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Oligodeoxynucleotide Decoy Therapy Blocks Type 1 Procollagen Transcription and The Prolyl Hydroxylase β Subunit Translation

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Abstract Persistent transforming growth factor-β1 (TGF-β1) exposure to lungs increases type 1 collagen synthesis and deposition resulting in excess fibrosis which leads to morbidity and possibly death. We now report using human embryonic lung fibroblasts in the presence of TGF-β1, a novel double-stranded (ds) DNA decoy with phosphorothioate (PT) linkages, containing the TGF-β cis-element found in the distal promoter region of the COL1A1 gene which silences COL1A1 gene expression. In a cell-free protein translation system, we have previously reported that collagen synthesis was inhibited by disulfide isomerase, the prolyl-4-hydroxylase (P-4-H) β subunit. By comparative proteomics dsdecoy therapy increased the levels of disulfide isomerase, the P-4-H β subunit. These findings taken together support the notion that the dsdecoy inhibits type 1 collagen synthesis at both the transcriptional and translational levels. J. Cell. Biochem. 103: 1066–1075, 2008.

Key words: oligonucleotide decoy; gene therapy; collagen synthesis; proteomics

The treatment of fibroblasts with transforming growth factor-β1 (TGF-β1) promotes the synthesis of type 1 collagen. The chronic exposure of TGF-β1 to lungs increases the deposition of type 1 collagen, causing excess fibrosis, which leads to morbidity and possibly death. The direct silencing of the procollagen α1 (1) (COL1A1) gene is a method of reducing type 1 collagen synthesis. We report with human embryonic lung fibroblasts in the presence of TGF-β1, a novel double-stranded (ds) DNA decoy with phosphoro-thioate linkages, containing the TGF-β cis-element found in the distal promoter region of the COL1A1 gene, blocks type 1 collagen synthesis. These decoys like glucocorticoids block the formation of the TGF-β activator protein/TGF-β element complex, thereby directly silencing COL1A1 gene expression. In a cell-free protein translation system, we have reported that collagen synthesis was inhibited by disulfide isomerase, the prolyl-4-hydroxylase (P-4-H) β subunit. By comparative proteomics dsdecoy therapy increased the levels of disulfide isomerase, P-4-H β subunit. These findings taken together support the notion that the dsdecoy inhibits type 1 collagen synthesis at both the transcriptional and translational levels.

Impairment of normal wound healing results from abnormal and excessive fibrogenesis. An over exuberance of fibrosis is caused by

This paper is dedicated to the memory of Professor Kenneth R. Cutroneo, who passed away in April of 2007. He devoted himself to medical education and biomedical research, specializing in the regulation of collagen gene expression and gene therapy.

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persistent profibrotic growth factors including fibroblast growth factor (FGF), platelet derived growth factor (PDGF), but most predominantly transforming growth factor-β1 (TGF-β1). Excessive fibrosis which causes scarring may result from the increased expression of the fibrillar type 1 collagen genes, proCOL1A1 and proCOL1A2, which are responsible for the synthesis of proα1 (1) and proα2 (1) polypeptides in the ratio of 2:1 which forms the stable triple helical type 1 collagen molecule in the extracellular space.

We have previously contrasted oligodeoxynucleotide (ODN) antisense and ODN sense gene therapies in wound healing and tissue fibrosis [Cutroneo and Chiu, 2000]. Treatment of injured ligaments with antisense decorin ODNs results in large collagen fibrils in early scarring and improves ligament functional healing [Nakamura et al., 2000]. Decorin is a small proteoglycan, which binds to TGF-β1. Antisense decorin but not sense phosphorothioate (PT) ODN's applied to the wound site decreased scarring which was associated with a decrease in TGF-β1 gene expression [Choi et al., 1996]. Antisense therapy has the disadvantage of having a short therapeutic half-life. Single-stranded antisense ODNs have natural unmodified phosphodiester bonds, which are extremely susceptible to degradation by intracellular nucleases. ODN decoys are ds containing modified phosphodiester bonds, which reduce their susceptibility to nuclease degradation.

Oligodeoxynucleotides provide a therapeutic strategy for regulating gene transcription. This therapy provides precise and effective modification of the expression of specific genes. Cis-element referred to as "decoy" ODNs have been reported to be powerful tools as a new class of antigene strategies for gene therapy and are presently under study for transcriptional regulation. Transfection of dsODNs corresponding to the cis-sequence will result in the attenuation of authentic cis-element with subsequent modulation of gene expression. This "decoy" strategy is a novel antigene therapy. It is also a powerful tool for the study of endogenous gene regulation in vivo as well as in vitro. Administration of NF-Kβ decoy ODNs into arthritic joints of rats with collagen-induced arthritis leads to an amelioration of this condition [Tomita et al., 1999]. It has also been shown that intramuscular injection of dsODN decoys to the NF-Kβ binding site inhibits cachexia in mice [Kawamura et al., 1999]. DsODN decoys may be novel drugs for cardiovascular disease [Morishita et al., 2001; Tomita et al., 2003]. For example, AP-1 decoy transfection in vivo dramatically prevented neointimal thickening in balloon-injured arteries, thus providing gene therapy to reduce restenosis [Kume et al., 2002]. AP-1 ODN decoys inhibited vascular smooth muscle cell (SMC) proliferation in vitro and neointimal formation in vivo [Ahn et al., 2002]. CpG-ODNs prevent acute inflammation by suppressing cytokine and cellular responses, but only subepithelial collagen deposition and not tissue fibrosis [Jain et al., 2002; Jain and Kline, 2004; Youn et al., 2004].

The elucidation of molecular and signaling pathways in eukaryotic cells is often achieved by targeting a regulatory element found in the promoter or enhancer region of eukaryotic genes using a ds (ODN) containing a specific cis-element. The dsODN decoy provides a therapeutic strategy to regulate gene transcription. ODN "decoys" are not only powerful tools as a class of gene therapies, but are presently under study for transcriptional regulation. Transfection of dsODN corresponding to the cis-sequence will result in the attenuation of cis-trans interaction, leading to the removal of a transfactor from the endogenous gene preventing its expression [Meisler et al., 1999]. One of the principle problems with decoys is their susceptibility to nuclease activity. PT dsdecoys and other modified decoys are presently in the pipeline, which are more potent, have better delivery and have longer duration of action. Our laboratory has developed linearized PT dsODN decoys containing the TGF-β1 element as a novel nonsteroidal antifibrotic for silencing or knocking out the expression of the COL1A1 gene and therefore type 1 collagen synthesis [Cutroneo and Ehrlich, 2006]. The sense strand has the following sequence: ATCCTGGC**TGCCCACGGCCAG**CCGGCC 3′ where the bolded sequence is the TGF-β element, which is found in the distal promoter of the COL1A1 gene and regulates the transcription of the wild-type COL1A1 gene by acting as a docking site for the specific transacting factor, TGF-β activator protein [Boros et al., 2005]. If the normal synthesis of COL1A1 is inhibited by PT dsdecoy therapy, this would result in preventing the synthesis of proα1 (1) and proα2 (1) polypeptides and the synthesis of type 1

procollagen, the immediate precursor of type 1 collagen arranged in a triple-helix, which is resistant to proteolytic degradation.

MATERIALS AND METHODS

Materials

All reagents used were of analytical grade. The rat monoclonal antibodies against the human type 1 collagen N-terminal propeptide, the mouse monoclonal antibodies raised against the human type 1 collagen C-terminal propeptide, goat antimouse IgG and goat antirat IgG used in the immunohistochemical studies were obtained from Chemicon (Temecular, CA). IMR-90 human embryonic lung fibroblasts were provided by ATCC (Rockville, MD). The PT sense and antisense ODNs were obtained from Operon (Huntsville, AL) and annealed as previously described [Boros et al., 2005]. The following were obtained from the designated vendors: penicillin, streptomycin, L-glutamine, phosphate buffered saline (PBS) (GIBCO-BRL, Grand Island, NY), DMEM with 4.5gm/L glucose (Hyclone Laboratories, Logan, UT), JET-PEI and lipofectin were obtained from Poly Plus (San Marcos, CA) and Invitrogen (Carlsbad, CA), respectively. As indicated in the figure legends either lipofectin or JETPEI was used as the transfecting agent. The consensus TGF-β element (TGC-CCA-CGG-CCA-G) containing wild-type PT dsODN decoys served as therapies. Scrambled TGF-β element (CAC-GAC-GGC-TGC-C) containing PT dsODN decoys or the five-base mutated TGF-β element (TGT-GCG-CGG-CCC-T) containing dsODN decoys were used as controls. PT dsODN decoys containing the five-base mutated TGF-β element or the scrambled sequence were used since these dsODNs did not bind to the TGF-β activator protein in gel mobility shift assays [Boros et al., 2005]. Also Meisler et al. [1999] demonstrated that the COL1A1 plasmid with the five-base mutation when transfected into fibroblasts demonstrated little, if any, transcription of the COL1A1 gene.

Cell Viability

Cell viability was measured by the naphthol blue black (NBB) staining. Cells grown on multi-well plates were fixed with 10% (v/v) formalin for 10 min and stained with NBB solution (0.05% (w/v) in 9% (v/v) acetic acid with 0.1M sodium acetate) for 30 min at room temperature. The cells were washed with water and the attached dyes were eluted with 0.05 N NaOH. The optical densities at 595 nm were measured using a plate reader (Sunnyvale, CA). No dramatic global change in protein expression was observed, suggesting that the decoy had a specific effect on collagen without exerting toxicity on lung fibroblasts.

Cell Culture and Transfection of dsODN Decoys

IMR-90 human lung embryonic fibroblasts were obtained from the ATCC. The cells were maintained in DMEM medium with 10% (v/v) fetal bovine serum at 37°C in a 5% CO_2 air atmosphere and grown to 90% confluence before transfection. Cells were covered with serum-free medium and transfected with 100 nM dsODN decoys using lipofectin (Invitrogen) or JETPEI (Poly Plus) according to the manufacturer's protocols. The incubation was continued for 6 h and the transfection medium was replaced with complete medium. After 18 h the cells were subjected to collagen staining or proteomic analyses. The uptake of the dsODN decoys was also monitored by transfection of fluorescein-labeled ds-ODNs.

Collagen Staining in Cell Culture

The collagen deposited on the cell layer in culture was stained with Sirius Red (Biocolor, Newtonabbey, Northern Ireland) as previously described [Tullberg-Reinert and Jundt, 1999]. Cells were washed with PBS and fixed with Bouin's fixative (prepared by mixing 15 ml saturated aqueous picric acid with 5 ml 35% (v/v) formaldehyde and 1 ml glacial acetic acid) for 10 min. The fixation fluid was removed and the culture plates were washed thoroughly with water. The cells were stained with Sirius Red stain (1 ml/well in a 24 well plate) for 1 h. The stain solution was removed and the stained cell layers extensively washed with 0.01 N hydrochloric acid. The collagen was deposited on the cell layer and stained red. The assays were performed in triplicate.

Two Dimensional Gel Electrophoresis (2-DE) and Mass Spectrometry

Cells were washed with PBS and lysed in a buffer containing 8 M urea, 4% (w/v) CHAPS, 60 mM dithiothreitol and subjected to two dimensional gel electrophoresis (2-DE). 2-DE was performed with IPGphor isoelectric

focusing (IEF) and electrophoresis system (GE Health Care, Piscataway, NJ). Protein lysates (100 μg of total protein) were mixed with rehydration solution containing 8 M urea, 4% (w/v) CHAPS, 60 mM dithiothreitol and 0.5% (v/v) IPG buffer (pH 3–10) and loaded on IPG strips (13 cm, pH range 3–11). The IEF conditions were: 30 V for 10 h (rehydration), 500 and 1,000 V for 1 h each, and 8,000 V for 10 h for a total of 9.53 kV·h. After IEF, the strips were incubated for 15 min in equilibration buffer (6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris-HCl, pH 6.8) containing 1% (w/v) dithiothreitol and then another 15 min with equilibration buffer containing 2.5% (w/v) iodoacetamide. For the second-dimension SDS–PAGE, the strips were transferred onto 12.5% (w/v) polyacrylamide slab gels containing 0.1% (w/v) SDS and electrophoresed.

Image Acquisition and Analysis

The silver stained gels [He et al., 2003] were scanned using an Image Scanner (Amersham Biosciences) operated by Lab Scan software. Intensity calibration was performed with an intensity step wedge prior to gel image capture. Image analysis was carried out by using the Image Master 2D Elite software 4.01. Image spots were initially detected, matched and then manually edited. Each spot intensity volume was processed by background subtraction and total spot volume normalization. The resulting spot volume percentage was used for comparison. Only up/downregulated spots (over ± twofold) between treatment groups were selected for analysis using mass spectrometry analysis after in-gel tryptic digestion.

In-Gel Tryptic Digestion

The excised protein spots were destained with a solution of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate. The gel slices were washed with 50 mM ammonium bicarbonate and dehydrated with acetonitrile. In gel tryptic digestion was carried by incubating the dried gel slices with an ammonium bicarbonate buffered solution of MS grade trypsin (Trypsin Gold, Promega, Madison, WI) at 37°C overnight.

Protein Identification by MALDI-TOF MS

The proteomic tryptic digests together with 1 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid (10 mg/ml) in 0.1% (w/v) trichloroacetic acid/35% (w/v) acetonitrile were spotted on the target plate and air dried. The resulting peptides were subjected to MALDI-TOF MS and MS/MS using Voyager-DE STR or 4700 proteomic analyzer (Applied Biosciences, Foster, City, CA). Internal mass calibration was achieved using procine trypsin autolysis digestion products. Protein identifications were performed with the Mascot program with a mass tolerance setting of 50 ppm. Positive identifications were accepted with a Mascot protein score over 50.

Data Analysis and Reproducibility

The analyses were performed three times. Protein spots with a threshold difference of twofold in normalized volumes between treatment groups were subjected to MS identification. The collagen assays were performed in triplicate. Shown are representative/microscopic images.

RESULTS

Under phase contrast microscopy the human fibroblasts on plastic demonstrated the same morphology as typical fibroblast (Fig. 1A). We next fluorescent dye tagged the TGF-β element PT dsODN decoys and incubated the tagged dsODN decoys in at least six experiments. For each experiment at least a field of 100+ cells were counted. The average uptake efficiency was over 30%.

The effects of various doses of TGF-β1 on total collagen secreted were determined by Sirius Red stain and light microscopy (Fig. 2). Fibroblast cultures were treated with either 100 nM TGF-β element containing dsODN decoys (WT) or the same dose of five-base mutated TGF-β element dsODN decoys (MT). A dose of 0.1 ng/ml of TGF-β1 was found to give optimal birefringence in human lung cultures receiving also the MT dsODN decoys (Fig. 2B). In other 0.1 ng TGF-β1 treated fibroblast cultures control mutated TGF-β element dsoligos were not added and no notable difference was observed in intensity of Sirius Red staining (data not shown).

Fibroblasts were treated with either JETPEI alone, TGF-β element dsODN decoys or scrambled TGF-β element dsODN decoys and examined immunohistochemically with monoclonal antibodies to either the N-terminal propeptide or the C-terminal propeptide of human proCOL1 the intracellular precursor of

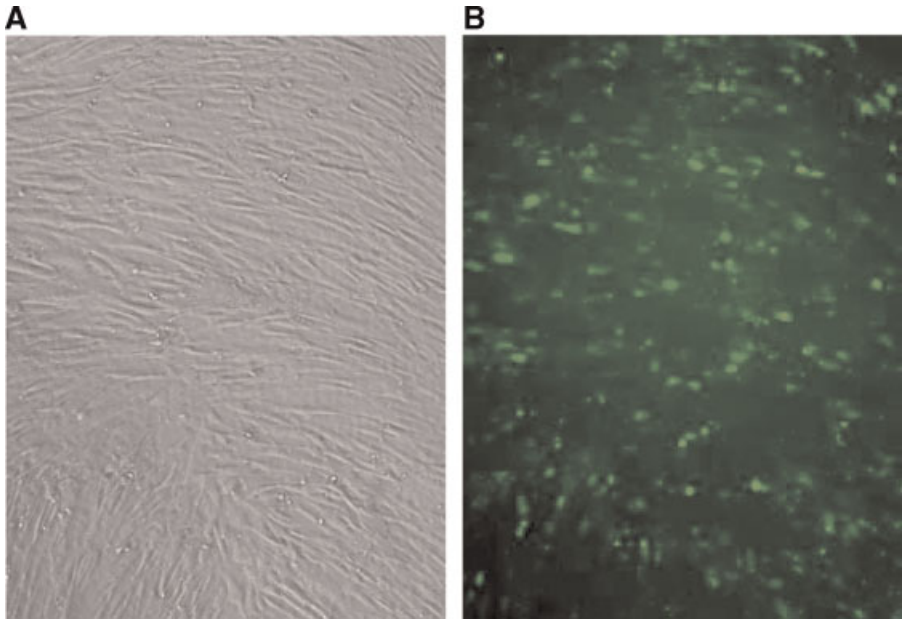

Fig. 1. **A**: Bright field micrograph of human lung fibroblasts in late log phase adhered to plastic. **B**: The cellular uptake of fluorescent tag conjugated to the TGF-β element containing phosphorothioate double-stranded dsODN decoys as examined by fluorescence microscopy. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

COL1 (Fig. 3). COL1 represents the majority of total collagen secreted by fibroblasts. The data demonstrate that the TGF-β element containing dsODN decoys significantly decreased the fluorescence intensity of human proCOL1 when either antibody was used as compared to the JETPEI treated alone fibroblasts or the scrambled TGF-β element dsODN decoy control treated fibroblasts.

We determined the possible effects of TGF-β element containing dsODN decoys on total intracellular proteins by 2-D PAGE followed by automated comparative proteomics. Lung fibroblasts were treated with either 100 nM WT or the same amount of MT. The cell lysates were subjected to 2-D PAGE and silver staining (Fig. 4), followed by image analysis. The gel regions containing the proteins of interest, which increased or decreased by twofold were excised and tryptic in-gel digested, followed by mass spectroscopy to identify the proteins and by searching the NCBI protein database. Table I gives the accession number of each intracellular protein up- or downregulated by WT treatment as compared to MT treatment of lung fibroblasts. This table also indicates the physical characteristics of migration on 2-D PAGE. As can be seen the proteins of interest have diverse functions in certain cell types, but not all may be relevant to human lung fibroblasts. These proteins include enzymes of glycolysis and tricarboxylic acid cycle, mitochondrial permeability regulator, endoplasmic reticulum chaperons, and cytoskeleton binding proteins. All but one of the intracellular proteins was significantly decreased in the WT treated fibroblasts as compared to MT treated cells. The one intracellular protein that was significantly increased was identified as protein disulfide isomerase (PDI). PDI is the β subunit of P-4-H (Fig. 5). This indicates specificity of effect of TGF-β element dsODN decoys on this protein.

DISCUSSION

Proteomic analysis of total intracellular proteins after 100 nM WT dsODN decoy treatment demonstrated an antiTGF-β1 effects on energetic enzymes in human lung fibroblasts. Previous reports indicate that TGF-β1 treatment results in increased glycolysis in total rat kidney cells [Boerner et al., 1985], an increase of glycolysis [Nowak and Schnellmann, 1996; Nowak et al., 1996], and a decrease of gluconeogenesis [Nowak and Schnellmann, 1996]. In preantral follicle cells TGF-β1 increases glycolysis and the tricarboxylic acid cycle enzymes [Roy and Terada, 1999]. In human embryonic

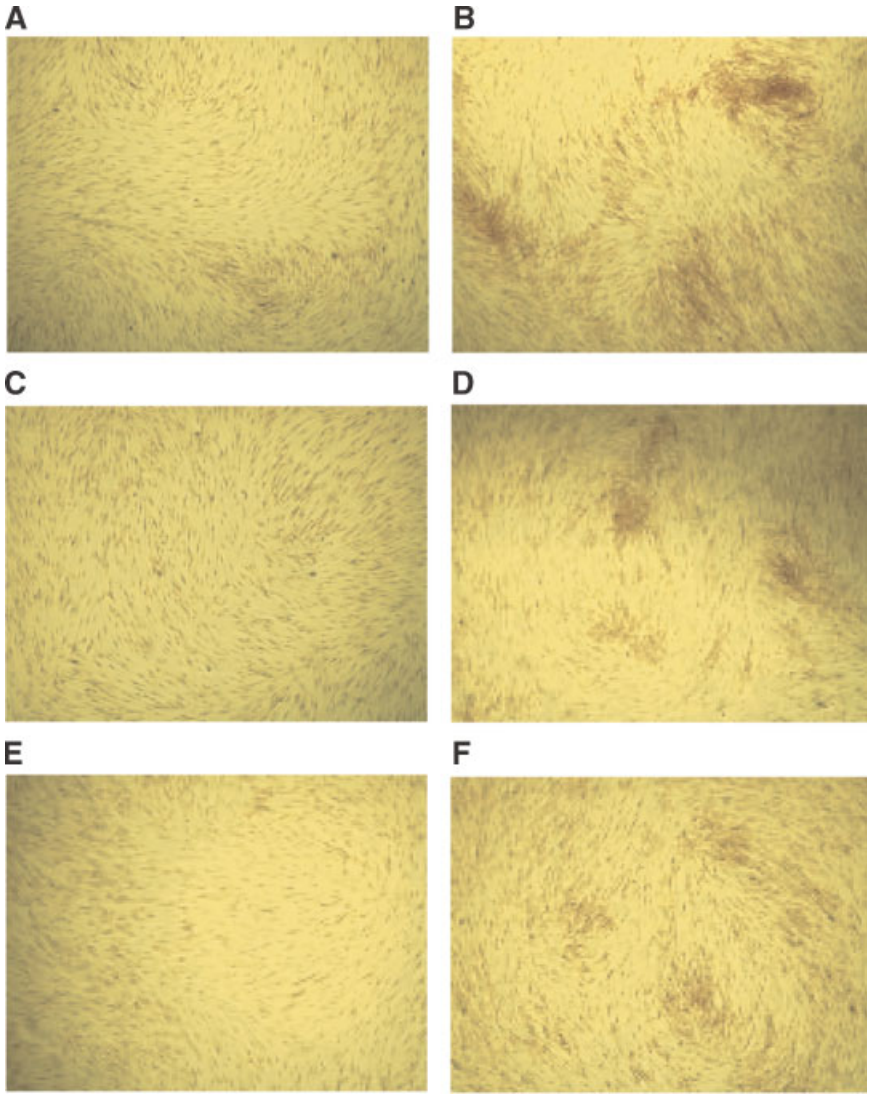

Fig. 2. The effects of 100 nM TGF-β element containing dsODN decoys (**left side**) and the five-base mutated TGF-β element containing dsODN decoys (**right side**) on fibroblasts treated with various concentrations 0.1, 0.03, and 0.01 nM of TGF-β1 from **top** to **bottom**. Total collagen secreted by the human lung fibroblast was stained with Sirius Red and cell cultures were examined under a light microscope. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

lung fibroblasts WT PT dsODN decoy treatment results in a decreased mitochondrial malate dehydrogenase. In human lung fibroblasts the WT PT dsODN decoys decrease the voltage-dependent anion channel 1 protein as compared to MT PT dsODN decoys. This protein which is involved in apoptosis is increased by TGF-β1 treatment of mammary epithelial cells [Kolek et al., 2003]. TGF-β1 increases transgelin gene expression in fibroblasts derived from benign keloid tumors [Satish et al., 2006]. WT PT dsODN decoys decreases this protein's expression. In normal fibroblasts TGF-β1 increases profilin, a protein of the contractile apparatus, involved in the transformation of fibroblasts to α-smooth muscle actin positive myo-fibroblasts [Malstrom et al., 2004]. The increased expression of all the above mentioned proteins by TGF-β1 may be due to the general pleotropic nature of this growth factor.

The expression of PDI, which is the β subunit of P-4-H (EC 1.14.11.2) is not only significantly but substantially increased in human lung fibroblasts treated with WT dsODN decoys. It is the only one increased by our standards. The fact that PDI is the only protein increased by

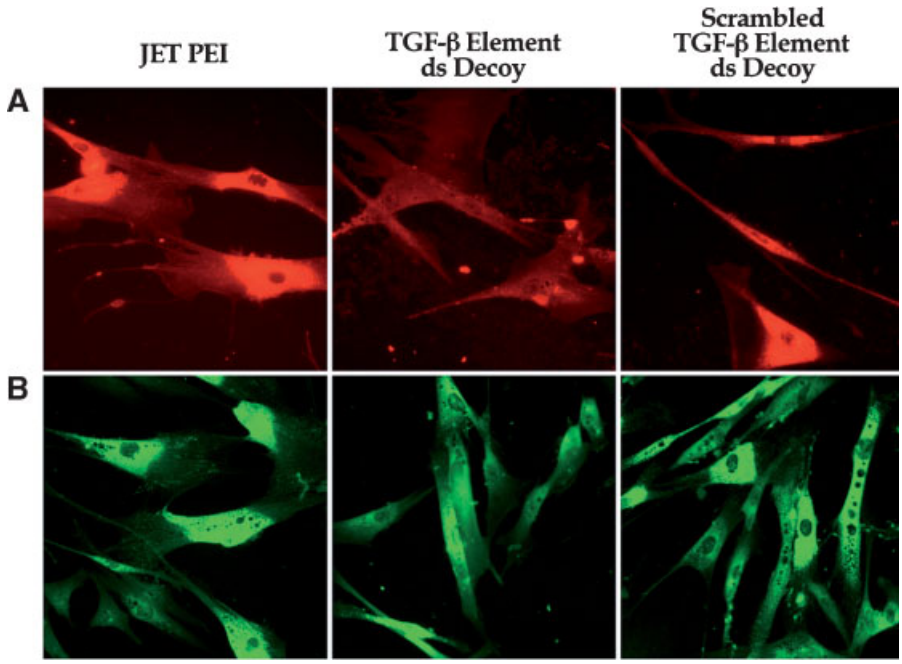

Fig. 3. Immunohistochemical studies of the effect of TGF-β element dsODN decoys and scrambled TGF-β element dsODN decoys on human proCOL1. Fibroblasts were seeded in Lab-Tek-well chamber slides at passage 8 in complete media and grown for 4 days. The cells were treated at time zero with either 100 nM TGF-β wild or scrambled dsODN decoys in the transfecting agent JETPEI media containing serum and then switched to serum deficient media as described in Section ''Materials and Methods.'' **A**: Reaction with rat monoclonal antibody to the N-terminal propeptide of human proCOL1; (**B**) reaction with mouse monoclonal antibody to the C-terminal propeptide of human proCOL1. For each experiment six images were averaged. Images were acquired using a Microfibre CCD (Optronics) mounted on a Nikon Microphot-SA with a 20× objective.

automated comparative proteomics indicates specificity not a cause effect relationship. As far as the TGF-β element dsdecoy decreasing all the other proteins this may result from the expression of these proteins being controlled by the TGF-β element/activator signaling pathway.

P-4-H sequence specifically hydroxylates certain prolyl resides in pro COL1A1 and proCOL1A2 polypeptides both co- and post-translationally. The formation of 4-hydroxyproline in fibrillar COL1 is necessary for the stability of the triple helix. Therefore, the holoenzyme P-4-H made up of α and β subunits

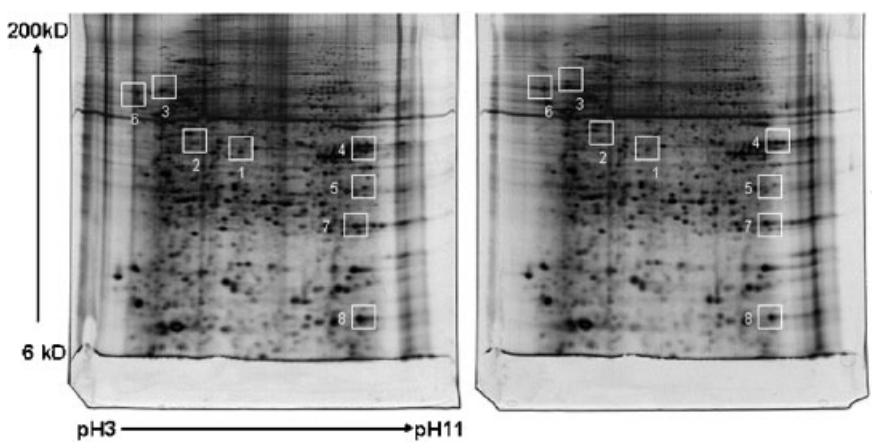

Fig. 4. 2-D-PAGE gel images of total intracellular proteins isolated from lung fibroblasts treated with either wild-type dsODN decoys (**right**) or five-base mutated dsODN decoys (**left**). Protein spots of interest are squared and zoomed in Figure 5.

TABLE I. Characteristics of the Proteins Which Significantly Changed by TGF-β Element Containing Double-Stranded Decoy ODNs as Determined by MALDI-TOF Mass Spectroscopy of the In-Gel Tryptic Digests

Protein ID	NCBI accession No.	Experimental mass (kDa)/pI	Peptide searched	Mascot protein score	Fold difference	Function
Lactate dehydrogenase B	13786848	36.6/5.7	6	174	−2.9	Glycolysis
Pyruvate kinase 3 isoform 1	14043291	57.9/7.9	14	196	−2.0	Glycolysis
Voltage—dependent anion channel 1	57093539	30.7/8.6	4	81	−2.0	Mitochondrial permeability
Mitochondrial malate dehydrogenase	1200100	35.5/8.9	8	155	−2.1	TCA cycle
Protein disulfide isomerase	35655	57.1/4.8	20	256	+2.9	ER chaperon
Calreticulin	14043949	48.1/4.2	15	401	−2.7	ER chaperon
Transgelin	12803567	22.4/8.4	17	266	−2.8	Actin binding
Profilin	4826898	15.0/8.4	8	313	−2.4	Actin binding

Fig. 5. 2-D-PAGE analysis of proteins that increased or decreased by at least twofold in Figure 4. From **top** to **bottom**: lactate dehydrogenase β↓, pyruvate dehydrogenase ↓, protein disulfide isomerase ↑, mitochondrial malate dehydrogenase ↓, voltage-dependent anion channel 1 ↓, calreticulin ↓, transgelin ↓, and profilin ↓. The 2-D gels on the **left** were from proteins of interest observed in this figure from cells treated with five-base mutated dsODN decoys and those on **right** from cells treated with wild-type element dsODN decoys.

is required for the synthesis of procollagen and native COL1 molecules extracellularly. Although PDI is increased this may ensure that enough holoenzyme is formed to allow for proper 4-prolyl hydroxylation and subsequent helix formation of the decreased number of

proCOL1 molecules synthesized in TGF-β element dsODN decoy treated human fibroblasts. Although our proteomic studies did not demonstrate significant expression of the α subunit of P-4-H, this subunit is extremely susceptible to proteolytic degradation [Guzman, 1988]. The purification of human P-4-H from placenta which is a protease-rich tissue does not result in the purified enzyme having a 1:1 α to β subunit ratio as compared to the purified chick embryo enzyme [Guzman et al., 1992]. This author also showed that in vitro the protease digested purified chick enzyme selectively loses α subunits [Guzman, 1988]. When holo-human purified placental P-4-H was submitted to denaturing PAGE, α subunits were specifically degraded as compared to the β-subunits. In our comparative proteomic studies during cell lysate preparation, the α subunits may have been susceptible to proteolytic degradation as compared to the more stable β-subunits, although proteomic analysis has limitations to detect all proteins.

McGee et al. [1971] and Stassen et al. [1974] noted a subunit or subunit precursor, which was widely distributed in tissues and was termed the "cross reacting protein" or CRP. Chen-Kiang et al. [1977] purified the CRP to homogeneity and found it was homologous to the smaller β immunogenic subunit of the holoenzyme and crossreacts with it.

In a cell-free lysate protein synthesizing system, the run-off of the holoenzyme by total neonatal rat lung polysomes and P-4-H antibody precipitated polysomes resulted in the holoenzymes synthesized having the same amounts and sizes of the α and β subunits as determined on denaturing PAGE [Rokowski et al., 1981]. This in vitro protein synthesizing system was run at 25°C and actively synthesizes a number of full-length proteins.

These studies demonstrated that total neonatal rat lung polysomes synthesized 37% protease-free bacterial collagenase digestible proline-labeled product while P-4-H antibody precipitated polysomes synthesized a negligible amount of collagenase digestible product in the cell-free run-off wheat germ lysate system. P-4-H activity has not only been localized in the cisternae of the rough endoplasmic reticulum, but associated with polysomes [Rokowski et al., 1981]. P-4-H activity resides in the α subunits of the holoenzyme and the immunogenicity resides the β subunits [Guzman, 1988]. The P-4-H rat skin antibody used was directed to the β-subunit and not the α subunit. The antibody precipitate lacked P-4-H activity and the antibody precipitated polysomes did not synthesize any appreciable amount of protease-free bacterial digestible product [Rokowski et al., 1981]. This interpretation of these previous findings is in accord with the present findings in which TGF-β element containing decoys significantly increased PDI which is the β-subunit of P-4-H [Guzman, 1988] that we believe inhibits the synthesis of type 1 procollagen synthesis in fibroblasts. However, as yet by direct rigorous experimentation we have not proved a cause/effect relationship. Immunohisto-chemical studies demonstrate that the TGF-β element containing PT dsODN decoy decreases fibroblast membrane associated proCOL1. This location of this precursor of native triple helical COL1 is in accord with previous flow cytometry and cell sorting studies [Breen et al., 1990]. This is all circumstantial evidence yet in total all these data taken together support our hypothesis.

The TGF-β element PT dsODN dsdecoy and its target initiation factor, the TGF-β activator protein, are specific for inhibiting the transcription of the proCOL1A1 gene and subsequently decreasing native COL1 secreted into the extracellular space. The TGF-β cis regulatory element is in the distal promoter and not proximal to the start site of transcription where numerous common transcription factors bind, such as Sp1. The use of antisense or decoys targeting this transcription factor would not be specific for inhibiting the synthesis of proCOL1 [Verrecchia et al., 2001; Chae et al., 2006]. In conclusion, the TGF-β element decoys since they act both at the transcriptional and translational levels to decrease proCOL1 synthesis have an added advantage.

Finally, transacting factors and regulation of gene expression through a cis-element in a gene's promoter region also involves co-activator(s) and co-inhibitor(s). Although the eight different genes in the present study code for distinct proteins through the same TGF-β activator/TGF-β element pathway it may be the co-activator that ultimately causes the expression of the PDI genes to increase while co-inhibitors cause the expression of the other genes to decrease.

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