Topical Application of a Peptide Inhibitor of Transforming Growth Factor-β1 Ameliorates Bleomycin-Induced Skin Fibrosis

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Transforming growth factor-β (TGF-β) plays a crucial role in the pathogenesis of skin fibrotic diseases. Systemic TGF-β inhibitors effectively inhibit fibrosis in different animal models; however, systemic inhibition of TGF-β raises important safety issues because of the pleiotropic physiological effects of this factor. In this study, we have investigated whether topical application of P144 (a peptide inhibitor of TGF-β1) ameliorates skin fibrosis in a well-characterized model of human scleroderma. C3H mice received daily subcutaneous injections of bleomycin for 4 wk, and were treated daily with either a lipogel containing P144 or control vehicle. Topical application of P144 significantly reduced skin fibrosis and soluble collagen content. Most importantly, in mice with established fibrosis, topical treatment with P144 lipogel for 2 wk significantly decreased skin fibrosis and soluble collagen content. Immunohistochemical studies in P144-treated mice revealed a remarkable suppression of connective tissue growth factor expression, fibroblast SMAD2/3 phosphorylation, and α-smooth muscle actin positive myofibroblast development, whereas mast cell and mononuclear cell infiltration was not modified. These data suggest that topical application of P144, a peptide inhibitor of TGF-β1, is a feasible strategy to treat pathological skin scarring and skin fibrotic diseases for which there is no specific therapy.

Key words: antagonists/fibrosis/skin/topical administration/transforming growth factor β
J Invest Dermatol 125:450–455, 2005

Excessive accumulation of extracellular matrix (ECM) proteins is the hallmark of fibrotic skin conditions such as hypertrophic scarring, keloids, and localized or systemic sclerosis (scleroderma). This process is dependent on the activation of ECM synthesis in interstitial fibroblasts that often develop into α-smooth muscle actin (α-SMA)-positive myofibroblasts (Jimenez *et al*, 1996; Jelaska and Korn, 2000). One of the key molecular factors involved in both processes is transforming growth factor-β (TGF-β), which is consistently overexpressed in most fibrotic diseases and displays a variety of profibrotic effects in fibroblasts (Querfeld *et al*, 1999; Schiller *et al*, 2004). Activation of TGF-β receptors leads to the activation of several kinase signaling cascades, leading to the phosphorylation of SMAD proteins as well as the activation of SMAD-independent kinases that collectively activate ECM synthesis and fibroblast growth and differentiation into myofibroblasts (Shi and Massague, 2003; Daniels *et al*, 2004). Connective tissue growth factor (CTGF) is a soluble mediator selectively and rapidly induced in fibroblasts by the action of TGF-β (Leask *et al*, 2004). CTGF has also been specifically detected in skin fibrotic diseases (Igarashi *et al*, 1996), and in animal models, it enhances and perpetuates the profibrotic effects of TGF-β (Frazier *et al*, 1996).

Although most fibrotic diseases are usually initiated by variable degrees of inflammation, anti-inflammatory therapies are ineffective in targeting chronic fibrotic diseases that represent an important group of disorders for which there is no specific therapy. TGF-β appears as an attractive target for the therapy of fibrotic diseases, and several anti-TGF-β strategies have been successfully assayed in animal models of fibrosis, including several murine models of scleroderma (McCormick *et al*, 1999; Yamamoto *et al*, 1999b; Zhang *et al*, 2003; Lakos *et al*, 2004). Systemic inhibition of TGF-β, however, raises important safety concerns, because this factor displays pleiotropic and potent effects in immunomodulation, inflammation, and tumor development (Akhurst, 2002). Consistently, in TGF-β1-deficient mice, skin scarring is reduced but they develop a severe wasting syndrome accompanied by a generalized inflammatory response and tissue necrosis, resulting in organ failure and death (Bottinger *et al*, 1997). Therefore, local rather than systemic TGF-β inhibition, or targeting of downstream factors involved in TGF-β profibrotic signaling represent alternative strategies for the development of anti-fibrotic therapies (Daniels *et al*, 2004; Lakos *et al*, 2004). Local inhibition of TGF-β has previously been attempted by the direct application of neutralizing antibodies on skin or corneal open wounds, but the application of antibodies or large peptides through the epidermal barrier appears to be an

Abbreviations: α-SMA, α-smooth muscle-actin; CTGF, connective tissue growth factor; PBS, phosphate-buffered saline; TGF-β, transforming growth factor-β

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unpractical approach (Jester *et al*, 1997; Brahmatewari *et al*, 2000).

We have previously reported that the peptide P144: TSLDASIIWAMMQN, encompassing aminoacids 730–743 (accession number Q03167, SwissProt) from human TGF-β1 type III receptor (β-glycan), was able to block the biological activity of TGF-β1 (Ezquerro *et al*, 2003). This peptide is derived from the membrane-proximal ligand-binding domain of β-glycan (Esparza-Lopez *et al*, 2001), and similar to soluble β-glycan (Lopez-Casillas *et al*, 1994), was able to interfere with TGF-β1 binding to its cellular receptors on Mv1Lu cells (Ezquerro *et al*, 2003). P144 prevented TGF-β1-dependent inhibition of Mv1Lu cell proliferation and, in cultured fibroblasts, it induced a concentration-dependent decrease on TGF-β1-dependent stimulation of a reporter gene under the control of human α2(I) collagen promoter (Ezquerro *et al*, 2003). Intraperitoneal administration of P144 also showed potent *in vivo* anti-fibrotic activity in the liver of rats receiving CCl_4 (Ezquerro *et al*, 2003). Its small size and highly lipophilic character may allow its local use by topical application in skin fibrotic diseases, thereby reducing potential systemic effects. To examine the potential anti-fibrotic effects of the topical application of this peptide *in vivo*, we have tested P144 on a lipogel vehicle in an animal model of skin sclerosis induced by bleomycin. This model reproduces most of the features of human scleroderma such as skin-inflammatory cell infiltration, vascular damage, mast cell activation, and prolonged skin fibrosis (Yamamoto *et al*, 1999c). In this model, previous studies have demonstrated that either the administration of anti-TGF-β antibodies or genetic SMAD3 deficiency ameliorates fibrosis development, strongly supporting a key role for TGF-β (Yamamoto *et al*, 1999b; Lakos *et al*, 2004).

Results

In order to study the anti-fibrotic effect of P144 (a peptide inhibitor of TGF-β1) on bleomycin-induced skin fibrosis, we measured the changes induced in mice treated with bleomycin for 4 wk with and without P144 administration. It was found that bleomycin-treated mice showed a marked increase of the collagen matrix of the dermis. The dermis showed an increase of thickness that partially replaced the subcutaneous fat when compared with phosphate-buffered saline (PBS)-treated mice (Fig 1*A*). An increase in the collagen matrix around the upper fascia of the paniculus carnosus muscle was also observed, and it was particularly

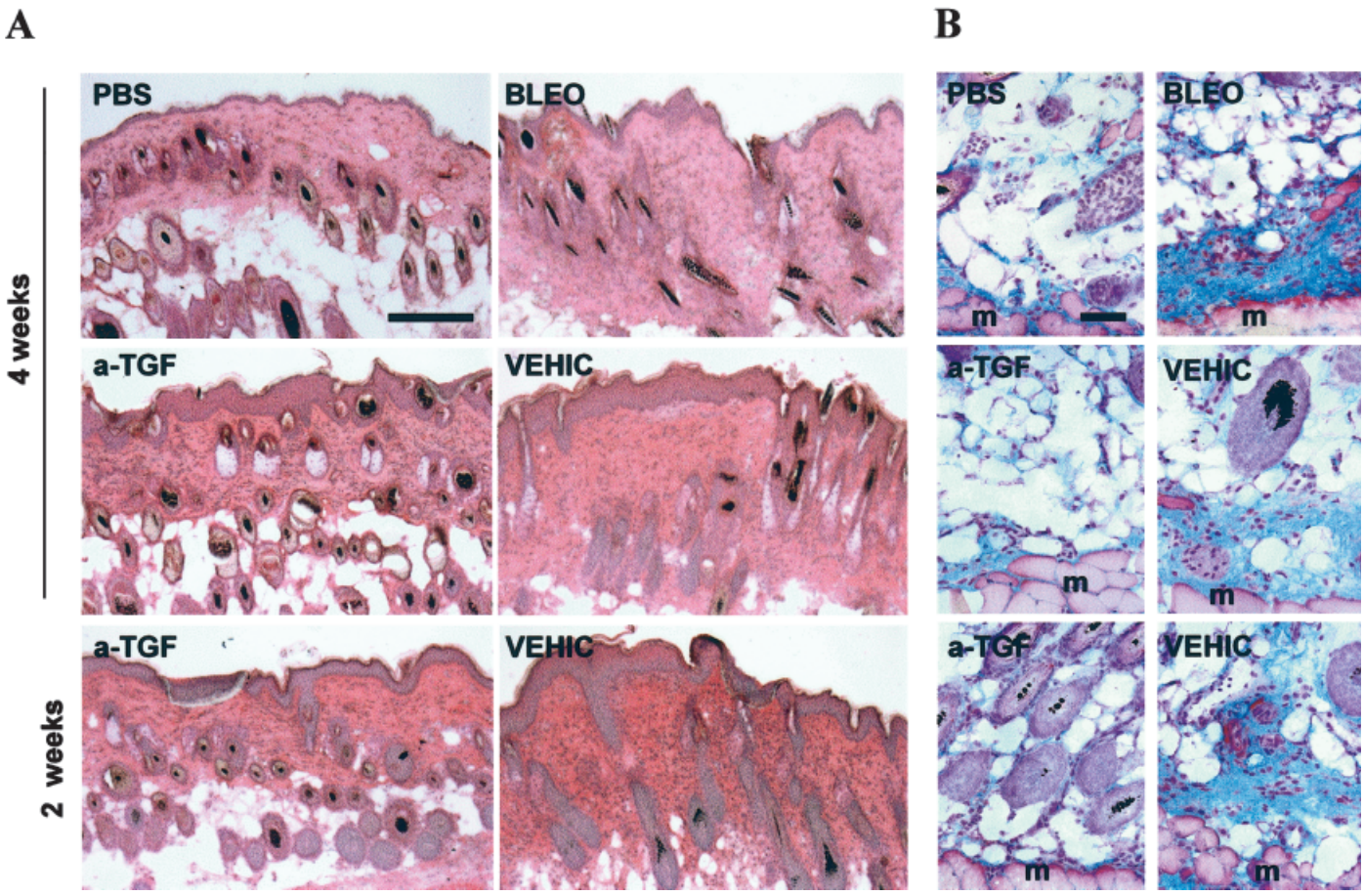

Figure 1
Histopathological evaluation of bleomycin-induced skin sclerosis in P144-treated mice. C3H mice received daily subcutaneous injections of bleomycin (BLEO), and were treated topically with P144 lipogel emulsion (a peptide inhibitor of transforming growth factor-β1 (TGF-β1)) or vehicle (VEHIC) for 4 wk, or for 2 wk after the 4 wk of bleomycin injections. Control mice were daily injected with phosphate-buffered saline (PBS). Skin sections show the hematoxylin–eosin-stained dermis in A, and the hypodermal area over the paniculus carnosus muscle (m) stained with Masson's trichrome in B. *Scale bars*: 200 μm (*A*) and 50 μm (*B*). Data are representative of 10 mice per group.

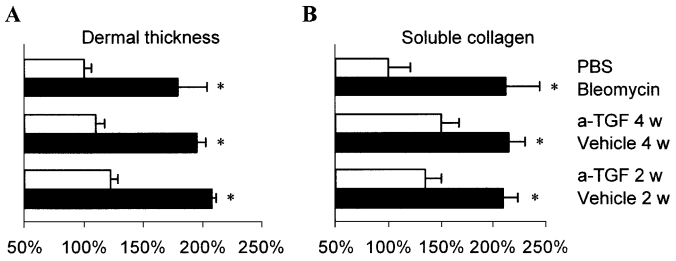

Figure 2
Effect of P144 lipogel emulsion administration on dermal thickness and soluble collagen content in bleomycin-treated mice. Dermal thickness was measured on digitalized images from hematoxylin–eosin-stained sections. Soluble collagen content was determined by the colorimetric Sircol method in pepsin-digested homogenates of skin biopsies. Three groups of mice were treated as follows: (i) phosphate-buffered saline (PBS) (*open bar*) or bleomycin (*filled bar*) for 4 wk, (ii) bleomycin plus P144 lipogel emulsion (*open bar*) or bleomycin plus vehicle: lipogel emulsion without P144 (*filled bar*) for 4 wk, and (iii) bleomycin only for 4 wk, treatment with P144 lipogel emulsion (*open bar*) or vehicle (*filled bar*) during 2 wk. Data represent mean ± SD of 10 mice per group, and the values of PBS-treated mice are set to 100%. $^{*}p < 0.05$.

evident in Masson's trichrome-stained sections of bleomycin-treated mice skin (Fig 1*B*). An abundant inflammatory infiltrate, mainly composed of mononuclear cells as well as an increased number of mast cells, many of them showing degranulation features, was also observed in bleomycin-treated mice (data not shown). Mice treated with P144 anti-TGF-β1 peptide showed a decrease of the dermal and hypodermal collagen area compared with vehicle-treated mice (Fig 1*A*, *B*). The thickness of the dermis was significantly decreased in P144-treated mice compared with vehicle-treated mice, which showed a thickness similar to that found in untreated mice (Fig 2). To confirm the histological observation of decreased fibrosis in P144-treated mice, we determined the pepsin-soluble collagen content of 4 mm punch skin biopsies by a colorimetric Sircol-based assay. This analysis showed a significant decrease of the soluble collagen content in P144-treated mice (Fig 2). Changes in the density of inflammatory cell infiltration, mast cell infiltration, or morphological changes of the epidermis were not observed in the P144 or vehicle-treated mice compared with those receiving only bleomycin injections (data not shown).

We also evaluated the effect of treating mice with established fibrosis after 4 wk of bleomycin injections, with daily topical P144 treatment for 2 wk. After 6 wk, fibrosis persisted in vehicle-treated mice, whereas mice treated with P144 for 2 wk showed a significant decrease of dermal thickness and collagen content (Figs 1 and 2).

To further characterize the cellular effects of neutralizing TGF-β1 with P144, we analyzed its effect on the development of α-SMA-positive myofibroblasts and fibroblast SMAD2/3 phosphorylation induced by bleomycin. In control mice, α-SMA-positive myofibroblasts were rarely observed, whereas an abundant number of these cells was observed after 4 wk of bleomycin injections (Fig 3). P144-treated mice showed a significant reduction in the number of α-SMA-positive myofibroblasts compared with vehicle-treated mice (Fig 3). We also observed an increase in the number of dermal fibroblasts displaying phosphorylated SMAD2/3 in a nuclear and cytoplasmic pattern in bleomycin-injected mice, confirming previous observations in this model (Takagawa *et al*, 2003). The number of phospho-SMAD2/3-positive fibroblasts was also significantly decreased in P144-treated mice compared with vehicle-treated mice (Fig 4).

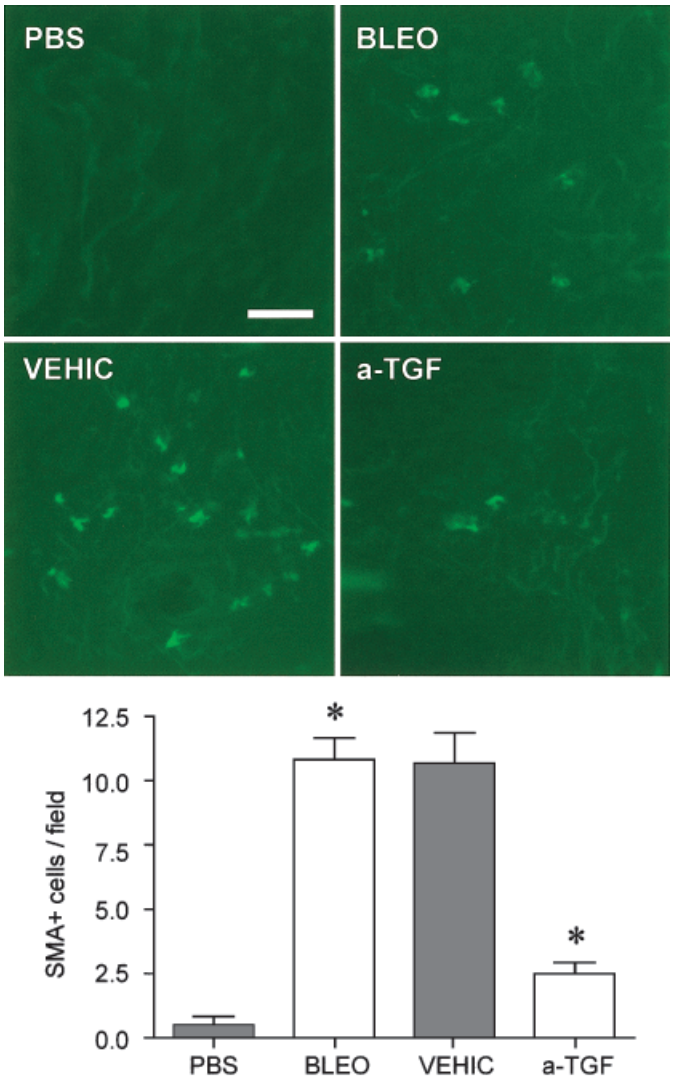

Figure 3
Immunofluorescent detection of myofibroblasts in skin sections of bleomycin-injected and P144 lipogel emulsion-treated mice. Sections were labeled with anti-α-smooth muscle actin (SMA)–fluorescein isothiocyanate (FITC) and examined under a fluorescence microscope. *Scale bar*: 40 μm. The mean ± SD number of SMA + cells per field is shown. Data are representative of 10 mice per group. $^{*}p < 0.05$.

To determine whether CTGF expression, a well-known downstream effector of TGF-β, is downregulated by P144 peptide in bleomycin-treated mice, we performed immunohistochemistry with L-20 polyclonal antibody. In our study, this antibody specifically recognized a single 38 kD protein, which was strongly induced by TGF-β1 treatment in cultured fibroblasts (data not shown). CTGF expression was strongly induced in fibroblasts and also in epidermis and hair follicle epithelial cells of bleomycin-treated mice (Fig 5). P144 treatment clearly decreased CTGF expression in the epidermis and hair follicles, compared with vehicle-treated mice, whereas fibroblast CTGF was still detectable after P144 therapy (Fig 5).

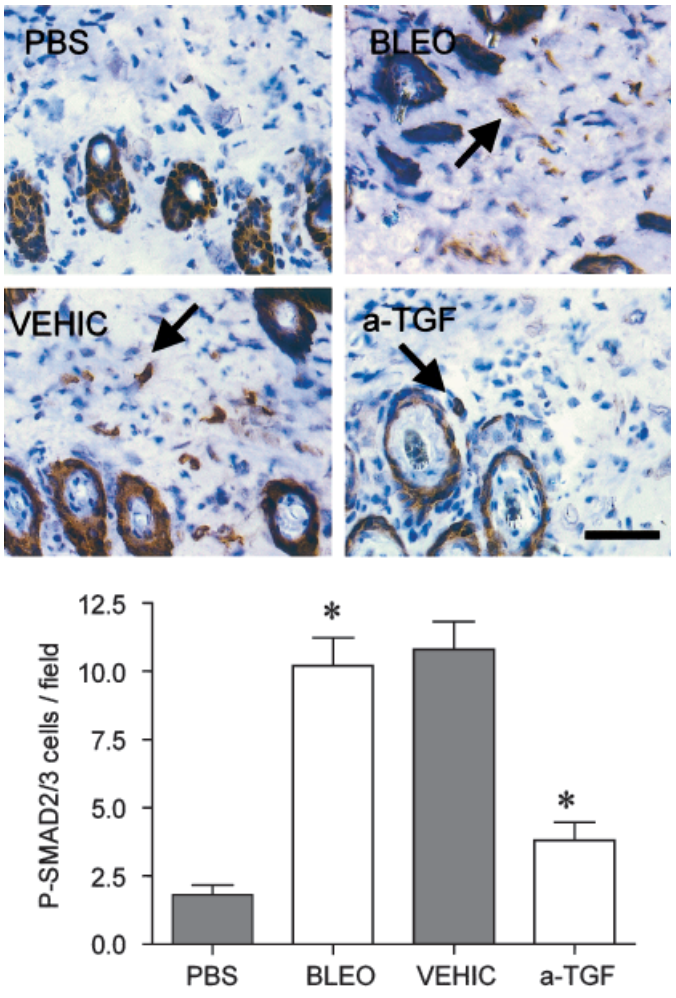

Figure 4
Immunohistochemical detection of phospho-SMAD2/3 in skin sections of bleomycin-injected and P144 lipogel emulsion-treated mice. Skin sections were labeled with a specific anti-phospho-SMAD2/3 antibody and developed with diaminobenzidine substrate (brown). Labeled fibroblasts are marked by arrows. Sections are hematoxylin counterstained. *Scale bar*: 50 μm. The mean ± SD number of phospho-SMAD2/3-positive fibroblasts per field is shown. Data are representative of five mice per group. $^{*}p < 0.05$.

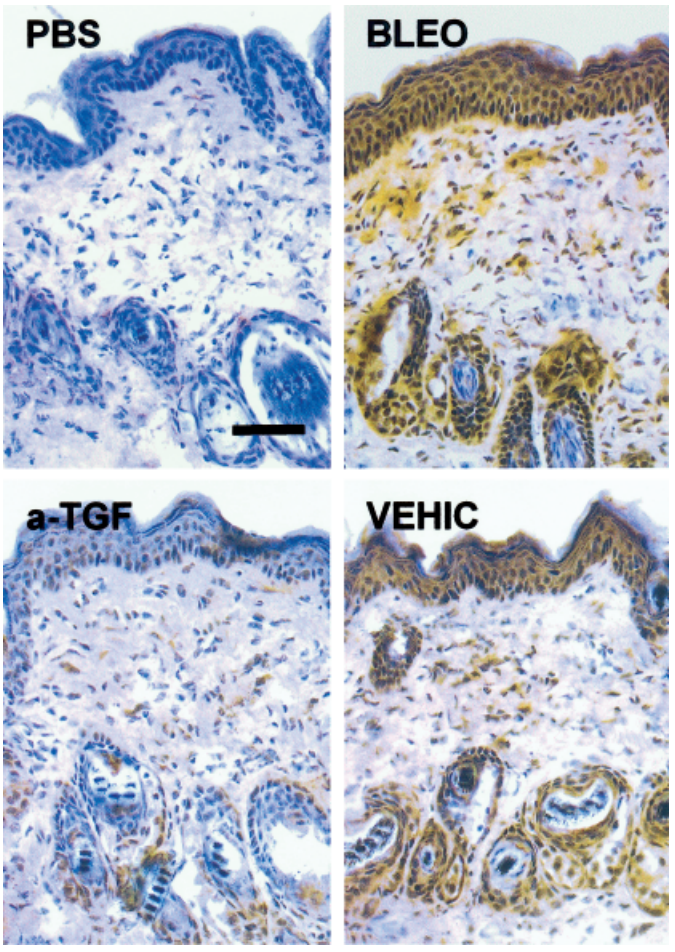

Figure 5
Immunohistochemical detection of connective tissue growth factor (CTGF) in skin sections of bleomycin-injected and P144 lipogel emulsion-treated mice. Skin sections were labeled with a specific anti-CTGF antibody and developed with diaminobenzidine substrate (brown). Sections are hematoxylin counterstained. Images are representative of 10 mice per group. *Scale bar*: 50 μm.

Discussion

The effectiveness of systemic strategies targeting TGF-β during the development of experimental skin fibrosis has been previously demonstrated. The natural human latency-associated peptide, and neutralizing anti-TGF-β1 antibodies have shown to prevent the development of skin fibrotic lesions effectively in different experimental models (McCormick *et al*, 1999; Yamamoto *et al*, 1999b; Zhang *et al*, 2003). These molecules are large enough to prevent its diffusion through the epidermal barrier. We have tested the feasibility of using a smaller lipophilic peptide, based on a conserved region of human type III TGF-β1 receptor, as a topical therapy for skin fibrosis.

Our data consistently show that daily application of this peptide for 4 wk in parallel to fibrogenic bleomycin subcutaneous injections prevents fibrosis. Furthermore, and more importantly, regarding human skin fibrotic diseases, established fibrosis was also significantly reduced following topical application of peptide P144 for 2 wk. Improvement of established skin fibrosis in this model by post-onset therapy has been previously demonstrated with systemic interferon-γ, or superoxide dismutase therapy but not with systemic TGF-β inhibitors (Yamamoto *et al*, 1999a, b, 2000). We decided to test topical application of P144 because it was thought that in the case of bleomycin-induced scleroderma, this would be more efficacious than systemic administration of this peptide inhibitor. Also, in the event of P144 being toxic, topical application might reduce toxic side-effects that might be encountered following systemic administration of P144.

In previous studies in the bleomycin-induced scleroderma model, treatment with systemic anti-TGF-β antibodies reduced fibrosis in parallel to a reduction in mast cell and inflammatory cell infiltration (Yamamoto *et al*, 1999b). The relevance of mast cells in skin fibrosis models is uncertain, because previous studies in mast cell-deficient mice have shown their dispensable contribution to fibrosis development (Everett *et al*, 1995; Yamamoto *et al*, 2001). Inflammatory cell infiltration plays an important role in the early stages of fibrosis development but its role is less clear at

later stages, where it can either resolve or persist independent of the progression of fibrosis. Indeed, at later stages, fibrosis usually progresses in the absence of significant inflammatory cell infiltration. Our data and similar data using latency-associated peptide in a model of graft *versus* host scleroderma, or in SMAD3-deficient mice challenged with bleomycin, suggest that fibrosis can be decreased by antagonizing TGF-β independent of inflammatory cell infiltration (Zhang *et al*, 2003; Lakos *et al*, 2004).

The mainstay of therapy for dermatological diseases remains topical therapy because it can readily target lesional skin decreasing systemic effects of the active principles; however, delivery of large peptides is limited by their size and physicochemical properties. We took advantage of the small size of P144 peptide and its lipophilic properties, which allowed for its application as a lipogel. Although dermal absorption of the peptide is yet to be demonstrated, our data suggest that topical application of this peptide efficiently interferes with TGF-β action on dermal fibroblasts as critical players of TGF-β profibrotic responses. Alternatively, its local accumulation in the epidermis could have potentially contributed to its anti-fibrotic effects. In this regard, cross-talk between the epidermis and the dermis during fibrosis development may occur, as profibrotic factors such as TGF-β and monocyte chemoattractant protein 1 (MCP-1) have been detected in the epidermal layer of fibrotic skin (Galindo *et al*, 2001; Flanders *et al*, 2002). Indeed, keratinocyte overexpression of TGF-β1 in transgenic mice induces dermal fibrosis (Ito *et al*, 2001; Yang *et al*, 2001; Chan *et al*, 2002). Interestingly, our study points to CTGF induction in skin keratinocytes of bleomycin-treated mice, which was reduced by topical anti-TGF-β therapy to a higher extent than in dermal fibroblasts. Although the role of epidermal CTGF has not been established in fibrosis, previous studies demonstrate that it is expressed by normal keratinocytes *in vivo* (Quan *et al*, 2002). Also, its downregulation by ultraviolet (UV) radiation has been linked to the reduction in procollagen synthesis induced by UV radiation (Quan *et al*, 2002).

The demonstration of the effectiveness of topical application of a peptide inhibitor of TGF-β1 provides a potentially fruitful strategy for the therapy of pathological scarring and skin fibrotic diseases. Experiments are being carried out to determine as to what extent P144 might be systemically absorbed through the skin. These experiments, together with a study of the potential toxicity of P144, will determine whether this peptide is suitable for human therapy.

Materials and Methods

Female C3H mice aged 6 wk were obtained from Harlan SL (Barcelona, Spain). Bleomycin (Sigma, Madrid, Spain) was dissolved in PBS at 100 μg per mL. Using a 27-gauge needle, 100 μL of filter-sterilized bleomycin or PBS was injected subcutaneously into the shaved back skin. Injections in the same site were administered daily for 4 wk. Mice were euthanized by CO_2 asphyxiation 24 h after the final injection. The back skin was removed and processed for histological examination, and 4 mm diameter punch biopsies were frozen for protein analysis. The study was approved by the ethical committee of Universidad Complutense de Madrid, Spain.

P144 peptide was originally developed and synthesized in our laboratory using the solid-phase method (Merrifield, 1963) and the Fmoc alternative (Atherton *et al*, 1989) as described previously (Borrás-Cuesta *et al*, 1991). P144 used in this study was purchased from Sigma-Genosys Ltd, Cambridge, UK. Peptide was at least 90% pure as per high-performance liquid chromatography and mass spectrometry. Two lipogel emulsions were prepared: a lipogel emulsion containing P144 and a control vehicle emulsion without P144. The vehicle lipogel emulsion was prepared by mixing the following components: 10 g dimethicone, 40 g liquid paraffin, 0.1 g chlorocresol, 0.5 g cetrimide, and 5 g ketostearic alcohol. This mixture was warmed to 70°C and emulsified with 44.4 g of distilled water (also at 70°C). The P144 lipogel emulsion was prepared in an identical manner, but the 44.4 g of water were replaced by a mixture of 44.28 of water plus 0.010 g of P144 previously dissolved in 100 μL of dimethyl sulfoxide.

Two groups of mice were given a daily application of either 100 μL of the P144 peptide lipogel preparation or control vehicle onto the shaved skin area during the 4 wk of bleomycin injections. Additional groups of mice received bleomycin injections for 4 wk, and thereafter, vehicle or P144 peptide were applied daily for 2 wk before sacrifice.

Pepsin-soluble collagen is an extractable fraction that represents recently synthesized collagen in tissues. It was quantified in 4 mm diameter punch biopsies of the back skin and adjusted by weight. Briefly, after skin homogenization, pepsin-soluble collagens were extracted overnight with 5 mg per mL pepsin in 0.5 mol per liter acetic acid. The soluble collagen content was determined using the Sircol Collagen Assay kit (Biocolor, Newtownabbey, Northern Ireland), according to the manufacturer's instructions.

Additional skin samples were snap-frozen in liquid nitrogen and embedded in optimal cutting temperature (OCT) medium for histological and immunohistochemical studies. Skin sections were stained with hematoxylin and eosin, Masson's trichrome, and toluidine blue for metachromatic staining of mast cells. Myofibroblasts were detected in skin sections by immunofluorescent labeling with an fluorescein isothiocyanate-labeled anti-α-SMA mAb (Sigma), and directly photographed under a Zeiss Axioplan-2 fluorescence microscope (Jena, Germany). For immunohistochemical detection of CTGF and phosphorylated-SMAD2/3, we used polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, California) and a biotin peroxidase-based method (ABC, Vector Laboratories, Burlingame, California). Slides were developed with diaminobenzidine chromogen and counterstained in Gill's hematoxylin.

For histomorphometrical analyses, three random fields of each skin biopsy were photographed and digitalized using a Spot RT CCD camera and Spot 4.0.4 software (Diagnostic Instruments, Sterling Heights, Michigan). The thickness of the dermis was measured, and the number of myofibroblasts, phosphorylated-SMAD2/3 positive fibroblasts, or mast cells per 400 × field were also counted on digitalized images.

This project was funded through the "UTE project CIMA," a grant from Instituto de Salud Carlos III (C03/02), and fellowships from the Fondo de Investigación Sanitaria to I. Gutierrez-Cañas and G. Palao.

DOI: 10.1111/j.0022-202X.2005.23859.x

Manuscript received February 3, 2005; revised April 15, 2005; accepted for publication May 13, 2005

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