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Promoter Competitors as Novel Antifibrotics That Inhibit Transforming Growth Factor-β Induction of Collagen and Noncollagen Protein Synthesis in Fibroblasts

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Abstract A single-stranded 27-mer phosphorothioate oligodeoxynucleotide (ssPT) containing the transforming growth factor-β (TGF-β) response element was synthesized. Rat fetal lung fibroblasts were stably transfected with the ColCat 3.6 plasmid, which contains a portion of the 5′-flanking region of the proα1(I) collagen gene linked to the chloramphenicol acetyltransferase (CAT) gene. The cells were transiently transfected with the modified oligodeoxynucleotides in both the presence and absence of bleomycin, a fibrogenic antineoplastic agent. At 50 μg ssPT, the bleomycin-induced increase in CAT activity was abrogated. The ability of ssPT to inhibit collagen synthesis in rat fetal lung fibroblasts was determined. Single-stranded PTs inhibited both collagen synthesis and noncollagen protein synthesis induced by TGF-β1, the mediator of the bleomycin fibrogenic effect. Inflamed granulation tissue fibroblasts were prepared from polyvinyl alcohol sponges implanted in the backs of rats. These fibroblasts were treated with various doses of ssPTs in the presence and absence of TGF-β1. Single-stranded PTs also blocked both the TGF-β1-induced increase in collagen synthesis and noncollagen synthesis in these fibroblasts. However, the TGF-β1-induced increase in collagen and noncollagen protein synthesis was not blocked by ssPTs containing a mutated TGF-β response element. In addition, ssPT did not significantly alter the basal levels of collagen and noncollagen protein synthesis in rat lung fibroblasts or in granuloma derived fibroblasts. Since dexamethasone was also able to block the TGF-β1-induced increase in collagen and noncollagen protein synthesis (Meisler et al., [1997] J. Invest. Dermatol. 108:285–289), these data indicate that phosphorothioate oligodeoxynucleotide antifibrotic agents mimic the inhibitory effect of glucocorticoids on collagen synthesis without the untoward side effects of these steroids. J. Cell. Biochem. 75:196–205, 1999. Published 1999 Wiley-Liss, Inc.†

Key words: phosphorothioate oligodeoxynucleotide; promoter competitor; antifibrotic agent; collagen synthesis; collagen gene expression; fibroblast; transforming growth factor-β1

Antisense oligodeoxynucleotides provide a novel therapeutic strategy to regulate the translation of genes. This therapy provides precise and effective modification of specific gene expression [Stein and Cohen, 1988; Szczylik et al., 1991; Morrison, 1991]. Antisense oligodeoxynucleotides have proved effective in reducing the expression of viral genes [Matsukura et al., 1987; Agrawal et al., 1989] and oncogenes [Gewirtz and Calabretta, 1988; Saison-Behmoaras et al., 1991] in tissue culture. One of the principal problems with antisense oligodeoxynucleotide therapy relates to nuclease sensitivity. Antisense compounds, particularly unmodified phosphodiesters [Wickstrom, 1986] have decreased stability within cells, which can reduce the potency and shorten the duration of the action of these antisense agents. The reduced stability of antisense oligodeoxynucleotides has been largely overcome by the use of backbone modified oligodeoxynucleotides that are more resistant to nucleases. Phosphorothioates appear to be more resistant to enzymatic digestion than the corresponding natural oligodeoxynucleotides [Wickstrom, 1992; Letsinger, 1993; Zon, 1993]. These modified oligodeoxynucleotides have a sulfur in place of one oxygen in the phosphodiester group of DNA.

Abbreviations used: TGF-β1, transforming growth factor-β1; CAT, chloramphenicol acetyltransferase; ssPT, single-stranded phosphorothioate oligodeoxynucleotide.

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The present study was conducted to determine the ability of a single-stranded 27- mer phosphorothioate oligodeoxynucleotide (ssPT) containing the TGF-β response element to regulate bleomycin and TGF-β1-induced collagen synthesis and proα1(I) collagen gene expression. The study demonstrates that the TGF-β response element located in the 5′-flanking region of the proα1(I) gene is a key determinant of procollagen gene expression. The specific effects on both collagen and noncollagen protein synthesis were determined by mutagenesis of the ssPT. Compared with a previous study from our laboratory [Meisler et al., 1997], these results demonstrate that these modified oligodeoxynucleotides mimic the effect of dexamethasone on collagen and noncollagen protein synthesis induced by TGF-β1. We propose that ssPTs, after transfection into the cell, compete with the TGF-β response element for binding to the TGF-β activator protein. This would prevent the TGF-β activiator protein from associating with the TGF-β response element, thereby inhibiting procollagen gene expression.

MATERIALS AND METHODS

Cell Culture

Rat fetal lung fibroblasts (RFL-6) were purchased from the American Type Culture Collection (CCL 192; ATCC, Rockville, MD). RFL-6 fibroblasts were grown in 90% (v/v) Eagle's minimum essential medium (BioWhittaker, Walkersville, MD) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS)(Hyclone Laboratories, Logan, UT), 100 U of penicillin per ml, 100 µg of streptomycin per ml, 292 µg of L-glutamine per ml, and 0.22% (w/v) sodium carbonate. Fibroblasts derived from granulation tissue were prepared from the outgrowth of polyvinyl alcohol sponges implanted in the dorsum of male Sprague-Dawley rats as previously described [Meisler et al., 1997]. The cells were maintained in 90% (v/v) Dulbecco's modified Eagle medium (DMEM)(4.5 µg glucose/L) (BioWhittaker) containing 10% (v/v) heat-inactivated FBS, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 292 µg of L-glutamine per ml. Fetal rat skin (FRS) fibroblasts were purchased from the American Type Culture Collection (CRL 1213; ATCC). The cells were grown in 90% (v/v) DMEM (1.0 glucose/L) containing 10% (v/v) heat-inactivated FBS, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 292 µg of L-glutamine per ml. Before treatment with bleomycin or TGF-β1, cells were washed twice with phosphate-buffered saline (PBS) (Gibco-BRL, Grand Island, NY) and placed in AIM V, a synthetic serum-free medium (Gibco-BRL). Lyophilized samples of TGF-β1 were reconstituted in 4 mM HCl containing 1 mg bovine serum albumin (BSA) per ml before addition to AIM V medium. This solution was added to controls without TGF-β1.

Stable Transfection

FRS fibroblasts were stably transfected using the calcium phosphate coprecipitation method [Chen and Okayama, 1987]. Plasmids were purified by double CsCl banding according to standard procedures. ColCat 3.6 plasmids (supplied by D. Rowe and A. Lichtler, University of Connecticut Health Science Center, Farmington, CT) containing the rat proα1(I) collagen promoter or constructs containing mutations of the proα1(I) collagen promoter were transfected into FRS cells [Lichtler et al., 1989; Ritzenthaler et al., 1991]. The pSV2neo plasmid was cotransfected for stable cell selection. Cells were selected using G418 (200 µg/ml). Polymerase chain reaction (PCR) mutagenesis of the TGF-β elements in ColCat 3.6 was performed as previously described [Nelson and Long, 1989; Meisler et al., 1995]. RFL-6 fibroblasts were also stably transfected with ColCat 3.6 and PSV2neo plasmids.

Transient Transfection

RFL-6 fibroblasts and granulation tissue fibroblasts were transiently transfected with single-stranded modified oligodeoxynucleotides known as phosphorothioates (phosphorothioate analogues of DNA having a sulfur in place of an oxygen as one of the nonbridging ligands bound to phosphorous). The phosphorothioate oligodeoxynucleotides were synthesized by Research Genetics (Huntsville, AL). The modified oligodeoxynucleotides contain the TGF-β response element sequence (5′-TGC CCACG GCCAG-3′). The complete sequence of the phosphorothioate oligodeoxynulceotides is listed in the legends to Figures 2 and 7. Using the calcium phosphate coprecipitation method [Chen and Okayama, 1987], the synthetic oligodeoxynucleotides were transiently transfected into cells stably transfected with ColCat 3.6 or into nontransfected cells. The cells were incubated at 37°C overnight, washed twice with phosphate-buffered saline (PBS), and placed in AIM V medium for

subsequent treatment with either TGF-β1 or bleomycin. Control cells were treated in the same manner as treated cells except for the absence of oligodeoxynucleotides in the transfection reaction. Alternately, the cells were incubated at 37°C for 4 h after transfection and shocked for 1 min in a 15 % (v/v) glycerol solution. The glycerol solution was removed, and the cells were washed twice with PBS before the addition of complete medium. The cells were incubated at 37°C overnight and then treated as stated above. The presence of transiently transfected oligodeoxynucleotides in nuclei was verified using flourescent microscopy; phosphorothioate oligodeoxynucleotides were synthesized with a 5′-fluorescein label (data not shown).

Cellular Collagen and Noncollagen Protein Synthesis

Granulation tissue fibroblasts and RFL-6 fibroblasts were plated at a density of 1.4×10^5 cells per 25-cm^2 flask. Ascorbic acid (Calbiochem, Los Angeles, CA) was added daily to cultures at a final concentration of 16 μM. The cells were transiently transfected with phosphorothioate oligodeoxynucleotides on day 4 of culture in serum-containing medium. Approximately 18 h later, the cells were washed twice with PBS and treated with TGF-β1 in AIM V medium; 24 h later, the cells were given ascorbic acid and 5-[^{3}H]proline (20 μCi/ml of medium; 25 Ci/mmol; Amersham Life Sciences, Arlington Heights, IL) for an addtional 1.5 h. The cells were collected by scraping, washed with PBS, and centrifuged at 1,200*g* for 10 min. The cells were washed two additional times with PBS and resuspended in 1.5 ml of 100 mM NaCl and 50 mM Tris-HCl, pH 7.5. The samples were homogenized in a Polytron ST homogenizer (Brinkman, Westbury, NY) for 45 s, boiled for 10 min, followed by homogenization for 30 s. Radioactive protein was digested with purified bacterial collagenase (Advanced Biofractures, Lynbrook, NY) to distinguish collagen from noncollagen protein [Newman and Cutroneo, 1978] and total protein concentrations were determined [Lowry et al., 1951].

Preparation of Cell Lysates and Assay of Chloramphenicol Acetyltransferase Activity

At 48 h after treatment with bleomycin, RFL-6 fibroblasts were placed on ice and washed three times with ice-cold PBS and harvested in 1 ml of 40 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA at 4°C [Gorman et al., 1982]. CAT activity was determined by modification of an established method [Neumann et al., 1987]. The same amount of protein was added to each reaction mixture contained in 50 μl of 100 mM Tris-HCl, pH 7.8. A total of 200 μl of 1.25 mM chloramphenicol in 100 mM Tris-HCl, pH 7.8, was added along with 0.5 μCi of [^{3}H]acetyl-CoA (200 mCi/mmol; Dupont NEN, Boston, MA) and 22.5 μl of 1 mM unlabeled acetyl-CoA (Sigma Chemical Co., St. Louis, MO). The entire reaction mixture was overlaid with liquid scintillation fluid (Econofluor-2, Dupont NEN). This assay allows multiple time point determinations by liquid scintillation counting. The acetylated chloramphenicol product is miscible in the aqueous-immiscible scintillation mixture. The time course activity of each sample was determined, and only activity in the linear range of the time course was used. Within each assay, data from the same time point were reported for all samples. Protein concentrations of cell lysates were determined [Lowry et al., 1951].

DNA Mobility Shift Assay

RFL-6 fibroblasts were grown to late log phase and transiently transfected with 5, 10, or 15 μg of ssPT for 18 h. Cells were washed twice with PBS and treated with TGF-β1 (5 μg/ml) for 24 h in AIM V serum-free media. Nuclear protein extracts were prepared by the method of Andrews and Faller [1991], and protein concentrations were determined [Lowry et al., 1951]. Single-stranded oligodeoxynucleotides containing the TGF-β element sequence (5′-TGC CCACG GCCAG-3′) were synthesized (Integrated DNA Technologies, Coralville, IA). A total of 20 μg of the single-stranded oligodeoxynuclotides was annealed with 20 μg of their complementary strands in 200 mM NaCl by heating to 95°C for 7 min and then slowly cooled to 4°C. The double-stranded oligodeoxynucleotides were stored at −20°C. The oligodeoxynucleotides were labeled with ^{32}P, using the 5′ DNA Terminus Labeling System (GibcoBRL). Gel shift binding reactions (20 μl) contained ^{32}P-end labeled double-stranded oligodeoxynucleotide (approximately 2.0 to 3.0×10^6 cpm/pmol); 10 μg of nuclear protein extract; 1.5 μg of poly (dI-dC) (Pharmacia, Piscataway, NJ); 90 mM KCl; 1 mM EDTA; 1 mM DTT and 5% glycerol. Reaction mixtures were incubated for 30 min at room temperature and separated on

6% polyacrylamide gels (19:1 acrylamide to bisacrylamide) in low-ionic-strength buffer (22.25 mM Tris borate, 22.25 mM boric acid , 500 μM EDTA, pH 8.0) for 4 h at 100 V at 4°C. The gels were air-dried and autoradiographed. The band intensities of the bound protein-DNA complexes were measured by densiometric analysis using a Shimadzu dual-wavelength scanner, model CS-930 (Shimadzu Corp., Kyoto, Japan).

Statistical Analysis

All results are expressed as means±SEM. Significance of differences in means was determined by Student's *t*-test.

RESULTS

Mutagenesis of the TGF-β Response Element(s) in the Proα1(I) Collagen Promoter Decreases Basal CAT Activity in Stably Transfected FRS Fibroblasts

In a previous study from our laboratory, we reported on the site-specific mutagenesis of the glucocorticoid response element (GRE) in the ColCat 3.6 plasmid and subsequent stable transfection of the mutated construct into FRS cells [Meisler et al., 1995]. Fibroblasts stably transfected with the construct in which the GRE (at −0.6 kb in the proα1(I) collagen promoter) was mutated (MG), produced little change in basal CAT activity compared with cells transfected with wild-type ColCat 3.6 (control) (Fig. 1). Ritzenthaler et al. [1991] demonstrated that TGF-β treatment of human lung fibroblasts transiently transfected with the ColCat 3.6 plasmid led to increased CAT activity. They located a nuclear factor-1 (NF-1)-like sequence approximately −1.6 kb upstream from the start site of transcription that was required for the stimulation of proα1(1) collagen promoter activity by TGF-β1. We refer to this sequence as the TGF-β response element. When fibroblasts were stably transfected with the ColCat 3.6 plasmid containing a mutated TGF-β response element (MT), basal CAT activity was almost totally negated (Fig. 1). When both the GRE and TGF-β response element were mutated in the same plasmid (DM), there was no further decrease in CAT activity than found with mutation of the TGF-β response element alone. Two other NF-1-like sequences, identified in the proα1(I) collagen promoter by Ritzenthaler et al. [1991] at approximately −2.3 kb and −2.9 kb upstream from the start site of transcription, competed

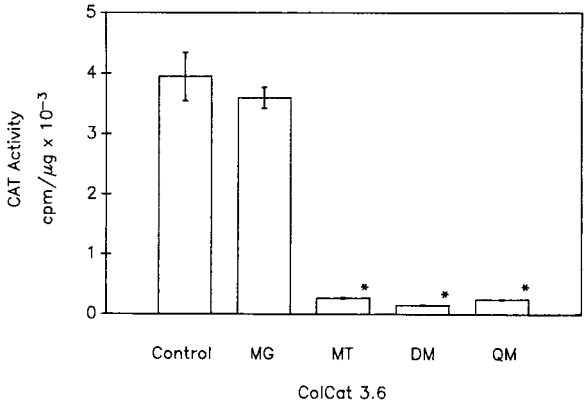

Fig. 1. The TGF-β response element in ColCat 3.6 is required for proα1(I) collagen promoter activity. Fetal rat skin fibroblasts were stably transfected with either ColCat 3.6 (control), ColCat 3.6 containing a mutated GRE (MG), ColCat 3.6 containing a mutated TGF-β response element (MT), ColCat 3.6 containing both a mutated GRE and a mutated TGF-β response element (DM), or ColCat 3.6 containing a mutated GRE, a mutated TGF-β response element and two additional mutated *cis*-elements which contain NF-1-like sequences (QM). Cells were grown to late log phase, and cell lysates were prepared and assayed for protein content and CAT activity. *Significantly different from control values at $P < 0.01$. Values represent the mean of four samples±SEM.

with the TGF-β response element for binding to nuclear protein in gel mobility shift assays. When a construct containing the mutated GRE, mutated TGF-β response element, and the two mutated NF-1-like sequences were stably transfected into FRS fibroblasts (QM), there was also no further decrease in basal CAT activity (Fig. 1). These mutagenesis studies indicated that in fibroblasts transfected with the ColCat 3.6 plasmid, the TGF-β response element was required for basal CAT activity. In addition, these studies indicate that these cells are producing TGF-β at a basal level that, through signal transduction, stimulates the pro α1(I) collagen genes.

Bleomycin Treatment of Fetal Rat Lung Fibroblasts Increases Nuclear Binding to the TGF-β Response Element Found in the 5′-Flanking Region of the Rat Proα1(I) Collagen Gene

A previous study from our laboratory demonstrated that bleomycin treatment of rat lung fibroblasts increased TGF-β mRNA in addition to increasing TGF-β protein [Breen et al., 1992]. In another study, our laboratory determined that in rat lung fibroblasts transfected with ColCat 3.6, the TGF-β response element was required for both bleomycin and TGF-β1 stimulaton of promoter activity [King et al., 1994].

Bleomycin is thought to increase TGF-β message causing synthesis of TGF-β protein, which then increases proα1(I) collagen gene expression, resulting in increased Type I procollagen synthesis.

Gel mobility shift assays were used to study the binding of nuclear proteins from control and bleomycin-treated RFL-6 fibroblasts to the TGF-β response element sequence (Fig. 2). RFL-6 fibroblasts were treated with bleomycin (1.5 μg/ml) for 24 h (Fig. 2A) or 48 h (Fig. 2B). Bleomycin treatment of nontransfected RFL-6 fibroblasts increased the binding of isolated nuclear proteins to the ^{32}P-labeled oligodeoxynucleotide containing the TGF-β response element sequence (5′-TGC CCACG GCCAG-3′) approximately 2-fold. In a previous study, TGF-β1 treatment of FRS fibroblasts increased binding of isolated nuclear protein to the oligodeoxynucleotide containing the TGF-β response element [Meisler et al., 1995].

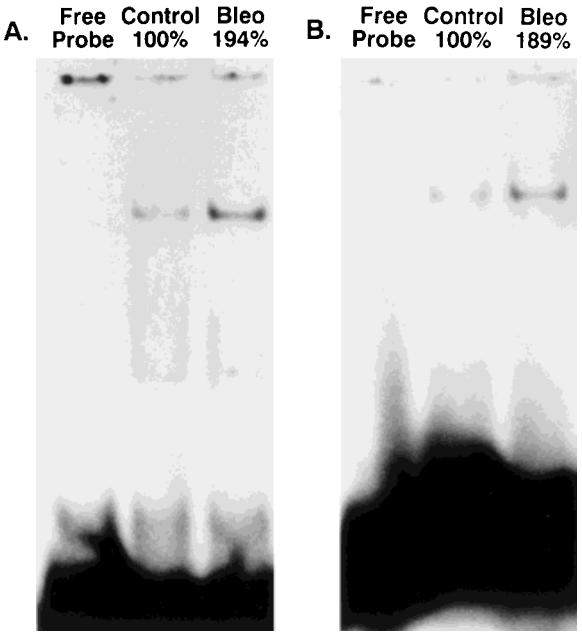

Fig. 2. Nuclear extracts isolated from bleomycin-treated RFL-6 fibroblasts demonstrate increased protein binding. Rat fetal lung fibroblasts were treated with bleomycin (1.5 μg/ml) for 24 h **(A)** or 48 h **(B)** in AIM V serum-free medium. Nuclear protein extracts were prepared according to the method of Andrews and Faller [1991]. In gel mobility shift assays, each mixture (20 μl) contained ^{32}P-end-labeled double-stranded oligodeoxynucleotide (1.9×10^6 cpm/pmol) containing the TGF-β response element sequence (5′-TGC CCACG GCCAG-3′), 10 μg of nuclear protein extract, and 1.5 μg of poly (dI-dC) in buffer described under Materials and Methods. The complete sequence of the 27-mer oligodeoxynucleotide used in the assays and found in the rat proα1(1) collagen promoter from positions −1636 to −1610 is 5′-AGCCTAACTGCCCACGGCCAGCGACGT-3′.

Effect of PPTs on the Bleomycin-Induced Increase of proα1(I) Collagen Promoter Activity in Stably Transfected Rat Fetal Lung Fibroblasts

RFL-6 fibroblasts, stably transfected with ColCat 3.6, were transiently transfected with ssPTs for 18 h before treatment with bleomycin. Bleomycin led to a significant increase in CAT activity, which was blocked by 50 μg of the ssPT containing the TGF-β response element (Fig. 3); 25 or 50 μg of the ssPT did not affect the basal level of CAT activity (Fig. 3).

Single-stranded ssPTs Block the TGF-β-Induced Increase of Collagen and Noncollagen Synthesis in Rat Fetal Lung Fibroblasts

RFL-6 fibroblasts were transiently transfected with 15 μg of the ssPT containing the TGF-β response element for 18 h before 24-h treatment with TGF-β1 (5.0 ng/ml). In the absence of ssPTs, TGF-β1 caused a 1.9-fold increase in collagen and a 2.0-fold increase in noncollagen protein synthesis (Fig. 4). In the presence of ssPTs, the increase in collagen and noncollagen synthesis caused by TGF-β1 was strongly suppressed. Collagen protein synthesis was returned to control values. The presence of ssPTs in the cells did not significantly

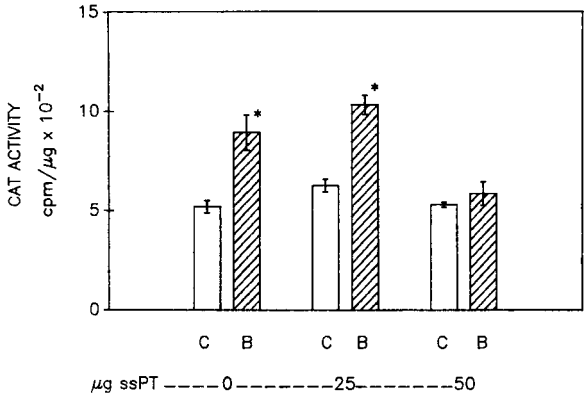

Fig. 3. Single-stranded PTs decrease the bleomycin-induced increase in rat proα1(I) collagen promoter activity in stably transfected fibroblasts. RFL-6 fibroblasts, stably transfected with ColCat 3.6, were transiently transfected with 0, 25 or 50 μg of the ssPT for 18 h. The cells were washed twice with PBS and incubated with bleomycin (1.5 μg/ml) in AIM V medium for 48 h. Cell lysates were prepared and assayed for CAT activity, expressed as cpm per μg protein. Open bars, control cultures; hatched bars, bleomycin-treated cultures. *Significantly different from respective control values at $P < 0.01$. Values represent the mean of three to four samples±SEM.

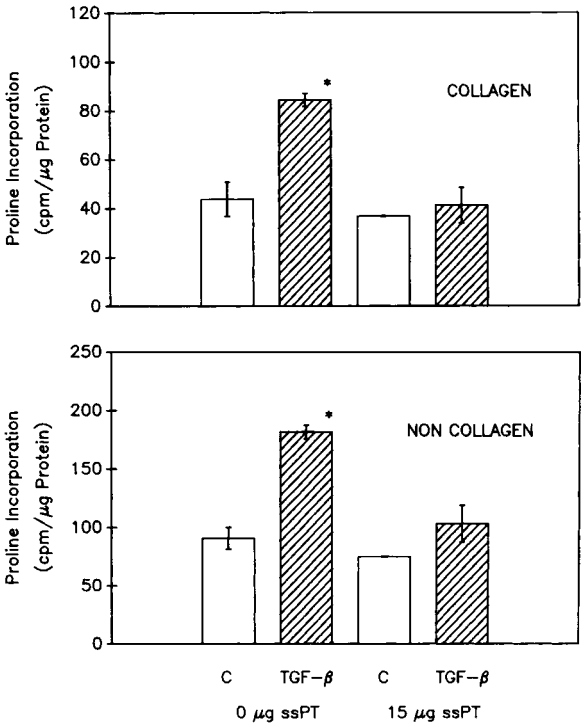

Fig. 4. TGF-β-induced increase in collagen and noncollagen protein synthesis returns to control levels when RFL-6 fibroblasts are transiently transfected with ssPTs. RFL-6 fibroblasts were transiently transfected with either 0 or 15 μg of the ssPT for 18 h. The cells were washed twice with PBS and treated with 5.0 ng of TGF-β1 per ml of AIM V medium for 24 h. Ascorbic acid and 5-[^{3}H]proline were added to cultures 1.5 h before harvesting of cells. The cells were collected by scraping, and incorporation of [^{3}H]proline into collagen and noncollagen protein was determined by collagenase digestion. Open bars, control cell cultures; hatched bars, TGF-β1-treated cultures. *Significantly different from control value at $P < 0.01$. Values represent the mean of three to four samples±SEM.

affect the basal level of collagen and noncollagen protein synthesis (Fig. 4). The increase of noncollagen protein synthesis in the presence of TGF-β1 could be due to the increase in the synthesis of noncollagen extracellular matrix proteins. TGF-β has been found to increase elastin in rat lung fibroblasts [McGowan and McNamer, 1990], fibronectin, and fibronectin receptor in human lung fibroblasts [Roberts et al., 1988], and tenascin isoforms in cultures from rat fetal lung tissue [Zhao and Young, 1995].

Single-Stranded Phosphorothioate Oligodeoxynucleotides Inhibit the TGF-β-Induced Increase of Collagen and Noncollagen Protein Synthesis in Rat Fibroblasts Derived From Granulation Tissue

A more detailed analysis of the effect of ssPTs that contain the TGF-β response element on collagen and noncollagen synthesis was carried out using granulation tissue fibroblasts. Cells were transiently transfected with increasing amounts of ssPTs for 18 h and then treated with TGF-β1 (5.0 ng/ml) for 24 h. In the absence of ssPTs, TGF-β1 caused a 2.8-fold increase in collagen and a 1.8-fold increase in noncollagen protein synthesis (Fig. 5). In the presence of 5, 10, and 15 μg of the ssPT, TGF-β1 caused a 2.1-, 1.6-, and 1.2-fold increase in collagen synthesis, respectively. The fold increase in collagen synthesis in the presence of TGF-β1 was based on the control values of cells transfected with the same amount of the ssPT. In the presence of 5, 10, and 15 μg of the ssPT, TGF-β1 caused a 1.9-, 1.4-, and 1.3-fold increase in noncollagen synthesis, respectively (Fig. 5). The observation that ssPTs blocked the TGF-β-induced increase of

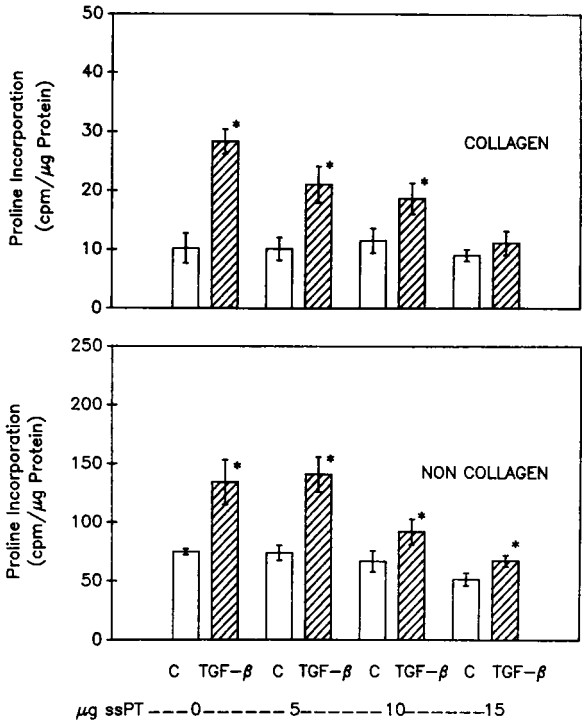

Fig. 5. Dose-response relationship of TGF-β-induced collagen and noncollagen protein synthesis to ssPT concentration in rat fibroblasts derived from granulation tissue. Granulation tissue fibroblasts were transiently transfected with either 0, 5, 10, or 15 μg of the ssPT for 18 h. The cells were washed twice with PBS and treated with 5.0 ng of TGF-β1 per ml of AIM V medium for 24 h. Ascorbic acid and 5-[^{3}H]proline were added to cultures 1.5 h before harvesting of cells. The cells were collected by scraping and incorporation of [^{3}H]proline into collagen and noncollagen protein was determined by collagenase digestion. Open bars, control cell cultures; hatched bars, TGF-β1-treated cultures. *Significantly different from respective control values at $P \leq 0.05$. Values represent the mean of three to five samples±SEM.

collagen and noncollagen protein synthesis but did not inhibit the synthesis of these proteins below basal values indicates that the ssPTs were not toxic to the cells. Thus, in normal fibroblasts (RFL-6) or in fibroblasts derived from granulation tissue, 15 μg of the ssPT containing the TGF-β response element only blocked the TGF-β-induced increase of collagen synthesis and noncollagen protein.

The specificity of ssPTs to abrogate the fibrogenic effect of TGF-β on collagen and noncollagen protein synthesis was shown by mutating the TGF-β response element sequence and subsequent transfection of the mutated ssPT into granulation tissue fibroblasts. The TGF-β response element sequence was changed from 5′-TGC CCACG GCCAG-3′ to 5′-GTA AACAT TAACT-3′. The rest of the sequence in the 27-mer phosphorothioate oligodeoxynucleotide remained the same. In the absence of ssPTs, TGF-β1 caused a 2.4-fold increase in collagen and a 2.3-fold increase in noncollagen synthesis (Fig. 6). When cells were transfected with the mutated ssPT (mssPT) followed by treatment with TGF-β1, there was a 2.4-fold increase in collagen synthesis and a 2.3-fold increase in noncollagen synthesis (Fig. 6).

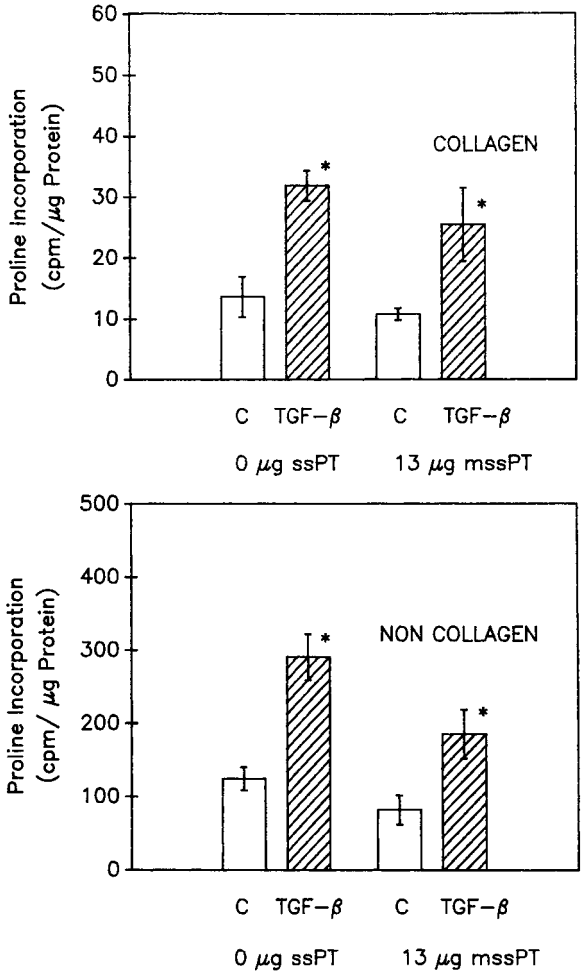

Fig. 6. Mutation of the TGF-β response element in ssPTs does not alter the TGF-β-induced increase in collagen and noncollagen synthesis in fibroblasts derived from granulation tissue. Granulation tissue fibroblasts were transiently transfected with 13 μg of the ssPT or 13 μg of the mssPT for 18 h. The ssPT contained the TGF-β response element sequence 5′-TGC CCACG GCCAG-3′. In the mssPT, the response element sequence was changed to 5′-GTA AACAT TAACT-3′. The sequences surrounding the TGF-β response element in the 27-mer ssPT and mssPT were identical. After transfection, the cells were washed twice with PBS and treated with 5.0 ng of TGF-β1 per ml of AIM V medium for 24 h. Ascorbic acid and 5-[^{3}H]proline were added to cultures 1.5 h before harvesting of cells. The cells were collected by scraping and incorporation of [^{3}H]proline into collagen and noncollagen protein was determined by collagenase digestion. Open bars, control cell cultures; hatched bars, TGF-β1-treated cultures. *Significantly different from respective control values at $P < 0.05$. Values represent the mean of three to four samples ± SEM.

Interaction of Phosphorothioate Oligodeoxynucleotides With the TGF-β Activator Protein

RFL-6 fibroblasts were transiently transfected with either 0, 5, 10, or 15 μg of ssPT for 18 h. Equal amounts (10 μg) of nuclear proteins prepared from TGF-β-stimulated fibroblasts were incubated with the ^{32}P-labeled double-stranded 27-mer oligodeoxynucleotide containing the TGF-β response element. As shown in Figure 7, increasing amounts of exogenous phosphorothioate oligodeoxynucleotide given in cellulo competed with the ^{32}P-labeled probe for binding to the TGF-β activator protein in vitro. Even though the fibroblasts were treated with TGF-β1, which results in an increase in TGF-β activator protein binding activity [Meisler et al., 1995], the phosphorothiate oligodeoxynucleotides were able to inhibit the binding of the TGF-β activator protein to the TGF-β response element.

DISCUSSION

Bleomycin sulfate is used clinically as a cancer chemotherapeutic agent for the treatment of several types of malignancies [Blum et al., 1973; Bennett and Reich, 1979]. Pulmonary fibrosis is the major conspicuous side effect of bleomycin therapy and results from an inflammatory response of the lung to injury. This occurs in four phases, including the initial injury and inflammatory phase, a proliferative

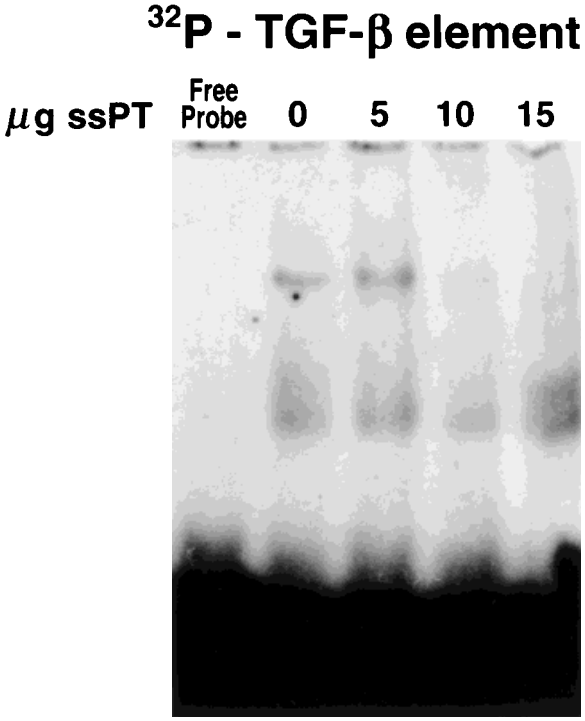

Fig. 7. Transient transfection of ssPTs decreases nuclear binding to the TGF-β response element. RFL-6 fibroblasts were transiently transfected with either 0, 5, 10 or 15 μg of the ssPT for 18 h. The cells were washed twice with PBS and treated with 5.0 ng of TGF-β1 per ml of AIM V medium for 24 h. Nuclear protein extracts were prepared according to the method of Andrews and Faller. [1991]. In gel mobility shift assays, each mixture (20 l) contained ^{32}P-end-labeled double-stranded oligodeoxynucleotide (2.4×10^6 cpm/pmol) containing the TGF-β response element sequence (5′-TGC CCACG GCCAG-3′), 10 μg of nuclear protein extract, and 1.5 μg of poly (dI-dC) in buffer described under Materials and Methods. The labeled probe was also incubated in reaction mixtures without nuclear protein (Free Probe). The complete sequence of the 27-mer-labeled oligodeoxynucleotide is 5′-AGCCTAACTGCCCACGGCCAGCGACGT-3′.

phase of connective tissue cells and other cells, and a remodeling- repair phase. The fibrogenic response is characterized by increased synthesis and deposition of collagen and other extracellular matrix proteins leading to the distortion of the pulmonary architecture. Both lung collagen content and collagen synthesis are increased by bleomycin treatment [Sikic et al., 1978; Laurent and McAnulty, 1983]. We have demonstrated that bleomycin treatment of chick skin and chick lung fibroblasts results in a specific increase in procollagen synthesis in the cell layer. It is mediated by elevated levels of polysomal type I procollagen mRNAs via a repartitioning of these mRNAs within the fibroblast [Sterling et al., 1983]. Dexamethasone was found to reverse the bleomycin-induced elevations of both cell layer procollagen synthesis and polysomal type I procollagen mRNAs [Sterling et al., 1983].

We have studied the role of TGF-β as a mediator of the fibrogenic effect of bleomycin. TGF-β stimulates fibroblasts and other reparative cells to synthesize collagen [Ignotz and Massagué, 1986; Appling et al., 1989] and thus may play an essential role in the production of lung fibrosis by the induction of extracellular matrix proteins. The increase in TGF-β mRNA in bleomycin-induced lung fibrosis precedes the increase of type I and type III procollagen mRNAs [Kelley et al., 1985]. Bleomycin treatment of rat lung fibroblasts increases TGF-β mRNA and secretion of TGF-β protein [Breen et al., 1992]. We recently showed that in bleomycin-treated rat lung fibroblasts, proα1(I) collagen promoter activity was stimulated through the TGF-β response element by intracellular and extracellular signalling [King et al., 1994]. When plasmids containing the rat proα1(1) collagen promoter linked to the CAT gene (ColCat 3.6) were transfected into rat lung fibroblasts, both bleomycin and TGF-β1 increased promoter activity. Rat lung fibroblasts transfected with a deletion construct that lacked the TGF-β response element did not respond to either bleomycin or TGF-β1. Furthermore, when ColCat 3.6 containing a mutated TGF-β response element was transfected into lung fibroblasts, the bleomycin and TGF-β-induction of CAT activity was greatly reduced [King et al., 1994].

Concomitant with the increase in collagen promoter activity, TGF-β1 also caused an increase in nuclear protein bound to the *cis*-element found within the proα1(I) collagen gene promoter [Ritzenthaler et al., 1991, 1993]. In the present study, we have shown an increase in binding to the same *cis*-element when nuclear protein was isolated from lung cells treated with bleomycin. Thus, both TGF-β and bleomycin treatment of rat lung fibroblasts result in an increase in the amount of TGF-β activator protein bound to the TGF-β response element.

Bleomycin increases TGF-β message in rat lung fibroblasts, causing the synthesis of the latent form of TGF-β. The latent TGF-β secreted by fibroblasts and other cell types in bleomycin-induced fibrotic lung is converted by extracellular proteases to active TGF-β. TGF-β is the prototype of a large superfamily, which

includes activins and bone morphogenetic proteins [Kingsley, 1994]. Members of the TGF-β superfamily signal through heteromeric complexes of type II and type I serine/threonine kinase receptors [Attisano and Wrana, 1996; Massagué and Weis-Garcia, 1996]. Through a unique signal transduction pathway, the binding of the ligand to the serine /threoine kinase receptors is connected ultimately to the binding of nuclear protein to DNA [Heldin et al., 1997]. In our system, treatment of fibroblasts with TGF-β1 results in the increased binding of TGF-β activator protein to the TGF-β response element sequence in the 5′-flanking region of the rat proα1(I) collagen gene [Meisler et al, 1995].

The present studies indicate that the fibrogenic effects of bleomycin and TGF-β may be neutralized by the addition of phosphorothioate oligodeoxynucleotides that contain the TGF-β response element. Although ssPT inhibited the TGF-β induced increase of collagen and noncollagen protein synthesis, the basal levels of synthesis of these proteins were not altered by treatment with these oligodeoxynucleotides. We previously showed that dexamethasone abrogated the fibrogenic effect of TGF-β in rat granuloma and in fibroblasts isolated from rat granulation tissue [Meisler et al., 1997]. The disadvantage of using systemic glucocorticoids to prevent fibrosis is the toxic side effects of these compounds. In addition, these steroids inhibit the basal levels of collagen and noncollagen protein synthesis [Sterling et al, 1983].

In the present study, we have found that in rat fetal lung fibroblasts and in granulation tissue fibroblasts, transient transfection of ssPTs blocked the fibrogenic effect of TGF-β. Single-stranded PTs may act by inhibiting the binding of the TGF-β activator protein to the TGF-β response element, thus inhibiting procollagen gene expression. Confirmation of this hypothesis was shown in Figure 7, where 10 μg and 15 μg of transiently transfected ssPT competed with the ^{32}P-labeled double-stranded 27-mer oligodeoxynucleotide containing the TGF-β response element for binding to the TGF-β activator protein. This mechanism is different from the way in which antisense oligodeoxynucleotides inhibit gene expression. Antisense oligodeoxynucleotides are designed to hybridize to a complementary target mRNA and cause a block at translation.

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