Therapies for Bleomycin Induced Lung Fibrosis Through Regulation of TGF-β1 Induced Collagen Gene Expression

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This review describes normal and abnormal wound healing, the latter characterized by excessive fibrosis and scarring, which for lung can result in morbidity and sometimes mortality. The cells, the extracellular matrix (ECM) proteins, and the growth factors regulating the synthesis, degradation, and deposition of the ECM proteins will be discussed. Therapeutics with particular emphasis given to gene therapies and their effects on specific signaling pathways are described. Bleomycin (BM), a potent antineoplastic antibiotic increases TGF-β1 induction, TGF-β1 gene expression, and TGF-β1 protein.Like TGF-β1, BM acts through the same distal promoter cis-element of the COL1A1 gene causing increased COL1 synthesis and lung fibrosis. Lung fibroblasts exist as subpopulations with one subset predominately responding to fibrogenic stimuli which could be a specific cell therapeutic target for the onset and development of pulmonary fibrosis.

molecule after the removal of the N and C terminal globular regions.

Nuclear P-4-H is a distinct enzyme from the P-4-H located in the cisternae of the RER (Hofbauer et al., 2003; Marxsen et al., 2004). The nuclear enzyme’s function is to hydroxylate specific prolyl residue(s) of hypoxia inducible factor-1, to ready this enhancer factor for ubiquinization, and subsequent 26 S proteosome degradation (Huang et al., 1998).

The synthesis of the different proCOL1 polypeptides is controlled by two separate pathways, the TGF-β-activating protein signaling pathway and the SMAD signaling pathway (Fig. 1). When TGF-β1 reacts with cell membrane receptors, the TGF-β activator protein signaling pathway is activated and the COL1A1 gene is transcribed. The transcription of the COL1A2 gene is via the SMAD pathway. Methods are available for reducing fibrosis advanced by excess TGF-β1 accumulation. Glucocorticoids diminish the synthesis of proCOL1 without affecting the degradation of pro COL1A1 and pro COL1A2 mRNAs (Cutroneo and Sterling, 2004). Silencing the signal pathways responsible for procollagen gene expression is another possible method for reducing COL1 synthesis associated with fibrosis. Cutroneo and Ehrlich [2006] reported the use of dsdecoys containing the TGF-β element in the phosphorothioate (PT) sense strands 5’-ATCCTGGCTGCCCACCGCCACGGCC-3’ where the underlined base sequence represents the consensus TGF-β element, as a novel nonsteroidal antifibrotic to control fibrosis. Can dsdecoys alter the levels of proCOL1 and collagen synthesis? The hallmark of chronic fibrosis is increased extracellular matrix (ECM) protein synthesis by fibrotic fibroblasts continually stimulated by TGF-β1. The most fibrous collagen is COL1 which forms hypertrophic scar tissue and in the case of vital organs such as the heart, liver, or lung, leads to morbidity and in some patients death.

**Bleomycin-induced pulmonary fibrosis**

TGF-β1 the major profibrotic growth factor during fibrogenesis is produced by fibroblasts, myofibroblasts, recruited SM cells, and macrophages. This growth factor is stored in platelets. Bleomycin (BM), the antineoplastic antibiotic, treatment of lung fibroblasts results in an increased transcription of TGF-β1, an increase of TGF-β1 gene expression, and increased TGF-β protein (Breen et al., 1992; Fig. 1). BM was shown to act through the same distal cis-element of the COL1A1 gene as TGF-β1 by intracellular and extracellular signaling (King et al., 1994; Fig. 1). An autocrine mechanism maintains homeostasis for fibroblasts to limit the fibrogenic response to BM and TGF-β1 (Cutroneo, 2006); through intracellular signaling by decreasing the Smad 3 transcription factor binding to a distinct SMAD binding element in the proximal promoter of the Smad 7 inhibitory gene (Fig. 1). The early increase of total lung TGF-β1 (i.e., 7 days post-intratracheal BM) results from TGF-β1 production by alveolar macrophages, which generate latent TGF-β1 complexed with the glycoprotein thrombospondin (TSP-1) associated with CD36. This associated complex is necessary for plasmin release of active TGF-β1 (Yehualaeshet et al., 2000). Alveolar epithelial cell (AEC) apoptosis is a prime factor in pulmonary fibrosis. The onset of AEC apoptosis by BM requires
TGF-β, fibrosis, and coll synthesis

TGF-β is the major profibrotic growth factor which stimulates fibroblast collagen production and deposition. The three known isoforms of TGF-β stimulate cultured fibroblast procollagen synthesis but are differentially expressed in BM-induced murine pulmonary fibrosis (Coker et al., 1997). However, these studies only determined gene expression and not protein profiles.

The SMAD signaling pathway transmits the signal from the TGF-β receptor 1 on the cellular membrane to the nucleus through the cytoplasm (for review see Heldin et al., 1997; Fig. 1). Increased transcription of the COL1A2 gene by TGF-β1 is determined by the binding of a Smad 3/Smad 4 to the TβRE cis-element in the proximal promoter region of this gene (Zhang et al., 2000; Fig. 1). Sp1 also binds to this complex. Recently, it has been shown that TGF-β1 cytosolic signals rely on a Smad 3 but not a Smad 2 dependent pathway (Kim et al., 2005). In addition, the TGF-β1 signaling level depends on the Smad 3/Smad 2 ratio. A Smad 3/Smad 4 complex is required for Smad 7 gene transcription (Fig. 1) by TGF-β1 which codes for this major inhibitory Smad 7 (Von Gersdorff et al., 2000).

TGF-β1 induction of the COL1A2 gene depends on a 25 bp cis-element in the proximal promoter of this gene. This is an AP-1 site and this sequence is essential for the TGF-β response which may not require Sp1 binding sites on this gene (Chung et al., 1996). Another study demonstrated that Sp1-1 but not Ap-1 is required for the upregulation of COL1A2 gene transcription (Greenwell et al., 1997). Treatment of fibroblasts with TGF-β1 or BM, the latter which acts on fibroblasts through TGF-β1, increases collagen synthesis and the amount of TGF-β1 activator protein/TGF-β1 element complex in gel mobility shift assays. Glucocorticoids decrease collagen synthesis and the transacting protein/TGF-β element complex (Fig. 1; Shukla et al., 1999).

Gene therapy for BM-induced lung fibrosis

There exist many viral vectors coupled with certain genes which attenuate BM-induced pulmonary fibrosis. BM-induced murine lung receiving an intratracheal injection of a human adenovirus vector carrying mouse Smad 7 cDNA resulted in significant decreases of pro COL1 mRNA and lung hydroxyproline content with no morphological evidence of fibrosis as compared to mice given Smad 6 cDNA (Nakao et al., 1999). A single adenovirus-decorin transgene treatment of BM-induced murine lung, decreased the fibrotic response as determined by lung hydroxyproline as compared to control virus (Kolb et al., 2001). Murine BM-induced pulmonary fibrosis was reduced after intra-venous injection of prostaglandin D2 synthetase cDNA-expressing fibroblasts (Ando et al., 2003). This treatment increased basic fibroblast growth factor (VEGF) and the procollagen mRNAs. After the transfer of the flt-1 gene in vivo into mouse skeletal muscle, this tissue served as a biofactory for anti-vascular endothelial growth factor (VEGF) since flt-1 is a specific receptor for VEGF and soluble flt-1 binds to VEGF and competitively inhibits it from binding to its receptors. This therapeutic strategy was used to attenuate BM-induced pulmonary fibrosis (Hamada et al., 2005). When rats were given intratracheal BM plus adenoviral vector expressing the tissue factor pathway inhibitor (TFPI), there was a decreased BM-induced procoagulant and thrombin generation, inhibition of pulmonary fibrosis, and decreased CTGF gene expression (Kijiyama et al., 2006). The elucidation of molecular and signaling pathways in eukaryotic cells is often achieved by targeting regulatory element(s) found in the promoter or the enhancer region of eukaryotic gene(s) using a double-stranded (ds) oligodeoxynucleotide (ODN) containing a specific cis-element. Our laboratory is focusing on dsODN decoys containing the TGF-β1 element found in the distal promoter of the 5′-flanking region of the proCOL1A1 gene as a novel nonsteroidal antifibrotic for achieving normal wound healing. Dsdecoys have been useful in searching and unraveling molecular mechanisms of the initiation of disease development. The failure of a specific transcription factor to bind to the gene’s cis-element may totally knock out specific gene expression. Our dsdecoys have the ability to either silence or totally knockout gene transcription depending upon decoy dose (Cutroneo and Ehrlich, 2006). Knockout of specific mice genes is an in vivo genomic method to observe the physiological, biochemical, and the molecular effect(s) for eliminating the expression of a specific gene(s) and how it alters physiology. However, often it renders a lethal progeny. With SiRNA therapies a transfection agent is required. However, no transfection agent is needed in vivo when locally using the naked linearized PT dsdecoy containing the TGF-β1 element to inhibit COL1. Therefore, there are major advantages of using linearized PT dsoligo decoy gene therapy for either silencing or knocking out specific gene expression. First, the degree of silencing may be controlled by PT dsdecoy dose. Secondly, our present in vivo studies demonstrate that transfection methods were not required to obtain biochemical effects of these PT dsdecoys (Cutroneo and Chiu, 2000; Boros et al., 2005).

The central issue is that PT dsdecoys or other modified decoys presently in the pipeline which are more potent, have better delivery, and a longer duration of action could contain the
involved in TGF-β resulting in transcription inhibition. We are focusing on aspects inhibit pulmonary fibrogenesis? In the future studies, we will determine if you specifically inhibit TGF-β synthesis without inhibiting total noncollagen protein synthesis stranded (ss) and the PT dsdecoys specifically decrease COL1 synthesis during fibrosis without inhibiting total noncollagen protein synthesis (Cutroneo and Chiu, 2000; Cutroneo and Boros, 2002). In future studies, we will determine if you specifically inhibit TGF-β1-induced synthesis by using these PT dsdecoys, will this inhibit pulmonary fibrogenesis?

Protein kinase C epsilon (PKC-ε) and lung fibrosis
PKC-ε signaling protein is involved in lung fibrogenesis. Following microvascular injury in vivo chronic exposure to thrombin results in the conversion of fibroblasts to myofibroblasts with the activation of PKC-ε being required (Bogatkevich et al., 2001). Associated with these myofibroblasts which resemble the phenotype of scleroderma lung fibroblast, protein-activated receptor expression is increased (Bogatkevich et al., 2005). Scleroderma lung tissue is association with inflammatory and fibroproliferative foci. Thrombin increases tenacin in lung fibroblasts which is mediated by PKC-ε. In fibrotic systemic sclerotic (SS) lung fibroblasts, thrombin decreased PKC-ε mediated tenacin secretion (Tourkina et al., 2001). SS lung fibroblasts in culture overexpressed collagen, contained more activated MEK/ERK as compared to normal fibroblasts (Tourkina et al., 2005). This same study demonstrated that antisense to PKC-ε resulted in concomitant decreases of collagen and PKC-ε. The cis-PKC-ε element in the distal promoter of the PKC-ε gene has a sequence very homologous differing to TGF-β cis-element found in the distal promoter of the COL1A1 gene by only two bases (Cutroneo and Ehrlach, 2006). Will PKC-element dsdecoys be as effective or more so in silencing or knocking out COL1 synthesis and attenuating BM-induced pulmonary fibrosis? Or will TGF-β element containing dsdecoys silence or knockout PKC-εsilion synthesis?

Lung fibroblast heterogeneity
There are many cell types involved in BM-induced pulmonary fibrosis including alveolar macrophages, type II AECs, neutrophils, eosinophils, myofibroblasts, both resident and collagen synthesizing fibroblasts derived from bone marrow progenitor cells (Hashimoto et al., 2004). Normal lung fibroblasts exist as a heterogenous population of cells (Phippis, 1992) which can be separated and isolated by flow cytometry and cell sorting based upon the presence of the glycophosphatidylinositol-linked protein on the cell surface.

THY-1(+) and THY-1(−) lung fibroblast subpopulations (Phippis, 1992). The subpopulations when treated with BM were assessed for active TGF-β1, Smad 3 phosphorylation, alpha-SM actin, and fibronectin expression (Zhou et al., 2004). THY-1(−) fibroblasts responded to BM treatment by increases in all four parameters, while the THY-1(+) cells did not show any stimulating effects. THY-1(-)C57 BL mice treated with BM showed a greater fibrotic response both histopathologically, greater TGF-β1 activation and collagen content (Hagedorn et al., 2005). Breen et al. [1990] based on fibroblast cell surface proCOL1 or proCOL3 previously separated by flow cytometry and cell sorting the heterogeneous population of lung fibroblasts into subpopulations, one mainly expressing the proCOL1A1 and the COL1A2 genes and the other population mainly expressing the COL3A1 gene. Breen et al. [1992] later demonstrated that the total lung fibroblast population treated with BM before flow cytometry and cell sorting could be separated into one population which responded to BM with increased proCOL1A1 and proCOL1A2 mRNAs which lasted for at least three passages (Breen et al., 1992). Would this population of lung fibroblasts make an excellent specific cell target for dsdecoy attenuation of BM-induced fibroblast proCOL1 synthesis during BM-induced pulmonary fibrosis?

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