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# Therapeutic effect of a peptide inhibitor of TGF-β on pulmonary fibrosis

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ABSTRACT

Pulmonary fibrosis encompasses several respiratory diseases characterized by epithelial cell injury, inflammation and fibrosis. Transforming growth factor (TGF)-β1 is one of the main profibrogenic cytokines involved in the pathogenesis of lung fibrosis. It induces fibroblast differentiation into myofibroblasts, which produce high levels of collagen and concomitantly loss of lung elasticity and reduction of the respiratory function.

In the present study, we have investigated the effects of P17 (a TGF-β inhibitor peptide) on IMR-90 lung fibroblast differentiation in vitro, as well as on the inhibition of the development of bleomycin-induced pulmonary fibrosis in mice.

It was found that in IMR-90 cells, P17 inhibited TGF-β1-induced expression of connective tissue growth factor and α-smooth muscle actin. In vivo, treatment of mice with P17 2 days after bleomycin administration decreased lung fibrosis, areas of myofibroblast-like cells and lymphocyte infiltrate. P17 also reduced mRNA expression of collagen type I, fibronectin and the fibronectin splice isoform EDA in the lung, and increased the expression of IFN-γ mRNA. Finally, therapeutic treatment with P17 in mice with already established fibrosis was able to significantly attenuate the progression of lung fibrosis.

These results suggest that P17 may be useful in the treatment of pulmonary fibrosis.



## 1. Introduction

Pulmonary fibrosis (PF) is a progressive lung disorder characterized by accumulation of extracellular matrix (ECM) proteins [1]. Unfortunately, despite its high impact on human health, no effective treatment has been yet developed. Pathogenesis of PF appears to result from a complex interaction between inflammatory cells, fibroblasts and lung parenchymal cells. Inflammatory cells produce profibrotic cytokines which cause fibroblast transformation, proliferation and accumulation of ECM proteins, causing tissue destruction and loss of lung functions [2]. One of the most relevant profibrotic cytokines in PF is TGF-β1, which plays a key role in the synthesis and accumulation of collagen and fibronectin in the lungs [3]. This suggests that TGF-β inhibition would enhance the efficacy of currently used therapies [4]. Indeed, a protective effect on the development of lung fibrosis has been described in different animal models when using anti-TGF-β antibodies, decorin or TGF-β soluble receptors [5–8].

Experimental evidence demonstrates that fibroblasts play a critical role in the wound-healing process and in the development of lung fibrosis [9]. When fibroblasts become activated, they proliferate and may differentiate into myofibroblasts, displaying smooth muscle cell morphology. Different factors secreted by the pulmonary epithelium after damage, including TGF-β1, are involved in these processes inducing enhanced synthesis of ECM proteins, especially collagen and fibronectin [10]. In fact, many in vitro studies have revealed that TGF-β1 promotes myofibroblast differentiation, induces expression of α-SMA in lung fibroblasts [11] and enhances the synthesis of the ECM. Interestingly, extra-domain A of fibronectin (EDA-FN), an isoform of fibronectin, is necessary for the induction of the myofibroblast phenotype by TGF-β1 in fibroblast cells. This isoform is de novo expressed during wound healing and plays an essential role in PF [12]. In addition, areas of fibroblastic foci at sites where TGF-β1 is expressed, as well as active ECM products, have been observed in lung tissue sections from patients with idiopathic lung fibrosis [13].

In the present study, we have investigated the effects of P17, a peptide inhibitor of TGF-β1, on the differentiation of fibroblasts into myofibroblasts in vitro, and in vivo, on the development of pulmonary fibrosis in mice. For these experiments, we have used

*Abbreviations:* α-SMA, alpha-smooth muscle actin; CTGF, connective tissue growth factor; ECM, extracellular matrix; EDA-FN, extra domain A of fibronectin; IFN-γ, interferon gamma; PF, pulmonary fibrosis; TGF-β1, transforming growth factor-β1.

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a model based on the administration of bleomycin to mice, which induces pulmonary fibrosis associated to upregulation of TGFβ1, accumulation of collagen and α-SMA-expressing myofibroblasts, resembling thus pulmonary lesions observed in patients [14–18].

## 2. Materials and methods

### 2.1. Materials

TGF-β1 inhibitor peptide P17, developed in our laboratory [19], as well as control peptide P301, encompassing amino acids 301–315 of HIV-1 gp120, which is unable to bind to TGF-β1, were purchased from NeoMPS, Inc. (Strasbourg, France). Peptide purity was at least 98% per high-performance liquid chromatography and mass spectrometry. Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN).

### 2.2. Human lung fibroblast culture

IMR-90 human lung fibroblasts (ATCC, Manassas, VA) were cultured in EMEM medium (Cambrex, Belgium) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, UK). For experiments, $6 \times 10^4$ cells/well were cultured in 6-well plates in 2 ml of medium and when they were 80% confluent, medium was replaced with serum-free EMEM for 48 h to induce quiescence. TGF-β1 (5 ng/ml) and P17 (100 μg/ml) were co-incubated for 90 min at 37 °C to allow binding of both molecules before addition to cell cultures. Quiescent cells were then stimulated with TGF-β1 and/or P17 for up to 6 or 24 h. After these incubation periods, fibroblasts were harvested for RT-PCR and Western blot analysis, respectively.

### 2.3. Model of bleomycin-induced pulmonary fibrosis

Female C57BL/6 mice (6–8 weeks of age) were obtained from Harlan SL (Barcelona, Spain) and maintained in accordance with the ethical committee of Universidad de Navarra (Spain) for the experimental use of animals. Lung injury was induced with 0.08 units/mouse of bleomycin sulfate (Sigma, Spain) diluted in 200 μl of PBS. Briefly, the suspension of bleomycin was instilled by pipetting into the nose of mice previously anesthetized with 2% isoflurane vapor. PBS was instilled in mice as a negative control for PF, following the same procedures. Peptide treatment was started two days after bleomycin challenge and it was administered intraperitoneally in 500 μl of PBS on alternate days. Mice received high (75 μg/mouse) or low (25 μg/mouse) doses of P17, or the high dose (75 μg/mouse) of control peptide P301. Lungs were obtained 15 or 26 days after bleomycin administration to evaluate profibrogenic gene expression or histological damage, respectively. To study the therapeutic effect of P17 in the development of fibrosis, it was administered daily ten days after bleomycin challenge, once fibrosis was established. Twenty-six days after bleomycin instillation, mice were sacrificed and lung samples were fixed in order to evaluate histological damage.

### 2.4. Real time PCR analysis in IMR-90 fibroblasts and lung tissue

Total RNA was purified using Ultraspec® RNA (Biotecx; Houston, TX) according to the manufacturer's instructions from IMR-90 fibroblasts and whole lung samples obtained from mice treated during 14 days. In this last case, lungs were kept frozen at −80 °C before manual homogenization and RNA extraction. Then, 1 μg of RNA was incubated with DNase I (2 U/μl) (Gibco-BRL) for 30 min at 37 °C. Then, samples were reverse-transcribed with the M-MLV Reverse transcriptase (Gibco-BRL) in the presence of RNaseOUT (Gibco-BRL) with random hexamers according to the manufacturer's protocol. Expression of human connective tissue growth factor (CTGF) mRNA induced by TGF-β1 in IMR-90 fibroblasts was measured by real time RT-PCR using an ICycler and IQ SYBR Supermix (Bio-Rad, Italy). Actin was used as endogenous reference housekeeping gene. For in vivo analysis, expression of murine Fibronectin, EDA-FN, Collagen type I and IFN-γ was measured in lungs, using Histone 3 as internal control. Primers designed are listed in Table 1. The amount of each transcript was expressed by the formula: $2^{\Delta Ct}$ ($\Delta Ct$ = Ct (control gene) − Ct (gene)), being Ct the threshold cycle number at which an increase in the signal associated with exponential growth of PCR products begins to be detected using Bio-Rad analysis software.

### 2.5. Western blot analysis in IMR-90 cells

To assess αSMA and CTGF expression, IMR-90 fibroblasts were lysed with RIPA buffer containing 10 U/ml leupeptin, 10 U/ml pepstatin, 1 mM PMSF, 1 mM $Na_3VO_4$ and 1 mM NaF. Then, equal amounts of protein were electrophoresed through 10% SDS polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were analyzed for expression of αSMA protein using anti-αSMA monoclonal antibody (2.6 μg/ml; Sigma–Aldrich) and horseradish peroxidase-labeled anti-mouse IgG (1:5000 dilution; GE Healthcare). CTGF was detected using rabbit anti-CTGF antibody (0.5 μg/ml; Abcam). Expression of actin was detected using rabbit anti-actin antibody (0.6 μg/ml; Sigma–Aldrich). In both cases a horseradish peroxidase-labeled anti-rabbit IgG was used (1:5000 dilution; GE Healthcare). Bands were visualized using Lumi Light plus Western blotting detection system (Roche Diagnostic, Germany) and Hyperfilm ECL (Amersham Biosciences, UK).

### 2.6. Whole lung histological analysis

Lungs were fixed by inflation with 4% paraformaldehyde overnight and dehydrated in 70% ethanol. Tissue was embedded in paraffin wax, 3 μm thick sections were prepared and stained with Masson's trichrome to measure collagen deposition. Percentage of fibrosis in the lung was evaluated by counting the number of pixels corresponding with stained collagen areas from digital images, using the Adobe Photoshop CS3 program as described [20]. To do this, six to seven random photomicrograhs (4×) per animal were captured, which covered almost 80% of the whole lung tissue section in each animal.

### 2.7. Immunohistochemical analysis in lung tissue

Dewaxed and rehydrated lung tissue sections were subjected to endogenous peroxidase inactivation with a blocking reagent

**Table 1**
Primer sequences used for real time RT-PCR.

| Gene | Sense primer (5′–3′) | Antisense primer (5′–3′) |
|---|---|---|
| muCollagen type I | TTTGGAGAGAGCATGACCGA | TGCTGTAGGTGAAGCGACTGTT |
| muFN | CTATCTATGCTGTGGAGGAG | GAGTTTGGTGGTCTGTTGTG |
| muEDA-FN | ACATTGATCGCCCTAAAGGAACT | TGTGGACTGGATTCCAATCAGGGG |
| muIFNγ | TCAAGTGGCATAGATGTGGAAGAA | TGGCTCTGCAGGATTTTCATG |
| muHistone 3 | AAAGCCGCTCGCAAGAGTGCG | CTCCTGCAAAGCAC |
| huCTGF | TGATTAGAGCCAACTGCCTG | GGTATGTCTTCATGCTGGTG |
| huActin | AGCCTCGCCTTTGCCGA | CTGGTGCCTGGGGCG |

mu: murine; hu: human.