TGF-β antagonist attenuates fibrosis but not luminal narrowing in experimental tracheal stenosis

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Running title: TGF-β antagonist reduces tracheal fibrosis

Funding: This work was supported by the Fondo de Investigación Sanitaria, Instituto de Salud Carlos III (FIS PI12/00646 and PI12/439), co-financed by FEDER (European Regional Development Fund).

Conflict of Interest: None.

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Abstract

Introduction/Objective: Acquired tracheal stenosis (ATS) is an unusual disease often secondary to prolonged mechanical trauma. ATS pathogenesis involves inflammation and subsequent fibrosis with narrowing of the tracheal lumen. TGF-β represents a pivotal factor in most fibrotic processes and therefore, a potential target in this context. The aim of this study is to analyze the role of TGF-β as a target for anti-fibrotic interventions in tracheal stenosis.

Methods: Human stenotic tracheobronchial tissues from patients with benign airway stenosis and normal controls from pneumonectomy specimens were analyzed. Tracheal stenosis was induced in adult NZ rabbits by a circumferential thermal injury to the mucosa during open surgery and re-anastomosis. Rabbits were treated postoperatively with a peritracheal collagen sponge containing a TGF-β peptide antagonist (p17) or vehicle. Fibrosis was determined by Masson’s trichrome staining, and α-SMA⁺ myofibroblasts, CTGF and p-Smad2/3 expression by immunohistochemistry.

Results: Human and rabbit stenotic tissues showed extensive submucosal fibrosis, characterized by significantly increased α-SMA⁺ myofibroblasts and CTGF expression. In human stenotic lesions, increased p-Smad2/3⁺ nuclei were also observed. p17 treatment significantly reduced the fibrotic thickness as well as the density of α-SMA⁺ myofibroblasts and CTGF⁺ cells in rabbit stenotic lesions but failed to improve the luminal area.

Conclusion: ATS is characterized by a TGF-β dependent fibrotic process but reduction of the fibrotic component by TGF-β1 antagonist therapy was not sufficient to improve tracheal narrowing, suggesting that fibrosis may not be the main contributor to luminal stenosis.
Keywords: Tracheal stenosis, TGF-β antagonist, animal model

Level of Evidence: N/A

Introduction

Acquired tracheal stenosis (ATS) is an unusual disease which main causes are related to mechanical stress by endotracheal intubation or tracheostomy tubes. Burns, trauma, previous surgery, and infectious or autoimmune inflammatory diseases may also be responsible for ATS. Since the initial experience reported by McDonald and Stocks regarding extended endotracheal intubation as a way of ventilatory support in pre-term neonates, the incidence of airway stenosis in children has increased substantially. A long-standing cuffed endotracheal tube may cause mucosal damage and secondary scarring resulting in ATS. The histologic changes observed in the mucosa include inflammation, granulation tissue formation and fibrosis with subsequent narrowing of the tracheal lumen.

The type of treatment to be instituted in a patient with ATS depends on the severity, morphologic type of the lesion, and the etiologic factors. An obstruction of 50% or more of the tracheal lumen is definitely symptomatic and requires surgical or endoscopic therapy. In selected cases, tracheal resection of the stenosis with end to end anastomosis may offer a good and permanent result. Endoscopic procedures, such as balloon dilation, laser photoablation or stenting may be indicated in selected cases. Overall, treatment failure for both surgical and endoscopic treatment may vary between 9% and 43% in different studies, due to severe complications such as anastomotic dehiscence, re-stenosis, airway perforation, bleeding, or tracheo-esophageal fistula.

The role of medical therapy is less clear. Local treatments with corticoids or mitomycin C have been empirically tried with variable results. Therefore, the study of the specific factors involved in the development of granulation tissue and fibrosis...
causing ATS may help to identify potential therapeutic targets. Although the precise pathomechanism is not known, a general response to injury or mechanical stress to the respiratory epithelium involves selective release of transforming growth factor-β1 (TGF-β1)\(^1\), one of the key molecular factors involved in fibrogenesis\(^1,12\). Several cellular and molecular pathways such as M2 macrophage dysregulation or activation of the IL-17 axis may initiate the process leading to excessive fibroproliferative responses, an emerging target for intervention\(^13,14,15\).

TGF-β signals through specific receptors that trigger a specific intracellular signalling cascade, leading to phosphorylation and activation of SMAD proteins. pSmad2/3 form a heteromeric complex with Smad4 and translocate into nucleus where they act as specific transcription factors, initiating normal or pathological scarring responses\(^16,17\). Therefore pSMAD detection is a surrogate marker for TGF-β activation and response. TGF-β response involves differentiation of stromal cells into high extracellular matrix ECM-secreting myofibroblasts, characterized by smooth muscle cell α-actin (α-SMA) expression\(^18,19\). TGF-β also induces the expression of connective tissue growth factor (CTGF) gene, a cysteine-rich protein that further enhances the pro-fibrotic effects of TGF-β and that is expressed in most fibrotic conditions\(^20-22\). Therefore, TGF-β represents a potential therapeutic target in different fibrotic conditions. TGF-β antagonistic antibodies or peptides have shown anti-fibrotic activity in a variety of experimental models of fibrosis\(^23-27\).

Several animal models for ATS secondary to different types of trauma that reproduce the formation of a granulation tissue with subsequent fibrosis and stenosis have been described\(^28-30\). We aim to identify the participation of TGF-β signalling in the pathologic process of human and experimental ATS and to evaluate the capacity of a TGF-β peptide antagonist to reduce fibrosis and tracheal stenosis in an animal experimental model.
Materials and Methods

Ethics statement

All human and animal procedures were approved by Clinical Research Committee and Animal Care and Use Committee of Hospital 12 de Octubre with protocol reference number PI12/00646, and carried out in accordance with the institutional guidelines.

Patients

Human tracheobronchial tissues were obtained by excision of stenotic lesions during elective therapeutic intervention of 7 pediatric or young adult patients with benign ATS (67% male and mean±SD age 20±7 years [11-28 years old]). Normal tracheobronchial tissues were obtained from 9 pulmonary resection specimens indicated by non-tracheal pathology (78% male and mean±SD age 63±4 years [58-70 years old]).

Animal model

We developed a model of tracheal stenosis in adult New Zealand White rabbits (Granja cunicola San Bernardo, Navarra, Spain). Briefly, an incomplete transverse incision of the trachea was performed under anaesthesia, followed by a circumferential thermal injury to the mucosa with electro-cauterization and suture. At 4 weeks the tracheal segment was excised, fixed in formaldehyde 4% solution and paraffin embedded. In preliminary sequential studies from 2 to 12 weeks after the intervention, the maximal stenosis was observed in rabbits sacrificed at 4 weeks.

To determine the degree of stenosis, the luminal areas at the site of injury and at the first distal non-involved tracheal ring were photographed and the stenotic/normal luminal area ratio calculated using ImageJ software (http://rsb.info.nih.gov/ij).

For the intervention study, a total of forty rabbits were divided into two groups (saline and p17 treatment). In addition, a group of ten rabbits was used as control of the surgical procedure.
Histology and immunohistochemistry

Submucosal fibrosis was evaluated as the maximal thickening of the submucosal collagen stained tissue on Masson’s trichrome stained tracheal sections, which was measured using ImageJ software. Tracheas were also stained with Safranin O to assess biochemical and structural integrity of cartilage.

Immunohistochemistry (IHC) was performed on deparaffinised and rehydrated tissues, after microwave heating in 1mM EDTA pH8 for antigen retrieval. Immunoperoxidase labelling was performed with mouse monoclonal anti-α-SMA antibody (1A4 clone, Sigma-Aldrich Química, Madrid, Spain), rabbit polyclonal anti-pSmad2/3 antibody and goat polyclonal L20 anti-CTGF antibodies (Santa Cruz Biotechnology, CA, USA) and developed by diaminobenzidine chromogen (Vector Laboratories, Burlingame, CA, USA). Rabbit and human tissues were stained with α-SMA and CTGF antibodies whereas pSmad2/3 antibody could only specifically recognize pSmad2/3 in human tracheal sections.

Immunostained sections were visualized on a Zeiss Scope.A1 microscope (Zeiss, Jena, Germany), photographed and digitalized using an AxioCam ERc 5S camera and ZEN lite 2012 software. The number of α-SMA and CTGF positive cells per area were determined.

Local treatment with p17 TGF-β antagonist

To confirm the involvement of TGF-β in the rabbit experimental model of tracheal stenosis, a peritracheal collagen sponge containing a peptide antagonist of TGF-β p17 (2mg/ml) or control vehicle (0.9% saline) was sutured around the trachea after anastomosis. p17 is a soluble hydrophilic peptide derived from a phage display peptide library. It is capable of blocking the effects of TGF-β1 both in vitro and in vivo and has demonstrated anti-fibrotic activity across different species. Four weeks after the surgical procedure, rabbits were sacrificed and tracheas were excised and paraffin embedded for histological studies.
**Statistical analysis**

Statistical differences were analyzed using Prism software v6.0 (GraphPad Software, San Diego, CA, USA). Quantitative data were analyzed by Mann–Whitney U-test and correlation analysis by Spearman’s rank test. P values less than 0.05 were considered significant.

**Results**

**Human tracheal stenotic tissue show features of TGF-β driven fibrosis**

To analyze the histopathological changes in the tracheobronchial tissue of patients with benign airway stenosis, we compared stenotic specimens with normal control tracheobronchial tissues. Histological examination confirmed an extensive submucosal fibrotic component, with an enhanced collagen stained area, as well as a variable inflammatory cell infiltration and epithelial hyperplasia in all cases of tracheal stenosis (Fig. 1).

To obtain evidence of TGF-β participation in the human submucosal fibrotic lesions, we analyzed by IHC the expression of several surrogate markers of TGF-β signalling. We first compared the presence of nuclear phosphorylated Smad2/3 (p-Smad2/3) in stenotic versus normal tracheobronchial tissues. In normal control tracheas, p-Smad2/3 positive nuclei were rarely observed, whereas in stenotic tissues a significant increase of p-Smad2/3 positive nuclei was observed in both tracheal epithelial and submucosal cells (Fig. 1).

The expression of the TGF-β inducible pro-fibrotic factor CTGF was also significantly increased in stenotic compared to normal tracheobronchial tissues. Whereas normal trachea showed minimal or absent CTGF immunostaining, an important increase in the number of CTGF expressing cells, most with stromal cell shape, in the submucosal fibrotic connective tissue (Fig. 1).

Finally, the presence of α-SMA+ myofibroblasts was analyzed. In normal tracheobronchial tissues, only vascular and smooth muscle structures showed α-
SMA⁺ expression. In stenotic tissues, an abundant infiltrate by α-SMA⁺ cells with stromal shape consistent with myofibroblasts was observed in the submucosal fibrotic area (Fig. 1).

**The rabbit model of tracheal stenosis reproduces the human fibrotic process**

Rabbit tracheal tissues undergoing surgical procedure showed a progressive luminal stenosis from 2 to 4 weeks that tended to spontaneously decrease at 12 weeks (Supp. Fig. S1). Tracheal stenosis was histologically characterized by a prominent fibrotic response in the submucosa, with a significant thickened collagen stained area by Masson’s trichrome (Fig. 2). Safranin O staining showed cartilage damage in stenotic areas, as patchy areas of reduced cartilage thickness and weaker proteoglycan staining (Supp. Fig. S2). In addition, epithelial hyperplasia and an abundant inflammatory infiltrate, mainly composed by mononuclear and mast cells, were evident in the stenotic lesion (Fig. 2). A significant increase in the number of α-SMA⁺ myofibroblasts and CTGF⁺ cells per area were also observed in stromal cells of stenotic tracheal tissues compared to control group (Fig. 2).

**Effect of TGF-β antagonist on experimental tracheal stenosis**

To evaluate the potential contribution of TGF-β to the development of submucosal fibrosis and tracheal stenosis, rabbits were treated with peri-tracheal collagen sponges containing the TGF-β inhibitor p17 peptide or vehicle control. Thirty four of 40 rabbits were analyzed at 4 weeks. The rest were excluded due to early mortality or signs of local infection. To quantify fibrosis, we measured the maximal thickness of the submucosal collagen stained area which was significantly reduced in p17 treated compared to saline treated group (Fig. 3). The densities of α-SMA⁺ myofibroblasts and CTGF⁺ cells per mm² were significantly reduced by p17 treatment (Fig. 3). Focal areas of reduced proteoglycan staining, and reduced cartilage thickness were similarly observed in p17 and control group (Supp. Fig. S2).
The degree of luminal stenosis was evaluated in both groups of rabbits as the stenotic/normal luminal area ratio. Despite the observed reduction in fibrosis, we did not observe a significant increase in the luminal area in p17 compared to saline-treated group (Fig. 4A and 4B). Since this data suggested that stenosis did not improve as much as the fibrotic component, we analysed the correlation between both variables, the collagen thickness and the luminal area. Although there was a trend to negative correlation between the luminal area and collagen thickness, the correlation coefficient was low (r=0.29) and non-significant (Fig. 4C).

Discussion

The investigation of the mediators involved in the development of the granulation tissue and fibrotic response responsible for the development of ATS is relevant to the identification of targets of pharmacological therapies. Non-specific anti-inflammatory strategies such as glucocorticoids have shown a limited therapeutic impact\cite{5,37} and therefore, more specific therapeutic targets are needed. However, obtaining mechanistic information is being limited by human tissue availability and data from appropriate animal models are also scarce.

Previous studies have identified an important submucosal fibrotic component in stenotic lesions, that may be the end-result of the activation of specific inflammatory pathways\cite{13,14,15}. However, the pathogenic relevance of common pro-fibrotic mediators such as the TGF-\(\beta\) pro-fibrotic pathway had not been addressed. Increased expression of TGF-\(\beta\) and VEGF in stenotic lesions has been described\cite{38-40}. TGF-\(\beta\) could be directly induced by mechanical pressure on the respiratory epithelium as suggested by data in cultured bronchial epithelial cells providing a link between mechanical stress and the inflammatory and stenotic response\cite{38}.

Our data confirm that there is an important fibrotic component in tracheal stenotic lesions where all surrogate markers of TGF-\(\beta\) signaling are present (p-Smad, CTGF
and α-SMA). In addition, these features were reproduced in the animal model of tracheal stenosis. After the surgical procedure, rabbit tracheal tissues showed marked submucosal fibrosis accompanied by an increase CTGF expression and α-SMA+ myofibroblasts. Therefore, this animal model provides a valid preclinical to test the impact of TGF-β antagonist.

We used a peptide antagonist of TGF-β that has demonstrated its ability to reduce fibrosis across different species, by interfering the binding of TGF-β to its receptors in vivo. p17 has shown potent anti-fibrotic effects by interfering TGFβ1 and β2 activity in a variety of animal models, either by systemic or local application\textsuperscript{24,26,27,34-36}. Although local application by direct surgical implantation of collagen sponges containing p17 is not a feasible strategy to treat human stenosis, it guaranteed inhibitor availability in this proof of concept study. Since the release of active peptide to tracheal tissue is difficult to determine, a large excess of p17 was empirically used, considering data from previous local and systemic studies. We assume efficient delivery because it resulted into significant reduction of fibrosis but perhaps higher concentrations could have a higher impact on tracheal stenosis.

Our data provide consistent evidence on the role of TGF-β in the pathogenesis of tracheal fibrosis. p17 showed significant anti-fibrotic effects associated with a decreased expression of α-SMA and CTGF, two processes directly induced by TGF-β. However, the significantly reduced accumulation of fibrotic tissue in tracheal submucosa was not accompanied by an improvement in the luminal tracheal stenosis. This suggests that other components such as inflammation, epithelial hyperplasia, or structural changes such as cartilage damage might contribute to the reduced tracheal lumen and remain unmodified despite anti-TGF-β effects on fibrosis. Indeed, we confirmed a poor and non-significant correlation between the degree of fibrosis and the tracheal luminal area in the rabbit experimental model.
Conclusions

In summary, our data show evidence of the relevance of TGF-β in the pathogenesis of fibrosis in a human and an animal model of ATS but also an insufficient therapeutic effect of a TGF-β blocking strategy, suggesting that the fibrosis is only a partial contributor to the reduction of the tracheal lumen in ATS.

Acknowledgements

We thank Vanessa Miranda for excellent technical assistance and Mª Dolores Blanco for help with study design. This work was supported by the Fondo de Investigación Sanitaria, Instituto de Salud Carlos III (FIS PI12/00646 and PI12/439), co-financed by FEDER (European Regional Development Fund).

Authors’ contributions

†JLAP and AU have equally contributed and share first authorship; JLAP, AU and JLP planned the project, analyzed the data, interpreted the results, performed statistical analysis and wrote the manuscript; JLAP, AU, IM, CMGH, MG and APG performed the experimental work; JLRP contributed to tissues processing and analysis; AMM contributed new reagents; all authors reviewed, revised and approved the manuscript for submission.
References


Figure legends

Fig. 1. Histopathological and immunohistochemical evaluation of human tracheal tissues. Representative images of Masson’s trichrome and quantitative analysis of p-Smad2/3, CTGF and α-SMA staining in control and stenotic tracheal tissues (Mean±SEM; *p<0.05, **p<0.01, ***p<0.001). Bar 100 µm in Masson's and α-SMA panels and 50 µm in p-Smad2/3 and CTGF panels.

Fig. 2. Histopathological and immunohistochemical evaluation of experimental airway stenosis model in rabbits. Representative images of rabbit control and stenotic trachea at 4 weeks after thermal injury with 25x magnification and insets with 200x magnification (Masson’s trichrome). Immunolabeling of α-SMA and CTGF positive cells in control and stenotic tracheal rings in an animal model of airway stenosis. Quantification of tracheal thickening and α-SMA and CTGF positive cells per area (Data shown are mean±SEM; **p<0.01). Bars 400 µm (25x Masson’s panels) and 50 µm (Masson’s insets, α-SMA and CTGF staining).

Fig. 3. Effect of p17 treatment on submucosal fibrosis and myofibroblasts and CTGF expression in rabbit experimental model. Rabbits were intraoperatively treated with p17 TGF-β antagonist or saline. Tracheal fibrosis was determined as the collagen area stained by Masson’s trichrome, and by the direct measure of tracheal thickening (upper panel). Representative images and quantification of α-SMA (middle panel) and CTGF (lower panel) positive cells per area. Bar 100 µm, insets bar 25 µm. (Data shown are mean±SEM; **p<0.01, ***p<0.001).

Fig. 4. Luminal stenosis in p17-treated rabbits. Representative images of saline-treated and p17-treated tracheas (A). Cross-section of control (right) and stenotic (left) trachea. Quantitative analysis of tracheal luminal area in saline or p17 treated
rabbits (B). Correlation analysis between the luminal area and collagen thickening (C).

**Supp. Fig. S1. Macroscopic analysis of rabbit tracheal sections undergoing surgical procedure.** Images show the area of maximal luminal stenosis (left rings) compared with the first distal uninvolved ring (right rings), at the indicated time points after surgery.

**Supp. Fig. S2. Evaluation of cartilage structure and proteoglycan staining in rabbit tracheal tissues.** Representative images of cartilage damage determined by safranina O stained area in control (left), saline (middle) and p17-treated rabbits (right) are shown. Bar 100 µm.