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Colon Cancer Cells That Are Not Growth Inhibited by TGF-β Lack Functional Type I and Type II TGF-β Receptors

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Objective
The authors determined the molecular mechanisms for the failure of transforming growth factor-beta (TGF-β) to inhibit the growth of SW1116 and SW48 colon cancer cell lines.

Background
Transforming growth factor-β is a bifunctional regulator of cell growth that typically stimulates proliferation of mesenchymal cells, but inhibits proliferation of normal epithelial cells. In the colon, TGF-β appears to arrest proliferation of enterocytes as they leave the intestinal crypt and move to the villus tip. Transforming growth factor-β actions are mediated by binding to heteromeric complexes of type I and type II TGF-β receptors. Loss of TGF-β responsiveness may contribute to uncontrolled cell growth and cancer.

Methods
The effects of TGF-β_1 on DNA synthesis were measured by incorporation of tritiated thymidine into DNA of cultures of moderately differentiated adenocarcinoma (SW48) and poorly differentiated adenocarcinoma (SW1116) colon cell lines and a mink lung epithelial cell line (CCL-64). The effects of TGF-β on the expression of *c-myc*, TGF-α, and TGF-β in SW48 cells, SW1116 cells, and CCL-64 cells (*c-myc* only) were measured by Northern blot analysis. Expression of TGF-β receptors in the cell lines was measured using competitive binding assays, receptor affinity labelling techniques, and reverse transcriptase-polymerase chain reaction.

Results
Incubation with TGF-β_1 (50 ng/mL) did not decrease serum-stimulated uptake of [^{3}H]-thymidine into actively growing cultures of SW48 or SW1116 cells, but suppressed DNA synthesis of actively growing CCL-64 cells by 90%. Similarly, incubation with TGF-β_1 (12 ng/mL) for 4 hours did not substantially alter the mRNA levels of *c-myc*, TGF-α, and TGF-β_1 in either colon tumor cell line, although levels of *c-myc* mRNA in CCL-64 cells were reduced by TGF-β_1 treatment.

Competitive displacement of [^{125}I]-TGF-β_1 binding detected high levels (16,500 TGF-β receptors per cell) of specific, high-affinity (200 pmol/L half-displacement) TGF-β receptors on CCL-64 cells. In marked contrast, very low levels of TGF-β_1 binding to SW1116 cells (250 receptors per cell) and SW48 cells (260 receptors per cell) were detected. Autoradiograms of CCL-64 cells affinity labelled with [^{125}I]-TGF-β_1 revealed the presence of type I, type II, and type III TGF-β receptors. No TGF-β receptors were identified on SW1116 cells, and only very low levels of the nonsignaling type III TGF-β receptors were detected on SW48 cells. Reverse transcriptase-polymerase chain reaction amplification detected mRNAs for type I, type II, and type III TGF-β receptors in CCL-64 cells, SW48 cells, and SW1116 cells.

Conclusions

These results suggest that the lack of growth inhibition by TGF-β in SW48 and SW1116 colon cancer cells may be caused by a lack of expression of functional TGF-β receptors.

Colon cancer is the most lethal malignancy of the gastrointestinal tract. Extensive research has focused on understanding the molecular alterations that contribute to development of colon cancer. Loss of function of several genes has been correlated with increased risk of colon cancer, including MCC (mutated in colon cancer), familial adenomatous polyposis, and the tumor suppressor gene, p53.[1] It also has been hypothesized that peptide growth factors and their receptors play important roles in the development of epithelial carcinomas, including those of the colon. One of these growth factors is transforming growth factor-beta (TGF-β).

In mammals, three structurally related TGF-β proteins have been identified and cloned. All three TGF-β proteins (TGF-β_1, TGF-β_2, TGF-β_3) are homodimers of approximately 25 kd and have similar biological properties. These include the modulation of cell growth, development, and differentiation. Of particular importance for tumor biology, TGF-β has been shown to be a bifunctional regulator of cell growth. Transforming growth factor-β typically inhibits growth of normal differentiated cells of ectodermal origin while stimulating growth of cells derived from the mesoderm.[2–5] The growth of some epithelial tumor cells is not inhibited by TGF-β, suggesting that loss of growth inhibitor response to TGF-β contributes to the development of cancer.

Three general concepts have been proposed to explain the loss of TGF-β growth inhibition observed in some epithelial cancers. First, all peptide growth factors act on cells through specific membrane receptors. Cancer cells may have totally lost expression of TGF-β receptors, which would prevent TGF-β action on cells. A few examples of tumor cells that have lost expression of all TGF-β receptors have been reported, and these include retinoblastomas and pheochromocytomas.[5,6] Second, tumor cells may only express the nonsignaling forms of the TGF-β receptors. Recently, cross-linking experiments have indicated that there are three distinct high-affinity TGF-β receptors (designated types I, II, and III) that are commonly expressed on the surface of cells. All three TGF-β receptors have been cloned and sequenced.[7–14] The type I and type II receptors, which are both serine/threonine kinases, are required and act in concert to mediate the growth-inhibiting actions of TGF-β. The type III receptor lacks an intracellular domain, and a possible function of this receptor is to bind and present ligand to the type II receptor.[15] More recent experiments have shown that on TGF-β binding, the type II receptor, which is a constitutively active kinase, is recognized by the type I receptor, which is then recruited into the complex and becomes phosphorylated by the type II receptor. This type II mediated phosphorylation of the type I receptor results in signal transduction to downstream substrates.[16] Third, the postreceptor signal-transducing mechanisms for the TGF-β system could be defective. These include the p53 and retinoblastoma tumor-suppressor proteins, and the *c-myc* proliferation-inducing protein.[4,17,18]

Previous experiments in colon carcinoma cell lines have shown that addition of TGF-β_1 or TGF-β_2 inhibited growth under conditions of continuous proliferation or serum-stimulation after quiescence of most well-differentiated and some moderately differentiated human colon carcinomas.[19,20] In contrast, TGF-β did not inhibit growth of poorly differentiated colon cancer cells. The failure of TGF-β to inhibit poorly differentiated colon carcinoma cells could not be explained by lack of TGF-β binding. However, it was not determined whether both type I and type II receptors were present. These results led previous authors to suggest that the stage of differen-

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tiation of the cancer cells may be related to their responsiveness to negative growth regulators, such as TGF-β.

To more precisely define the role of TGF-β in colon cancer, we examined the biochemical aspects of TGF-β on two colon cancer cell lines—a moderately differentiated adenocarcinoma (SW48) and a poorly differentiated adenocarcinoma (SW1116). A mink lung epithelial cell line (CCL-64) that is highly growth-inhibited by TGF-β was used as a control. We tested the effect of TGF-β_1 on DNA synthesis of the three cell cultures under conditions of both continuous serum stimulation and serum induction of quiescent cells. Our results show that both the moderately differentiated SW48 cells and the poorly differentiated SW1116 colon cancer cells were not growth-inhibited by TGF-β. Similarly, the levels of mRNAs for *c-myc*, TGF-α, and TGF-β were not reduced by treatment with 12 ng/mL of TGF-β_1 for 4 hours, as determined by Northern blot analysis. To elucidate the molecular mechanisms for the failure of these cells to respond to TGF-β, the expression of TGF-β receptors was measured by competitive binding assays, receptor affinity-labelling techniques, and reverse transcriptase-polymerase chain reaction (RT-PCR). Although RT-PCR detected mRNAs for all three receptor types in all three cell lines, no protein receptor types were identified in SW1116 cells and only nonsignaling type III protein receptors were identified in SW48 cells, by affinity-labelling techniques. Thus, our results add additional data to support the hypothesis that functional type I and type II receptors are required for TGF-β to inhibit mitosis in epithelial cells and that lack of functional type I and type II receptors may contribute to uncontrolled cellular proliferation.

MATERIALS AND METHODS

Cell Culture

The two human colon carcinoma cell lines, SW1116 and SW48, were obtained from American Type Culture Collection. The SW1116 cells were designated by Leibowitz as poorly differentiated, and the SW48 cells were designated as moderately differentiated, based on the cytologic staining and growth characteristics.[21] All cell types were grown at 37 C in humidified air in chemically defined medium (CDM), which consisted of equal parts of Medium 199, Ham's F-10, and Dulbecco's modified Eagle's medium containing 25 mmol/L Hepes, pH 7.4, 1 mmol/L sodium bicarbonate, and antibiotic drugs, and was supplemented with 10% newborn calf serum. Cells were passaged with trypsin/edetic acid at twofold dilution.

DNA Synthesis Assays

DNA synthesis was determined by measuring the incorporation of [^{3}H] thymidine using a modification of a method previously described.[22] Briefly, cells were grown to approximately 80% confluency in 24-well plates in CDM supplemented with 10% fetal calf serum. The cells were washed three times with CDM without serum. The cells were then divided into two experimental arms. One arm was made quiescent by incubation for 24 hours in serum-free CDM. In the second arm, each 24-well plate was divided into four groups of six wells each. Control cells (group 1) were incubated at 37 C for 24 hours in CDM without serum containing 4 μCi/mL [^{3}H] thymidine. The other three groups of cells were incubated for 24 hours in CDM containing 10% fetal calf serum, [^{3}H] thymidine at a concentration of 4 μCi/mL (group 2) and either 12 ng/mL TGF-β (0.5 nmol/L; group 3), or 50 ng/mL (2 nmol/L) TGF-β_1 (group 4). These concentrations were chosen because they are the reported upper and lower limits of the dose response curve for the inhibition of fibroblasts by TGF-β.[23] Recombinant human TGF-β_1 was provided by the Bristol Myers Squibb Company (Seattle, WA). The cells in the second experimental arm were divided and treated as aforementioned, after an initial 24-hour incubation in serum-free CDM to make them quiescent. After incubation with [^{3}H] thymidine for 24 hours, cells in both arms were treated with 10% trichloroacetic acid to precipitate DNA, washed with methanol, and solubilized with 1 mL of 0.2 mol/L sodium hydroxide. Radioactivity was measured with a liquid scintillation counter. Cell numbers were determined using a Coulter counter model ZM (Coulter Electronics, Inc., Hialeah, FL).

Northern Blot Analysis of Colon Cell RNA

Cells were grown to approximately 80% confluency in 150-cm^2 flasks in CDM supplemented with 10% newborn calf serum. The media was replaced with either fresh CDM, with 10% serum, or CDM with 10% serum containing 12 ng/mL (0.5 nmol/L) TGF-β_1, and the cells were incubated for 4 hours at 37 C. Total RNA was then isolated using the guanidinium isothiocyanate method as described by Chirgwin.[24] Approximately 30 μg of total RNA was electrophoresed through a 1% agarose-formaldehyde gel and blotted by diffusion in 10 × SSC (1 × SSC = 1.5 mol/L sodium chloride, 0.15 mol/L sodium citrate) onto Hybond N+ membrane (Amersham, Arlington Heights, IL). The membrane was then hybridized sequentially with [α-^{32}P] dCTP-labelled cDNA probes for *c-myc*, TGF-α, and TGF-β_1. Hybridization was performed overnight at 65 C in 10 mL of 250 mmol/L sodium phosphate buffer, pH 7.4, and 1% bovine serum albumin. The blots were then washed at 60 C in 20 mmol/L phosphate buffer, pH 7.4, with 0.1% sodium dodecyl sulfate, and exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) with intensifying

screens at −80 C. After each hybridization, the blots were stripped in 0.1 × SSC and 0.1% sodium dodecylsulfate at 95 C for 5 minutes.

All cDNA probes were generated by restriction endonuclease digestion of cesium chloride purified recombinant clones, purified by agarose gel electrophoresis, and labeled with [α-^{32}P] dCTP using the Prime-It random-primed labeling kit (Stratagene, La Jolla, CA). The TGF-α probe was a gift from Dr. G. Plowman (Bristol Myers Squibb Co., Seattle, WA) and encodes amino acids 17 through 160 of the human TGF-α precursor. The *c-myc* probe encodes the C-terminus, beginning at amino acids 178.[25] The TGF-β_1 probe was obtained from American Type Culture Collection. The TGF-β_1 probe encodes the entire coding sequence for TGF-β_1 and includes 779 base pairs of 5′ untranslated sequence and 179 base pairs of 3′ untranslated sequence, including a polyA tract of 37 base pairs. Quantification of autographic bands was performed using the Millipore Video Image Densitometer (Millipore Corporation, Bedford MA), and RNA sample levels loaded in each well were assessed by the intensity of ethidium bromide staining of the ribosomal RNA bands.

Quantification of TGF-β Binding by Colon Cells

Levels of TGF-β receptors were measured by competitive binding of [^{125}I]-TGF-β_1 to intact cells as described previously.[26] Briefly, TGF-β_1 was labelled with [^{125}I] by the modified chloramine-T method to a specific activity of 32 μCi/μg.[27] The colon cells were grown to initial confluency in CDM containing 10% serum. Binding to the CCL-64 cells and SW1116 cells was performed using 24-well plates. Because the SW48 cells attach lightly to dishes, cells were dislodged from confluent culture flasks, and binding was performed with cells in 12 × 75 mm polypropylene tubes. The cells were washed with binding buffer (128 mmol/L NaCl, 5 mmol/L KCl, 5 mmol/L $MgSO_4$, 1.2 mmol/L $CaCl_2$, 50 mmol/L Hepes, pH 7.5, 2 mg/mL bovine serum albumin) and allowed to equilibrate in binding buffer for 30 minutes at 4°C. The buffer was then removed and unlabeled TGF-β_1 was added to each well at concentrations ranging from 10 nmol/L to 1 pmol/L. [^{125}I]-TGF-β (100 pmol/L) was immediately added to each well, and the cells were incubated at 4 C overnight. The cells were washed five times with binding buffer, solubilized for 40 minutes at 4 C in 0.5 mL of 1% Triton X-100, 10% glycerol in 50% binding buffer, and the levels of radioactivity of the solubilized cells were measured with a gamma counter. Specific binding was calculated and transformed by the method of Scatchard, and the affinity and level of TGF-β receptors were calculated from the Scatchard plot.

Affinity Labeling of TGF-β Receptors

Affinity labeling of TGF-β_1 receptors was performed by chemical cross-linking of [^{125}I]-TGF-β_1 to cells, as described previously.[26] Briefly, the two colon cell lines and the CCL-64 cells were grown to confluency in 150-cm^2 tissue culture flasks and washed three times in binding buffer, as described for the competitive binding assay. [^{125}I]-TGF-β_1 (35 pmol/L) was bound in the absence (total binding) or presence (nonspecific binding) of 5 nmol/L unlabeled TGF-β_1 overnight at 4 C. After washing three times with binding buffer, [^{125}I]-TGF-β_1 was chemically cross-linked to the receptors with 150 μl of 27 mmol/L disuccinimidyl suberate in 30 mL of binding buffer without bovine serum albumin for 15 minutes at 4 C. After a brief rinse at 4 C with detachment buffer (0.25 mol/L sucrose, 10 mmol/L Tris, 1 mmol/L edetic acid, pH 7.4, and 0.3 mmol/L phenylmethylsulfonyl fluoride, the cells were scraped from the flask at 4 C in 1 mL of detachment buffer. The cells then were pelleted by centrifugation at 12,000 × g for 2 minutes, solubilized in a minimal volume of solubilization buffer (125 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L edetic acid, pH 7.0, 1% Triton X-100) with 10 μl/mL of protease inhibitors (1 mg/mL leupeptin, 10 mg/mL soybean trypsin inhibitor, and 30 mmol/L phenylmethylsulfonyl fluoride) and incubated overnight at 4 C with inverted mixing. Insoluble cell debris was then removed by centrifugation for 15 minutes at 12,000 × g. Supernatants were added to an equal volume of electrophoresis buffer (100 mmol/L Tris, pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate, 0.05% bromophenol blue and 100 mmol/L dithiothreitol), and samples were analyzed by 6% sodium dodecylsulfate polyacrylamide gel electrophoresis.[28] The gels were stained with Coomassie brilliant blue, dried, and exposed to XAR-5 film with one intensifying screen.

Reverse Transcriptase-Polymerase Chain Reaction Amplification of RNA

Total RNA was isolated from cells that were grown to 80% confluency in 150-cm^2 flasks by the guanidinium isothiocyanate method[24] as described for Northern blot analysis. Oligonucleotide primers were synthesized at the University of Florida Interdisciplinary Center for Biotechnology Research on an Applied Biosystems division of Perkin Elmer, 380B nucleic acid synthesizer (Applied Biosystems, Foster City, California). One microgram of total RNA was reverse transcribed into cDNA in 20 μL of reaction buffer (10 mmol/L Tris-HCl pH 8.3, 50 mmol/L $CaCl_2$, 2 mmol/L $MgCl_2$), containing 200 μM each of dCTP, dATP, dGTP, and dTTP, 0.3 units/μL RNasin (Life Technologies), 0.025 μg/μL oligo d(T) (Life Technologies), and 10 units/μL M-MLV reverse

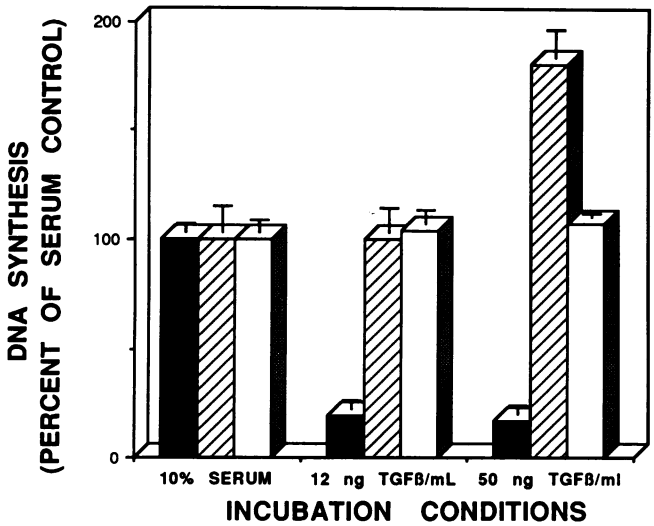

Figure 1. The effect of TGF-β_1 on DNA synthesis of colon cells is shown. Mink lung epithelial cells (CCL-64) (closed boxes), moderately differentiated (SW48) (open boxes) and poorly differentiated (SW1116) (hatched boxes) colon cancer cells were treated with 12 ng TGF-β/mL or 50 ng TGF-β/mL in the presence of 10% newborn calf serum and analyzed for their ability to incorporate [^{3}H] thymidine into DNA, as described in the materials and methods section.

transcriptase (Life Technologies, Gaithersburg, MD) at room temperature for 10 minutes, then at 42 C for 1 hour, then 94 C for 5 minutes. The polymerase chain reaction was performed in the same tube by adding 30 μL of reaction buffer containing 200 μM each of dCTP, dATP, dGTP, and dTTP, 2.5 units Taq polymerase (Perkin Elmer, Norwalk, CT) and 100 pmol of each of the 5′ and 3′ oligonucleotide primers. The PCR amplification reactions were run for one cycle at 94 C for 5 minutes, 58 C for 2 minutes, and 72 C for 3 minutes, and 40 cycles at 94 C for 1 minute, 58 C for 2 minutes, and 72 C for 3 minutes. The PCR products were digested with Sau3A1 (type I), HinclI (type II), or BamHl (type III) restriction endonucleases using buffers and conditions recommended by the manufacturers.

PCR Oligonucleotide Primers

The nucleotide sequences of the oligonucleotide primers were as follows:

TGF-β type I receptor (Alk-5) primers: 5′ primer: 5′ ACAGCATGTGTATAGCTGAAA 3′; 3′ primer: 3′ TCCGTGAGGCAGAGATTTATC 3′.

TGF-β type II receptor primers: 5′ primer: 5′ TGTGTTCCTGTAGCTCTGATG 3′; 3′ primer; 5′ GATCTTGACTGCCTCTGTCTC 3′.

TGF-β type III receptor primers: 5′ primer: 5′GAACTCAAGATAGCAAGAAACA 3′; 3′ primer: 5′GATGGTTATGAAGATCTGGAGT 3′.

RESULTS

Effect of TGF-β on Incorporation of [^{3}H]-thymidine into Colon Cells

As shown in Figure 1, TGF-β_1 at concentrations of 12 ng/mL (0.5 nmol/L) and 50 ng/mL (2 nmol/L) significantly ($p = 0.02$) inhibited by 90% the incorporation of [^{3}H]-thymidine into actively growing CCL-64 (mink lung epithelial) cells. In addition, TGF-β_1 also blocked [^{3}H] thymidine incorporation into quiescent CCL-64 cells re-exposed to serum (data not shown). Thus, TGF-β_1 inhibited DNA synthesis in actively growing and serum-stimulated quiescent CCL-64 cells. Addition of TGF-β_1 did not inhibit the mitogenic response of SW1116 cells (data not shown). SW1116 cells were not inhibited by 24 hours of treatment with 12 ng (0.5 nmol/L) of TGF-β_1 ($p = 0.83$) and actually were stimulated by 50 ng of TGF-β_1 for 24 hours ($p = 0.01$). SW48 cells also were not inhibited by 24 hours of treatment with 12 ng or 50 ng TGF-β (Fig. 1). Therefore, SW48 cells and SW1116 cells were not inhibited by incubation with TGF-β_1.

The Effects of TGF-β on the mRNA Levels of c-*myc*, TGF-α, and TGF-β in Colon Cells

Increased synthesis of *c-myc* and TGF-α mRNA and protein have been found to be associated with increases in cell proliferation. To assess whether the expression of these proliferation associated genes in the colon cancer cells was affected by treatment with TGF-β, we also treated CCL-64 cells, SW48 cells, and SW1116 cells with 12 ng (0.5 nmol/L) of TGF-β_1 for 4 hours, isolated total RNA and analyzed the RNA by Northern blots. As shown in Figure 2A, the mRNA levels of *c-myc*, TGF-α, and TGF-β were not substantially reduced by TGF-β treatment in both the moderately differentiated SW48 cells and the poorly differentiated SW1116 cells. However, the levels of mRNA for *c-myc* in CCL-64 cells were substantially reduced by treatment with 12 ng of TGF-β for 4 hours (Figs. 2A and 2B). The SW1116 cells make very low levels of TGF-β mRNA (Fig. 2A), which may relate to the lack of functional TGF-β receptors on these cells.

Quantitation and Affinity Labeling of TGF-β Receptors in Colon Epithelial Cells

Competitive radioreceptor binding assays were performed on CCL-64 cells, SW48 cells, and SW1116 cells. Figure 3 shows that high levels (16,500 receptors per cell) of high-affinity (half-displacement = 200 pmol/L) TGF-β receptors were detected on CCL-64 cells. In contrast, extremely low levels of TGF-β receptors were detected

on SW48 cells (260 receptors per cell) and SW1116 cells (250 receptors per cell).

To further characterize the TGF-β receptors expressed by colon epithelial cells, [^{125}I]-TGF-β was chemically cross-linked to CCL-64 cells, SW48 cells, and SW1116

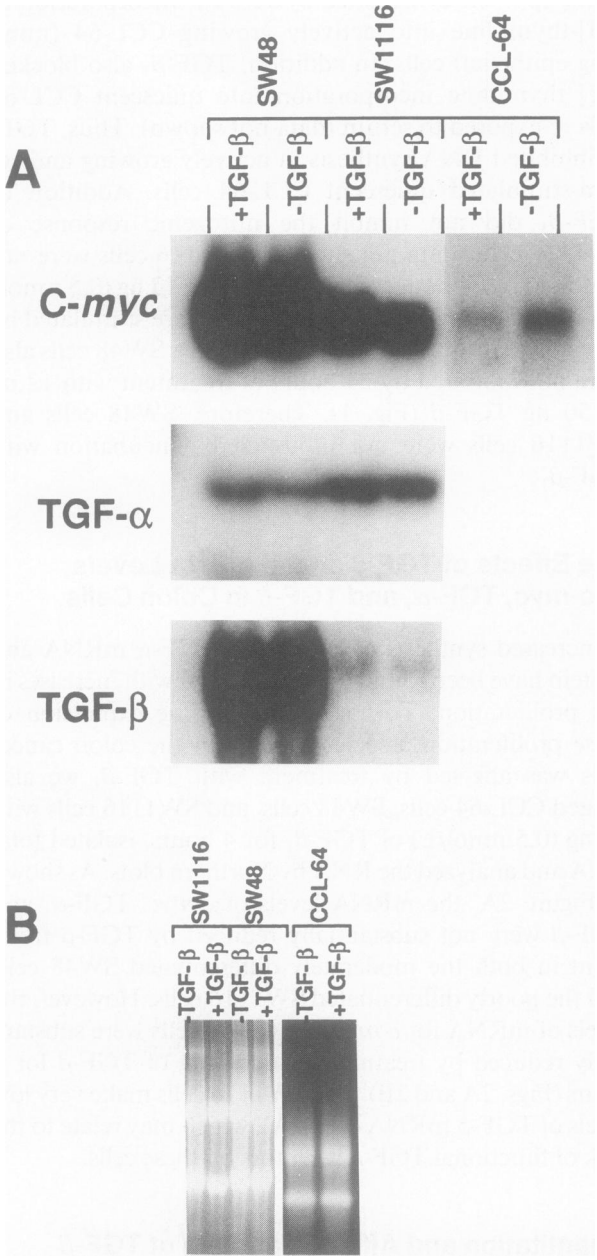

Figure 2. The effect of TGF-β_1 on mRNA levels of *c-myc*, TGF-α, and TGF-β genes is shown. (A) Northern analysis of *c-myc*, TGF-α, TGF-β expression is compared with ethidium bromide stained RNA (B) in mink lung epithelial cells (CCL-64), moderately differentiated (SW48), and poorly differentiated colon cancer cells treated (+TGF-β) or untreated (−TGF-β) with 12 ng/mL of TGF-β. Total RNA was isolated, electrophoresed through a 1% agarose-formaldehyde gel (B) and blotted onto Hybond N+ membrane and probed sequentially with [α-^{32}P] dCTP-labeled cDNA probes for *c-myc*, TGF-α, and TGF-β.

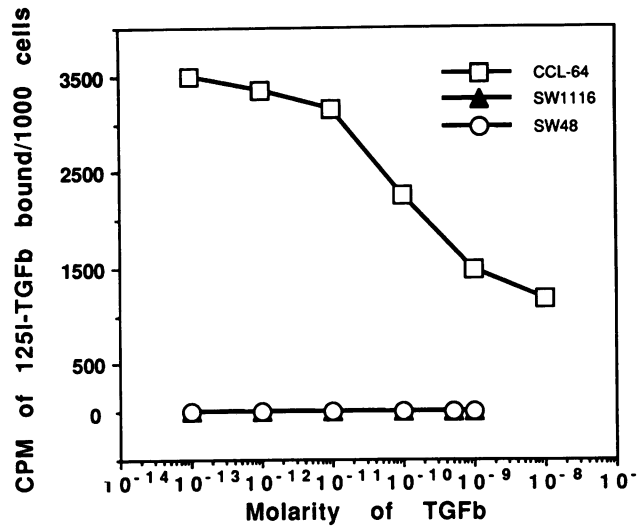

Figure 3. Competitive binding of [^{125}I]-TGF-β_1 to colon cells is shown. Colon cells were grown to confluency in 24-well plates (CCL-64 and SW1116 cells) or 12 × 75 polypropylene tubes (SW48 cells) in CDM containing 10% bovine calf serum. The cells were washed and allowed to bind to [^{125}I]-TGF-β in the presence of unlabeled TGF-β, ranging from 10 nmol/L to 1 pmol/L. Cell numbers were determined using a Coulter counter, and the binding was determined per 10^3 cells.

cells (Fig. 4). To control for nonspecific binding of [^{125}I]-TGF-β, each cell type also was incubated in the presence of 5 nmol/L TGF-β before addition of [^{125}I]-TGF-β (Fig. 4 lanes marked NSB).

As shown in Figure 4, CCL-64 cells have high levels of type I, type II, and type III receptors (molecular weights of TGF-β-receptor complexes of 68, 85, and 280 kd, respectively (Fig. 4, lanes 1 and 2). Figure 4 also shows that SW48 cells produce low levels of type III TGF-β receptors, but no discernible levels of type I or type II receptors. Cross-linking studies on SW1116 cells could detect only a faint band of approximately 80 kd. This band does not correspond to any TGF-β receptor type previously described. Additionally, this band is not competed for by addition of 5 nmol/L of unlabeled TGF-β, suggesting that it represents low affinity, nonspecific binding of TGF-β (Fig. 4, lanes 5 and 6). These results support aforementioned results obtained by the competitive binding studies.

RT-PCR of Type I, Type II, and Type III TGF-β Receptor mRNA

To determine if mRNA for each of the three TGF-β receptors was made in the colon cells, total RNA was isolated from actively growing cells and cells treated for 4 hours with 12 ng of TGF-β_1. Reverse transcriptase-polymerase chain reaction was performed on this RNA using oligonucleotide primers made from the published DNA sequences of the human type I,[9] type II,[12] and type

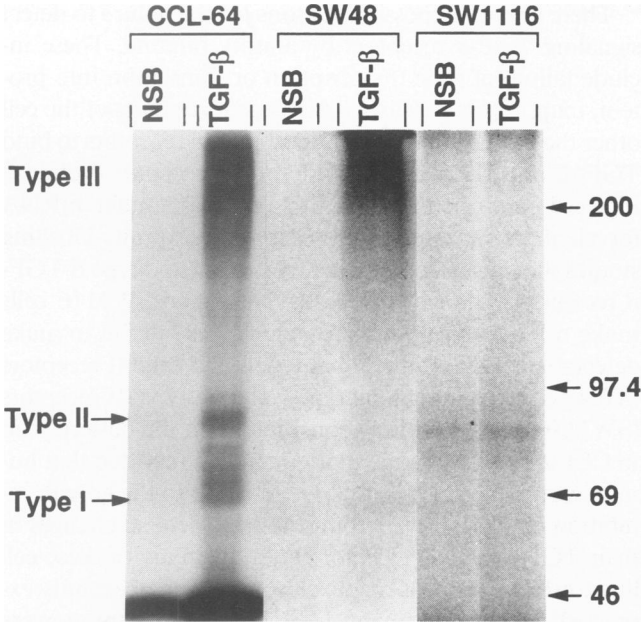

Figure 4. Cross-linking of [^{125}I]-TGF-β to the surface of CCL-64 cells, SW48 cells, and SW1116 cells is shown. [^{125}I]-TGF-β (35 pmol/L) was cross-linked chemically to colon cells in the absence (lanes marked TGF-β) or presence of 5 nmol/L of unlabeled TGF-β to detect nonspecific binding (lanes marked NSB) using 27 mmol/L disuccinimidyl suberate. After cross-linking, the cells were subjected to 6% sodium dodecylsulfate polyacrylamide gel electrophoresis, then exposed to XAR-5 film using one intensifying screen. The expected positions of the type I, II, and III receptors are indicated by arrows on the left. The numbers and arrows on the right represent the sizes and positions of molecular weight markers.

III[14] TGF-β receptors. As shown in Figure 5A, RT-PCR of total RNA isolated from each cell type revealed that both the colon cancer cell types and the CCL-64 mink lung epithelial cells make mRNA for all three TGF-β receptor types. Figure 5B shows that Sau 3A1 (type I), HincII (type II), and BamHI (type III) restriction endonuclease digestion of the RT-PCR amplicon products from SW1116 cells and SW48 cells generated fragments with the predicted sizes, confirming the specificity of the PCR amplification reaction. Because the nucleotide sequence of mink TGF-β receptor mRNAs is not known, demonstration of the specificity of the CCL-64 amplicons is not possible by restriction endonuclease digestion. Therefore, the undetectable levels of type I and type II TGF-β receptors on SW48 cells and the undetectable levels of type I, type II, and type III receptors on SW1116 cells, as observed by the [^{125}I]-TGF-β affinity-labelling studies, are not because of lack of receptor mRNA.

DISCUSSION

Transforming growth factor-β is a potent inhibitor of epithelial cell proliferation *in vitro*.[1] Specifically, TGF-β has been shown to inhibit growth of mouse and human keratinocytes[29] and normal jejunal crypt cells of the small intestine (IEC-6 cells).[30] This suggests that in the small intestine, TGF-β may function to arrest proliferation of enterocytes as they leave the intestinal crypt and then maintain the terminally differentiated phenotype as they move to the villus tip.[31] Experimental data also suggest that growth of colon epithelial cells is modulated by both the stimulatory effects of epidermal growth factor and TGF-α, and the inhibitory effects of TGF-β.[32] Pertubation of these growth regulatory pathways may lead to neoplastic transformation of colon cells.[33] Consistent with this hypothesis is the finding that the growth of most cultured colon carcinomas is not inhibited by TGF-β, although the growth of some moderately differentiated colon adenocarcinoma cell lines previously were reported to be inhibited by TGF-β.[19,20,34,35] To confirm and expand these observations, we studied the effects of TGF-β on two colon cancer cell lines—one moderately differentiated adenocarcinoma (SW48), and one poorly differentiated adenocarcinoma (SW1116). The mink lung epithelial cell line CCL-64 cells, which is highly

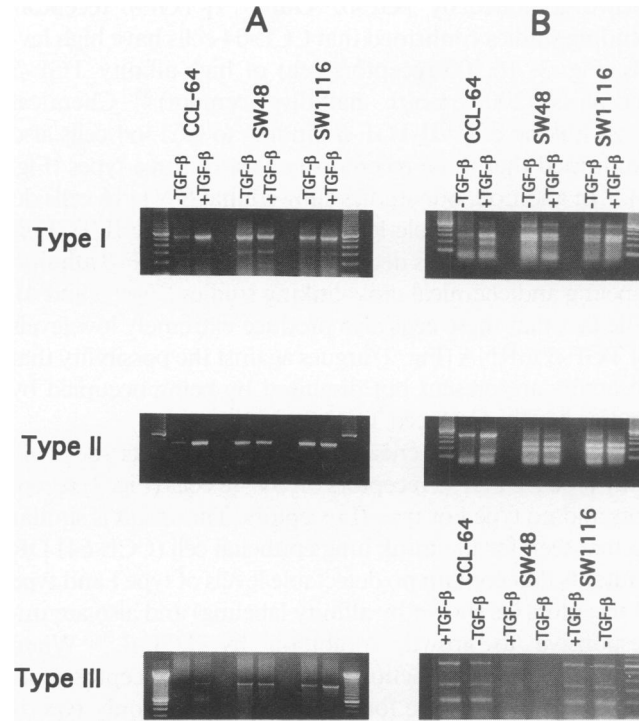

Figure 5. Identification of type I, II, and III TGF-β receptor mRNA by RT-PCR is shown. Total RNA was isolated from CCL-64, SW48, and SW1116 cells treated with 12 ng of TGF-β for 4 hours (+TGF-β) or left untreated (−TGF-β), reverse transcribed into DNA, then amplified by PCR using the oligonucleotide primers described in materials and methods. Half the amplified DNA was electrophoresed through a 2% agarose gel (A) and half was digested with Sau 3A1 (type I), Hinc II (type II), and BamHI (type III) before 2% agarose gel electrophoresis (B).

growth-inhibited by TGF-β_1, was used as a positive control.[36] We predicted that the moderately differentiated colon cancer cells possibly would be growth-inhibited by TGF-β_1 whereas the poorly differentiated colon cancer cells would be unaffected. Our experiments showed that growth of both colon cell types, as measured by DNA synthesis, was unaffected by TGF-β. However, TGF-β_1 treatment of CCL-64 mink lung epithelial cells blocked DNA synthesis at 12 ng (0.5 nmol/L) and 50 ng/mL (2 nmol/L) (Fig. 1), as described previously, demonstrating the biological activity of the TGF-β_1 preparation.[35] Similarly, the expression of the proliferation associated gene *c-myc* was reduced by 4 hours of treatment with 12 ng of TGF-β_1 in CCL-64 cells, but not in the two colon cancer cell lines (Fig. 2). The mRNA levels for TGF-α and TGF-β in SW48 and SW1116 cells also were not reduced by treatment with 12 ng of TGF-β_1 for 4 hours. Thus, our experiments show that SW48 cells and SW1116 cells are resistant to the growth inhibitory effects of TGF-β_1.

It has been suggested that either a lack of functional TGF-β receptors or postreceptor defects (signal transduction) are the most likely explanations for cells becoming unresponsive to growth inhibition by TGF-β. Retinoblastoma cells lack functional TGF-β receptors and are not growth-inhibited by TGF-β.[6] Our [^{125}I]-TGF-β receptor-binding studies confirmed that CCL-64 cells have high levels (Fig. 3, 16,500 receptors/cell) of high-affinity TGF-β receptors (200 pmol/L half-displacement).[36] Chemical cross-linking of [^{125}I]-TGF-β binding to CCL-64 cells also confirmed that these receptors consist of three types (Fig. 4).[37] In addition, our studies showed that SW1116 cells do not produce detectable levels of type I or type II TGF-β-signaling receptors, as determined by [^{125}I]-TGF-β affinity-labeling and chemical cross-linking studies (Figs. 3 and 4). The fact that these cells also produce extremely low levels of TGF-β mRNA (Fig. 2) argues against the possibility that receptors are present but disguised by being occupied by endogenously produced TGF-β.

Affinity-labeling, cross-linking experiments detected only type III TGF-β receptors on SW48 cells (Fig. 4) receptors and no type I or type II receptors. This result is similar to that seen for the mink lung epithelial cell (CCL-64) DR mutants that contain no detectable levels of type I and type II receptors (as shown by affinity labeling) and also are unresponsive to growth inhibition by TGF-β.[36] When transfected with a functional TGF-β type II receptor gene, the DR mutants were found to produce not only type II receptors, but also type I receptors.[38] Similarly, SW48 cells may have functional type I receptors, but these receptors may be unable to bind TGF-β in the absence of functional type II receptors. Consistent with this hypothesis was the finding that DR mutants were found to make a structurally defective type II receptor that was inactive in affinity binding studies.[39]

There are many possible reasons for the failure to detect signaling TGF-β receptors by affinity binding. These include failure of gene transcription or translation into protein, inappropriate delivery of receptors to parts of the cell other than the cell surface, the inability of receptors to bind TGF-β, or a shortened half life of the receptor protein.[40] Figure 5 confirms that both these cell types make mRNA for all three receptor types, despite the affinity-labelling studies showing a lack of functional type I and type II TGF-β receptors. The fact that both SW48 and SW1116 cells make mRNA for all three receptor types but fail to make detectable levels of functional type I and type II receptors (SW48 cells) or functional type I, II, and type III receptors (SW1116 cells) is entirely consistent with the pattern seen in CCL-64 DR mutants. It also has been reported that human gastric cancer cells that are resistant to the growth inhibition by TGF-β were found to have genetic changes in their TGF-β type II receptor genes. In many of these cell lines, mRNA still was made, although often aberrantly expressed, and the resultant TGF-β type II receptors were found to have structural abnormalities, which resulted in their inability to bind TGF-β.[41]

The cloning and sequencing of the three types of TGF-β receptors have resulted in the elucidation of their mechanism of activation. Both the type I and type II receptors are serine/threonine kinases that mediate the functions of TGF-β. The type III receptor has a minimal cytoplasmic domain and thus, does not appear to be involved in signal transduction. Transforming growth factor-β binds directly to receptor II, which is a constitutively active kinase. Once bound, the TGF-β is recognized by the type I receptor, which cannot bind TGF-β in the absence of the type II receptors. The type I receptor then forms a complex with the type II receptor and is phosporylated by type II receptor kinase activity. Once phosphorylated, the type I receptor can then propagate a signal downstream via its own serine/threonine kinase.[42] Thus, neither the type I or type II receptors can generate a growth inhibiting signal unless they bind TGF-β and form a heterodimer with the other receptor. The type III receptor may concentrate TGF-β and present it to the type II receptor, also forming a tertiary complex.[15,43]

Our results strongly support this model. Both the SW48 and SW1116 cells do not express functional type II receptors and are not growth-inhibited by TGF-β_1. Thus, our experiments have shown that these two colon cancer cell lines are not growth-inhibited by TGF-β, and the original state of differentiation of these colon cancer cell lines did not predict their responsiveness to TGF-β. The most likely reason for this lack of growth inhibition is that neither of these cell types has the normal component of functional TGF-β receptors. These data are further supported by the failure of TGF-β to affect mRNA levels of second messenger proteins, which normally are inhibited by

TGF-β. Thus, disregulation of TGF-β receptors may contribute to the morphologic properties of these malignant, transformed cells.

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Discussion

DR. COURTNEY M. TOWNSEND, JR. (Galveston, Texas): President McDonald, Fellows, and Guests, this is a carefully done study that was very wonderfully presented, and it explains, at least in part, the reason that TGF-β does not appear to have any inhibitory effect on the growth of two human colon cancer cell lines. The authors have very carefully analyzed all of the complete pathway of receptor and ligand signal transduction mechanisms that could be involved and found evidence only by reverse transcriptase PCR analysis. Is there any evidence of receptor gene expression, e.g., binding?

All three types of receptors are found by that very sensitive analysis.

That is a sensitive analysis and may provide false information, so the first question is: Have you validated the PCR analysis by Southern analysis and/or sequencing of the PCR product?

I think the most fascinating part of the paper is that not only did they not find inhibition, but that they found stimulation of the SW1116 cells, as shown in the graph and the slide in which DNA synthesis was evaluated.

The thesis was this peptide would inhibit growth and, therefore, that was not mentioned further. However, in the cross-linking studies, also an 80-kd band was found in the SW1116 cells. Does this suggest then that there is another as yet unidentified TGF-β receptor? What do you plan to do further to investigate what, at least to me, is a fascinating phenomenon, because there is an effect of TGF-β on these cells, suggesting some kind of receptor and intact signal transduction mechanism is involved?

Do you think this suggests that the cell lineage for the SW1116 is different because it is known that TGF-β stimulates mesenchymal cells? Or is it possibly a further example of the undifferentiated state of this cell line, and that more primitive mechanisms are involved?

I appreciate the opportunity to discuss the paper. Thank you.

DR. JEFFREY A. NORTON (St. Louis, Missouri): I enjoyed this excellent paper and presentation. I would just like to ask two relatively simple questions:

Is the lack of a functional TGF-β receptor on a colon epithelial cell sufficient to transform this cell into cancer?

If the mRNA is present and expressed in these colon cancer cells, as the PCR suggests, why isn't the receptor functional? What are the mechanisms to render the TGF-β receptors nonfunctional in these cell lines?

DR. RAVI S. CHARI (Durham, North Carolina): Thank you, Dr. McDonald. Dr. Copeland, I enjoyed this paper as well. As discussed, TGF-β is a multifunctional polypeptide whose effects on epithelial cells appears to be growth inhibitory. Changes in the receptor profile may be responsible for some of the loss of growth inhibition in cancer.

We have examined the changes in TGF-β receptor profile in the setting of hepatic fibrosis, hepatic regeneration after partial hepatectomy and carcinogenesis in a rat model as well as in human hepatocellular carcinoma.

Consistently in these models, using RNase, protection assay and ligand binding, we have seen a 30% to 60% change in receptors, but we have never observed an absolute zero. Furthermore, we have seen that it appears to be at the transcriptional level that this is occurring.

The loss of TGF-β growth inhibition by loss of receptors has been described by Brian Carr in Harvey Lodish's laboratory. There, they have shown, using a hepatoma cell line, that the growth inhibition could be restored by transfecting with a Type II TGF-β receptor. Further, in Dr. JoAnn Massaguće's laboratory, they have demonstrated that when they transfected cells with apparent loss of both the Type I and Type II receptors with Type II receptor, there was not only the recurrence of binding to the Type II receptor, but a restoration of the functional Type I receptor, implicating that binding of the Type I receptor is dependent on a functional Type II receptor.

I have the following questions then:

Do you think that there was a decrease in both the Type I and Type II receptors, or only a loss of functional Type II, rendering the Type I receptor unable to bind TGF-β?

Two, you have demonstrated that there was a decrease in all three receptors. Have you used an internal control to show that this is not a pan receptor phenomena whereby all receptors are decreased in the cell line?

Three, relating to some of the previous data I mentioned, have you thought of transfecting the unresponsive cells with the Type II or Type I gene to re-establish growth inhibition?

Four, have you looked at actual human specimens to verify whether this cell culture model is an actual phenomena?

And, finally, can you specify the region used for your PCR analysis?

I enjoyed the paper. I'd like to thank the Society for the privilege of discussing this paper.

DR. SALLY L. D. MACKAY (Closing Discussion): Dr. McDonald, Dr. Copeland, Fellows, and Guests of the Association. I would also like to thank the discussants for their questions and comments.

First, Dr. Townsend asked if we had validated the PCR by Southern blotting or by DNA sequencing. DNA sequencing was not performed, but we did do restriction endonuclease digest on the PCR-amplified fragments.

These experiments showed that the correct restriction endonuclease pattern was obtained for each of the three types of receptors except for the CCL-64 cells. The DNA sequence has not been determined for mink epithelial CCL-64 cell TGF-β receptors. Therefore, in mink lung epithelial cells, the DNA sequences that the endonucleases recognize are not necessarily present. Thus, you don't get the same digestion patterns.