The line is continuously maintained in a completely defined serum-free medium (Wan et al., 1988) and has retained differentiated characteristics in tissue culture including basolateral polarity and the ability to form transport domes (Chantret et al., 1988). The parental cell line does not readily form colonies in soft agarose and is only weakly tumorigenic in athymic nude mice (Brattain et al., 1984). The cell line displays all three types of known TGF-β receptors or binding proteins and like most well-differentiated colon carcinoma cell lines is inhibited by the addition of exogenous TGF-β to growing cultures (Hoosein et al., 1989). Another characteristic of differentiated colon carcinoma cell lines is the ability of exogenous growth factors to stimulate quiescent cells to undergo DNA synthesis, while quiescent poorly differentiated cell lines are unresponsive to exogenous growth factors (Mulder and Brattain, 1989). TGF-β attenuates the growth factor–mediated stimulation of DNA synthesis in quiescent FET cells (Mulder et al., 1990a,b). Thus, it would be expected that constitutive expression of TGF-β_1 antisense mRNA would result in significant biological changes with respect to differentiation, growth, and tumorigenicity of FET cells if TGF-β_1 was contributing to the control of these biological properties in an autocrine manner.

This report describes the biological consequences of molecular abrogation of TGF-β_1 expression in vitro and in vivo. The constitutive expression of TGF-β_1 antisense mRNA in FET cells increased anchorage-independent growth and enhanced tumorigenicity in athymic mice.

Materials and Methods

Cells and Culture

The FET human colon carcinoma cell line was established in vitro from a primary tumor as previously described (Brattain et al., 1984). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in McCoy's 5A medium (Gibco Laboratories, Grand Island, NY) supplemented with 4 μg/ml transferrin, 10 ng/ml EGF, and 20 μg/ml insulin (serum-free medium) as previously described (Boyd et al., 1988). Mink lung epithelial cells (ATCC CCL64) were purchased from American Type Culture Collection (Rockville, MD) and maintained in McCoy's 5A medium supplemented with 10% FBS.

Transforming Growth Factor-βs and Antibodies

Human recombinant TGF-β_1 was provided by Dr. Tony Purchio from Oncogen (Seattle, WA). Porcine platelet TGF-β_2 was purchased from R & D System, Inc. Both TGF-β_1 and TGF-β_2 were stored in 4 mM HCl and 1 mg/ml BSA at a concentration of 2,000 ng/ml before being diluted to the final desired concentration with tissue culture medium.

Chicken anti–porcine TGF-β_1 neutralizing antibody was purchased from Oncomembrane (Seattle, WA). The antibody is >90% pure as determined by SDS-PAGE. No cross-reaction with TGF-β_2 has been observed. Rabbit anti–porcine TGF-β_2 neutralizing antibody, purified by protein A chromatography was purchased from R & D System Inc. (Minneapolis, MN). This antibody does not cross react with TGF-β_1.

Construction of Human TGF-β_1 cDNA Clone and Antisense Expression Vector RLDN-RSVAS

Total RNA was prepared from human monocytes by the guanidinium isothiocyanate method (Chirgwin et al., 1979) and poly(A)$^+$RNA was purified by oligo (dT)-cellulose chromatography (Aviv et al., 1972). cDNA was prepared using a cDNA synthesis kit (Boehringer Mannheim Diagnostics, Inc., Houston, TX) and cloned into lambda ZAP arms (Strategene, San Diego, CA). Approximately 1×10^6 clones were screened (Wood et al., 1985) using oligonucleotides specific for TGF-β_1 sequences (Derynck et al., 1985). One positive clone was isolated and confirmed by DNA sequencing (Sanger et al., 1977) to be human TGF-β_1. This clone was digested with NarI and NcoI. The resulting 1.7-kb fragment (which includes ~400 bp of 5' untranslated region, 83 bp of 3' untranslated region, and 1,173 bp of coding region) was inserted in opposite orientation into the expression cassette of the plasmid vector RLDN. The RLDN vector was originally constructed by J. Trill and M. Reff (Smith, Kline, and Beechman Pharmaceuticals, Philadelphia, PA) for the stable integration, amplification, and expression of heterologous proteins in mammalian cells. In this vector, transcription of the cDNA is controlled by the Rous sarcoma virus long-terminal repeat promoter (Gorman et al., 1982) while a termination signal is provided by the bovine growth hormone 3'-flanking sequence (Pfarr et al., 1985). This vector also contained a bacterial neomycin phosphotransferase gene (NEO) expression cassette for G418 selection (Southern et al., 1982). The human TGF-β_1 antisense expression vector was designated RLDN-RSVAS.

Cell Transfection and Isolation of Stable Transfectants

FET cells (2×10^6) harvested at mid-log phase growth were transfected either with the RLDN–RSVAS plasmid (5 μg) or with the plasmid vector RLDN (5 μg). Electroporation was carried out at 250 V, 960 μFD with a gene pulser (Bio-Rad Laboratories, Cambridge, MA). Cells were allowed to grow for 2 d before being subjected to selection for the ability to grow in medium containing 600 μg/ml geneticin (G418 sulfate, Gibco Laboratories). Stable cell clones resistant to G418 sulfate were obtained after 2–3 wk growth with the drug-containing medium. These populations of transfectants (~10^3 individual clones) were pooled, expanded, and designated as FET NEO and FET RSVAS, respectively.

Southern Blot Hybridization Analysis

DNA isolation, restriction endonuclease digestion, 0.8% agarose gel electrophoresis, and Southern blotting onto Nytran membrane (Schleicher & Schuell, Inc., Keene, NH) were all performed as described by Sambrook et al. (1989). Labeling of probe, hybridization, and wash conditions were performed as described for RNA analysis below.

Northern Blot and RNase Protection Analyses

Total RNA was prepared from cultured cells by the guanidium isothiocyanate method (Chirgwin et al., 1979). Poly(A)$^+$RNA was isolated by one pass of total RNA through a poly (dT) column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Poly(A)$^+$RNA (10 μg) was electrophoresed on 1.2%/2.2 M agarose-formaldehyde gels, transferred to Nytran membranes (Scheicher & Schuell, Inc., Keene, NH) and hybridized to appropriate ^{32}P-labeled (Multiprime Labeling Kit; Amersham Corp., Arlington Heights, IL) cDNA probes as previously described (Mulder, 1991). The cDNA probes used were a 1.2-kb EcoRI fragment of simian TGF-β_1 cDNA (Sharples et al., 1987), a 2.2-kb EcoRI fragment of human TGF-β_2 cDNA (provided by K. Coleman, Bristol-Myers Squibb Co.), and a 700-bp PstI–

EcoRV fragment of human TGF-β_3 (provided by N. Stirpe, Bristol-Myers Squibb Co.).

RNase protection experiments were performed as described by Zinn et al. (1983). A 243-bp PvuII-pvuII fragment of TGF-β_1 cDNA was isolated from the RLDN-RSVAS expression vector and inserted into the pGEM3Z(−) plasmid (Promega Biotec, Madison, WI). The orientation of the insert in the resulting plasmid was determined by DNA sequencing (United States Biochemical, Cleveland, OH). This plasmid was linearized by EcoRI and used as a template to synthesize the sense strand TGF-β_1 RNA probe by SP6 RNA polymerase. DNase I was used to digest any remaining DNA template. Determinations of TGF-β_1 antisense levels in total RNA samples (50 μg) were performed with 5×10^5 cpm of labeled probe. Dried RNA was dissolved in 30 μl of RNA hybridization buffer (80% formamide, 400 mM NaCl, and 1 mM EDTA), denatured at 90°C for 10 min, and hybridized at 50°C overnight. RNase digestion buffer (300 μl) containing RNase A (40 μg/ml) and RNase T1 (3 U/ml) was used to degrade unhybridized probe at 37°C for 30 min. RNases were inactivated by adding 20 μl of 10% SDS, 50 μg of proteinase K, and incubating at 37°C for 15 min. The RNA was extracted with phenol/chloroform and analysis was performed by electrophoresis in 6%/8 M urea-polyacrylamide gels.

Growth Assays

Growth curves were performed in 96-well plates in 0.1 ml of serum-free medium with inocula of either 10^2 or 10^3 cells. Relative cell number was determined using the MTT assay (Carmichael et al., 1987). The values are means ± standard deviations derived from 16 wells.

For growth inhibition assays by TGF-β_1, FET NEO and FET RSVAS cells were inoculated into 96-well plates at 10^2 or 10^3 cells per well in 0.1 ml of growth medium containing varying concentrations of TGF-β_1. The growth inhibition was measured using the MTT assay as described above.

Mitogenesis assays were performed as previously described (Mulder and Brattain, 1989). Cells were plated in serum-free medium and allowed to grow to saturation density and rendered quiescent by addition of fresh basal McCoy's 5A medium for a 5-d starve period. Cells were then treated with either fresh basal McCoy's 5A medium, fresh basal McCoy's 5A medium together with the TGF-β_1 antibody (10 μg/ml), fresh basal McCoy's 5A medium plus TGF-β_1 (5 ng/ml), and fresh basal McCoy's 5A medium with both TGF-β_1 (5 ng/ml), and TGF-β_1 antibody (10 μg/ml). After 22 h, [³H]thymidine was added for 1 h, followed by determination of TCA-precipitable radioactivity. Values are means of three samples.

Soft Agarose Assay

To study the effects of TGF-β_1 antisense expression on anchorage-independent growth the FET transfectants, FET RSVAS cells, and RLDN-transfected control cells (FET NEO) were compared for clonogenic potential in semisolid medium. Cells were suspended at 10^4 cells/ml in 1 ml of 0.4% sea plaque agarose in McCoy's 5A serum-free medium and plated on top of 1 ml of 0.8% agarose in the same medium in 35-mm culture plates. Plates were incubated for 2.5–3.0 wk at 37°C with 5% CO_2 in a humidified incubator and stained with 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride (Sigma Chemical Co., St. Louis, MO) as described by Schaeffer and Friend (1976) to determine viability and to enhance the photographic contrast of the colonies from the agarose.

TGF-β Assays

Total TGF-β levels in serum-free conditioned medium from FET cells were determined with the CCL64 mink lung growth inhibition bioassay (Danielpour et al., 1989). Conditioned media collected at 72 h were tested for the ability to inhibit CCL64 cell growth by the MTT assay in comparison with the standard growth inhibition curves of the CCL64 cells by the TGF-β_1 and TGF-β_2, respectively. Neutralization of the conditioned media by the TGF-β_1 neutralizing antibody (10 μg/ml) or TGF-β_2 antibody (10 μg/ml) was carried out to determine specificity. The amount of active TGF-β present was determined using untreated conditioned medium, while the amount of total TGF-β (including active and latent forms) was determined using acid-treated conditioned medium. The cell numbers were determined when the conditioned media were harvested. Acidification of the media was performed as described by Arteaga et al. (1990).

Subcutaneous Inoculations of Tumor Cells

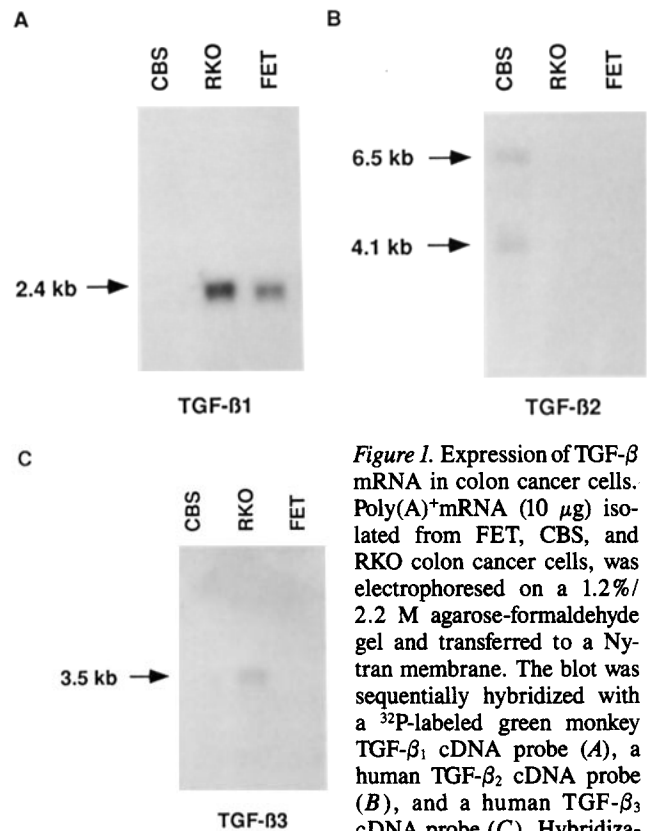Nude (nu-nu) mice (8–12-wk-old females) were purchased from the Jack-

Figure 1. Expression of TGF-β mRNA in colon cancer cells. Poly(A)⁺mRNA (10 μg) isolated from FET, CBS, and RKO colon cancer cells, was electrophoresed on a 1.2%/2.2 M agarose-formaldehyde gel and transferred to a Nytran membrane. The blot was sequentially hybridized with a ³²P-labeled green monkey TGF-β_1 cDNA probe (A), a human TGF-β_2 cDNA probe (B), and a human TGF-β_3 cDNA probe (C). Hybridization was carried out as described in Materials and Methods. A 2.4 kb TGF-β_1 mRNA (A), 6.5 kb and 4.1 kb TGF-β_2 mRNA (B), and a 3.5 kb TGF-β_3 mRNA (C) are indicated.

son Laboratories (Bar Harbor, ME). Tumorigenicity dose response curves were generated for the control FET cell line (FET NEO) and the antisense TGF-β_1 cell line (FET RSVAS). Mice (five in each group) were injected subcutaneously in the flank with either 1.25×10^5, 2.5×10^5, 0.5×10^6, 1×10^6, or 2×10^6 cells per animal, and the number of animals with tumors (0.5 cm³) in each group was scored every 2 wk.

Results

FET Cells Express TGF-β_1 mRNA but Not TGF-β_2 or TGF-β_3 mRNAs

Expression of TGF-β_2 and/or TGF-β_3 could potentially complicate the interpretation of results in TGF-β_1 antisense transfected cells since these factors have a high degree of homology (Massagué, 1990) and might interact with TGF-β_1 antisense mRNA. Furthermore, TGF-β_2 and TGF-β_3 have similar effects on target cells and can affect each other's expression (Graycar et al., 1989; Bascom et al., 1989). Consequently, there was concern that TGF-β_1 antisense effects could be masked by compensatory action of TGF-β_2 and TGF-β_3. FET colon carcinoma cells were chosen for these studies because they expressed high levels of TGF-β_1 mRNA (Fig. 1). Probes for human TGF-β_2 and TGF-β_3 did not detect mRNA for these genes under the same Northern blot conditions (10 μg poly(A)⁺mRNA). FET cells secreted a total of 40 ng/10⁶ cells per 72 h TGF-β_1 into tissue-culture medium as determined by bioassay with ∼10% of the polypeptide in the active form (4.7 ng/10⁶ cells per 72 h). It was
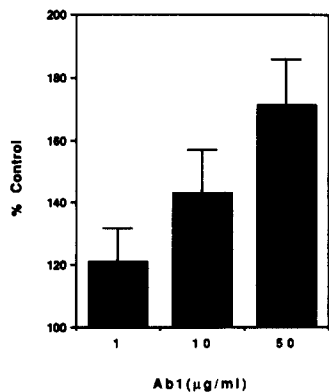
Figure 2. The stimulation of proliferation of FET cells after treatment with neutralizing antibody to TGF-β_1 in a proliferation assay. FET cells were treated with the indicated amounts of TGF-β_1 neutralizing antibody, cultured for 4 d and subjected to MTT assay. The growth stimulation of FET cells is represented by the percentage increase of cell growth relative to untreated cells. The values are means ± standard deviations derived from 16 wells.
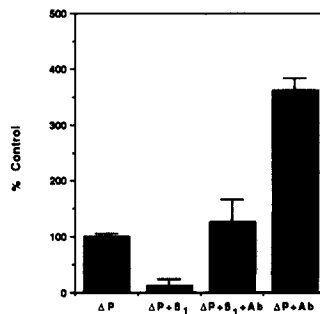

Figure 3. The stimulation of DNA synthesis in quiescent FET cells by TGF-β_1 antibody. Cells were made quiescent and then released from quiescence by the addition of fresh medium or medium plus the indicated treatment. DNA synthesis was determined as described in Materials and Methods. The abbreviations used are: *P*, addition of fresh basal McCoy's 5A medium; *P+β_1*, addition of fresh basal McCoy's 5A plus TGF-β_1 (5 ng/ml); *P+β_1+Ab*, addition of fresh basal McCoy's 5A medium, TGF-β1 (5 ng/ml), and TGF-β_1 neutralizing antibody (10 μg/ml); *P+Ab*, addition of fresh basal McCoy's 5A medium plus TGF-β_1 neutralizing antibody (10 μg/ml).

not possible to determine active TGF-β_2 protein within the limits of the bioassay (<3 ng/10^6 cells per 72 h) and there is currently no methodology for the specific detection of TGF-β_3 levels in tissue culture medium or cells. An additional advantage of FET cells for these studies was their continuous maintenance in a completely defined serum-free medium (Wan et al., 1988), thus eliminating any serum effects with respect to exogenous TGF-β_1 or TGF-β binding proteins.

TGF-β_1 Is a Negative Autocrine Growth Regulator in FET Cells

TGF-β_1 has been shown to be a negative growth regulator in a number of colon carcinoma cell lines including FET cells. This negative regulation has been shown to occur at the level of cell proliferation (Hoosein et al., 1987, 1989) and the initiation of DNA synthesis from quiescence (Mulder et al., 1990a,b). Therefore, if TGF-β_1 were acting as a negative autocrine growth regulator in the FET cells (as suggested by the levels of active TGF-β_1 secreted into FET cell medium), one would expect that neutralizing antibody to TGF-β_1 would have the effects of increasing FET proliferation and increasing the amount of DNA synthesis in cells released from quiescence.

Neutralizing antibody specific for TGF-β_1 was found to increase cell proliferation in a dose-dependent manner from 1–50 μg/ml when added to the growth medium of FET cells (Fig. 2). As in other studies with human cancer cells (Arteaga et al., 1990; Hafez, 1990), the increase in proliferation obtained was rather modest as only a 1.75-fold stimulation was obtained at the highest antibody concentration used. The antibody was also effective in stimulating DNA synthesis in FET cells released from quiescence by the addition of fresh medium (Fig. 3). In contrast to the proliferation results, the addition of neutralizing antibody at the same time as fresh medium addition showed an increase of almost fourfold in [^{3}H]thymidine incorporation. Addition of exogenous TGF-β_1 (5 ng/ml) along with the neutralizing antibody brought the levels of [^{3}H]thymidine incorporation back down to control levels for untreated cells. Quiescent cells treated with exogenous TGF-β_1 (5 ng/ml) at the same time as addition of fresh medium showed only 15% as much incorporation of [^{3}H]thymidine into DNA as untreated control cells.

These two experiments showed that FET cells are negatively regulated by TGF-β_1 in an autocrine manner. The demonstration of autocrine TGF-β_1 activity in FET cells

was essential to the selection of this cell line as a recipient for the TGF-β_1 antisense expression vector.

Expression of TGF-β_1 Antisense mRNA

The expression vector (RSVAS) contains a 1.7-kb fragment of the human TGF-β_1 cDNA which was inserted in an antisense orientation into the mammalian expression vector RLDN. Transcription of the insert is driven by the Rous Sarcoma virus LTR promoter placed upstream. The bovine 3' flanking sequence provides a termination signal for RNA processing (Fig. 4). FET cells were transfected with the vector as described in Materials and Methods and colonies selected by growth in G418. Resistant colonies to G418 were obtained within 2–3 wk. We decided to perform our experi-
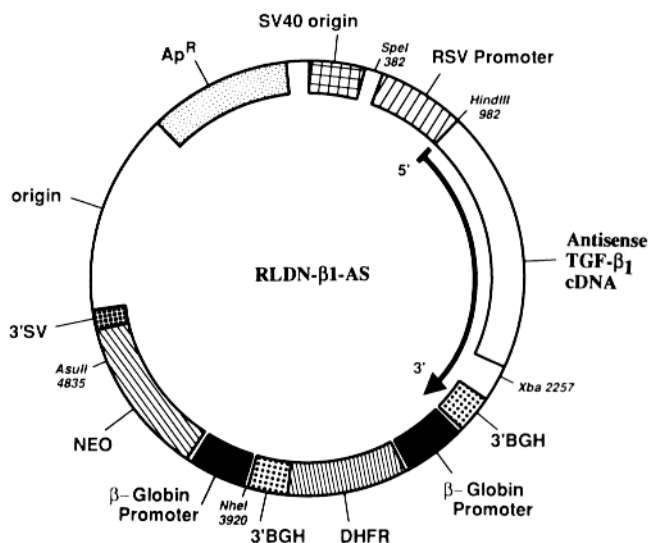

Figure 4. Human TGF-β_1 antisense expression vector, RLDN-RSVAS. The expression vector contains a 1.7-kb fragment of the human TGF-β_1 cDNA which was inserted in an antisense orientation into the mammalian expression vector RLDN. Transcription of the insert is driven by the Rous sarcoma virus long terminal repeat promoter. The bovine growth hormone 3'-flanking sequence provides a termination signal for RNA processing. A bacterial neomycin phosphotransferase gene (*NEO*) expression cassette allows G418 selection.

ments with pooled colonies of transfected cells designated FET RSVAS. Controls were pooled colonies of cells transfected with the vector without the TGF-β_1 antisense insert and designated FET NEO. We decided to use pooled colonies for our experiments because of previous work which showed a high degree of heterogeneity with respect to TGF-β_1 response among clones of cells from parental colon carcinoma cell lines (Mulder et al., 1988). This heterogeneity which also extends to TGF-β expression would be a complicating factor in the analysis of any single clone for TGF-β_1 autocrine effects.

Expression of the TGF-β_1 antisense mRNA was determined by a specific RNase protection assay which generated a 232-bp protected fragment from a 243-bp sense RNA probe synthesized by SP6 polymerases in vitro. The levels of protected fragments from pooled FET RSVAS and three individual clones (A, B, and I) are shown in Fig. 5. As expected, no protection is seen in FET NEO or parental FET cells. Cells expressing the antisense mRNA had a fivefold reduction of TGF-β_1 mRNA expression as indicated by densitometry of the Northern analysis shown in Fig. 6. TGF-β_1 protein levels were reduced from a level of ~40 ng/ml per 72 h/10^6 cells in FET NEO cells to a level below the detection limits of the assay in FET RSVAS cells.

Southern blot hybridization analysis with the TGF-β_1 cDNA probe (1.2-kb EcoRI fragment of simian TGF-β_1 cDNA) confirmed the presence of the TGF-β_1 antisense expression vector in the FET RSVAS cells. KpnI digestions of DNA showed a 651-bp fragment which was specific to FET RSVAS cells and clone B cells (data not shown).

Repression of TGF-β_1 Expression Reduces Lag Time in Cultures of FET Cells

Uncoupling of the TGF-β_1 autocrine loop by neutralizing antibody led to increased proliferation of FET cells as shown above. Therefore, disruption of the autocrine loop by reduction of TGF-β_1 mRNA expression should have similar effects on the growth of FET cells. It should be noted that previous work documenting the effects of TGF-β_1 neutralizing antibody on the growth of cancer cells (Arteaga et al., 1990; Hafez et al., 1990) involved one point proliferation assays similar to the proliferation assay presented in Fig. 2 above. The differences seen in proliferation could be due to an increase in proliferation rate (reduced doubling time) or due to a decrease in the time required to achieve logarithmic growth in tissue culture (reduced lag time). Constitutive reduction of TGF-β_1 expression in RSVAS transfected FET cells permitted us to determine whether uncoupling the TGF-β_1 autocrine loop affected doubling time or lag time by generating growth curves instead of one point assays.

Growth curves for FET RSVAS and FET NEO were generated in 96-well plates at inocula of 10^2 and 10^3 cells, respectively (Fig. 7, A and B). FET NEO cells show a period of 8 d before the termination of lag phase at an inoculum of 10^2 cells (Fig. 7 A) and 5 d at an inoculum of 10^3 cells (Fig. 7 B). In contrast, FET RSVAS cells showed a period of 5 d before the termination of lag phase at an inoculum of 10^2 cells (Fig. 7 A) and showed no lag period at all when 10^3 cells were inoculated. Once lag phases were completed no major differences were observed in the growth rates of FET NEO and FET RSVAS cells. These results indicate that the
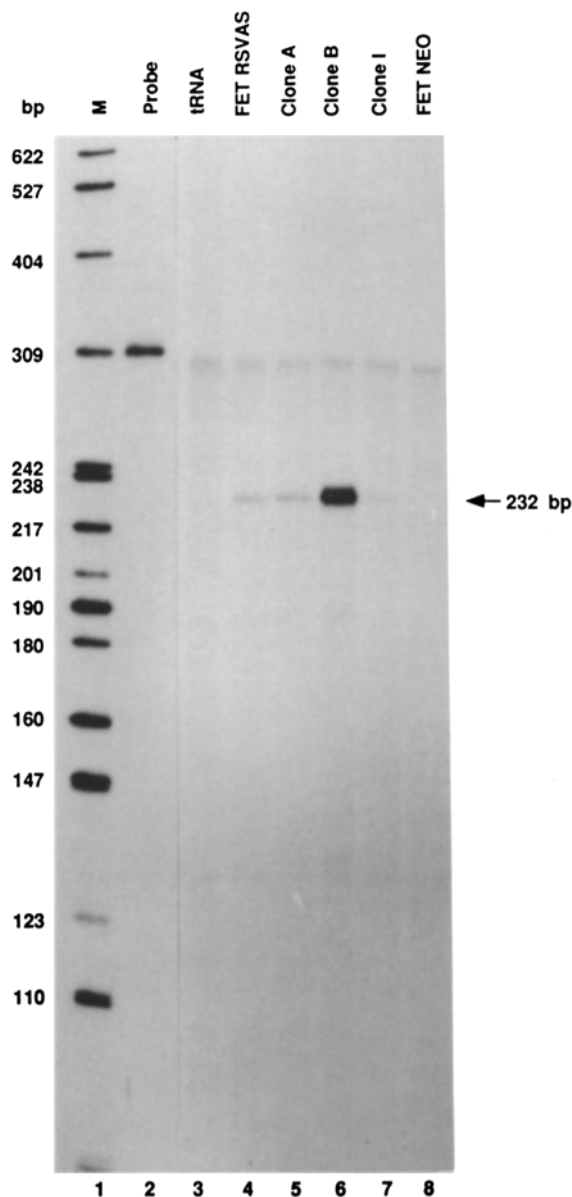

Figure 5. Detection of antisense TGF-β_1 RNA by RNase protection assay. A 243-bp PvuII–PvuII TGF-β_1 cDNA fragment was cloned into plasmid pGEM3Z (−) to generate the RNA probe. A uniformly radiolabeled ^{32}P-sense strand RNA probe was synthesized in vitro by SP6 RNA polymerase. Excess probe was hybridized in solution with 50 μg of total cellular RNA from TGF-β_1 antisense-transfected FET cells (*FET RSVAS*), TGF-β_1 antisense-transfected FET clone I, clone A, clone B, vector RLDN-transfected FET cells (*FET NEO*) or tRNA, and then digested with RNase as described in Materials and Methods. The protected RNA probe was fractionated by electrophoresis through a 6%/8 M urea polyacrylamide gel. All RNA from TGF-β_1 antisense-transfected FET cells protected an expected 232-bp fragment (lane *4, FET RSVAS*; lane *5, clone A*; lane *6, clone B*, and lane *7, clone I*). RNA from FET NEO cells and tRNA were unable to protect the probe (lane *8* and lane *3*). A pBR322 Msp cut marker (New England Biolabs, Inc., Beverly, MA) and full-length undigested probe are shown in lanes *1* and *2*, respectively. The position expected for a 232-bp nucleotide fragment is indicated.
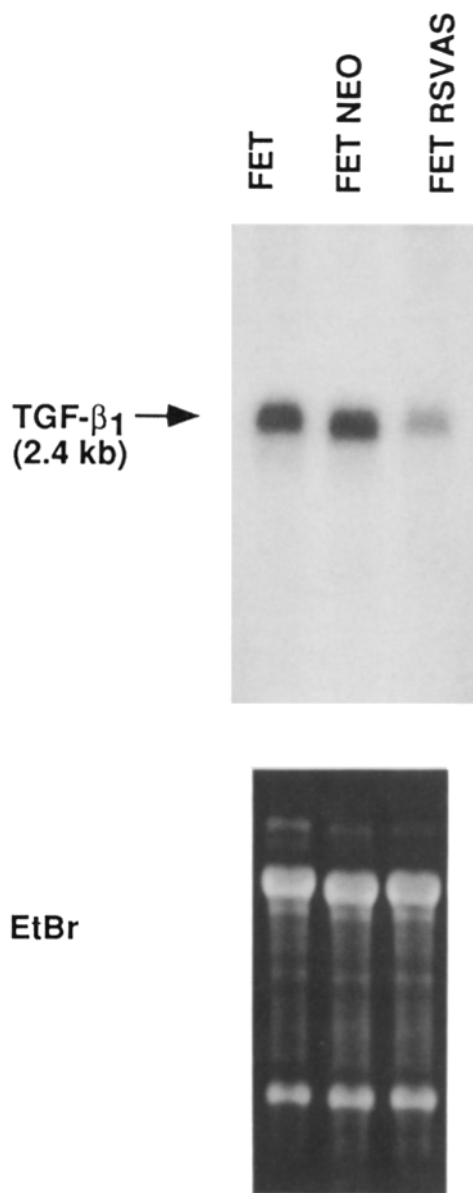
Figure 6. Northern analysis of TGF-β_1 RNA transcripts. Poly(A)$^+$-RNA (10 μg) from parental FET, plasmid vector RLDN and the TGF-β_1 antisense expression vector transfected cells were electrophoresed on a 1.2%/2.2 M formaldehyde-agarose gel, transferred into a Nytran filter, and hybridized to a ^{32}P-labeled TGF-β_1 cDNA probe. Ethidium bromide staining of the gel is shown.

Figure 7. Growth curves for FET NEO and FET RSVAS cells. Cells were plated at initial numbers of 10^2 (*A*) or 10^3 (*B*) in 96-well plates. Relative cell growth was estimated by the measurement of absorbance at 540 nM after the MTT assay carried out at the indicated times. Values are means $\pm$ standard deviations derived from 16 wells.

major effect of autocrine TGF-β_1 on the growth of FET cells in tissue culture is to reduce the lag time rather than to increase the rate of proliferation. Northern analysis of c-*myc* expression showed no difference in levels of expression between FET NEO and FET RSVAS cells indicating that constitutive repression of TGF-β_1 did not affect long-term steady-state levels of this competence gene (data not shown).

These findings are consistent with the effects of exogenous TGF-β_1 on the proliferation of FET cells. One-point proliferation analyses have shown that exogenous TGF-β_1 can inhibit FET cells as well as several other colon carcinoma cell lines by as much as 60% (Hoosein et al., 1989). Growth curves were performed to determine whether the effect is on
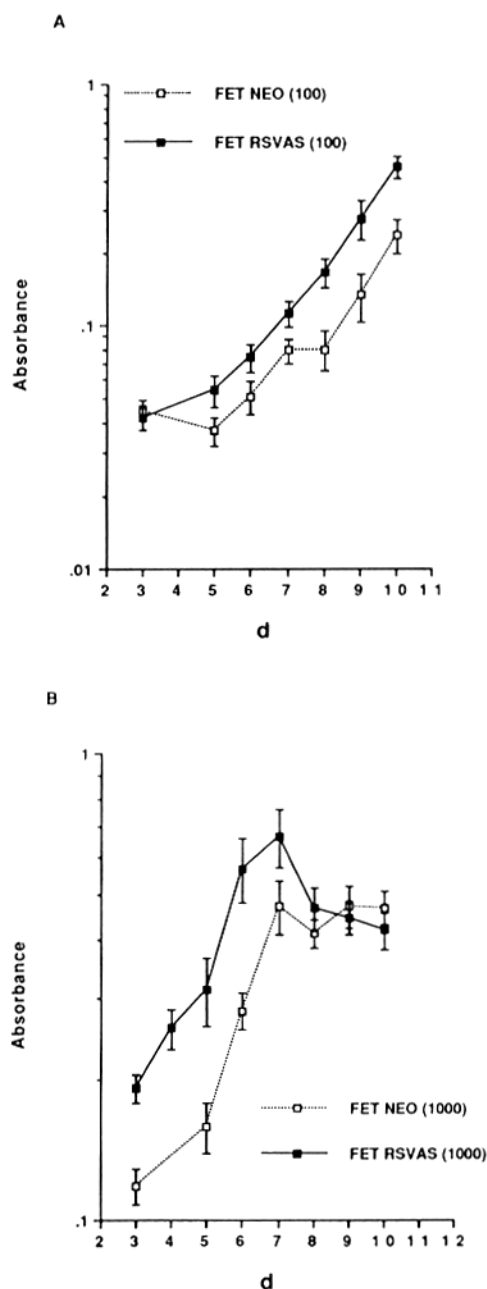
growth rate or lag time. Results were similar to those described above for FET RSVAS cells vs. FET NEO cells. Exogenous TGF-β_1 caused an extension of lag time relative to untreated cells at an inoculum of 100 cells. TGF-β_1 was less effective in causing increased lag time at higher initial inocula (data not shown).

Reduction of endogenous TGF-β_1 expression in FET RSVAS cells should lead to an altered response to exogenously added TGF-β_1 relative to FET NEO cells. The removal of endogenous TGF-β_1 would lead to the expectation that more exogenous TGF-β_1 would have to be added
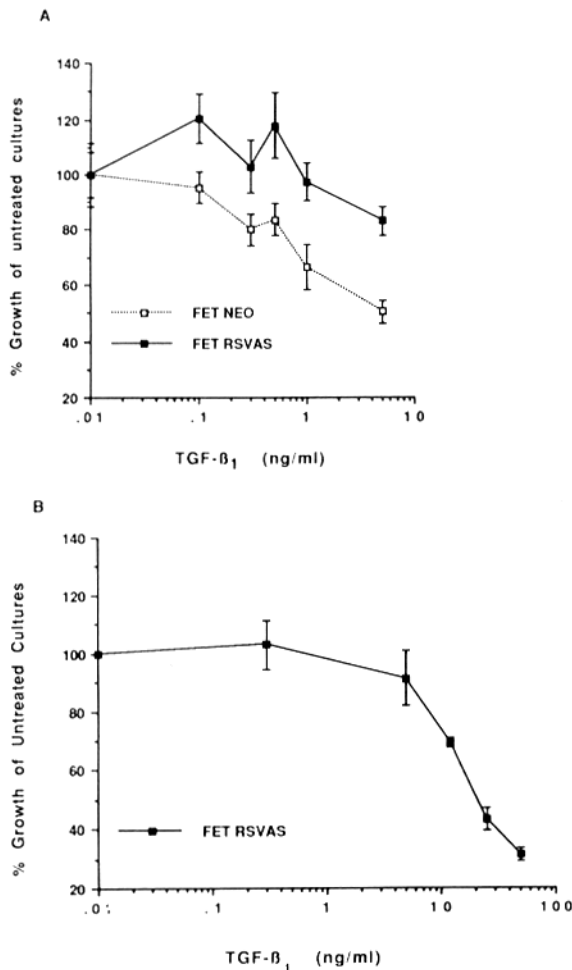
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Figure 8. Dose response of FET RSVAS and FET NEO cells for growth inhibition by exogenous TGF-β_1. (*A*) FET NEO and FET RSVAS cells were plated into 96-well plates at 10^2 or 10^3 cells per well in growth medium containing the indicated concentrations of TGF-β_1 (from 0.1 ng/ml to 5 ng/ml). After 4 d, the cell growth was measured by the MTT assay. The growth inhibition is presented as percentage growth of untreated cultures. Values are the means ± standard deviation derived from 16 wells. (*B*) Response of FET RSVAS cells to exogenous TGF-β_1 in the presence of high concentrations of TGF-β_1 (from 0.3 ng/ml to 50 ng/ml).

to FET RSVAS cells to achieve the same level of inhibition as that obtained in FET NEO cells. Consequently, dose response curves were performed to compare the response of FET RSVAS and FET NEO cells to exogenous TGF-β_1 (Fig. 8, *A* and *B*). FET NEO cells showed significant levels of inhibition at as little as 0.2 ng/ml TGF-β_1 with 50% inhibition of proliferation at 5 ng/ml (Fig. 8 *A*). As expected FET RSVAS cells required more exogenous TGF-β_1 to achieve similar levels of inhibition. Only 15% inhibition was observed at 5 ng/ml in FET RSVAS cells (Fig. 8 *A*) while 50% inhibition of proliferation required the addition of 25 ng/ml of exogenous TGF-β_1 (Fig. 8 *B*).

Repression of TGF-β_1 Enhances the Tumorigenicity of FET Cells

Anchorage-independent growth in semisolid medium and the formation of xenografts in immunocompromised mice are generally regarded as two of the most useful parameters

in assessing the malignancy of human cells. Since TGF-β_1 acts as a negative regulator of growth and is known to affect the activity of tumor suppressor genes such as retinoblastoma protein (Laiho et al., 1990), it was hypothesized that the uncoupling of the TGF-β_1 autocrine loop might lead to the expression of a more aggressive phenotype than control cells with an intact autocrine loop. Consequently, we compared the anchorage-independent growth and tumorigenicity of FET RSVAS cells with those of FET NEO cells.

The FET cell line has been shown to grow relatively poorly in soft agarose (Brattain et al., 1984) with cloning efficiencies in the range of 1-2% at inocula of 10^3-10^4 cells. We compared anchorage-independent growth of FET NEO and FET RSVAS cells at inocula ranging from 10^2 to 10^4 cells. Results from an inoculum of 10^4 cells are shown in Fig. 9. The cloning efficiency of FET NEO cells ranged from 1.4 to 1.8% in three plates at this inoculum while FET RSVAS cells showed cloning efficiencies ranging from 5.2 to 6.9% in the same experiment. Average cloning efficiencies were 1.5 and 6.1%, respectively. In addition to the fourfold increase in cloning efficiency, the FET RSVAS cells formed significantly larger colonies. The maximal size for FET NEO colonies was on the order of 50 cells while approximately half of the FET RSVAS colonies had colonies with four- to sixfold more cells as estimated by size.

FET cells had previously been shown to be weakly tumorigenic as relatively high inocula were required for subcutaneous tumor formation in athymic nude mice and when tumors did form there were relatively long periods of latency before their appearance (Brattain et al., 1984). Increased clonogenicity in soft agarose by the FET RSVAS cells suggested that disruption of the TGF-β_1 autocrine loop might also lead to increased tumorigenicity. To test this hypothesis tumor formation in athymic mice was tested at several inocula of FET NEO and FET RSVAS cells ranging from 1.2×10^5 to 2×10^6. FET NEO cells were tumorigenic only at 2×10^6 cells as all animals injected at this inoculum formed tumors within 4 wk of subcutaneous injection. Similar results were obtained with FET RSVAS cells. The time to reach a tumor size of 0.5 cm in diameter was the same for both control and FET RSVAS cells (data not shown) suggesting similar growth rates at high initial inocula. Inocula below 2×10^6 FET NEO cells did not give rise to any tumors within 6 wk of injection (Table I). In contrast at this inoculum of FET RSVAS cells, 80% of animals had tumors by 4 wk and 100% by 6 wk (Table I). At the next lowest inoculum (5×10^5) 40% of the animals had tumors by 6 wk (Table I). Lower inocula did not give rise to any tumors within 6 wk. The B clone of FET RSVAS cells which expressed high levels of antisense TGF-β_1 (see Fig. 5) was more aggressive as all animals injected with 10^6 cells showed tumors of 1 cm^3 within 4 wk. These results indicated a significant increase in the tumorigenic potential of cells in which the TGF-β_1 autocrine loop had been uncoupled by the expression of TGF-β_1 antisense mRNA and showed that the negative autocrine loop is operational in vivo as well as in vitro.

Discussion

Others have proposed that the TGF-βs could act as autocrine-negative growth factors (Tucker et al., 1984). The recent availability of TGF-β neutralizing antibodies has led to the
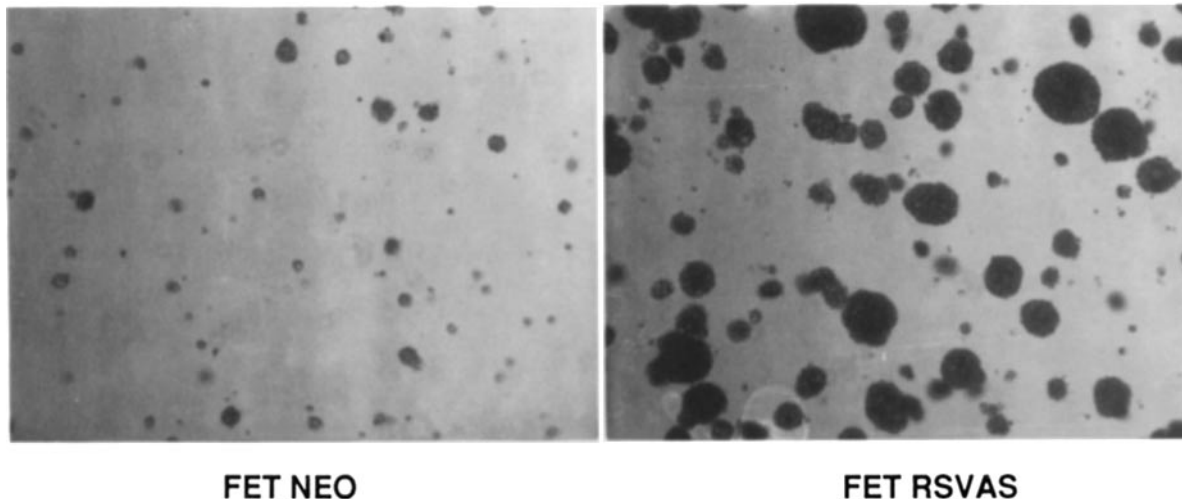
FET NEO **FET RSVAS**

Figure 9. Autocrine effect of TGF-β_1 on anchorage-independent growth. FET RSVAS and FET NEO cells were seeded at 10^4 cells/ml in 0.4% agarose in the serum-free medium and cultured for 2.5 wk at 37°C with 5% CO_2 in a humidified incubator. Plates were stained with a vital tetrazolium dye and photographed as described in Materials and Methods.

development of data which support this hypothesis (Arteaga et al., 1990; Hafez et al., 1990). However, it has been difficult to interpret results because most of the TGF-β secreted by cells in tissue culture is in a latent form (Lawrence et al., 1985). It is not clear whether the low levels of activated TGF-β_1 (usually ≤10%) produced by cell lines are a tissue culture artifact or whether negative autocrine loops are functional in an in vivo environment. Thus far, the neutralizing antibody approaches have used increased growth in tissue culture, increased mitogenesis, and increased clonogenicity cells as the expected end point of assays for negative TGF-β autocrine function. Not surprisingly, the levels of proliferative increase in these investigations have been modest (Arteaga et al., 1990). One explanation for the modest effects seen previously is that TGF-β is not the only negative autocrine factor present (Arteaga et al., 1990). Furthermore, studies thus far reported on TGF-β_1 neutralizing antibody have not dealt with the issue of whether the cells under investigation express TGF-β_2 and/or TGF-β_3 and whether the neutralizing antibody has any effect on the expression of these members of the TGF-β family. Compensatory or even basal expression of other members of the TGF-β family could reverse the effect of removing TGF-β_1 from the system.

To overcome some of the difficulties described above we have taken the approach of reducing endogenous TGF-β_1

Table I. Tumorigenicity of FET Cells Expressing TGF-β_1 Antisense in Athymic Nude Mice

Cell Line	Inoculum ($\times 10^6$ cells)	Tumors at 2 wk	Tumors at 4 wk	Tumors at 6 wk
FET NEO	1.0	0/5	0/5	0/5
FET RSVAS	1.0	0/5	4/5	5/5
FET NEO	0.5	0/5	0/5	0/5
FET RSVAS	0.5	0/5	0/5	2/5

8-12-wk-old nu–nu female mice were injected subcutaneously with either 0.5 × 10^6 or 1 × 10^6 FET NEO or FET RSVAS cells. Number of animals with tumors in each group within the indicated time are presented.

expression through the constitutive expression of an antisense mRNA to TGF-β_1. This approach allowed for the generation of more extensive in vitro proliferation data as well as the unique ability to identify any negative autocrine function of TGF-β_1 in vivo. In addition, the recipient colon carcinoma cell line, FET, was selected for these investigations because it was shown to express relatively large amounts of TGF-β_1 without expressing TGF-β_2 or TGF-β_3. Moreover, reduction of TGF-β_1 mRNA by transfection did not lead to the expression of either TGF-β_2 or TGF-β_3 mRNA (data not shown). Consequently, the selection of FET cells for these experiments circumvented potential complications which might arise from other members of the TGF-β family in the interpretation of results.

FET cells had several other advantages for these studies. These included their continuous maintenance in a fully defined, serum-free, tissue culture medium, which eliminated any potential effects of TGF-β binding proteins such as α macroglobulin (Danielpour et al., 1989) or effects of unknown positive or negative growth factors in serum which could confuse the interpretation of results. Furthermore, FET cells had the benefit that they were poorly growing in semisolid medium and did not readily form tumors in nude mice. As such, these cells, which have been classified as relatively unaggressive compared to other colon carcinoma cell lines (Brattain et al., 1984; Chantret et al., 1988; Mulder and Brattain, 1989), offered a relatively sensitive model system for alterations in tumorigenic properties.

Initially, we provided evidence that FET cells had a negative TGF-β_1 autocrine loop as shown by an increase in proliferation of 175% and an increase of mitogenesis in the range of 400% (see Figs. 2 and 3) in response to neutralizing antibodies. These results were similar to those which had been obtained with other human cancer cells including those derived from lung (Keski-Oja et al., 1987), breast (Arteaga et al., 1990), and colon (Hafez et al., 1990).

Cells expressing the TGF-β_1 antisense mRNA (FET RSVAS) also showed similar results as single-point proliferation assays indicated that elimination of TGF-β_1 expression resulted in a 200–250% increase in cell number. However,

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constitutive uncoupling of the TGF-β_1 negative autocrine loop in FET RSVAS cells also allowed us to determine whether the change in proliferation was due to decreased doubling time or an increase in lag time by generating detailed growth curves. The results clearly indicated that the major effect of the TGF-β_1 negative autocrine loop in FET cells was on increased lag time rather than decreased doubling time. Interestingly, the effect of exogenous TGF-β_1 on untransfected parental FET cells is essentially identical.

The length of lag time for cells in tissue culture is generally regarded to be a function of their ability to condition their growth medium with positive growth factors such as TGF-α. Once a critical concentration of positive factors is achieved, cells go into a logarithmic growth phase. One way in which TGF-β_1 may be acting as a negative regulator in this system would be to subdue the expression of positive factors which condition the medium. This would have the effect of delaying the achievement of a critical concentration of positive factors. Another piece of evidence which is consistent with this explanation is the reduced effect of TGF-β_1 on the inhibition of proliferation and repression of c-*myc* expression in colon cancer cells as initial cell density is increased (Mulder and Brattain, 1988). Similarly, FET RSVAS cells in log phase growth had the same c-*myc* levels as control cells in log phase growth. Finally, Mulder et al. (1990*b*) have shown that TGF-β_1 treatment of FET cells represses growth factor mediated induction of TGF-α mRNA in quiescent cells.

Exogenously added TGF-β_1 (5 ng/ml) was able to counteract the effect of neutralizing antibody on FET cell proliferation and DNA synthesis. Dose response curves comparing the inhibition of FET NEO and FET RSVAS cells by TGF-β_1 indicated that 3–5 ng/ml TGF-β_1 was required before inhibition of FET RSVAS cells was detected. Equivalent inhibition of FET NEO cells was obtained in the range of 0.1–0.3 ng/ml TGF-β_1 indicating that the expression of antisense TGF-β_1 mRNA had a net effect of reducing autocrine TGF-β_1 activity by 3–5 ng/ml over the 5-d period of the proliferation assay. These results are in relatively good agreement with the requirement for 5 ng/ml TGF-β_1 to overcome neutralizing antibody effects at an antibody concentration giving a similar increase in proliferation in parental FET cells as obtained with the RSVAS cells. It is also in good agreement with the levels of active TGF-β_1 detected in conditioned medium of FET cells (4.7 ng/ml) over a 3-d period.

The differences in anchorage-independent growth between FET NEO and FET RSVAS cells were much more dramatic (see Fig. 9) than the differences in proliferation. While the difference in cloning efficiency was fourfold, there was also a substantial difference in the size of the colonies obtained. The difference in lag time between FET NEO and FET RSVAS cells could explain part of the difference in cloning efficiency in this experiment, but does not seem to be adequate to fully explain the dramatic differences observed. The assay was performed over the course of 3 wk at an inoculum of 2 × 10⁴ cells, conditions which would seem likely to be beyond lag time effects. Secondly, as colonies grow to the size and density seen in FET RSVAS in Fig. 9, there is a significant nutrient-deprivation effect which retards further growth. This suggests that potential differences between the two cell types could be greater than those found in this assay.

Repression of TGF-β_1 mRNA expression also led to a higher level of tumorigenicity in athymic nude mice as judged by 100% tumor formation at 10⁶ cells relative to the complete lack of tumor formation by control cells at this inoculum. There was also some tumor formation at lower inocula for the FET RSVAS cells. These results are the first demonstration that TGF-β_1 can function as a negative autocrine factor in vivo as well as in tissue culture assays. The differences in tumor formation by FET cells and FET RSVAS cells would not seem to be adequately explained by a simple difference in lag time generated by the presence of TGF-β_1 in the FET NEO cells since parental FET cells were never found to be tumorigenic at inocula of 10⁶ cells even when mice were observed for up to 60 d.

FET RSVAS cells showed a fivefold reduction in mRNA levels of TGF-β_1 after transfection with the antisense vector. Efforts to demonstrate TGF-β_1 antisense mRNA by Northern analysis were unsuccessful and, therefore, a specific RNase protection assay was developed which did show that antisense mRNA was expressed. Consequently, it was not possible from the available data to determine how much expression of the antisense mRNA was necessary to achieve the fivefold reduction of TGF-β_1 sense mRNA. Furthermore, duplex formation of sense and antisense mRNA in the cytoplasm was not demonstrated (data not shown). Consequently, it is not clear whether the TGF-β_1 levels are reduced in FET cells as a result of sense–antisense binding or whether some other mechanism specifically involving the expression of TGF-β_1 is operational in these cells as has been shown in other systems (Inouye, 1989; Chadwick, 1988; Kokha et al., 1989). However, the ability of exogenous TGF-β_1 to reverse and neutralizing antibodies to mimic the effects of antisense expression indicate the specificity of the TGF-β_1 antisense expression vector.

We have developed a large bank of colon carcinoma cell lines with a broad spectrum of biological properties (Brattain et al., 1984). FET cells represent one extreme of cells in this spectrum. As indicated above, they are not tumorigenic at low inocula and have low cloning efficiencies in soft agarose (Brattain et al., 1984). Cell lines at the other extreme of the biological spectrum form tumors in 100% of animals at inocula of 10⁶ cells. Tumors generated at 10⁶ cells grow to 1 cm³ within 3 wk. Furthermore, these biologically aggressive cells have cloning efficiencies of ~10% in soft agarose. Consequently, it appears that the effect of uncoupling the TGF-β_1–negative autocrine loop in FET cells leads to the generation of significantly more aggressive properties, but does not result in properties which are as extreme as those indicated above for the most aggressive colon carcinoma cell types. Uncoupling of the loop leads to a cell type which is intermediate between the two extremes. This is not surprising since it is likely that there are other negative growth regulators contributing to the properties of FET cells. Conversely, there may be higher levels or more types of positive factors operational in the most aggressive cell types.

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