

AP-1 transcription factor decoy reduces the TGF- β 1-induced cell growth in scleroderma fibroblasts through inhibition of cyclin E

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Abstract. The transforming growth factor- β (TGF- β) signaling pathway plays a key role in the abnormal accumulation of type I and III collagen of scleroderma. Activator protein-1 (AP-1) is a key regulatory protein in TGF- β 1-induced type I collagen synthesis. However, it is largely unknown whether AP-1 is involved in the cell proliferation of fibroblasts in scleroderma. In this study, we investigated the effects of the AP-1 oligo-deoxynucleotide (ODN) decoy on TGF- β 1-induced cell growth in scleroderma fibroblasts. To investigate the inhibition of AP-1 ODN decoy on the growth rates of scleroderma fibroblasts through the regulation of cell cycle regulatory proteins, we transfected the AP-1 ODN decoy on scleroderma fibroblasts and analyzed the cell cycle regulatory proteins by RT-PCR and Western blot analysis. We found that the growth rates of normal fibroblasts and scleroderma fibroblasts showed similar rates. It is noteworthy that the scleroderma fibroblasts grew more rapidly than normal fibroblasts in the presence of TGF- β 1. Moreover, the transfection of AP-1 decoy ODN into scleroderma fibroblasts resulted in the down-regulation of the growth rates by the down-regulation of cyclin E. These results collectively suggest that AP-1 ODN decoy can down-regulate the growth rates of scleroderma fibroblasts, thus implying that AP-1 ODN decoy is a promising therapeutic tool for overcoming scleroderma.

Introduction

Scleroderma is a generalized disease which affects connective tissue in the skin and internal organs (1,2). Although its precise molecular pathogenesis is still unclear, perturbation of transforming growth factor (TGF)- β signaling pathways in

fibroblasts is a key event in the development of scleroderma (3-6). TGF- β is a major profibrotic cytokine, which regulates multiple cellular functions including differentiation, proliferation and induction of synthesis of extracellular matrix proteins (7). In human skin, TGF- β stimulates not only the synthesis and secretion of the major extracellular matrix proteins, especially type I collagen, but also the growth of dermal fibroblasts (7,8). Activator protein-1 (AP-1) modulates the expression of type I collagen gene in skin fibroblasts (9,10). Notably, AP-1 also stimulates cell cycle progression through the modulation of positive cell cycle regulators such as cyclin D1 and cyclin E (11). We reported that AP-1 oligodeoxynucleotide (ODN) decoy transfection inhibits the expression of type I collagen in scleroderma fibroblasts or keloid fibroblast (12,13). However, it is not known whether inhibition of AP-1 binding by AP-1 decoy ODN transfection prevents the growth of scleroderma fibroblasts. In this study, we investigated whether the AP-1 decoy ODN modulates the growth rates of scleroderma fibroblasts through the regulation of cell cycle regulatory proteins.

Materials and methods

Cell culture. Primary cultures of normal human fibroblasts (n=3, mean age: 33) and scleroderma fibroblasts (n=3, mean age: 34) were established from left-over adult skin following cosmetic surgery and being subcultivated on plastic culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μ g/ml) and amphotericin B (1 μ g/ml). The cells were maintained in a humidified 5% CO₂ - 95% air incubator at 37°C. Cells from the third and sixth passages were used in all experiments and viability was determined by trypan blue exclusion.

TGF- β 1 treatment. Confluent normal and scleroderma fibroblasts in 100 mm²-diameter petri dishes were treated with TGF- β 1 (5 ng/ml) for 24 h in the absence of FBS. TGF- β 1 was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) and was used after dilution in 95% ethanol.

Synthesis of ODN and selection of sequence targets. The AP-1 decoy ODN used in this study was kindly provided by Dr In Kyu Lee (Kyungpook National University School of

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Table I. DNA sequences of PCR primers used in this study.

Name		Primer sequences	Product size (bp)
GAPDH	Sense	5-CGTCTTCACCACCATGGAGA-3	300
	Antisense	5-CGGCCATCACGCCACAGTTT-3	
Cyclin D1	Sense	5-ACCTGGATGCTGGAGGTCTG-3	402
	Antisense	5-GAACTTCACATCTGTGGCACA-3	
Cyclin E	Sense	5-GGAAGGCAAACGTGACCGTT-3	638
	Antisense	5-GGGACTTAAACGCCACTTAA-3	
Rb	Sense	5-ATGTCAGAGAGAGAGCTTGG-3	579
	Antisense	5-GTGCACTCCTGTTCTGACCT-3	
CDK2	Sense	5-CGGGATCCGGCGCCATGTCAGAAC-3	509
	Antisense	5-CGGGAATTCGTGGGCGGATTAGGG-3	
CDK4	Sense	5-ACGTGACGTGTCAGGACCTT-3	336
	Antisense	5-GTTCTTGCTCCAGGCTGAGT-3	

Medicine). The construction and stability of AP-1 decoy ODN were described previously (14).

The sequences of circular dumbbell AP-1 decoy ODN (CDODN) against AP-1 binding sites and mismatched AP-1 decoy ODN (MODN, as a control) are as follows: (note, consensus sequences are underlined): CDODN: 5'-GGATCC ATGACTCAGAAGACGACACACGTCTTCTGAGTCAT-3'. MODN: 5'-GATCCAAATCTCAGAAGACGACACACGTCTTCTGAGATTT-3'. ODNs were annealed for 2 h with a steady temperature decline from 80 to 25°C. T4 DNA ligase (1 unit) was added to the mixture, followed by incubation for 24 h at 16°C to generate a covalently ligated dumbbell-shaped decoy ODN molecule. CDODN comprises of 2 loops and 1 stem containing two AP-1 consensus sequences in tandem.

Decoy ODN transfection using Lipofectamine Plus®. AP-1 decoy ODN was transfected with normal and keloid fibroblasts for 24 h prior to TGF-β1 treatment using Lipofectamine Plus (Gibco-Brl, USA). The cells were seeded at a density of 1-2x10⁵ cells/100 mm dish with 2 ml DMEM and incubated overnight prior to transfection. At 40-60% confluency, a complex of decoy ODN (4 μg) and Lipofectamine Plus reagent (20 μl) was added to the washed cells and they were incubated in a serum-free medium for 15 min.

Electrophoretic mobility shift assays (EMSA). The AP-1 decoy ODN was 5'-end labeled with [³²P]-ATP using T₄ polynucleotide kinase and purified by ethanol precipitation. Binding reactions were carried out in a buffer containing 10 mM Tris HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 10 mM MgCl₂, 10% glycerol, 0.05% NP-40, and 2 mg of poly (dI-dC) on ice for 15 min. The assay mixture was incubated with radiolabeled decoy ODN for 30 min at 4°C. After 6 x strength dye solution (0.1% bromophenol blue, 30% glycerol) was added, the mixture was immediately loaded and electrophoresed on a non-denaturing 6% polyacrylamide gel in 0.25 x TBE (1 x TBE: 89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA) for 2 h at 200 V. The gels were dried in a vacuum dryer at 80°C for 1.5 h and autoradiographed on a Fuji RX X-ray film.

Northern blot analysis. Total RNA was isolated using the RNAzol™ B (Biotecx laboratories Inc., Houston, TX, USA) according to the manufacturer's instructions and quantitated by spectrophotometry. Extracted RNA was analyzed by northern hybridization with various ³²P-labeled cDNA probes. The [³²P]-cDNA-mRNA hybrids were visualized by autoradiography, and steady-state levels of mRNA were quantitated by laser densitometry (LKB Instruments, Bromma, Sweden). All quantitated mRNA levels were standardized to GAPDH mRNA levels in the same samples and all experiments were performed in triplicate.

Western blot. Cells were lysed in lysis buffer (10 mM Tris (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, PMSF (10 μg/ml), aprotinin (10 μg/ml), leupeptin (10 μg/ml), 5 mM phenanthroline, and 28 mM benzamidinium-HCl) for 30 min on ice. Lysates were clarified by centrifugation. Lysates were quantitated using the Bradford assay (Life Science Co., CA, USA) with bovine serum albumin as a reference standard. Proteins (35 μl) were resolved by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gels) and transferred to immobilon-P transfer membrane (Millipore Co., MA, USA). After incubation with primary antibodies, proteins were visualized by incubation with horseradish peroxidase-conjugated secondary antibodies, followed by ECL according to the manufacturer's instructions (Amersham Life Science Co., Buckinghamshire, UK). Primary antibodies against Rb (PharMingen Co., CA, USA), cyclin E, cyclin D1, cyclin dependent kinase 2 (CDK2), CDK4 and β-actin (Santa Cruz Co., CA, USA) were applied at optimized concentrations. The membrane was continuously incubated with appropriate secondary antibodies coupled to horseradish peroxidase, and developed in the ECL Western detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from cultured skin fibroblasts using the RNAzol B (Biotecx laboratories Inc.) according to the manufacturer's instructions and quantitated by spectrophotometry. One microgram of total RNA was reverse

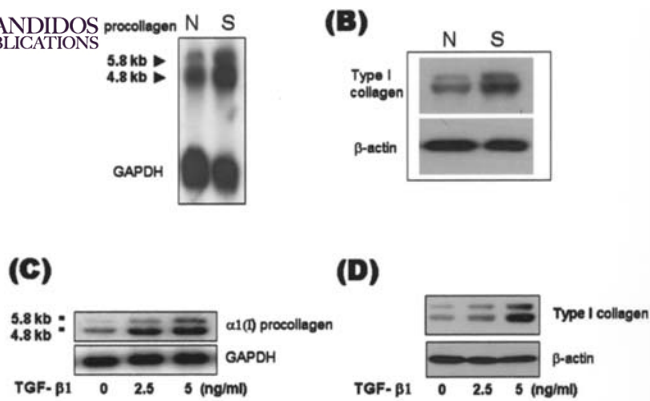


Figure 1. Expressions of type I collagen mRNA and protein in normal (N) and scleroderma (S) fibroblasts. Cells were cultured in the normal media without TGF- β 1 (A,B) or with TGF- β 1 (C,D). Cell lysates were prepared for Northern blot (A,C) and Western blot (B,D) analysis. Similar results were found in two different experiments.

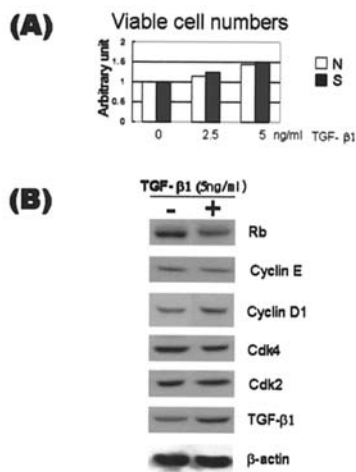


Figure 2. Increased viable cell number by TGF- β 1 treatment in scleroderma fibroblasts. Viable cells were counted using trypan blue exclusion assay 48 h after TGF- β 1 treatment with various concentrations in normal (N) and scleroderma (S) fibroblasts (A). Proteins were also extracted 48 h after TGF- β 1 treatment in scleroderma fibroblasts, and cell cycle regulatory proteins were analyzed by Western blot analysis (B).

transcribed using M-MLV Reverse Transcriptase (Promega Co., Madison, WI, USA). The PCR reaction was carried out under the conditions recommended by the manufacturer's instructions (Takara Co., Otsu, Japan). Briefly, 50 μ l of a reaction mixture including 2.5 units of Taq polymerase (Takara Co.), 5 μ l of 10 x buffer, 1.5 mM MgCl₂, 200 μ M dNTPs, 1 μ l of first-strand cDNA, and each 25 pmol of primer, were subjected to 28 PCR cycles (denaturation at 94°C for 1.5 min, annealing at 58°C for 1 min, and polymerization at 72°C for 1 min). The primer sequences used in this study are described in Table I.

Results

Effect of TGF- β 1 on expression of type I collagen in scleroderma fibroblasts. To confirm the established

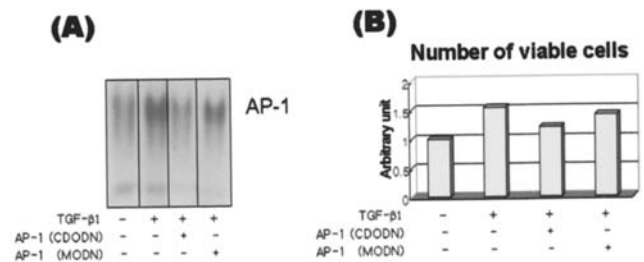


Figure 3. Inhibition of AP-1 DNA binding activity in scleroderma fibroblasts after AP-1 decoy CDODN transfection. The cells were transfected with AP-1 decoy CDODN or MODN, and these cells were cultured for 24 h. AP-1 DNA binding activity (A) and the viable cell numbers (B) were analyzed by EMSA and trypan blue exclusion assay, respectively. Similar results were found in two different experiments.

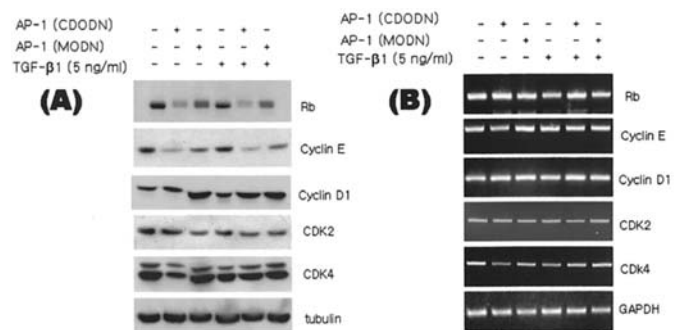


Figure 4. Expressions of cell cycle regulatory genes in scleroderma fibroblasts after AP-1 decoy ODN treatment. The cells were transfected with AP-1 decoy CDODN or MODN and then the cells were cultured in the absence or presence of TGF- β 1 for 24 h. Cell lysates were prepared for Western blot (A) and RT-PCR (B) analysis. Similar results were found in two different experiments.

scleroderma fibroblast cell lines, the expression of type I collagen was analyzed by Northern blot and Western blot analysis. Expressions of type I collagen mRNA and protein were markedly increased in scleroderma fibroblasts compared to normal cells (Fig. 1A and B). To determine the effect of TGF- β 1 on expressions of type I collagen mRNA and protein in scleroderma fibroblasts, cells were treated with various concentrations of TGF- β 1 for 24 h. As shown in Fig. 1C, expression of type I collagen mRNA dramatically increased in a dose-dependent manner. Increased expression of type I collagen mRNA was clearly visualized 24 h after TGF- β 1 (5 ng/ml) treatment. In agreement with Northern blot, an increased expression of type I collagen protein was observed in TGF- β 1-treated scleroderma fibroblasts (Fig. 1D).

Effect of TGF- β 1 on cell proliferation rates in scleroderma fibroblasts. To determine the effect of TGF- β 1 on the proliferation rates of scleroderma fibroblasts, viable cell counts were performed using trypan blue exclusion assay. We examined the proliferation rates of normal and scleroderma fibroblasts. Normal and scleroderma fibroblasts showed similar proliferation rates (data not shown). Notably, TGF- β 1-treated normal and scleroderma fibroblasts showed increased proliferation rates compared to non-treated cells, respectively (data not shown). However, the TGF- β 1-treated scleroderma

fibroblasts grew more rapidly than the TGF- β 1-treated normal fibroblasts (Fig. 2A).

To investigate the effect of TGF- β 1 on expressions of G1 cell cycle regulatory proteins in scleroderma fibroblasts, we performed immunoblotting of whole cell lysates using the anti-pRb, cyclin E, cyclin D1, CDK4, CDK2, and TGF- β 1 antibodies. As shown in Fig. 2b, the expressions of pRb, cyclin E, CDK2, and CDK4 were not changed in the TGF- β 1-treated fibroblasts. It is noteworthy that the expressions of cyclin D1 and TGF- β 1 were increased in TGF- β 1-treated scleroderma fibroblasts (Fig. 2B).

Effect of AP-1 decoy ODN on the TGF- β 1-induced cell proliferation in scleroderma fibroblasts. To confirm the growth inhibitory effect of AP-1 decoy ODN, we transfected the CDODN or MODN into TGF- β 1-treated scleroderma fibroblasts. The AP-1 binding activity in TGF- β 1-treated-scleroderma fibroblasts was markedly decreased by CDODN transfection (Fig. 3A). The CDODN-transfected cells, but not the MODN-transfected cells, showed a decrease in cell numbers compared to TGF- β 1-treated cells, implying that the AP-1 decoy ODN modulates growth signals even under the influence of TGF- β 1 (Fig. 3B).

Modulation of cell cycle regulatory proteins in scleroderma fibroblasts by AP-1 decoy ODN. To investigate the effect of AP-1 decoy ODN on expression of G1 cell cycle regulatory proteins, we performed immunoblotting of whole cell lysates using the anti-pRb, cyclin E, cyclin D1, CDK4, and CDK2 antibodies. As shown in Fig. 4A, the expressions of pRb and cyclin E proteins were markedly decreased by the CDODN transfection. The expressions of cyclin D1, CDK2, and CDK4 were found to be similar among cells. Notably, the expressions of G1 cell cycle regulatory genes such as pRb and cyclin E mRNA were not changed by CDODN transfection (Fig. 4B).

Discussion

Alterations in the TGF- β signaling pathway have been proposed for key molecular events in the fibrosis of scleroderma skin lesions (4,5,15). TGF- β regulates cell replication and differentiation, bone formation, angiogenesis, cell cycle progression and cellular migration (16-18). TGF- β stimulates the proliferation of fibroblasts, implying that downstreams of TGF- β correlates with signal pathways of cell cycle progression. AP-1, one of down-stream signal molecules of TGF- β , is implicated in the transcriptional regulation of a wide range of genes participating in cell proliferation and ECM production under TGF- β signaling cascades (19,20). A new technique has been developed to inhibit target gene expression based on DNA technology such as antisense oligodeoxynucleotides (ODN), small interfering RNA (siRNA), ribozyme and decoy ODN (21-23). In this study, in order to develop a molecular therapeutic strategy based on decoy ODN, we investigated whether the AP-1 decoy ODN inhibits the growth of scleroderma fibroblasts. In the present study, we found that the scleroderma fibroblasts grew more rapidly than normal fibroblasts in the presence of TGF- β 1. Notably, AP-1 decoy ODN transfected-scleroderma fibroblasts showed

decreased cell growth rates through the down-regulation of cyclin E.

Cell cycle progression is regulated by two protein classes, the cyclins and their kinase partners, CDKs (24,25). Two families of cyclins are successively activated during the G1 phase, and thus also named as G1 cyclins (26,27). G1 cyclins are composed of D-type and E-type cyclins. The D-type cyclins assemble with their catalytic partners, CDK4 and CDK6, followed by cyclin E interacting with its catalytic partner, CDK2. The subsequent cyclin-CDK complexes drive cell cycles from the G1 phase into the S phase by the phosphorylation of Rb. Rb is a central component of a transcriptional complex that inhibits the expression of many genes whose products are necessary for the G1/S transition and S phase (28,29). Therefore, cyclin D1 and cyclin E are the key regulatory proteins in the progression of G1/S transition phases. Several studies have indicated that the activation of AP-1 correlates with accelerated cell cycle progression through the up-regulation of cyclin D1 and cyclin E (30,31). Increased expressions of cyclin D1 and cyclin E were also observed in endometrial cancer or breast cancer showing increased AP-1 activities (32,33). Furthermore, signaling pathways concerning the proliferation of fibroblasts may be associated with an increased production of type I collagen synthesis. Recent findings suggest that TGF- β 1-induced connective tissue growth factor (CTGF) plays an important role in collagen synthesis and the proliferation of fibroblasts (34). In the case of cardiac fibroblasts, growth factors such as platelet-derived growth factor, insulin-like growth factor and fibroblast growth factor 2, play an important role in the regulation of fibroblast replication and collagen production (35,36). In this study, it was investigated whether the AP-1 decoy ODN decreases the proliferation of scleroderma fibroblasts. The proliferation rates of AP-1 decoy ODN transfected scleroderma fibroblasts were about 2-fold decreased through the down-regulation of cyclin E expressions, followed by dephosphorylation of Rb protein. Further efforts are needed to evaluate the molecular mechanisms involved in AP-1 decoy ODN-induced down-regulation of cyclin E expressions.

In conclusion, findings of the present study demonstrate that AP-1 decoy ODN inhibits not only the proliferation of scleroderma fibroblasts but also the expression of type I collagen, thereby suggesting that gene therapy using AP-1 ODN decoy may be used as a promising strategy for the regulation of collagen synthesis and cell growth. Further studies are required in order to prove that the blockade of the transcriptional factor AP-1 by AP-1 decoy ODN is sufficient in blocking the progression of scleroderma *in vivo*.

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