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

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

We have previously reported that the peptide P144: TSLLASIIWAMMQN, 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 CCl4 (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-inflammarory 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 1A). An increase in the collagen matrix around the upper fascia of the paniculus carnosus muscle was also observed, and it was particularly...
evident in Masson’s trichrome-stained sections of bleomycin-treated mice skin (Fig 1B). 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 1A, 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).

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 number of the 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).

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 L20 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).
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-β antibodies have shown to prevent the development of skin fibrotic lesions effectively in different experimental models (McCor- mick 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
ultraviolet (UV) radiation has been linked to the reduction in whether this peptide is suitable for human therapy. 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.

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 chlororcesol, 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 g of water plus 0.10 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 Axiosplan-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.

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**References**


**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 CO2 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.


