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ARTICLES

Evidence for TGF-ß1 and Bleomycin Intracellular Signaling through Autocrine Regulation of Smad 3 Binding to the Proximal Promoter of the *Smad 7* Gene

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Abstract Both Bleomycin and TGF-β1 increase the transcription of the *COL1A1* gene. Bleomycin acts through TGF-β1. Bleomycin stimulates the COL1A1 promoter through the distal TGF-β response element by intracellular and extracellular signaling. As demonstrated in this manuscript, Bleomycin's intracellular signaling can be explained by a decrease of Smad 3 transcription factor binding to the SBE located in the proximal promoter of the inhibitory Smad 7 gene. This would result in TGF-β1-induced activated SMADS, which would result in more collagen. Bleomycin's extracellular signaling results from the secretion of more latent TGF-β produced by lung fibroblasts and cleaved to active TGF-β extracellularly. Since the TGF-β genes are auto-induced in human embryonic IMR-90 lung fibroblasts, this study indicates an autocrine mechanism to maintain homeostasis in vivo for fibroblasts and other cell types, which produce TGF-β1 to limit the fibrogenic response to TGF-β1 and Bleomycin. J. Cell. Biochem. 97: 933–939, 2006.

Key words: Smad transcription factors; Smad 3 transcription factor; Smad 3 binding element (SBE); Smad 7; Smad 7 gene; TGF-ß signaling; bleomycin

TGF-ßs are a group of a super family of proteins, which have a broad array of biological activities controlling development by SMAD signaling [Luukko et al., 2001; Ito et al., 2002; Xu et al., 2003], for rev. see Cutroneo, 2005 through regulating genes and thereby determining a cell's phenotype. The Smad proteins are the intracellular mediators for TGF-ß-mediated signaling. TGF-ß occupies the TGF-ß RII, which results in the TGF-ß RI's intrinsic serine/threonine kinase activities to phosphorylate the transcription factors Smad 2 and Smad 3. These phosphorylated Smads form a complex with the unphosphorylated transcription factor Co-Smad 4. This complex then translocates from the cytoplasm into the nucleus [Luo et al., 2003; Venkatesan et al., 2004]. TGF-ß has been shown to increase the activation of Smad 3 through phosphorylation, the gene expression of Smad 3 mRNA, Smad 3 protein expression, and Smad 3 translocation into the nucleus by TGF-ß1 treatment in a dose and time dependent manner in stromal as well as epithelial cells [Luo et al., 2003]. In the nucleus the Smads complex with co-activators or co-inhibitors regulate the expression of specific genes for development and homeostasis. The Smad signaling pathway reflects intracellular trafficking not only by acting as TGF-ß1 effectors but also as signal transducers intracellularly for drugs that act through TGF-ß1 such as Bleomycin [King et al., 1994], which causes lung fibrosis [Tryka, 1987].

Bleomycin treatment results in DNA damage, which is amplified by growth hormone [Cianfarani et al., 1988]. This effect is more marked by insulin-like growth factors I and II. These growth factors stimulate p53 protein expression, which by causing DNA repair may counteract the effect of insulin-like growth factors.

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Smad 6 and Smad 7 act as intracellular antagonists of TGF-ß signaling. Anti-sense Smad 7 increases TGF-ß1 and its signaling through the Smad pathway [Afrakhte et al., 1998]. The inhibitory Smads form autoinhibitory feedback loops to regulate the responses to TGF-ßs [Miyazono, 2000; Park, 2005]. Smad 7 stability associates and blocks the Smad 2 phosphorylation [Hayashi et al., 1997]. The C-terminal domain of the inhibitory Smads is the effector part of this inhibitory Smad 7 protein while the N-terminal domain confers species specificity [Souchelnytskyi et al., 1998]. Various regulatory factors have been shown to interact with Smad 7. YAP65 potentiates Smad 7′s inhibitory activity [Ferrigno et al., 2002]. TGF-ß induction of Smad 7 gene transcription results from a synergism between the transcription factors TFE 3 and Smad 3 [Hua et al., 2000]. To maintain homeostasis there must exist tight regulatory controls for the inhibitory Smads through transcription and post-translational protein degradation. In addition, the activated TGF-ß RI receptor is depohsphorylated by Smad 7 [Shi et al., 2004].

The post-translational mode of protein degradation involves Smad 7 binding Smufs to form an E3 ubiquitin ligase complex, which increases TGF-ß1 receptor and Smad 7 degradation by the proteosomal and lysosomal pathways [Kavsak et al., 2000; Ebisawa et al., 2001; Murakami et al., 2003]. Thus, Smad 7/Smurf RF complex formation complex and subsequent endocytosis of the TGF-ß1 receptor is of utmost importance in the autoinhibitory action of Smad 7 on the TGF-ß signal transduction pathway [Di Guglielmo et al., 2003]. TGIF, a novel E3 ubiquitin ligase, is responsible for the degradation of NEDD 4-2 activator TGF-ß RI and Smad 2 [Seo et al., 2004], which signals the degradation of activated TGF-ß RI and the transcription factor Smad 2 by uqiquitinization and subsequent proteosomal/lysosomal degradation [Kuratomi et al., 2005].

Bleomycin has previously been shown to signal intracellularly [King et al., 1994]. Since Bleomycin acts through TGF-ß1 [Breen et al., 1992] and TGF-ß1 induces Smad proteins, the molecular mechanism by which Bleomycin and TGF-ß1 regulates the level of inhibitory Smad 7 protein may be by modulating Smad 3 binding to the SBE [Brodin et al., 2000] located in the proximal promoter of the Smad 7 gene was investigated. An autocrine mode of modulating Smad 3 binding to the SBE located in the proximal promoter is proposed for both TGF-ß1 and Bleomycin.

MATERIALS AND METHODS

Cells and Materials

Human IMR-90 embryonic lung fibroblasts were obtained from ATCC (Rockville, MD). The following materials were purchased from the designated companies: AMI V synthetic media, polynucleotide T4 kinase, penicillin, streptomycin, L-glutamine, phosphate buffered saline (PBS) (GIBCO-BRI, Grand Island, NY); fetal bovine serum (FBS), DulBecco's modified Eagle's essential media (DMEM) with high glucose (Hyclone Laboratories, Logan, UT); ^{32}P ATP (3,000 Ci/mmol) (New England Life Sciences, Boston, MA); G-25 Micro Spin columns (Boehringer Mannheim, Indianapolis, IN); Smad 3 binding element (SBE), sense and antisense oligodeoxynucleotides (Geneka Biotechnology, Montreal, Quebec, Canada); bovine serum albumin (BSA), plasmin isolated from human plasma, poly dI-dC (Sigma, St. Louis, MO; Smad 3 peptide, Smad 3 antibody (Santa Cruz, CA); TGF-β1 (R&D Systems, Minneapolis,MN); Bleomycin was a generous gift from Bristol-Myers Squib. All other reagents were of analytical grade.

Cell Culture

Human embryonic lung fibroblasts were maintained in Eagle's minimal essential media with Earle's balanced solution, 10% (v/v) FBS at 37°C under an atmosphere of 5% CO_2 and air. The lung fibroblasts were grown to late log phase in DMEM containing high glucose, 10% (v/v) FBS, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 292 μg of glutamine/ml in 100 mm petri dishes. The cells adhered to plastic were washed gently with both PBS and Aim V media.

Drug Preparation and Treatment

The cells were treated with either an equal volume of vehicle, Bleomycin (1.5 μg/ml) or TGF-β1 (12.5 ng/ml). Bleomycin was solubilized in PBS. TGF-β1 was solubilized in 4 mM HCl containing 1 mg BSA/ml. This same vehicle was used to treat control cultures without TGF-β1 added. All cell cultures were treated in Aim V media at the end of each experiment, that is,

either after 15 min or 24 h. The cells were collected by scraping.

Preparation of Nuclear Extracts

The nuclear extracts from 10^6 cells were prepared by a slight modification of the method of Dignam et al. [1983]. After nuclear protein extraction the sample was dialyzed at 4°C with agitation for 60 mm against 20 mM HEPES (pH 7.9), 100 mM KCl, 1 mM $NaCl_2$, 20% (v/v) glycerol, 0.5% (w/v) DTT and 1 mM PMSF. The nuclear extract protein concentration was determined by the Bio-Rad's Bradford assay (Hercultes, CA), using BSA as standard. The nuclear extract was stored at −80°C until use in gel mobility shift assays.

Preparation of Wild Type Double-Stranded Oligo Probes

Equal amounts of the sense and the antisense probes were suspended in the annealing buffer (50 mM Tris-HCl (pH 7.5), 1 M NaCl, 100 mM Mg Cl_2, 0.2 mM EDTA, and 10 mM DTT). This sample was placed in a boiling water bath for 30 min and then brought to the annealing temperature of 60°C for 1 h. The sample was slowly brought to room temperature and stored in aliquots at −20°C.

Radiolabeling of the Double-Stranded Wild Type Oligo Probes

Fifty nanograms of unlabeled probes were combined with 2:l of 10× kinase buffer, 40 μCi ^{32}P-ATP, 10 U of T4 polynucleotide kinase brought up to a final volume of 20 μl with water in a microfuge tube. The sample was incubated at 37°C for 30 min followed by the addition of 5 μl of a 1% (w/v) SDS and 100 mM EDTA to stop the kinase reaction. The sample was vortexed and spun down in a microfuge. The labeled probes were passed through a Micro G25 Spin column per instruction of the supplier. The final radioactivity of the purified probe was 1×10^5CPM/μl.

Gel Mobility Shift Assays

Ten micrograms of nuclear extract, 0.5 ng labeled SBE Smad 3/Smad 7 oligo probe, 20 mM HEPES (pH 7.9), 50 mM KCl, 4 mM $MgCl_2$, 62.5 ng/μl poly dI-dC, 1 mM EDTA, and 1 mM DTT were added to a microfuge tube at 4°C, the Smad 3 antibody (8 μg) only, the cold Smad 3/Smad 7 probe (50 ng), or Smad 3 antibody plus the blocking peptide were incubated for 20 min at 4°C. Each premix was added in the order to the nuclear extract mix as described above. Each entire mixture was loaded onto a 5% (w/v) polyacrylamide gel and run at 210 V for approximately 1 h at 4°C. The air-dried gels were autoradioraphed for different times.

RESULTS

As can be seen in Figure 1 the SBE (5′ GTCTAGAC-3′) is located at −124 from the start site of transcription in the proximal promoter region of the Smad 7 gene. To prove that the ^{32}P-labeled double-stranded Smad 3/Smad 7 oligo binds to Smad 3 and forms a complex, a competitive gel mobility shift assay using cold double-stranded Smad 3/Smad 7 oligo and Smad 3 antibody was performed. The nuclear extracts was prepared from human IMR-90 embryonic lung fibroblasts treated for 24 h with TGF-β1 as described under Materials and Methods. Figure 2 indicates that the binding of Smad 3 ^{32}P-labeled SBE double-stranded oligo is competed out by the homologous unlabeled double-stranded oligo. This Smad 3/SBE labeled complex is also supershifted by a Smad 3 polyclonal antibody. The Smad3 blocking peptide inhibited the supershifting by the Smad 3 antibody alone.

Bleomycin decreased the binding of the Smad 3 transcription factor to the SBE ^{32}P-labeled double-stranded oligo in a time-dependent manner. This phenomenon was observed in three separate experiments done on different days (Fig. 3). As a ^{32}P-labeled homologous control sequence 5′-CAGGACA-3′ with 3′ and 5′ flanking regions was run. This double-stranded labeled oligo, which did not contain this homologous sequence to the CAGA motif, did not form a complex (data not shown). Alternatively the nuclear extract isolated from TGF-β1 treated human embryonic lung fibroblasts in three separate experiments when submitted to gel mobility shifts on different days showed a time-dependent increase in Smad 3 transcription factor binding to the ^{32}P labeled SBE binding oligo (Fig. 4). As before the ^{32}P labeled homologous element oligo formed no complex with the Smad 3 transcription factor (data not shown).

DISCUSSION

Bleomycin is an antineoplastic glycopeptide therapeutic used in the treatment of head and neck carcinomas, lymphomas and testicular

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-204   AATAGCTTTTAGAAACCCGATCTGTTGTTTGCGAAACACAATCGCTTTTT
                                  SBE
-154   TTTTTTTTTTAAAGCGACAGGGTGTCTAGACGGCCACGTGACGAGGCCGG

-104   AGCCGGGCGCGCCACTGCGCAGTGGAACCAGCCGAGCATAGGGCCGGAGG

 -54   GGGGGGTCCGGGGGGCCGGGGAGGAGGCGGCGCCGGTCCGGCGCTGGGGG

  -4   CGGGGGAGGGAAGGGGGAGGAAGGGGGAGGGAAGGGGGCGGGGGCGGGAG

+47    GCTTGCGGGAGGCGGTGAGCCCCGGGCACACTCGCTTGCTGCTCGGCGCA
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Fig. 1. The base sequence of a portion of the 5′ flanking region and a small section of the translated region of the Smad 7 gene. SBE, the Smad 3 binding element (SBE) in the proximal promoter region. The arrow indicates the start site of transcription.

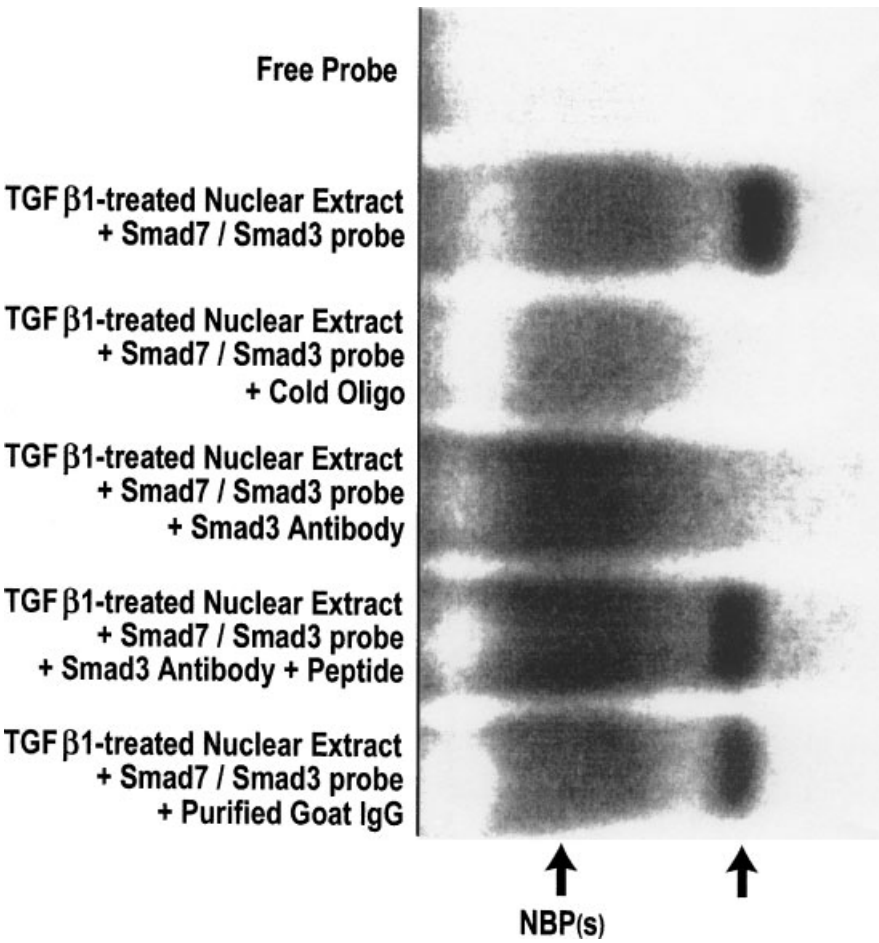

Fig. 2. The effect of cold double-stranded oligo, the Smad 3 antibody, the Smad 3 blocking peptide and purified IgG on DNA binding proteins isolated from the nuclei of IMR-90 human embryonic lung fibroblasts treated with TGF-β1 (12.5 mg/ml) for 15 min. Cell culture and gel mobility shift assays were performed as described under Materials and Methods. Competition and supershift assays were run with excess cold Smad 7/Smad 3 oligo and polyclonal Smad 3 antibody. The blocking peptide was used to prevent supershifting by the Smad 3 antibody. Purified goat IgG was used as a supershift control. The Smad 7/Smad 3 oligo sense sequence is 5′-CAGGGTGTCTAGACGGCCAC-3′. NBP(s), non-specific binding protein(s).

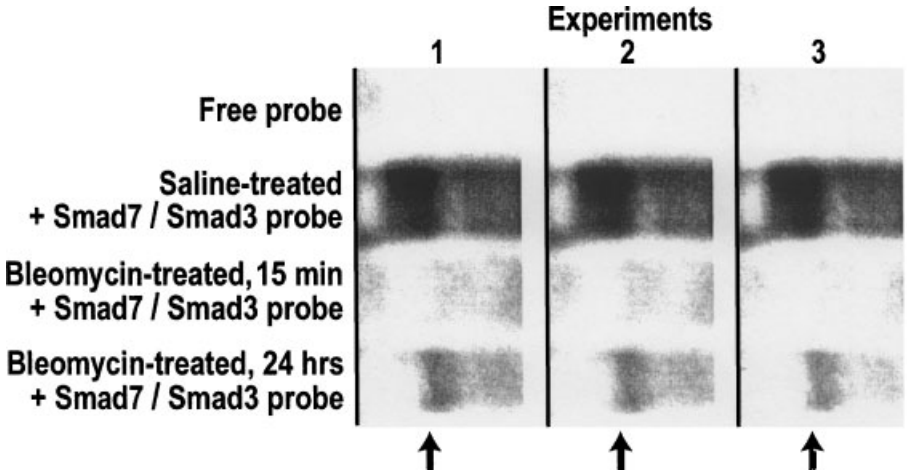

Fig. 3. The temporal response of Smad 3 binding to the Smad 7/Smad 3 probe in Bleomycin-treated cells. IMR-90 human embryonic lung fibroblasts were either treated for 15 min or 24 h with Bleomycin (1.5 μg/ml) in three separate experiments (1,2, and 3) which were done on different days. The nuclei were isolated, DNA binding proteins were prepared and gel mobility shift assays were run as described under Materials and Methods.

carcinomas, which has as its limiting toxic side effect lung fibrosis. This therapeutic agent has been shown to increase TGF-β1 mRNA, TGF-β1 gene transcription, and total TGF-β activity [Breen et al., 1992]. King et al. [1994] demonstrated that this drug increased the transcription of the *COL1A1* gene through the TGF-β response element by intracellular and extracellular signaling. Bleomycin's intracellular signaling can be explained by a decrease of Smad 3 transcription factor binding to the SBE, which is located in the proximal promoter of the inhibitory Smad 7 gene, resulting in decreased transactivation of this gene. This would result in TGF-β1-induced activated SMADS, which would lead to more collagen. Bleomycin extracellular signaling results from more latent TGF-β produced by lung fibroblasts, which is cleaved to the active form by proteases secreted from these cells into the media associated with the cell membrane. TGF-β would bind to the TGF-β RII, which would cause phosphorylation of Smad 2 and 3 by the Type I receptor, forming a complex with Co-Smad 4, and subsequently this

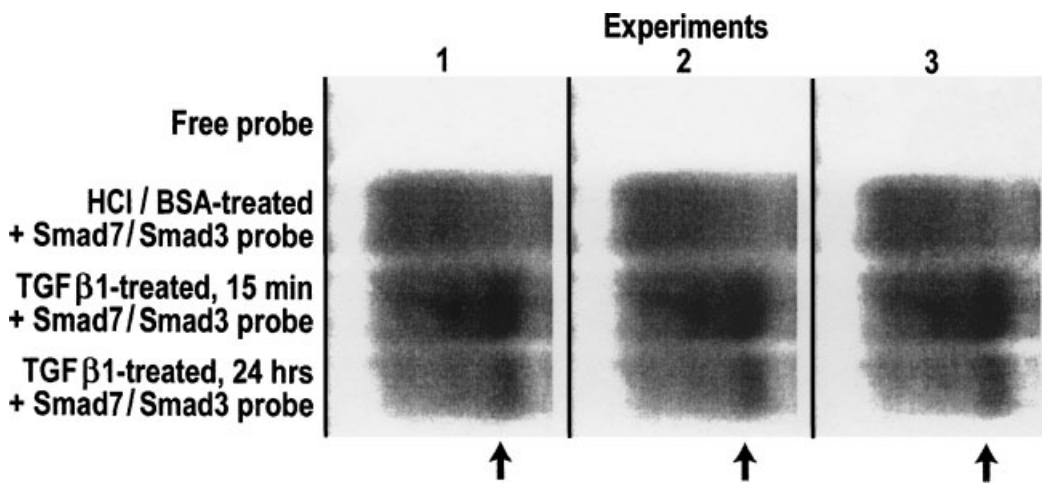

Fig. 4. The temporal response of Smad 3 binding to the Smad 7/Smad 3 probe in TGF-β1-treated cells. Embryonic human lung fibroblasts were treated with HCb-BSA or 12.5 mg, AIMV V media, or TGF-β1 solubilized in this solution (see Materials and Methods). The fibroblasts were treated for either 15 min or 24 h in three separate experiments (1, 2, and 3) done on different days. The nuclei were isolated, DNA binding proteins were prepared and gel mobility shift assays were run as described under Materials and Methods.

complex would be translocated into the nucleus turning on the transcription of the ECM protein genes [for review see Cutroneo, 2005]. TGF-β1 is a very potent profibrotic growth factor [Roberts et al., 1986]. As with Bleomycin, fibroblasts treated with TGF-β1 increase the transcription of the *COL1A1* gene by the TGF-β activator protein binding to TGF-β element in the distal promoter region of this gene [Ritzenthaler et al., 1993].

The TGF-β genes are auto-induced in human embryonic IMR-90 lung fibroblasts [Kelley et al., 1993]. To maintain homeostasis in vivo, fibroblasts and other cell types, which produce TGF-β1 must have a feedback mechanism to limit the fibrogenic response to TGF-β1 resulting from the autoinduction of the TGF-β1 gene by TGF-β1. The present study indicates a time-dependent increase of Smad 3 transcription factor binding to the SBE, which would cause an increased transactivation of the Smad 7 gene and more of the inhibitory Smad 7. This would cause a feedback mechanism for inhibition of the TGF-β1 by Smad 3 signaling, thus resulting in decreased COL1.

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