Eur Respir J, 1994, 7, 515–518
DOI: 10.1183/09031936.94.07030515
Printed in UK - all rights reserved

European Respiratory Journal
ISSN 0903 - 1936

RAPID COMMUNICATION

Treatment by human recombinant soluble TNF receptor of pulmonary fibrosis induced by bleomycin or silica in mice

P.F. Piguet, C. Vesin

Treatment by human recombinant soluble TNF receptor of pulmonary fibrosis induced by bleomycin or silica in mice. P.F. Piguet, C. Vesin. ©ERS Journals Ltd 1994.

ABSTRACT: Intratracheal instillation of bleomycin (0.08 U) or silica (2 mg) to mice leads, after 15 days, to a patchy pulmonary fibrosis associated with a significant increase of the lung hydroxyproline. Since tumour necrosis factor (TNF) seems to be an important mediator in pulmonary fibrosis, we wondered whether this fibrosis might be prevented by a new TNF-α antagonist.

Infusion of a 55 kD human recombinant soluble TNF receptor rsTNFR-β, at a rate of 20 μg·day^{-1}, prevented the bleomycin/silica induced increase of lung hydroxyproline content, as measured 15 days after instillation. Infusion of rsTNFR-β was also effective in the treatment of an established fibrosis, *i.e.* administered 25 or more days after instillation of bleomycin or silica, since it reduced lung collagen content. Recombinant soluble TNFR-β had no significant influence on the number of cells, mostly macrophages, recovered by bronchoalveolar lavage. The examination of histological sections indicated that the rsTNFR-β reduced the proportion of areas of damaged lung and, in silicosis, the formation of nodules with a rich collagen content.

This study suggests that rsTNFR-β might be useful in the therapy of pulmonary fibrosis.

Eur Respir J., 1994, 7, 515–518.

Dept of Pathology, Hôpital Cantonal Universitaire de Genève, Geneva, Switzerland.

Correspondence: P.F. Piguet
Dept of Pathology
Hôpital Cantonal Universitaire de Geneve
1 rue M. Servet
1211 Geneva 4
Switzerland

Keywords: Bleomycin
pulmonary fibrosis
silica
tumour necrosis factor
tumour necrosis factor receptor

Received: September 15 1993
Accepted after revision November 5 1993

This work is supported by grant no. 31-28855.91 from the Swiss National Science Foundation.

Pulmonary fibroses are diseases characterized by an increase of the proteins of the extracellular matrix within the alveolar septa, notably the collagens, generally associated with the growth of fibroblast-like interstitial cells [1]. The growth and collagen secretion of interstitial cells is modulated by several cytokines, one of these being tumour necrosis factor-α (TNF-α) (as reviewed in [2, 3]). In pulmonary fibrosis, TNF-α is apparently an important mediator, since bleomycin or silica-induced pulmonary fibroses in mice are associated with a marked up-regulation of the TNF-α messenger ribonucleic acid (mRNA) levels, and since these diseases can be prevented by anti-TNF-α antibodies [4, 5].

Recently, a new generation of TNF antagonists has been derived from the extracellular domain of the TNF receptors (TNFR). Two types of TNF receptors have been described in either mouse or human, a 55 and a 75 kD receptor (also called TNFR-β and TNFR-α respectively), which are variably expressed in different cell types (as reviewed in [6]). Both are capable of binding TNF-α and of blocking the effects of TNF-α *in vitro* [7]. These antagonists are also effective *in vivo*, since, in animal models, they influence the course of diseases in which TNF-α is an important mediator, such as lipopolysaccharide(LPS)- induced mortality [8], or collagen arthritis [9].

In this work, we treated two types of pulmonary fibrosis, elicited in mice by the intratracheal instillation of bleomycin or silica, with an infusion of human recombinant soluble TNFR-β (rsTNFR-β). This treatment was effective not only in preventing the development of pulmonary fibrosis but also influencing an established fibrosis.

Methods

Mice

CBA/Ca and C57BL/10 (B10) mice were purchased from OLAC Ltd (Blackthorn, UK) and bred for 3–4 generations in our animal facilities. F1 hybrids were raised in the animal house and experiments were performed with 3–4 month old male CBA×C57BL10 F1 mice, weighing 25–29 g.

Bleomycin and silica administration

Bleomycin (Lundbeck, AVS, Copenhagen, Denmark), dissolved in Hank's balanced salt solution (HBSS) and 0.08 U in 0.1 ml, was injected intratracheally in nembutal anaesthetized mice. This procedure induced some morbidity, manifested by a loss in body weight and mortality on day 15 after instillation, ranging between 0–5%

in different experiments [4]. Silica particles (DQ 12, size <5 μm, were a kind gift from M. Reisner, Steinkohlenbergbauverein, Essen, FRG). About 2 mg silica in 0.1 ml were injected intratracheally. This treatment did not induce detectable loss of body weight or mortality.

rsTNFR-β

The complementary deoxyribonucleic acid (cDNA) encoding the extracellular domains of the human TNFR-β were amplified by the polymerase chain reaction. The cDNA was expressed in chinese hamster ovarian (CHO) cells and the protein was purified by affinity chromatography [7]. Recombinant soluble TNR-β (55 kD) or its excipient was introduced into Alzet osmotic minipumps (No. 2001 or 2002, Palo Alto, Ca, USA) and these pumps were implanted *i.p.*

Microscopy

The lungs were fixed by the intratracheal administration of glutaraldehyde (2% in 0.1 M cacodylate buffer, pH 7.4). Paraffin embedded sections were stained with haematoxylin and eosin (H & E) and silver staining (Gomori). Four sections across the hilus of different lobes for each individual mouse were examined and the proportion of damaged parenchyma was determined by point counting.

Lung hydroxyproline content

Determination of lung hydroxyproline content was performed according to established procedures [10]. In brief, the lungs were submitted to an acid hydrolysis and the hydrolysates were neutralized and extracted with phenol-chloroform-isoamyl-alcohol to clarify the aqueous phase. Hydroxyproline concentration was determined colorimetrically [10].

Lung bronchoalveolar lavage (BAL) cells

About 2 ml of saline was instilled within the trachea using a 25 cm hydrostatic pressure and the fluid was recovered by gravity. The suspension was centrifuged to recover the BAL cells.

Statistical evaluation

Means were compared using a non-parametric Mann Whitney U-test [11].

Results

Effect of rsTNFR-β infusion on the lung hydroxyproline content

After bleomycin or silica instillation, mice were treated with a continuous rsTNFR-β infusion. In both models this infusion decreased the lung collagen content, as detected on day 14 after instillation (table 1). These results are comparable to those obtained previously with an injection of a rabbit anti mouse TNF-α immunoglobulin G (IgG), at a dosage of 2 mg every 5–7 days [4, 5].

Table 1. – rsTNFR-β prevent silicosis or bleomycin-induced pulmonary fibrosis

Instillation	Treatment	BAL cells $\times 10^4$	H-proline μg/lung
None	None	2 (1)	79 (8)
Silica	Excipient	6 (1)	102 (8)
Silica	rsTNFR-β	7 (3)	83 (8)*
Bleomycin	Excipient	12 (4)	157 (18)
Bleomycin	rsTNFR-β	15 (5)	109 (24)*

Data are presented as mean (±SD) of the values obtained on day 14 after bleomycin or silica instillation in groups of 8–10 mice. Antagonist or its excipient was administered by an osmotic mini-pump, at a rate of 20 μg·day during days 7–14 after instillation. *: sig. different from the excipient treated group ($p<0.001$). rsTNFR-β: recombinant soluble tumour necrosis factor receptor-β; BAL: bronchoalveolar lavage; H-proline: hydroxyproline.

Following instillation of bleomycin or silica, the lung collagen content increased between day 5–10 and, subsequently, remained stable at a higher level for several weeks. We, therefore, explored whether the treatment was effective not only in preventing the accumulation of collagen but also in influencing an established fibrosis. As seen in Table 2, the rsTNFR-β was capable of decreasing the lung collagen content when given 25 or more days after instillation (table 2).

Effect of rsTNFR-β on the BAL cells

This treatment had little influence on the alveolar exudate, since the number of BAL cells was only slightly modified (table 1). No significant change could be detected in treated mice in the percentage of macrophages, lymphocytes and polymorphonuclear leucocytes in the BAL cells.

Table 2. – Treatment of established pulmonary fibrosis with rsTNFR-β

Instillation	Infusion μg·lung^{-1}	H-proline
None	None	88 (12)
Bleomycin	Excipient	121 (22)
Bleomycin	rsTNFR-β	110 (19)*
Silica	Excipient	126 (16)
Silica	rsTNFR-β	92 (14)*

Data are presented as the mean (±SD) of the values obtained in groups of six mice. Bleomycin-instilled mice were treated on days 25–32, and those with silica during days 30–37 with rsTNFR-β at a rate of 20 μg·day^{-1} and sacrificed at the end of the experiment. *: sig. different from the excipient treated group ($p<0.001$). For abbreviations see legend to table 1.

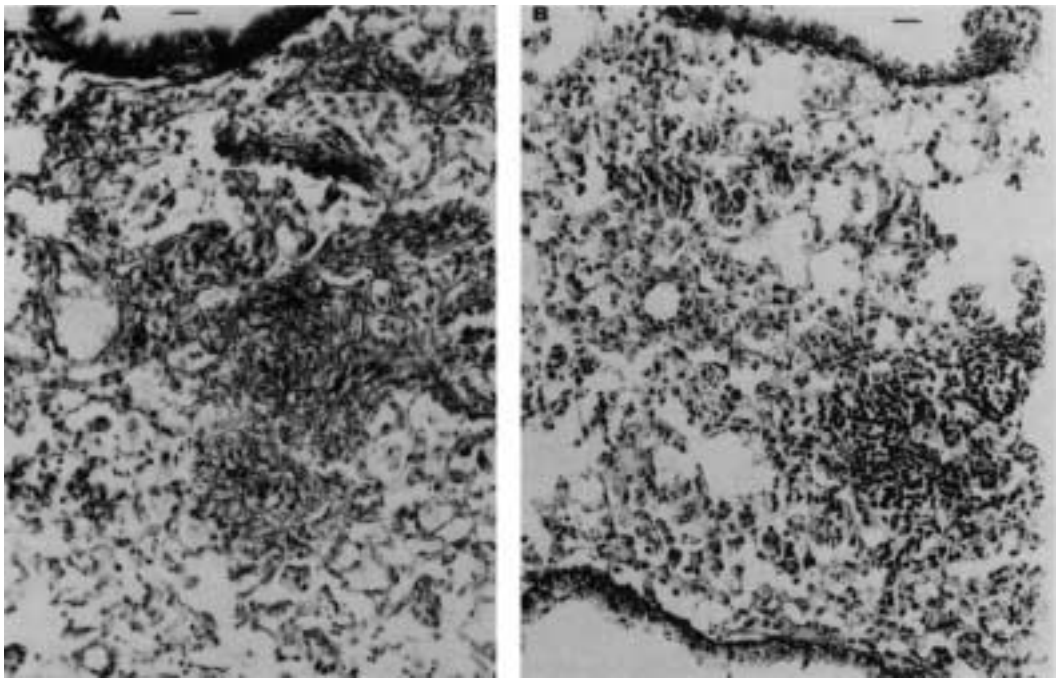

Fig. 1. – Silica-induced alveolitis in mice treated with the excipient (A) or the rsTNFR-β (B) A). In the vicinity of a bronchus (top), the alveolitis lead to a condensation of the parenchyma, rich in fibrils B) There is only alveolitis, without organization and fibrils. Recombinant solubleTNFR-β or its solvent were administered at 55 μg·day^{-1} on day 30–37 after silica instillation. rsTNFR-β: recombinant soluble tumour necrosis factor receptor. (Silver staining; scale bar = 60 μm).

Histological evaluation

Silicosis. Instillation of silica leads, as seen on day 15 thereafter, to a plurifocal alveolitis with some degree of nodular organization which predominates along the bronchioles, as shown in our earlier work [12]. In lungs from mice treated as those in table 1, the percentage area showing various degree of alveolitis was reduced by a 7 day treatment with rsTNFR at 20 μg·day^{-1}, from 28 (14)% to 18 (10)% (mean±SD) for five mice treated with the excipient or the rsTNF, respectively. Nodules were poorly formed and less dense in rsTNFR treated mice. In an experiment performed with an infusion of 55 μg·day^{-1} over 7 days, the alveolitis was still present but no silicotic nodule could be detected in the lungs from three treated mice (fig.1).

Bleomycin. Intratracheal instillation of bleomycin leads to a plurifocal fibrosing alveolitis, which predominates in the subpleural regions. In five mice treated with rsTNFR-β at 20 μg·day^{-1} (as shown in table 1), the area showing alterations of various degrees of severity were moderately reduced by the treatment, from 28 (23)% to 24 (16)% for excipient or rsTNFR treated mice, respectively. The alveolitis probably resulted in less remodelling and less fibrogenesis in mice treated with rsTNFR, compared to controls, although this could not be evaluated quantitatively.

Discussion

The present results demonstrate that the rsTNFR-β can effectively prevent silica or bleomycin-induced pulmonary fibrosis, as observed previously with anti-TNF-α antibodies [4, 9]. They further extend these observations by showing that the capacity of TNF antagonists to decrease the lung collagen content is not limited to a peculiar phase of these diseases.

In vivo, the capacity to inactivate TNF-α rests on the formation of a TNF-α/rsTNFR complex, which inhibits the binding of TNF-α to its cellular receptors and eventually promotes the clearance and degradation of the complex. The rsTNFR-β, being of a relatively small size, is rapidly eliminated by the kidney, when injected as a bolus dose. To obviate this drawback, a fusion protein, made of the extracellular domain of the rsTNFR 55 kD fused to human gamma-3 IgG was prepared and was successfully used to prevent LPS-induced toxicity by a single injection [8]. Pulmonary fibroses are subacute or chronic diseases for which one week seems to be the minimal time of treatment, with regard to the slow turnover rate of the lung collagen [13]. Therefore, to ensure a permanent presence of the antagonist, we used a cotinuous infusion, rather than repeated injections. In these conditions, an infusion of the rsTNFR-β, at 20 μg·day^{-1}, induced a significant reduction of the lung hydroxyproline and was of similar efficacy to the rsTNFR 55-human gamma-3 IgG fusion protein (not shown). Since the fusion protein presents the risk of being more immunogenic in mouse and, therefore, more rapidly eliminated, the present study was performed mainly with the rsTNFR-β. A dosage of 20 μg·day^{-1} of the rsTNFR-β is certainly an effective dose, but may not be optimal. Indeed, in a group of silicotic mice treated with 55 μg·day^{-1}, the influence on the size of the nodules was apparently more pronounced (fig. 1).

TNF-α is recognized today as a major mediator of inflammatory reactions and one might, therefore, suspect that in diseases where inflammation and collagen deposition are closely associated, it acts by decreasing the alveolar inflammation. However, TNF-α antagonists did not significantly decrease the alveolar exudate (table 1), and the histological study as well as the evaluation of the lung hydroxyproline content suggest that they decrease the lung collagen deposition more than the alveolitis. This possibility is strongly supported by the observation that TNF-α antagonists also decrease the lung collagen content in normal mice, *i.e.* act independently of inflammation (unpublished observation).

It is likely that the accumulation of collagen within the alveolar septa contributes to the respiratory deficiency of pulmonary fibrosis, which responds poorly to current therapy. The present experiments indicate that the treatment of these diseases with a TNF-α antagonist might be helpful, whatever the stage of the disease. Anti-TNF antibodies are certainly effective antagonists, but their useful period of administration is seriously limited by the immunogenicity of mouse monoclonal antibodies (MoAbs) when injected into humans [14]. Thus, rsTNFR-β, being of human origin and, therefore, non-immunogenic, may possibly provide a new and promising therapy for pulmonary fibrosis.

Acknowledgement:: The authors are grateful to W Lesslauer and co-workers in F. Hoffmann-Laroche Ltd, Basle, Switzerland, for their gift of rsTNFR and to G. Leyvraz, P. Henchoz, J Stalder and Le Minh Tri for the preparation of the histological sections.

References

1. Colby TV, Churg AC. Patterns of pulmonary fibrosis. *Pathol Ann* 1986; 21: 277–309.
2. Kovacs EJ. Fibrogenic cytokines: the role of immune mediators in the development of scar tissue. *Immunol Today* 1991; 12: 17–23.
3. Piguet PF. Cytokines involved in pulmonary fibrosis. *Int Rev Exp Pathol* 1993; 34b: 173–181.
4. Piguet PF, Collart MA, Grau GE, Kapanci Y, Vassalli P. Tumor necrosis factor/cachectin plays a key role in bleomycin-induced pneumopathy and fibrosis. *J Exp Med* 1989; 170: 655–663.
5. Piguet PF, Collart MA, Grau GE, Sappino AP, Vassalli P. Requirement of tumor necrosis factor for development of silica-induced pulmonary fibrosis. *Nature* 1990; 344: 245–247.
6. Loetscher H, Steinmetz M, Lesslauer W. Tumor necrosis factor: receptors and inhibitors. *Cancer Cells* 1991; 3: 221–226.
7. Loetscher HR, Gentz R, Zulauf M, *et al*. Recombinant 55 kDa tumor necrosis factor (TNF) receptor. Stoichio metry of binding to TNF-alpha and TNF-beta and inhibition of TNF activity. *J Biol Chem* 1991; 266: 18324–18329.
8. Lesslauer W, Tabuchi H, Gentz R, *et al*. Recombinant soluble TNF receptor proteins protect mice from LPS-induced lethality. *Eur J Immunol* 1991; 21: 2883–2886.
9. Piguet PF, Grau GE, Vesin C, *et al*. Evolution of the collagen arthritis in mice is arrested by treatment with anti-tumour necrosis factor (TNF) antibody or a recombinant soluble TNF receptor. *Immunology* 1993; 77: 510–514.
10. Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. *Arch Biochem Biophys* 1961; 93: 440–447.
11. Mann HB, Whitney DR. On a test of whether one or two random variables is stochastically larger than the other. *Ann Math Statist* 1947; 18: 50–60.
12. Piguet PF, Vesin C, Rosen H, Grau GE. Antibody to the leukocyte integrins CD-11a or CD-11b prevent or cure pulmonary fibrosis elicited in mice by bleomycin or silica. *Am Rev Respir Dis* 1993; 147: 435–441.
13. Laurent GJ. Rates of collagen synthesis in lung, skin and muscle obtained *in vivo* by a simplified method using 3-H proline. *Biochem J* 1982; 206: 535–544.
14. Piguet PF, Chang HR, Tiberghien P, Hervé P. Use of antibodies to cytokines. *In* : Oppenheim J, ed. The Clinical Applications of Cytokines: Role in Pathogenesis, Diagnosis and Therapy 1993; Oxford University Press; Chapter 34, 257–262.