Bleomycin Stimulates Pro-α1(I) Collagen Promoter through Transforming Growth Factor β Response Element by Intracellular and Extracellular Signaling*

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Sandra L. King*, Alexander C. Lichtler†, David W. Rowe‡, Ronglin Xie‡, George L. Long‡, Marlene P. Absher*, and Kenneth R. Cutroneo‡

From the Departments of †Biochemistry and §Medicine, College of Medicine, University of Vermont, Burlington, Vermont 05405 and the $Departments of Pediatrics and Medicine, University of Connecticut Health Center, Farmington, Connecticut 06032

The role of transforming growth factor β as a mediator of the fibrogenic effect of bleomycin in lung has been investigated at the transcriptional level. Several constructs containing the rat pro-α1(I) collagen promoter fused to the chloramphenicol acetyltransferase gene were transfected into rat lung fibroblasts. Both bleomycin and transforming growth factor β1 increased promoter activity in fibroblasts transfected with constructs containing the transforming growth factor β response element. Fibroblasts transfected with a deletion construct that lacks this response element did not respond to either bleomycin or transforming growth factor β1. Anti-transforming growth factor β1-neutralizing antibodies did not block the increase in promoter activity induced by bleomycin, suggesting intracellular signaling. Mutation of the transforming growth factor β response element greatly reduced the bleomycin effect, which also interferes intracellular signaling. In addition, plasmin added to the media greatly enhanced bleomycin stimulation of promoter activity demonstrating that transforming growth factor β mediates the bleomycin effect through extracellular signaling.

Bleomycin is an antineoplastic agent used in the treatment of certain squamous cell carcinomas, testicular carcinomas, and malignant lymphomas (1-3). Although bleomycin has been shown to be beneficial in the treatment of these cancers without suppressing bone marrow function, pulmonary fibrosis is its most significant adverse effect (1-3). Insufficient bleomycin hydrolase activity in the lung may contribute to the development of bleomycin-induced pulmonary fibrosis (4).

Sequential morphological and biochemical changes in lung collagen metabolism in bleomycin-treated animals have been described extensively (5-10). In fibrotic lung, collagen content may be increased due to a selective increase in collagen synthesis in fibroblasts. Both bleomycin and transforming growth factor β1 increased collagen synthesis in fibroblasts (13-21). The increase in type I procollagen mRNAs in TGF-β1-treated fibroblasts is blocked by actinomycin D (14). It remains controversial as to whether cycloheximide inhibits (17, 18) or does not alter (14, 22) the effect of TGF-β1 on type I procollagen mRNAs in fibroblasts. Stability of the pro-α1(I) collagen mRNA may also be affected by TGF-β1 (18).

We have previously demonstrated that bleomycin treatment of rat lung fibroblasts increases TGF-β1 mRNA in addition to increasing TGF-β protein production (23). Increased levels of TGF-β mRNA have also been shown to increase prior to the increase of type I procollagen mRNA levels in bleomycin-induced fibrotic lung in vivo (24-26). TGF-β response elements have been identified in the pro-α1(I) and pro-α2(I) collagen genes (27, 28). Whereas both TGF-β response elements resemble nuclear factor 1 sites, the pro-α1(I) element also contains an activator protein 2 consensus site (27-29). Ritzenthaler et al. (29) have since shown that TGF-β1 stimulation of the rat pro-α1(I) collagen promoter through the TGF-β response element does not utilize nuclear factor 1 or activator protein 2 proteins. The present studies demonstrate that TGF-β is a mediator of the fibrogenic effect of bleomycin at the transcriptional level and that the TGF-β response element is required for bleomycin stimulation of the pro-α1(I) collagen promoter. The bleomycin regulation of procollagen gene expression is mediated at least in part through intracellular signaling.

MATERIALS AND METHODS

Plasmids—The ColCat 3.6, ColCat 2.4, and ColCat 0.9 plasmids were generously provided by Dr. D. Rowe. These plasmids contain 3.6 (-3521 to +115), 2.4 (-2,295 to +115), and 0.9 kilobases (-944 to +115) of the rat pro-α1(I) collagen gene fused to the chloramphenicol acetyltransferase (CAT) reporter gene (30, 31).

1 The abbreviations used are: TGF-β1, transforming growth factor β1; CAT, chloramphenicol acetyltransferase; HGH, human growth hormone; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; EMEM, Eagle’s minimum essential medium.
The M<sub>cc</sub>3.6 plasmid containing 3.6 kilobases of the rat pro-a(I) collagen gene with a site-specific mutated TGF-β response element was prepared by Dr. R. Xie following the method of Nelson and Long (32).

The pXGH5 plasmid was purchased from Nichols Institute Diagnostics, San Juan Capistrano, CA. The pXGH5 plasmid contains the mouse metallothionein I promoter and human growth hormone (HGH) sequences for the plasmid vector pUC12 (33). pXGH5 plasmid efficiency in cells transiently transfected with pXGH5 and one of the CAT plasmids was monitored by HGH secreted into the media.

The PSV2-neo plasmid, which confers resistance to gentamicin in stable transfectants, was also used (34).

**Other Materials**—Bleomyacin sulphate (Bleomaxene) was generously provided by Bristol-Myers, Evansville, IN. TGF-β1 isolated from porcine platelets was purchased from R&D Systems, Minneapolis, MN. Turkey anti-human TGF-β1-neutralizing antibody was purchased from Collaborative Biomedical Products/Benton Dickinson, Bedford, MA. Non-specific rabbit anti-chicken/turkey IgG (H&L) antibody was purchased from Zymed Laboratories, San Francisco, CA. Plasmid isolated from human plasma was purchased from Sigma.

**Cell Culture**—Adult rat lung fibroblasts (RL-90) were used for transient transfection experiments (35). Fetal rat lung fibroblasts (RFL-6) purchased from the American Type Culture Collection (Rockville, MD) were used for stable transfection experiments. Complete media consisted of Dubison's minimal essential media (Biowhittaker, Inc., Walkersville, MD) supplemented with 5% (v/v) fetal calf serum (Hyclone, UT). 87 units/ml penicillin, 87 µg/ml streptomycin, 254 µg/ml L-glutamine, and 0.19% (w/v) sodium bicarbonate (Life Technologies, Inc.). Aim V serum-free medium was purchased from Life Technologies, Inc.

**Transient Transfection**—Adult rat lung fibroblasts were transiently transfected with either 20 µg of ColCat 3.6 or M<sub>cc</sub>3.6 plasmid plus 0.5 µg of pXGH5 plasmid by a modified method of Graham and van der Eb (36, 37). The cells were grown to about 60% confluence. The cell culture media were changed 2-4 h before transfection. A DNA-calcium phosphate precipitate was prepared by first combining DNA, distilled water, and calcium chloride and then adding this mixture dropwise to 2 x BES-buffered saline solution while shaking. The DNA-calcium phosphate precipitate was allowed to stand at room temperature for 30-45 min before 1 ml of the precipitate was distributed over the cell surface. The cells were incubated at 37 °C for 4 h, shocked for 1 min in a 15% (v/v) glycerol solution, and washed with phosphate-buffered saline, and media were added. On the day after transfection, the media were changed, and the cells were then treated as described in the figure legends.

**Stable Transfection**—Fetal rat lung fibroblasts were stably transfected using 20 µg total of a 9:1 molar ratio of either ColCat 3.6, ColCat 2.4, or ColCat 0.9 plasmid to pSV2-neo plasmid following a modification of the procedure described by Chen and Okayama (38, 39). The cells were grown to 50% confluence. DNA, calcium chloride, and 2 x BES-buffered saline were combined. One milliliter of this mixture was distributed over the cell surface. The cells were incubated overnight at 37 °C in a 5% CO<sub>2</sub> incubator, during which time a DNA-calcium phosphate precipitate formed. Twenty-four hours after transfection, the cells were washed twice with phosphate-buffered saline and fed with complete media. Within 48 h, selection for stably transfected cells was begun using 200 µg/ml geneticin (Life Technologies, Inc.). During the 14-21-day selection period, the culture media was changed, and gentamicin was added every 4 days.

**Human Growth Hormone Assay**—The Allegro<sup>®</sup> HGH Transient Gene Expression Assay System was purchased from Nichols Institute Diagnostics and used as described by the manufacturer. The concentration of HGH in the media was determined by extrapolation from a standard curve for each assay.

**<sup>3</sup>H-CAT Assay**—CAT activity of transiently transfected cell extracts was determined using a modification of the <sup>3</sup>H-CAT assay described by Gorman et al. (40). The cells were placed on ice and washed 3 times with cold phosphate-buffered saline. Washed cells were incubated for 5 min on ice in TEN solution (40 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 150 mM NaCl) and then collected. The cell suspensions were microcentrifuged at 4 °C for 2-3 min. The cell pellets were suspended in 250 mM Tris-HCl, pH 7.8, and lysed using at least three freeze-thaw cycles. The lysates were microcentrifuged at 4 °C for at least 5 min to remove cellular debris. The supernatants were then assayed for protein concentration and CAT activity. During the enzymatic reaction, the <sup>3</sup>H-acetylated chloramphenicol diffusing into the liquid scintillation mixture was measured at different time intervals. Counts/min obtained from the linear portion of the curve were used to calculate counts/min/µg of protein.

**RESULTS**

**Bleomycin and TGF-β1 Increase Rat Pro-a(I) Collagen Promoter Activity**—Adult rat lung fibroblasts transiently cotransfected with ColCat 3.6 and pXGH5 plasmids. Fibroblasts were treated with either 1.5 µg/ml bleomycin in EMEM containing 8% fetal calf serum or 2.0 ng/ml TGF-β1 in EMEM containing 4% fetal calf serum for 48 h. Media samples were assayed for HGH activity, and cell extracts were assayed for CAT activity using the <sup>3</sup>H-CAT assay. Normalized CAT activity was calculated as (cpm acetylation/µg of protein/ng of HGH/ml of medium). Values represent the mean of two samples or the mean ± S.E. Values marked * are significantly different from the control at p < 0.05. Blank bars, values to be read off of the left-hand axis; striped bars, values to be read off of the right-hand axis. ▲, two single values.

**Stimulation of Rat Pro-a(I) Collagen Promoter by Bleomycin**—Adult rat lung fibroblasts stably cotransfected with ColCat 3.6 and pXGH5 plasmids. Fibroblasts were treated with 1.5 µg/ml bleomycin in EMEM containing 8% fetal calf serum for 48 h. Media samples were assayed for HGH activity, and cell extracts were assayed for CAT activity using the <sup>3</sup>H-CAT assay. Normalized CAT activity was calculated as (cpm acetylation/µg of protein/ng of HGH/ml of medium). Values represent the mean of two samples or the mean ± S.E. Values marked * are significantly different from the control at p < 0.05. Blank bars, values to be read off of the left-hand axis; striped bars, values to be read off of the right-hand axis. ▲, two single values.

**EXOGENOUS TGF-β, Antioxidants, and/or Other Compounds in Serum-Containing Media May Be Masking the Stimulation of the Collagen Promoter by Bleomycin**—The effect of TGF-β1, antioxidants, and/or other compounds in serum-containing media may be masking the stimulation of the collagen promoter by bleomycin. Additional transient transfection experiments were performed using various serum concentrations and serum-free media. Bleomycin (1.5 µg/ml) produced the following fold-increase in collagen promoter activity in EMEM containing 8% fetal calf serum, 4% fetal calf serum, 0.1% bovine serum albumin, or AIM V serum-free media respectively: 1.5, 1.4, 2.9, and 2.7 (Fig. 2). AIM V serum-free media were utilized in the remaining transfection experiments.

**Stimulation of Pro-a(I) Collagen Promoter by Bleomycin and/or TGF-β1 in Stably Transfected Rat Lung Fibroblasts**—Although the full-length rat pro-a(I) collagen promoter-CAT construct, ColCat 3.6, expressed sufficient CAT activity in transiently transfected fibroblasts, the two deletion constructs, ColCat 2.4 and ColCat 0.9, did not. It was necessary to use fetal rat

**FIG. 1.** Bleomycin and TGF-β1 increase rat pro-a(I) collagen promoter activity. Adult rat lung fibroblasts were transiently cotransfected with ColCat 3.6 and pXGH5 plasmids. Fibroblasts were treated with either 1.5 µg/ml bleomycin in EMEM containing 8% fetal calf serum or 2.0 ng/ml TGF-β1 in EMEM containing 4% fetal calf serum for 48 h. Media samples were assayed for HGH activity, and cell extracts were assayed for CAT activity using the <sup>3</sup>H-CAT assay. Normalized CAT activity was calculated as (cpm acetylation/µg of protein/ng of HGH/ml of medium). Values represent the mean of two samples or the mean ± S.E. Values marked * are significantly different from the control at p < 0.05. Blank bars, values to be read off of the left-hand axis; striped bars, values to be read off of the right-hand axis. ▲, two single values.
lung fibroblast cell lines stably cotransfected with either the ColCat 3.6, ColCat 2.4, or ColCat 0.9 plasmid with the pSV2-neo plasmid.

ColCat 3.6 stable transfecants responded to bleomycin and TGF-β1 in AIM V serum-free media. Bleomycin (1.5 μg/ml), TGF-β1 (5.0 ng/ml), and a combination of bleomycin and TGF-β1 increased collagen promoter activity by 3.2-, 3.5-, and 4.1-fold, respectively (Fig. 3). The combination of bleomycin and TGF-β1 did not greatly increase collagen promoter activity as compared with either treatment alone.

ColCat 3.6 and ColCat 2.4 stable transfecants treated with bleomycin (1.5 μg/ml) in AIM V serum-free media showed increased collagen promoter activity of 3.2- and 3.7-fold, respectively (Fig. 4). However, ColCat 0.9 stable transfecants did not respond to bleomycin treatment. Since each of the deletion constructs was a different stable cell line, the basal levels of CAT activity cannot be compared from cell line to cell line. ColCat 3.6 and ColCat 2.4 stable transfecants treated with TGF-β1 (5.0 ng/ml) in AIM V serum-free media demonstrated increased collagen promoter activity of 3.5- and 3.1-fold, respectively (Fig. 5). However, ColCat 0.9 stable transfecants did not respond to TGF-β1 treatment. ColCat 3.6 and ColCat 2.4 contain the TGF-β response element located approximately 1600 bases upstream from the transcription start site of the rat pro-α1(I) collagen promoter, whereas ColCat 0.9 does not. These data suggest that the TGF-β response element may be necessary for stimulation of the rat pro-α1(I) collagen promoter by bleomycin.

**Bleomycin Stimulation of Rat pro-α1(I) Collagen Promoter Is Mediated by Intracellular and Extracellular Signaling—**Adult rat lung fibroblasts were transiently cotransfected with ColCat 3.6 and pXGH5 plasmids. Fibroblasts were exposed to either bleomycin (1.5 μg/ml) or TGF-β1 (2.5 ng/ml) in AIM V serum-free media in either the absence of antibody, the presence of nonspecific antibody (20 μg/ml), or the presence of anti-TGF-β1-neutralizing antibody (20 μg/ml) (Fig. 6). Fibroblasts grown in the absence of antibody produced a 2.0- and 4.3-fold increase in collagen promoter activity for bleomycin and TGF-β1, respectively. Nonspecific antibody did not block the bleomycin or the TGF-β1 response, producing a 3.6- and 7.9-fold increase in collagen promoter activity, respectively. However, anti-TGF-β1 neutralizing antibody negated the TGF-β1 effect but did not inhibit the bleomycin effect, which indicates intracellular signaling. The TGF-β was added directly to the cell media at a specific concentration that would be neutralized by the TGF-β antibody. The neutralizing antibody, when added to the media, did not block the bleomycin response since bleomycin treatment of these cells results in the secretion of latent TGF-β. Since the TGF-β-neutralizing antibody did not abrogate the bleomycin effect on CAT activity when added to the cell media,
we propose that some of the latent TGF-β produced by bleomycin treatment is activated intracellularly, possibly by lysosomal enzymes (43, 44).

In another experiment designed to further investigate intracellular signaling, adult rat lung fibroblasts were transiently cotransfected with either the ColCat 3.6 or M₄CC3.6 plasmid plus the pXGH5 plasmid. ColCat 3.6 contains the TGF-β response element, TGCCCCAGCCCAAGGCG (the underlined bases are not actually contained in the wild-type sequence, as confirmed by Dr. D. Rowe). M₄CC3.6 contains the mutated TGF-β response element, TGTGGCGGCGGCCTGCG (bold-face letters indicate mutated bases). The mutations are based on the results of oligonucleotide competition experiments performed by Ritzenthaler et al. (28, 29). ColCat 3.6-transfected fibroblasts exposed to either bleomycin (1.5 µg/ml) or TGF-β1 (5.0 ng/ml) in AIM V serum-free media produced a 2.6- and 4.5-fold increase in collagen promoter activity, respectively. However, in fibroblasts transfected with the mutated plasmid M₄CC3.6, the bleomycin effect and the TGF-β1 effect were greatly reduced, as shown by 1.3- and 2.5-fold increases in collagen promoter activity (Fig. 7). Again different stably transfected cell lines were used for ColCat 3.6 and mutated ColCat 3.6. In light of the TGF-β-neutralizing antibody data (Fig. 6) and the data in Fig. 7, bleomycin appears to stimulate, at least in part, the rat pro-α1(I) collagen promoter through intracellular signaling. The bleomycin and TGF-β1 response may not be completely blocked in M₄CC3.6-transfected fibroblasts due to the presence of other TGF-β response elements within the 5'-flanking region of the pro-α1(I) collagen gene (28).

Adult rat lung fibroblasts were transiently cotransfected with ColCat 3.6 and pXGH5 plasmids. Fibroblasts were treated with bleomycin (1.5 µg/ml, 48 h) and/or plasmin (0.1 units/ml) for the last 24 h (Fig. 8). Plasmin has been shown to convert latent TGF-β to active TGF-β and may be a physiological activator of TGF-β under certain conditions (43, 45, 46). Collagen promoter activity was increased 2.3-, 2.1-, and 4.1-fold by bleomycin, plasmin, and a combination of bleomycin and plasmin, respectively. These data indicate that the large amount of TGF-β released into the media by rat lung fibroblasts in response to bleomycin may be activated by plasmin and in turn increase CAT activity by extracellular signaling.

A comparison cannot be made between the data in Figs. 3 and 8. The data in Fig. 3 are from stable transfectants, while the data in Fig. 8 are from transient transfectants. In addition, although bleomycin treatment of these cells increases the secretion of TGF-β by 4-fold, it is in the latent form and must be activated to be quantified in the mink cell growth inhibition assay (23).

**DISCUSSION**

The morphological and biochemical changes occurring during bleomycin-induced pulmonary fibrosis have been extensively studied. The molecular mechanisms involved in this complex process have yet to be elucidated. The synthesis and deposition of type I collagen and other extracellular matrix proteins occurs during the development of pulmonary fibrosis. Elevations of steady-state mRNA levels for pro-α1(I), pro-α2(I), and pro-α1(III) collagens; fibronectin; elastin; and TGF-β occur in vivo (24-26, 47-49) and in cultured lung fibroblasts (23) subsequent to bleomycin treatment. Molecular mechanisms responsible for the development of bleomycin-induced pulmonary fibrosis may involve transcriptional activation of extracellular matrix protein genes.

DNA-bleomycin interactions have been extensively studied. In complex with iron and activated oxygen (50), bleomycin produces single and double DNA strand breaks in purified DNA.
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(50–53), in cell culture (51, 54), and in lung (49). The observed DNA breakage occurs in a sequence-specific manner, GC > GT > GA > GG (50–53). DNA repair occurs by both short and long patch repair (55). Tertiary DNA structure has been shown to affect both DNA strand scission and repair (54, 56–58). Although the net level of DNA damage has been associated with sensitivity to bleomycin-induced pulmonary fibrosis, no link has been established between DNA damage and transcriptional activation of extracellular matrix protein genes (49).

We have investigated two mechanisms that may contribute to elevations of type I collagen mRNAs and collagen synthesis in fibroblasts exposed to bleomycin. Polysomal partitioning of type I procollagen mRNAs could account for an early increase in type I collagen synthesis in response to bleomycin during DNA breakage and repair (11, 12). The present studies demonstrate an increase in collagen gene transcription due to stimulation of the pro-a1(I) collagen promoter through a TGF-β response element that is mediated by intracellular and extracellular signaling.

Both bleomycin and TGF-β1 increase rat pro-a1(I) collagen promoter activity in transiently and stably transfected rat lung fibroblasts. Secreted antibodies added to the media, we were able to completely block TGF-β as a mediator of the fibrogenic effect of bleomycin at bleomycin treatment (23-26). We have previously shown in lung fibroblasts that bleomycin increases TGF-α mRNA and TGF-β protein production (23). We proposed that at this time TGF-β was a mediator of the fibrogenic effect of bleomycin at the transcriptional level, which the present transfection studies strongly support.

TGF-β response elements have been located in the pro-a2(I) and pro-a1(I) collagen promoters (27, 28). The TGF-β response element in the rat pro-a1(I) promoter is located approximately 1600 bases upstream from the transcriptional start site. We utilized ColCat 3.6 and two pro-a1(I) deletion constructs, ColCat 2.4 and ColCat 0.9, to study the effects of bleomycin and TGF-β1 on promoter activity in transfection studies. ColCat 3.6 and ColCat 2.4 contain the TGF-β response element, whereas ColCat 0.9 does not. In addition, we have prepared a plasmid containing the mutated TGF-β response element, M,CC3.6. We have demonstrated that the TGF-β response element is required for both bleomycin and TGF-β1 stimulation of the rat pro-a1(I) collagen promoter.

We have also demonstrated that intracellular and extracellular signaling mediates the bleomycin stimulation of the rat pro-a1(I) collagen promoter. Utilizing anti-TGF-β1 neutralizing antibodies added to the media, we were able to completely block stimulation of promoter activity by exogenous TGF-β1 (although we were unable to lessen the bleomycin-induced stimulation of promoter activity, suggesting that intracellular signaling may be involved). In addition, mutation of the TGF-β response element in the rat pro-a1(I) collagen promoter suggests that intracellular signaling may play a role in stimulation of the rat pro-a1(I) collagen promoter by bleomycin.

In contrast to human lung fibroblasts, rat lung fibroblasts have been shown to synthesize and secrete significant amounts of TGF-β (61). Since we have shown that bleomycin treatment stimulates TGF-β production by fibroblasts (29), a positive feedback loop could also increase procollagen mRNA transcription and collagen synthesis. The secreted TGF-β could be activated and interact with TGF-β receptors on the fibroblasts. Rat lung fibroblasts possess type I, II, and III TGF-β receptors, subsets of which appear to have a higher affinity for TGF-β1 than TGF-β2 (62).
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