A synthetic peptide from transforming growth factor β type III receptor inhibits liver fibrogenesis in rats with carbon tetrachloride liver injury

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Abstract

Transforming growth factor β1 (TGF-β1) is a pleiotropic cytokine, which displays potent profibrogenic effects and is highly expressed in fibrotic livers. For this reason, development of TGF-β1 inhibitors might be of great importance to control liver fibrogenesis as well as other undesired side effects due to this cytokine. Potential peptide inhibitors of TGF-β1 (derived from TGF-β1 and from its type III receptor) were tested in vitro and in vivo using different assays. Peptides P11 and P12, derived from TGF-β1, and P54 and P144, derived from its type III receptor, prevented TGF-β1-dependent inhibition of MV1Lu proliferation in vitro and markedly reduced binding of TGF-β1 to its receptors. P144 blocked TGF-β1-dependent stimulation of a reporter gene under the control of human α2(I) collagen promoter. Intraperitoneal administration of P144 also showed potent antifibrogenic activity in vivo in the liver of rats receiving CCl$_4$. These rats also showed a significant decrease in the number of activated hepatic stellate cells as compared with those treated with saline only. These results suggest that short synthetic peptides derived from TGF-β1 type III receptor may be of value in reducing liver fibrosis in chronic liver injury.
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1. Introduction

Transforming growth factor β (TGF-β) is a family of cytokines, which regulate the balance between cell mass and extracellular matrix (ECM). Three members of the TGF-β family (TGF-β1, TGF-β2 and TGF-β3) are expressed in mammalian cells and tissues [1], and their amino acid sequences are greater than 98% conserved among species. These isoforms share many of their biological activities and are able to stimulate their own synthesis and that of their receptors [2]. TGF-β receptors have been categorized into five types: types I, II and III are the most important because of their widespread expression in many cell types. Types I and II form a transmembrane complex that is able to transduce the signal inside the cell, whereas type III captures TGF-β1 and presents it to the complex formed by types I and II receptors. In addition, soluble type III receptor acts as a regulator of extracellular levels of active TGF-β1 (reviewed in Ref. [3]).

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TGF-β participates in a number of biological processes including cell growth and differentiation, formation of ECM and modulation of the immune response [1,4–7]. The biological effects of TGF-β may be different in vitro and in vivo and are influenced by the functional status of the target cell, by the interaction of the cells with ECM components and by the presence or absence of other cells in the extracellular milieu [8].

TGF-β regulates the equilibrium between the epithelial tissue and ECM. TGF-β increases the deposition of collagen and other ECM proteins by directly stimulating expression of these genes and by inhibiting the synthesis of collagenases [9]. TGF-β also enhances the production of inhibitors of collagen degradation, such as plasminogen-activator inhibitor type 1 and tissue inhibitor of metalloprotease [9]. In chronic viral hepatitis, the expression of TGF-β in the liver closely correlates with liver fibrogenic activity [10,11]. Because of the potent biological effects mediated by TGF-β, it is clear that pharmacological intervention to control TGF-β activity may be of importance to influence a diversity of pathological processes including liver fibrosis. In the present study, we demonstrate that short peptide sequences derived from TGF-β type III receptor effectively block several TGF-β1-mediated actions in vitro and in vivo. They decrease activation of hepatic stellate cells (HSCs), an ameliorate liver fibrosis in rats treated with CCl_4 to produce cirrhosis.

2. Results

Synthetic peptide inhibitors of TGF-β1 binding to its cell receptors were selected as follows: (i) overlapping peptides encompassing the sequence of TGF-β1 and the extracellular region of type III TGF-β1 receptor and (ii) peptides from the TGF-β1 type III receptor predicted to bind to TGF-β1 according to a computer program developed in our laboratory (see Section 4). The activity of these peptides was tested using MV1Lu cells incubated with 200 pg/ml of TGF-β1, which inhibits MV1Lu cell growth by 80–90% (data not shown), and 200 µg/ml of peptide. Peptide concentrations above 200 µg/ml were considered of no biological relevance. Peptides with an activity of 40% or more (MV1Lu cell assay) are shown in Table 1. All these peptides also inhibited binding of biotinylated TGF-β1 to its receptors in the flow cytometry assay. Peptide P54 from rat TGF-β1 type III receptor and P144 from the equivalent region of human type III receptor were among the most active.

Since P54 and P144, from rat and human TGF-β1 type III receptors, respectively, showed significant TGF-β1 inhibitory activities in the two systems used, and might, therefore, possess potential therapeutic value, we tested their ability to reduce TGF-β1-induced collagen expression. Using a fibroblast cell line, stably transfected with a chimeric plasmid containing the TGF-β1-inducible collagen α2(I) promoter linked to chloramphenicol acetyltransferase (CAT) gene [12,13]. It could be argued that the use of HSCs might be more appropriate to assess collagen promoter activity. However, we decided to use the transfected fibroblast cell line because this line was available, and we wished to confirm, by a method other that the MV1Lu cell assay, that the most active peptides were indeed able to block the activity of TGF-β1. It was found that P54 and P144 inhibited CAT expression in a dose-dependent manner (Fig. 1).

Liver fibrosis is associated with increased levels of TGF-β in hepatic tissue in a diversity of pathological conditions both in humans [11,14] and in experimental animal models [10,15]. To study whether TGF-β1 peptide inhibitors might be of value in reducing collagen deposition in chronic liver injury, we tested the effect of P144 in a model of liver fibrosis in the rat, induced by exposure to CCl_4 [16]. We tested the human type III

Table 1

Inhibition of human TGF-β1 by synthetic peptides from TGF-β1 and TGF-β1 type III receptors using two different assays

Peptide	Sequence of peptide	Region of parent protein	% Inhibition of TGF-β1	
			MV1Lu cell assay[b]	Flow cytometry assay[c]
P11	HANFCLGPCPYIWSLA	(318–332) Hum TGF-β1	40	96
P12	FCLGPCPYIWSLDTA	(321–334) Hum TGF-β1	96	56
P106	SNPYSAFQVDIIVDIA	(245–259) Rat TGF-β1 type III rec	40	94
P54	TSLDATMIWTMMA	(730–742) Rat TGF-β1 type III rec	97	90
P144	TSLDASIWAMMQNA	(729–742) Hum TGF-β1 type III rec	80	95

[a] The numbers in brackets correspond to the amino acid region of the corresponding protein. Accession numbers through PubMed databases for Hum TGF-β1, Rat TGF-β1 type III receptor and Hum type III receptor are: XP008912, NP058952 and JC1350, respectively.

[b] Inhibition of TGF-β1 by peptides was quantitated by comparing the growth of MV1Lu cells cultured in the presence of 200 pg/ml of human TGF-β1 with or without 200 µg/ml of peptide (see Section 4).

[c] Inhibition of TGF-β1 by peptides was quantitated by measuring the binding of Avidin–FITC to biotinylated human TGF-β1 bound to TGF-β1 receptors from MV1Lu cells with or without 100 µg/ml of peptide (see Section 4).

The alanine (in bold) was added at the C-terminal end of all peptides for synthesis convenience. Only those peptides with an activity ≥40% are shown.
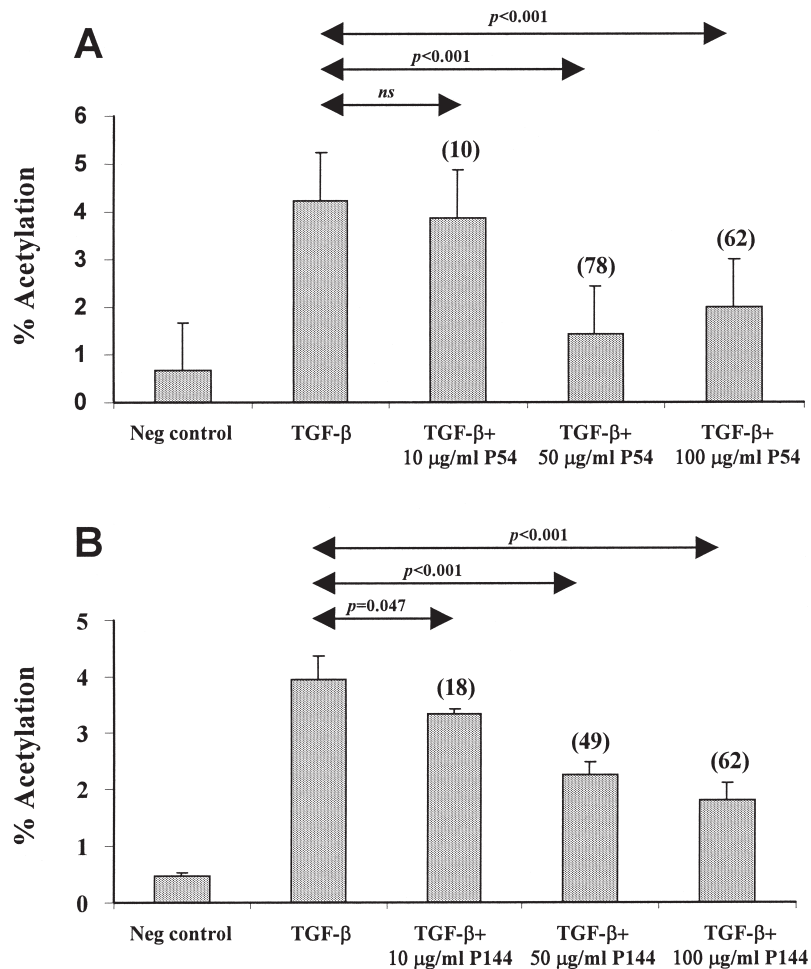
Fig. 1. Effects of P54 (A) and P144 (B) on the inhibition of the TGF-β1-inducible reporter gene CAT driven by collagen α2(I) promoter. Bars indicate the percentage of acetylation of radioactively labeled chloramphenicol by CAT in the presence or absence of different concentrations of inhibitor peptides. Experiments were carried out in triplicate. The percentage of inhibition of CAT expression (in brackets) was calculated by measuring acetylation in the presence of different concentrations of P54 or P144 according to the formula % inhibition = 100 − 100 × (% acetylation in the presence of TGF-β1 and inhibitor peptide − % basal acetylation)/(% acetylation in the presence of TGF-β1 − % basal acetylation).

receptor peptide P144 instead of the rat peptide P54, as we are interested in using the TGF-β1 peptide inhibitors in future therapy experiments in humans. Since P144 and P54 have only a few conservative differences in their sequence, we reasoned that the human peptide P144 would be active in rats. If that were so, we would validate the potential utility of P144 in humans. Rats receiving CCl_4 inhalations for 11 weeks (with a rest during week 5) were treated with 70 μg of P144 i.p. on alternate days during 6 weeks starting at week 6. A control group of rats received CCl_4 and saline instead of peptide. Fibrosis was measured by two methods: (a) estimation of the ratio between collagen and total protein of stained sections by spectrophotometry [17] and (b) evaluation of collagen deposition by image analysis in sections stained with Sirus red (see Section 4). As shown in Fig. 2, the ratio of collagen to total protein in rats receiving CCl_4 was significantly lower in rats treated with P144 than in rats treated with saline only. The antifibrogenic effect of P144 was confirmed by image

analysis of the liver specimens. As shown in Fig. 3A, B, collagen deposition was markedly reduced in CCl_4-treated rats that received P144, as compared with the corresponding control group receiving saline only.

TGF-β has an antiproliferative action in MV1Lu cells. However, TGF-β is the most important initiating cytokine so far identified in cirrhosis because it promotes collagen synthesis and stimulates proliferation of HSCs [18,19]. In the carbon tetrachloride-induced experimental cirrhosis, HSCs undergo transformation during the process of fibrosis. During these changes, the following events take place: (a) robust mitotic activity in areas developing new parenchymal fibrosis; (b) shift from the resting-state lipocyte phenotype to a transitional myofibroblast phenotype; and (c) increased capacity for synthesis and secretion of ECM. Because activation of HSCs is associated with the expression of α-smooth muscle actin (α-SMA) [20], and this activation is the crucial event in fibrosis [21], immunostaining of α-SMA has been used to quantify
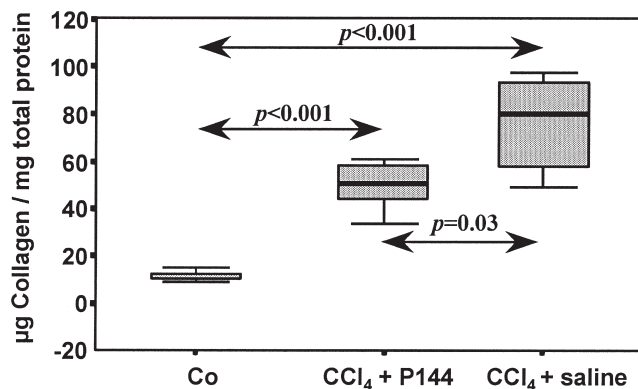
Fig. 2. Quantification of collagen in liver sections from rats subjected to CCl$_4$ inhalation (weeks 1–11 with a period of rest during the whole week 5). One group of eight rats (CCl$_4$ + P144) was treated with P144 (70 µg i.p. from week 6 to week 11) and another group of eight rats received saline (CCl$_4$ + saline). Co corresponds to eight healthy rats not subjected to CCl$_4$ inhalation. Values are represented using a box and whisker plot, the height of the box being the interquartilic range and the thick line, the median. The whiskers correspond to the highest and lowest observations within 1.5 times the interquartilic range from the edges of the box in both directions.

the number of activated HSCs in experimental cirrhosis in close relationship with fibrosis.

In order to assess whether P144 was able to inhibit HSC activation, we analyzed α-SMA by immunohisto-chemistry in liver sections from rats, subjected to CCl$_4$ inhalation, which received either P144 or saline. We found that the number of α-SMA positive cells was 1059 ± 330 per 30 high power fields in rats treated with saline ($n = 7$) and 458 ± 63 in those treated with P144 ($n = 8$), resulting in a $p < 0.05$ between groups (Fig. 4A). The α-SMA immunoreactivity was mainly found in bands of fibrous tissue surrounding parenchymal nodules. Fig. 4B shows representative sections of the liver from the two groups of rats, in which a clear reduction of α-SMA reactivity can be observed in those treated with P144.

3. Discussion

TGF-β1 plays a critical role in the deposition of increased amounts of collagens and other ECM proteins, which accompany the development of liver cirrhosis [11]. TGF-β1 is expressed in endothelial, hematopoietic and connective-tissue cells [9]. In cirrhotic liver, HSCs are the main source of this cytokine [22,23]. HSCs release TGF-β1 after activation by lipid peroxidation products generated in various forms of hepatic injury including viral, autoimmune and metabolic liver diseases and alcohol abuse. TGF-β, in turn, causes HSC activation and production of ECM proteins [24]. Liver fibrosis leads to portal hypertension by distorting liver architecture. It also affects liver function by altering hepatocyte gene expression as a result of the interaction of the hepato-

cellular membrane with an abnormal ECM [25]. Thus, efforts to control liver fibrosis by blocking TGF-β1 activity might be a useful therapeutic approach in cirrhotic patients or patients with liver conditions evolving to cirrhosis.

In the present work, we have identified five peptides, which are able to block the binding of TGF-β1 to its cell receptor and antagonize TGF-β1 activity in vitro. Two of these peptides (P11 and P12) are derived from the TGF-β1 sequence and are encompassed by a longer peptide previously described [26]. The other three peptides (P54, P106 and P144) are from the sequence of the extracellular region of type III TGF-β1 receptor. Peptides P54 and P144 correspond to equivalent regions of rat and human type III TGF-β1 receptors, respectively; they were predicted as a potential binder to TGF-β1 by a computer program developed in our laboratory. Both P54 and P144 were able to block the binding of TGF-β1 to its receptors, to counteract the antiproliferative effects of TGF-β1 on MV1Lu cells and to inhibit TGF-β1-induced activation of collagen gene expression in a fibroblast cell line. Most importantly, treatment with P144 at low doses caused a significant inhibition of HSC activation (Fig. 4A, C) and a marked reduction in liver fibrosis (Figs. 2 and 3) in a rat model of chronic exposure to CCl$_4$. All these data show that P144 is able to inhibit TGF-β1 activity both in vitro and in vivo, acting as a potent antifibrogenic compound.

In a previous study, George et al. [27] showed that soluble type II TGF-β1 receptor (generated by fusion of the extracellular region of type II receptor with the Fc domain of human IgG1) was very efficient at neutralizing TGF-β1 activity in vitro. This fusion protein at doses of 5 mg/kg reduced liver fibrosis in rats (400 g body weight) subjected to liver injury by laparotomy and high ligation of the bile duct. In this study, we demonstrated that a dose as low as 70 µg of the 15-mer peptide P144, given to rats (300 g body weight) on alternate days, decreased fibrogenesis significantly during chronic exposure to CCl$_4$. Thus, the two strategies to inhibit TGF-β1 (based on type II or type III receptors, respectively) appear to be effective in reducing liver fibrosis. Further studies will be needed to analyze the relative potency of these two forms of antifibrogenic intervention. Type III receptor is a non-signaling proteoglycan cytokine receptor, which functions by binding TGF-β1 and presenting it to the types I and II receptors [9]. A great proportion of TGF-β1 is found in a latent form attached to the ECM. Activation of TGF-β1 occurs by interaction of this latent form with certain ECM glycoproteins, such as trombospondin 1 [28], or by the effect of some proteases, such as plasmin [29–31]. In summary, we have shown that short synthetic peptides can be used to inhibit TGF-β1 activity in vitro and in vivo. These compounds, or their modifications,
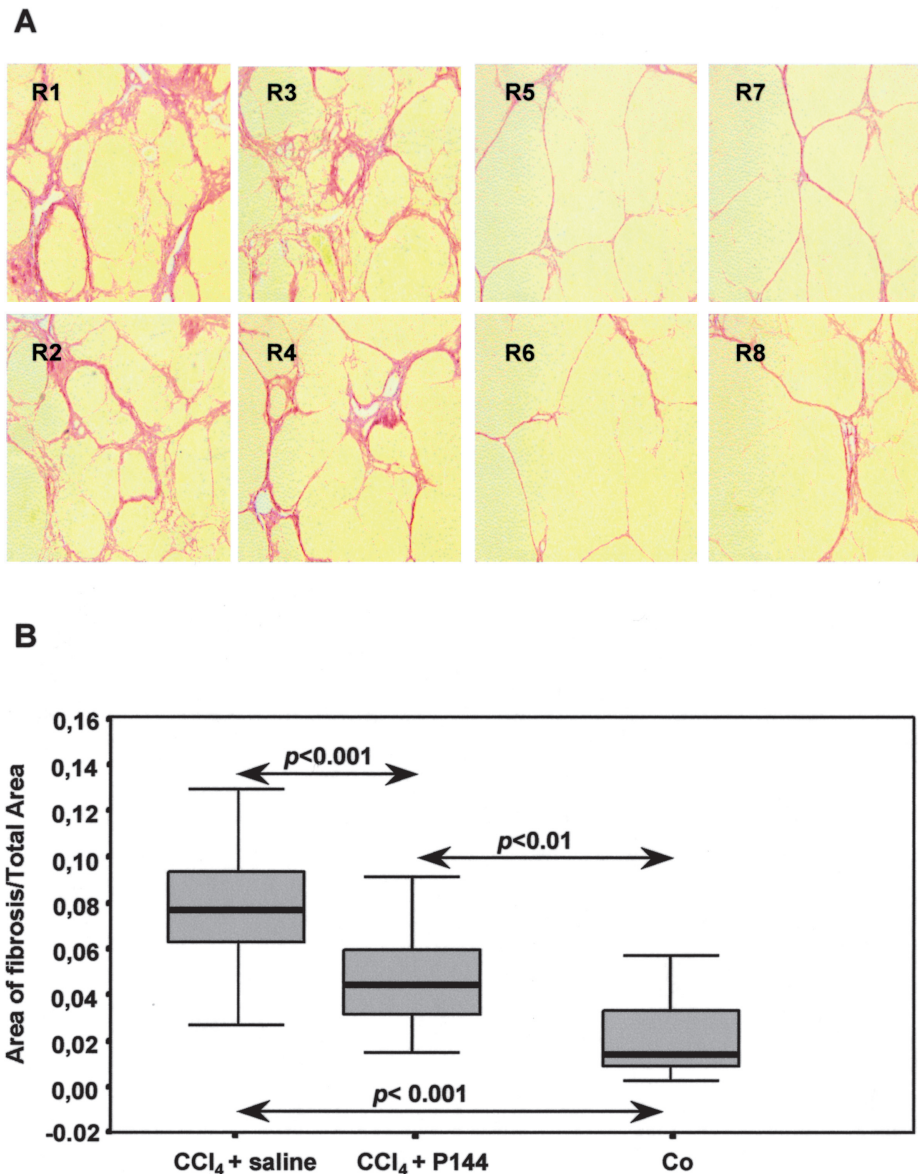
Fig. 3. Analysis of collagen in liver sections from rats submitted to CCl₄ inhalation and treated with P144 (eight rats) or with saline (eight rats). (A) Liver sections (10 ×) stained with Sirus red from rats submitted to CCl₄ inhalation. R1–R4 and R5–R8 correspond to eight representative sections from rats treated with saline only or with peptide P144, respectively. (B) Analysis of the ratio between the area of fibrosis and the total area of the image from liver sections: (CCl₄ + saline) rats treated with saline only ($n = 8$); (CCl₄ + P144) rats treated with P144 ($n = 8$). Co corresponds to eight normal rats not submitted to CCl₄ inhalation nor to any other kind of treatment. Values are represented using a box and whisker plot, the height of the box being the interquartilic range and the thick line the median. The whiskers correspond to the highest and lowest observations within 1.5 times the interquartilic range from the edges of the box in both directions.

might be of future interest to diminish fibrosis in processes, such as wound healing, chronic inflammatory reactions or liver disease evolving to cirrhosis. Also, because TGF-β1 is a pleiotropic cytokine and has potent immunomodulatory functions [7], its blockage for a prolonged period might have unwanted side effects. For this reason, extensive toxicity studies should be carried out before using any TGF-β1 inhibitors in humans. In the event that peptide p144 were found to be toxic, in future experiments it could be envisaged to administer a vector expressing P144 having tropism for

the liver (i.e. recombinant adenovirus). This might allow to diminish the circulating levels of p144 without decreasing the concentration at the site required.

4. Materials and methods

4.1. Selection of potential peptide inhibitors of TGF-β1

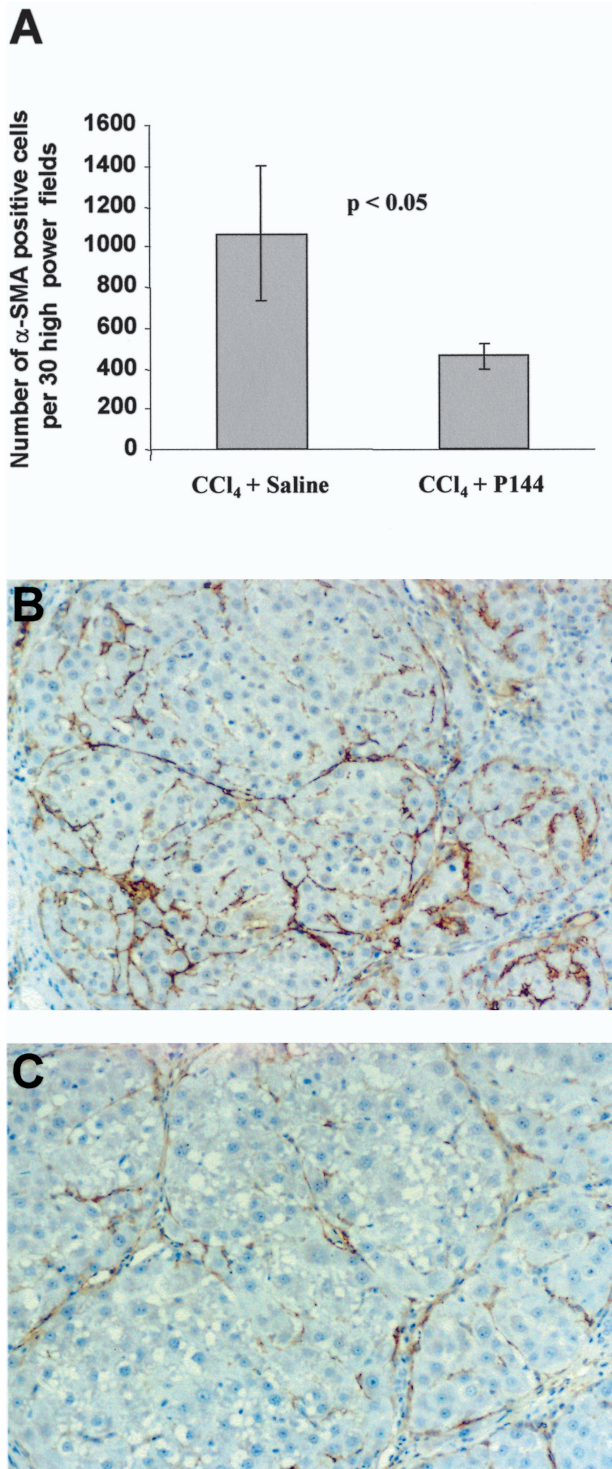Potential peptide inhibitors of TGF-β1 were chosen using two different strategies: (i) synthesizing overlapping

Fig. 4. Activated HSCs in the liver of rats subjected to CCl_4 inhalation and treated with saline or P144. (A) The number of α-SMA positive cells (presented as mean ± SD) per 30 random high power fields was determined. A significant decrease in the number of activated HSCs was observed in the group of rats treated with CCl_4 + P144 as compared with those that received saline; (B) representative liver section immunostained with antiα-SMA from rats treated with CCl_4 + saline (×200) and (C) representative liver section from rats treated with CCl_4 + P144 showing a lower number of positive cells stained for α-SMA (×200).

peptides encompassing the whole sequence of TGF-β1, and amino acids 44–418 from the extracellular region of TGF-β1 type III receptor and (ii) predicting peptides from TGF-β1 type III receptor (amino acids 1–853), which might bind to TGF-β1. Predictions were made using a program developed in our laboratory, which assigns potential interactions between two peptides (one from TGF-β1 and the other from a receptor protein of TGF-β1) based on the hydrophilicity/hydrophobicity and the net charge of the amino acid side chains from both peptides. Basically, the program calculates a putative score of interaction between sequential blocks of 12 amino acids from both proteins. To calculate the score of interaction between two blocks, the hydrophilicity of amino acid at position 1 from the first block is multiplied by the hydrophilicity of amino acid at position 1 from the second block, and so on till the amino acid at position 12. The sum of these 12 products constitutes the score of interaction between the two blocks. These calculations were carried out using the amino acid hydrophilicity scale of Hopp and Woods [32]. Since in the scale of Hopp and Woods, the value of the hydrophilicity of hydrophilic amino acids is positive, while the value of hydrophobic amino acids is negative, higher scores are obtained when two amino acids of the same type are compared. However, since charged amino acid side chains may attract or repel depending on their charge, the products of the hydrophilicities between amino acid side chains having the same sign of charge, Lys and Lys, Arg and Arg, Lys and Arg, Asp and Asp, Glu and Glu and Asp and Glu, were arbitrarily made negative to penalize the interaction between these repelling side chains. All other products were left unchanged.

4.2. Peptide synthesis

Peptides were synthesized by the solid phase method [33] using the Fmoc alternative [34] as previously described [35]. Peptides were at least 80% pure as per HPLC.

4.3. Inhibition of MV1Lu cells by TGF-β1 and assay of peptide inhibitors of TGF-β1

TGF-β1 inhibits the growth of MV1Lu cells in vitro [36]. Thus, inhibition of TGF-β1 by peptides re-establishes cell growth. MV1Lu cells (ATCC) were cultured to subconfluence in complete medium (RPMI 1640 containing L-glutamine and supplemented with 5% fetal calf serum and antibiotics) at 37 °C and 5% CO_2 in 162 cm^2 flasks (Costar Corporation, CA, USA). Cells (5×10^3/well) were cultured in a 96 flat well plate (Costar Corporation) at 37 °C, 5% CO_2 overnight to allow cell adhesion. Different peptide concentrations (or 200 μg/ml for initial screening assays) plus TGF-β1 (200 pg/ml) were added to the wells. After 24 h incubation, 1 μCi of

(methyl-^{3}H)-thymidine (Amersham Life Science, Buckinghamshire, UK) was added per well and incubated for 12 h. Cells were harvested (Titertek Cell Harvester Inc., Sterling, USA) and radioactivity measured on a β-scintillation counter (Beta plate system, LKB, Uppsala, Sweden). As positive and negative controls, we used MV1Lu cells grown in the absence or presence of TGF-β1, respectively. Inhibition of TGF-β1 was calculated using the formula

% inhibition

$$= 100 \times (\text{cpm peptide tested} - \text{cpm negative control})/$$
$$(\text{cpm positive control} - \text{cpm negative control})$$

Negative control represents cells in the presence of TGF-β1, but without peptide, whereas positive control corresponds to cells without TGF-β1 or peptide.

4.4. Study of the inhibition of binding of TGF-β1 to its receptors by flow cytometry

Peptide inhibition of TGF-β1 binding to its receptors was studied by flow cytometry using the Fluorokine TGF-β-biotin kit (R&D Systems) according to manufacturer's instructions. Briefly, MV1Lu cells were incubated with peptide (100 μg/ml), in the presence of biotinylated TGF-β1 for 1 h. Avidin–FITC was then added and the fluorescence measured. As positive and negative controls, we measured cell fluorescence after adding, biotinylated TGF-β1 and Avidin–FITC, or Avidin–FITC only, respectively. Inhibition was calculated using the formula

% inhibition

$$= 100 - ((\text{mf peptide} - \text{mf negative control})$$
$$\times 100/(\text{mf positive control} - \text{mf negative control}))$$

mf peptide is the mean fluorescence of cells having bound biotinylated TGF-β1 in the presence of peptide that was tested; mf negative control: mean fluorescence of cells in the absence of biotinylated TGF-β1; mf positive control: mean fluorescence of cells having bound biotinylated TGF-β1 in the absence of peptide tested.

4.5. Effect of the TGF-β-inhibiting peptide on the stimulation of collagen α2(I) promoter function by TGF-β1

Primary human fetal skin fibroblast cells (CF37) stably transfected with a chimeric plasmid containing the TGF-β1-inducible collagen α2(I) promoter linked to CAT gene were used as previously described [12,13]. Briefly, fibroblasts were grown at 37 °C and 5% CO$_2$ for 3 h in Dulbecco's modified Eagle's medium containing 4.5 g/l of glucose supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and 150 μg/

ml of hygromicin B. After washing with PBS, cells were suspended in fresh medium containing inhibitor peptide tested, BSA 0.2%, FBS 0.4%, TGF-β1 (8 ng/ml) (Boehringer) and incubated in six-well plates for 67 h. Cells were trypsinized and centrifuged at 14,000 rpm for 1 min. The pellet was re-suspended in 100 μl of ice-cold 0.25 M Tris, pH 7.5 and lysed by three freeze–thaw cycles. Cytoplasmic extracts isolated at 4 °C and 14,000 rpm for 5 min were incubated at 37 °C with acetyl CoA and 2 μl (200 μCi/ml) of ^{14}C-labeled chloramphenicol for 1 h. Acetylation was stopped adding 1 ml of ethyl acetate and centrifuging at 14,000 rpm at 4 °C for 1 min. The ethyl acetate phase was vacuum-dried overnight and extracted with fresh ethyl acetate (30 μl). Mono-acetylated and non-acetylated chloramphenicol from these extracts were separated by thin-layer chromatography in chloroform–methanol 19 : 1 (v/v) using sheets of plastic-backed, silica gel 1B (J.T. Baker) for 1 h. Percentage of acetylation was calculated using the 860 PhosphorImager Storm (Molecular Dynamics) apparatus and the Imagequant program.

% acetylation

$$= (\text{counts in mono} - \text{acetylated species})$$
$$\times 100/(\text{counts in mono} - \text{acetylated species}$$
$$+ \text{counts in non} - \text{acetylated chloramphenicol}).$$

Percentage inhibition of TGF-β1 by peptides was calculated according to

% inhibition

$$= 100 - 100 \times (\% \text{ acetylation in the presence of}$$
$$\text{TGF-β1 and peptide} - \% \text{ basal acetylation})/$$
$$(\% \text{ acetylation in the presence of TGF-β1}$$
$$- \% \text{ basal acetylation}).$$

4.6. Immunohistochemistry and quantification of activated HSC

Immunohistochemical staining for α-SMA [20] was performed on formalin-fixed and paraffin-embedded tissues. Sections were deparaffinized with xylene and treated with 0.01 M citrate buffer (pH 6) in a microwave (800 W) at maximum power for 5 min. Specimens were then incubated at room temperature with a monoclonal antibody to α-SMA (Dako A/S, Copenhagen, Denmark). Antibody detection was carried out using the Envision kit (Dako) following the manufacturer's instructions. Slides were counterstained weakly with Mayer's hematoxylin, dehydrated and mounted. Negative controls were performed using PBS instead of the primary antibody. Estimation of the number of antiα-SMA immunoreactive was done by a blinded observer counting positive cells in 30 random high power fields

(× 400). Counting of α-SMA positive cells was carried out in the entire field without making any distinction if the positive cells were around or inside the nodules.

4.7. Induction of liver fibrosis by CCl₄

Hepatic fibrosis was induced in Wistar rats by inhalation of CCl₄ [37] twice a week as previously described [16]. Rats received CCl₄ until end of week 11 (with a rest during week 5) and injected 70 μg of the TGF-β1-blocking peptide i.p. on alternate days from the beginning of week 6 until the end of week 11. A control group of rats received saline instead of the peptide. All animals were treated according to the guidelines from our institution (CIFA, Pamplona, Spain).

4.8. Analysis of collagen in liver sections

Deposition of collagen in the liver was evaluated as follows. (i) Liver sections were stained with Fast green and Direct red and the stain eluted with methanol and sodium hydroxide. Collagen and total protein were estimated by spectrophotometry at 540 and 630 nm, respectively [17]. (ii) Image analysis of Sirus red-stained liver sections using polarized light microscopy and a green filter was performed.

4.9. Statistical analysis

Statistical analysis was carried out using the computer program SPSS for Windows. Comparison between groups was carried out using the Kruskal–Wallis test followed by Mann–Whitney's *U* test. The statistical analysis of inhibition of CAT with synthetic peptides, was done using the Shapiro–Wilk test ($p = 0.773$) followed by the Dunnett test.

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