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Effect of pentoxifylline on the degradation of procollagen type I produced by human hepatic stellate cells in response to transforming growth factor-β_1

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1 Pentoxifylline (PTF) may act as a potential antifibrogenic agent by inhibiting cell proliferation and/or collagen deposition in cell type(s) responsible for the accumulation of extracellular matrix. The aim of the present study was to investigate at which level PTF may affect synthesis and degradation of type I collagen in human hepatic stellate cells (HSCs), a key source of connective tissue in fibrotic liver.

2 Procollagen type I synthesis and release were evaluated in cells maintained in serum free/insulin free medium for 48 h and then stimulated with transforming growth factor-β_1 (TGF-β_1) for different time periods in the presence or absence of PTF. TGF-β_1 caused an upregulation of procollagen I mRNA levels with a peak increase after 3–6 h of stimulation. This effect was followed by an increase in both the cell associated and the extracellular levels of the corresponding protein, with a peak effect at 9–12 h after the addition of TGF-β_1. Co-incubation with PTF slightly but consistently reduced basal as well as stimulated procollagen I mRNA levels, with negligible effects on the cell-associated expression of the corresponding protein. Conversely, PTF dose-dependently reduced procollagen type I levels detected in supernatants from unstimulated and stimulated cells.

3 Pulse-chase experiments employing L-[^{3}H]-proline revealed that PTF was able to induce significantly the degradation of procollagen, mainly in the extracellular compartment. We next analysed the effect of PTF on the major pathway involved in type I collagen degradation. PTF did not affect the expression of metalloproteinase 1 (MMP-1) mRNA both in basal and stimulated conditions, whereas it markedly reduced the expression of tissue inhibitor of metalloproteinase 1 (TIMP-1) mRNA. Accordingly incubation with PTF increased the levels of 'activated MMP-1' in cell supernatants in both basal and stimulated conditions.

4 These results suggest that the antifibrogenic action of PTF on human HSCs is mainly mediated by extracellular collagen degradation rather than by a reduction of collagen synthesis.

Keywords: Collagen degradation; cyclic AMP; extracellular matrix (ECM); hepatic stellate cells (HSCs); liver fibrosis; matrix metalloproteinase (MMP); pentoxifylline (PTF); tissue inhibitor of metalloproteinase-1 (TIMP-1); transforming growth factor-β_1 (TGF-β_1); platelet-derived growth factor (PDGF)

Introduction

Liver fibrosis is the common consequence of different liver diseases characterized by chronic liver tissue damage. Several studies performed over the past 10 years have clearly shown that this process is the consequence of the chronic activation of extracellular matrix (ECM) producing cells, and particularly of hepatic stellate cells (HSCs, also referred to as Ito cells, lipocytes, fat-storing cells), leading to cell proliferation and increased deposition of ECM components (Friedman, 1993; Pinzani, 1995). Decreased degradation of the newly synthesized ECM is also considered a key factor in this process (Iredale *et al*., 1992; Arthur, 1994).

Pentoxifylline (PTF), a methylxanthine derivative, is currently employed in the treatment of peripheral vascular disorders because of its putative effects on erythrocyte deformability and tissue oxygen delivery (Ward & Clissold, 1987). Most of PTF activity is ascribed to its activity as a prototype phosphodiesterase inhibitor leading to increased intracellular cAMP levels (Stefanovich *et al*., 1974). More recently, studies performed in dermal fibroblasts and in two animal models of hepatic fibrosis, have suggested that PTF may act as an antifibrogenic agent (Peterson, 1993; Boigk *et al*., 1995). This potential therapeutic action has been attributed to both inhibition of ECM-producing cell proliferation and reduced deposition of ECM components, mainly collagen type I, by these cell types. Along these lines, PTF has been shown to inhibit platelet-derived growth factor (PDGF)-stimulated proliferation of cultured human HSCs (Pinzani *et al*., 1996). This action, largely dependent upon the inhibition of PDGF-stimulated activation of extracellular signal-regulated kinase (ERK) and of the related downstream signalling pathways, is likely to indirectly reduce ECM deposition through the inhibition of PDGF-driven proliferation of ECM-producing cells. Importantly, interference with this key features of PDGF signalling appears strictly related to the increase in intracellular cAMP levels caused by PTF. Furthermore, other recent studies have indicated that PTF is capable of retarding the transdifferentiation of rat HSCs towards their activated 'myofibroblast-like' phenotype (Windmaier & Gressner, 1996).

In addition to this 'indirect' action, other studies suggest a direct anti-fibrogenic effect of PTF. Overall, the limited available data obtained in dermal fibroblasts indicate that treatment of this cell type with pharmacological doses of PTF may result in a reduced constitutive secretion of collagen type I, and that this effect is possibly related to both reduced synthesis and increased degradation (Berman *et al*., 1989; Duncan *et al*., 1995). However, it is still unclear at which level PTF may exert its action(s) in the complex cascade of intracellular and extracellular events leading to collagen synthesis, secretion, assembly and degradation.

Hepatic stellate cells isolated from human liver and activated in culture have been shown to synthesize and secrete

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large amounts of ECM components, including collagen type I. The latter, together with collagen type III, constitutes the major architectural component of the so-called 'fibrillar' matrix characteristic of all fibrogenic disorders. In human HSCs, the constitutive production of procollagen type I is significantly enhanced by stimulation with transforming growth factor-β_1 (TGF-β_1) (Casini *et al*., 1993), a growth factor with well-established pro-fibrogenic properties and the expression of which appears strictly related to the progression of hepatic fibrosis (Castilla *et al*., 1991; Milani *et al*., 1991).

The present study was specifically designed to elucidate at which level PTF may affect synthesis and degradation of type I collagen in human HSCs, both in basal culture conditions and after stimulation with TGF-β_1.

Methods

Reagents

Primary and horseradish peroxidase-conjugated secondary antibodies and the chemiluminescent ECL Western blotting detection reagents were obtained from Amersham (Arlington Heights, IL). A specific rabbit anti-monkey polyclonal antibody directed against the procollagen I N-terminal propeptide (pIp) was kindly provided by Dr D. Schuppan (Klinikum B. Franklin, Berlin, Germany). L-[^{3}H]proline (specific activity 85–130 Ci mmol^{-1}) was purchased from Amersham, Dialysis was performed in a Slide-A-Lyzer^{TM} dialysis cassette system from Pierce (Rockford, IL). Activated MMP-1/TIMP-1 complex human ELISA system was provided by Amersham. Transforming growth factor-β_1 purified from human platelets was provided by Collaborative Research Inc. (Bedford, MA). Highly purified pentoxifylline was kindly provided by Dr G. Rinaldi (Hoechst Italia S.p.A., Milan, Italy). All other reagents were of analytical grade.

Isolation and culture of human HSCs

Human HSCs were isolated from wedge sections (10–20 g) of normal human liver unsuitable for transplantation as previously reported (Pinzani *et al*., 1992; Casini *et al*., 1993). Briefly, after a combined digestion with collagenase/pronase, HSCs were separated from other liver non-parenchymal cells by ultracentrifugation over gradients of stractan (Larex-LO, Larex International Co., Tacoma, WA). Cells were cultured on plastic dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) in Iscove's modified Dulbecco's medium supplemented with 0.6 U ml^{-1} insulin, 2.0 mmol l^{-1} glutamine, 0.1 mmol l^{-1} non-essential aminoacids, 1.0 mmol l^{-1} sodium pyruvate, antibiotic/antimycotic solution containing 10 000 U penicillin, 10 mg streptomycin and 25 μg amphotericin B/ml (GIBCO BRL Laboratories, Grand Island, NY) and 20% (v/v) foetal bovine serum (Imperial Laboratories, Andover, U.K.). The cells were grown at 37°C in a humidified 95% O_2 and 5% CO_2 atmosphere. Extensive characterization was performed as described elsewhere (Casini *et al*., 1993). Experiments described in this study were performed on cells between first and third serial passages (1:3 split ratio, i.e. after trypsinisation of a confluent cell monolayer, cells are plated in three plastic culture dishes identical to the original one) using two independent cell lines. Human HSCs, in our culture conditions, showed transmission electron microscopy features of 'transitional' cells (smooth cell surface with slender nucleus, flattened profiles of rough endoplasmic reticulum interposed among numerous lipid droplets) and 'myofibroblasts-like' cells. These phenotypical changes are currently considered analogous to those observed *in vivo* following chronic liver injury (Mak & Lieber, 1988).

Northern blot analysis

Experiments were performed in human HSCs grown to confluence in 100 mm Petri dishes (at a density of 1×10^6 cells/dish in complete culture medium). Confluent cells were incubated for 48 h in SFIF medium and then simulated with the appropriate experimental conditions for the indicated timepoints. Total RNA was isolated from HSCs by the guanidinium isothiocyanate-phenol-chlorophorm method using RNAzol^{TM} (Biotecs Labs, Houston, TX). Total RNA were fractionated by 1% agarose-3% formaldehyde gel, transferred to a GeneScreen filter (DuPont NEN, Wilmington, DE) and immobilized by baking for 2 h at 80°C. Filters were prehybridised and then hybridised at 42°C for 18 h with 10^6 c.p.m. ml^{-1} of ^{32}P-labelled complementary DNA (cDNA) probe. The 1600 bp *Pst*I-*Pst*I complementary DNA insert of *pαlRl* coding for rat α1(I) procollagen (Genovese *et al*., 1984), the 735 bp *Sst*I-*Eco*RI cDNA fragment of pCllase 1 obtained from American Type Culture Collection (ATCC 57685), coding for human matrix metalloproteinase (MMP)-1 (Whitham *et al*., 1986), and the 900 bp cDNA fragment coding for human tissue inhibitor of metalloproteinase (TIMP)-1 (Docherty *et al*., 1985) were labelled with [^{32}P]deoxycytidine 5′-triphosphate (New England Nuclear, Milan, Italy) to a specific activity of 2 to 5×10^8 c.p.m. μg^{-1} of DNA, by using a random priming kit (Amersham). Simultaneously (Figure 1a) or after removal of the various probes (Figures 5a and 6a), the same filters were hybridised with a ^{32}P-labelled probe encoding for the ribosomal protein 36B4 (Basset *et al*., 1990). Filters were then washed to a maximal stringency of $0.1 \times$SSC/0.1%

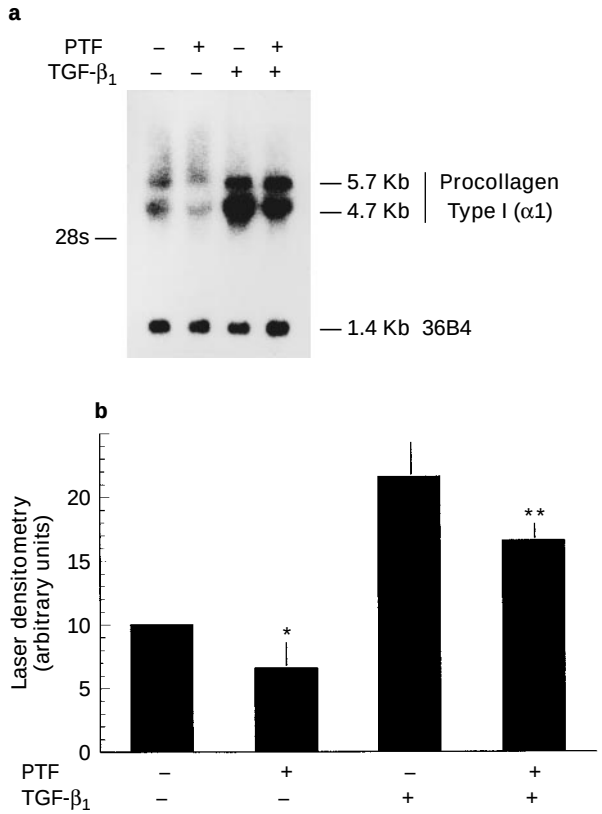

Figure 1 Effect of pentoxifylline (PTF) on procollagen type I mRNA expression in basal conditions and after stimulation with TGF-β_1 in human HSCs. (a) Northern blot analysis of 10 μg of total RNA fractionated by 1% agarose-formaldehyde gel electrophoresis. Experiments were performed on confluent cells after a 48 h incubation period in serum-free/insulin-free medium. Incubation time with PTF alone or with TGF-β_1 (1.0 ng ml^{-1}) with or without PTF co-incubation was 3 h. PTF was used at the dose of 170 μM. Representative blot of three different experiments. (b) Depicts relative procollagen type I mRNA expression normalized for 36B4 in the same blot, with the value of the control condition assigned a value of 10. Mean ± s.d. of values obtained from the three experiments performed. TGF-β_1 vs control: $P<0.001$; *, **$P<0.05$ vs control and TGF-β_1 alone, respectively.

sodium dodecyl sulfide (SDS) at 65°C and exposed to Kodak XAR-5 film at −70°C. The hybridisation bands were quantitated by scanning laser densitometry (Hoefer Scientific Instruments, San Francisco, CA) and normalized to the 36B4 bands.

Western blot analysis

Identical amounts of protein (20 μg), assayed by the Bradford method, were separated by 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and electroblotted on a polyvinylidene-fluoride (PVDF) membranes (Immobilon P, Millipore Corp., Bedford, MA). The membranes were blocked overnight with 2% gelatin in 0.1% PBS-Tween 20, and sequentially incubated at room temperature with an anti-monkey procollagen I N-terminal propeptide (pIp) (Becker *et al*., 1986); after 3 × 10 min washes in PBS and 0.1% Triton X-100, the membranes were incubated for 1 h at room temperature with the horseradish peroxidase-conjugated secondary antibodies (Amersham). Detection, after washing the membranes, was carried out using enhanced chemiluminescence according to the manufacturer's protocol (ECL, Amersham).

Determination of type I collagen levels in cell supernatants

Type I collagen content in culture media was determined by a previously described enzyme-linked immunoassay method (ELISA) (Casini *et al*., 1993), by using the same specific rabbit polyclonal antibody, an anti-monkey procollagen I N-terminal propeptide (pIp). For these experiments, confluent human HSCs in 100 mm Petri dishes (10^6 cells/well) were incubated for 48 h in SFIF medium. Standard and samples were assayed in triplicate. The detection limit was 2.0 ng/well. Results are expressed as nanograms of collagen per micrograms of cellular DNA.

Collagen degradation assay

Collagen degradation was measured by following the remaining collagen-bound radioactivity in a pulse-chase experiment. Confluent HSCs in 100 mm Petri dishes (1×10^6 cells/dish) in complete Iscove's medium with insulin and 20% FBS were then incubated in SFIF medium for 48 h. After extensive washing (× 3) with 10 ml each low-proline medium (DMEM/F12, Sigma Chemical Co., St. Louis, MO), cells were pulsed with 40 μCi L-[2,3,4,5-^{3}H]proline in 5 ml of pulse medium (DMEM/F12 containing 100 μg ml^{-1} β-aminopropionitrile and 100 μg ml^{-1} sodium ascorbate, for 2 h at 37°C). Pulse medium was then removed and, after extensive washing (× 3) with pre-warmed (37°C) 1 × PBS, was replaced with 5 ml of chase medium (identical to the pulse medium, except that 1 mM unlabelled L-proline was substituted for L[2,3,4,5-^{3}H]proline). Experimental conditions (TGF-β_1, 1.0 ng ml^{-1}, with or without PTF, 170 μM) were added at this time. At time 0, 6 and 12 h after the addition of chase medium and experimental conditions, the incubation was terminated, the supernatants removed and temporarily stored at −80°C. Cell monolayers were scraped with a rubber policeman in 5 ml of ice cold (lysis buffer) 1 M NaCl/50 mM Tris buffer, pH 7.4, and sonicated (3 pulses at 50 W for 10 s each). Cell media or cell layer suspensions were then subjected to dialysis in Slide-A-Lyzer™ dialysis cassettes 3 × against 2–5 litres of 3% acetic acid. After dialysis, non-collagenous proteins were digested with porcine pepsin (0.2 mg ml^{-1} of 3% acetic acid) for 4 h at 18–22°C. The digested samples (collagen-bound radioactivity, due to pepsin-resistant material) were then further dialysed 3 × against 3% acetic acid until no significant radioactivity was detected in the dialysate. The resultant radioactivity present in the dialysis cassettes was finally counted in a beta-counter. Collagen degradation was expressed according to the following formula:

$$\%\ \text{collagen degradation} = (\text{cpm at time}_0 - \text{cpm at time}_x) : \text{cpm at time}_0,$$

where time$_x$ was relative to 6 or 12 h determinations.

According to this procedure, positive values express collagen degradation, whereas negative values express net collagen synthesis (total synthesis minus degradation).

Activated MMP-1 assay

Human HSCs were grown to confluence in 24-well dishes in complete medium and then maintained in SFIF medium for 48 h. Cells were then incubated with TGF-β_1, 1.0 ng ml^{-1}, with or without PTF, 170 μM, for 6, 12 and 24 h. At each time point, cell supernatants were centrifuged at 3000 rpm for 10 min, and an aliquot temporarily stored at −80°C. Activated MMP-1 levels were assayed using the newly developed MMP-1/TIMP-1 complex human ELISA (Amersham, Arlington Heights, IL, U.S.A.), according to the instructions provided by the manufacturer.

Statistical analysis

Results, relative to the number of experiments indicated, are expressed as mean ± s.d. Statistical analysis was performed by one-way analysis of variance (ANOVA) and, when the F value was significant, by Duncan's test.

Results

The effects of PTF on the sequential steps related to both procollagen type I synthesis and secretion, namely mRNA abundance, cell-associated and extracellular protein levels were firstly evaluated. Unless otherwise specified, PTF was employed at a standard dose of 170 μM, corresponding to the EC_{50} for PTF-induced increase in intracellular cAMP levels and the IC_{50} for the inhibitory effect of PDGF-induced mitogenesis (Pinzani *et al*., 1996).

Effects on procollagen type I mRNA

As shown in Figure 1, incubation of human HSCs, maintained for 48 h in serum free/insulin free medium (SFIF), with PTF produced a modest, but consistent reduction of both basal and TGF-β_1-stimulated procollagen type I mRNA levels (approximately 37% in basal, and 19% in stimulated conditions). This effect, observed after 3 h of incubation with PTF alone or PTF plus TGF-β_1, was also evident after 1 h incubation when, however, the stimulatory effect of TGF-β_1 on procollagen type I mRNA was less remarkable ($P < 0.01$, data not shown).

Effects on procollagen type I cell-associated and extracellular levels

Figure 2 illustrates the effects of PTF on cell-associated procollagen type I levels evaluated by Western blot analysis. As expected, incubation of HSCs with TGF-β for 12 h increased cell-associated levels of procollagen type I protein. At the dose employed (170 μM), PTF did not affect significantly the levels of procollagen type I α_1 and α_2 chains (105 and 95 kDa) both in basal conditions and after stimulation with TGF-β_1. However, in the same experimental conditions, PTF (80 and 170 μM) was able to induce a remarkable reduction of both basal and stimulated procollagen type I levels in cell supernatants (Figure 3).

Effect on the degradation of newly synthesized collagen

In order to better define the potential effect of PTF on collagen degradation, confluent monolayers of HSCs maintained in

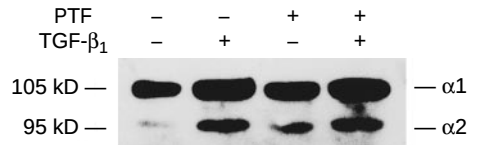

Figure 2 Effect of pentoxifylline (PTF) on cell-associated procollagen type I expression in basal conditions and after stimulation with TGF-β_1 in human HSCs. Western blot analysis of 15 μg of protein obtained from cell lysates separated by 7.5% SDS-PAGE. Experiments were performed on confluent cells after a 48 h incubation period in serum-free/insulin-free medium. Incubation time with PTF alone or with TGF-β_1 (1.0 ng ml^{-1}) with or without PTF co-incubation was 12 h. PTF was used at the dose of 170 μM. Representative blot of two different experiments.

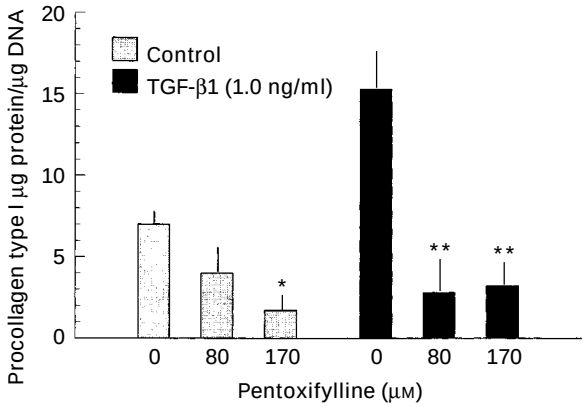

Figure 3 Effect of pentoxifylline (PTF) on procollagen type I levels in cell supernatants in basal conditions and after stimulation with TGF-β_1 in human HSCs. Confluent cells were incubated in serum-free/insulin-free (SFIF) medium for 48 h. After extensive washing cells were incubated in fresh SFIF medium with the addition of PTF alone or TGF-β_1 (1.0 ng ml^{-1}) with or without PTF co-incubation for 12 h. Analysis was performed with a specific ELISA (see Methods for details). Data, expressed as micrograms of protein per microgram of cellular DNA, are mean values $\pm$ s.d. of three experiments performed in quadruplicate. TGF-β_1 vs control: $P<0.001$; *$P<0.001$ vs control values; **$P<0.001$ vs TGF-β_1 alone.

SFIF medium for 48 h were stimulated with 1.0 ng ml^{-1} of TGF-β_1 for 6 and 12 h in the presence or absence of PTF 170 μM. The degree of collagen degradation was measured by following the remaining collagen-bound radioactivity (pepsin-resistant material) in pulse-chase experiments with by L-^{3}H-proline. This technique, allows to better explore the correlation between total collagen synthesis and degradation (Rodemann & Bayreuther, 1984). Cell layers and cell supernatants were separately evaluated to assess the net effect of PTF in the two compartments. As shown in Figure 4, relative to a representative experiment, PTF enhanced collagen degradation in both compartments. In the cell associated compartment, the difference in percentage newly synthesized collagen degradation between cells incubated or not with PTF was evident only after 12 h of incubation (31% vs -2%, respectively). The amount of degradation observed in this compartment at 6 h, in both experimental conditions, probably reflects the basal level of degradation that occurs rapidly and spontaneously and has been calculated to be around 15% (Bienkowski, 1984). As shown (black bars), stimulation with TGF-β_1 induced an evident and time-dependent increase in the % amount of newly synthesised collagen released in cell supernatants (68.6% and 127.6%, at 6 and 12 h, respectively). At the same time points, the release of collagen in response to TGF-β_1 was markedly decreased by the co-incubation with PTF (white bars) (23.0% and 57.5%, at 6 and 12 h, respectively). As synthesis of new collagen was calculated as the net result of total synthesis minus actual degradation, these results suggest that PTF is

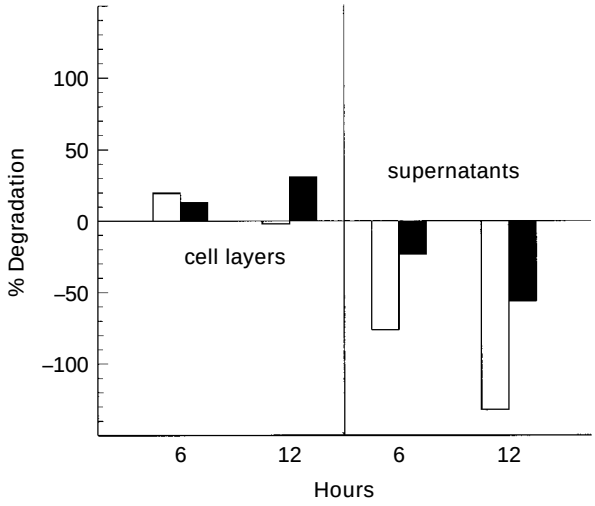

Figure 4 Effect of pentoxifylline (PTF) on the degradation of newly synthesized collagen in human HSCs. Collagen degradation was evaluated by following the remaining collagen-bound radioactivity in pulse-chase experiments employing L-[^{3}H]proline (see Methods for details). Cell layers and cell supernatants were handled separately in order to evaluate net effect of PTF in the two compartments. Experiments were performed for the indicated time points in cells incubated with TGF-β_1 (1.0 ng ml^{-1}) alone (white bars) or together with PTF (170 μM) (black bars). According to the formula used for evaluation of the results (see Methods), positive values express effective degradation, whereas negative values express net synthesis (total synthesis minus effective degradation). Representative experiment of two different ones.

effectively able in enhancing collagen degradation in cell supernatants.

Effects on MMP-1 and TIMP-1 mRNA expression

In agreement with this observation, the potential effects of PTF on the major constituents of the system regulating procollagen type I degradation, i.e. MMP-1, also known as interstitial collagenase, and TIMP-1, were then evaluated.

As shown in Figure 5, activated human HSCs maintained in SFIF medium for 48 h showed low but consistently detectable MMP-1 mRNA levels (lane 1). No regulation of this basal expression was observed by stimulation with either TGF-β_1 for 3 and 6 h (lanes 4 and 5) or with 20% foetal bovine serum for various time points (not shown). Incubation of HSCs with PTF did not produce any variation of the MMP-1 mRNA levels in both basal conditions and after stimulation with TGF-β_1 (lanes 2, 3 and 6, 7, respectively). Although the evidence provided by these experiments is limited to the evaluation of MMP-1 mRNA levels, it appears unlikely that PTF could exert relevant effects at this level.

Consequently, the expression and possible regulation of TIMP-1 mRNA in activated HSCs was analysed. As shown in Figure 6, activated human HSCs maintained in SFIF medium for 48 h clearly expressed TIMP-1 mRNA (lane 1), thus confirming the report of Iredale *et al.* (1992). Interestingly, incubation with TGF-β_1 for 6 h induced a significant upregulation of this basal expression (lane 5). Incubation with PTF induced a significant reduction of both basal and stimulated TIMP-1 mRNA expression: reduction of basal expression, already evident at 3 h (lane 2), was maximal after 6 h (lane 3), whereas it was clearly evident only for the TGF-β_1-induced upregulation observed at 6 h (lane 7).

Effects on 'activated' MMP-1 in cell supernatants

To further substantiate this possibility we analysed the levels of activated MMP-1 present in cell supernatants of both control and stimulated HSCs following incubation with PTF for var-

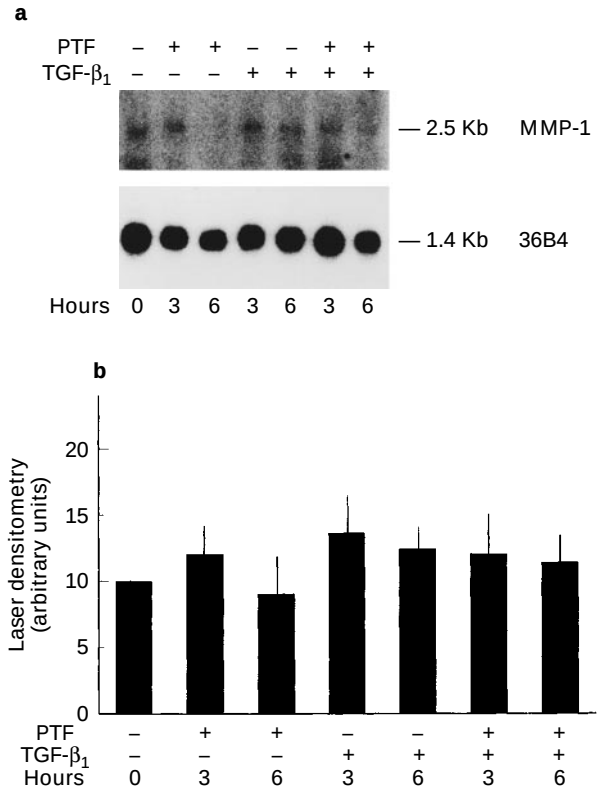

Figure 5 Effect of pentoxifylline (PTF) on MMP-1 mRNA expression in basal conditions and after stimulation with TGF-β_1 in human HSCs. (a) Northern blot analysis of 25 μg of total RNA fractionated by 1% agarose-formaldehyde gel electrophoresis. Experiments were performed on confluent cells after a 48 h incubation period in serum-free/insulin-free medium. Cells were incubated with PTF alone or with TGF-β_1 (1.0 ng ml^{-1}) with or without PTF for the indicated time points. PTF was used at the dose of 170 μM. Representative blot of two different experiments. (b) Depicts relative MMP-1 mRNA expression normalized for 36B4 in the same blot, with the value of the control condition assigned a value of 10. Mean $\pm$ s.d. of values obtained from the two experiments performed. No statistically significant differences were observed between all experimental conditions tested in this group of experiments.

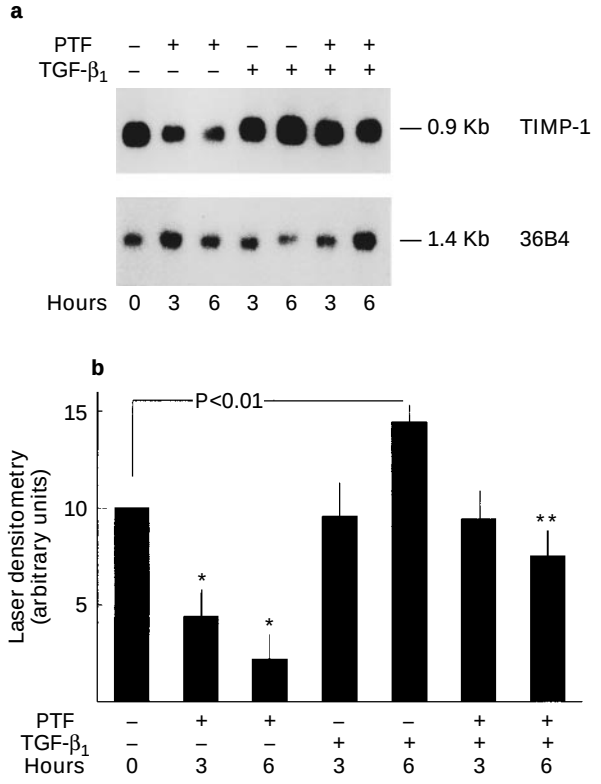

Figure 6 Effect of pentoxifylline (PTF) on TIMP-1 mRNA expression in basal conditions and after stimulation with TGF-β_1 in human HSCs. (a) Northern blot analysis of 10 μg of total RNA fractionated by 1% agarose-formaldehyde gel electrophoresis. Experiments were performed on confluent cells after a 48 h incubation period in serum-free/insulin-free medium. Cells were incubated with PTF alone or with TGF-β_1 (1.0 ng ml^{-1}) with or without PTF for the indicated time points. PTF was used at the dose of 170 μM. Representative blot of two different experiments. (b) Depicts relative TIMP-1 mRNA expression normalized for 36B4 in the same blot, with the value of the control condition assigned a value of 10. Mean $\pm$ s.d. of values obtained from the two experiments performed. $*P<0.001$ vs control values; $**P<0.001$ vs TGF-β_1 alone.

ious time periods, by using a newly developed ELISA system. This assay recognises MMP-1/TIMP-1 complex, i.e. activated MMP-1 that has subsequently been complexed with the specific inhibitor TIMP-1, and shows several advantages over the current bioassays (i.e. zymography), that rely on the enzyme's biological activity to degrade collagen. Indeed these assays require the activation of MMP-1 in a sample where inhibitor is also present, thus leading to artifactual underestimation of the effective enzyme activity. In addition, bioassays also lack specificity as they cannot distinguish between MMP-1 and MMP-8 and the inhibitors TIMP-1,2,3 and α_2-macroglobulin. As shown in Figure 7, incubation with PTF increased the amount of activated MMP-1 at any time point tested both in basal conditions and after stimulation with TGF-β_1, with the effect becoming statistically significant after 12 and 24 h of incubation.

Discussion

In the first part of the present work, the effects of PTF on the sequential steps leading to procollagen type I synthesis and secretion were evaluated. The observation that PTF, at the standard dose of 170 μM, was able to reduce both basal and stimulated procollagen type I mRNA expression is in agreement with the work of Duncan *et al.* (1995). These authors, however, described a more marked inhibition of basal procollagen type I mRNA level in human dermal fibroblasts, possibly because of the remarkably higher concentration of PTF (approximately 3.4 mM) employed.

Of particular interest, in this first group of experiments, is the discrepancy observed between the effect of PTF on cell-associated and extracellular procollagen type I. Overall, it seems unlikely that PTF acts mainly at the level of procollagen type I synthesis. Indeed, regardless of the absence of effect on cell-associated protein levels, the modest reduction of procollagen type I mRNA levels is likely insufficient to explain the marked decrease of the relative protein levels in cell supernatants. It is therefore conceivable to hypothesise an effect of PTF on the complex series of events leading to collagen accumulation following intracellular synthesis.

In an initial study, performed in dermal fibroblasts, Berman *et al* (1989) reported that PTF, as doses ranging from 340 μM to 3.4 mM, was able to reduce serum-driven extracellular total collagen accumulation possibly by increasing total collagenase activity in cell supernatants. However, due to the limitations of the methodology utilised (e.g. total collagen production assayed by ^{3}H-proline incorporation into collagenous proteins, collagenase production measured by solubilization of ^{3}H-labelled rat tail collagen fibrillar gels), this study was not sufficient to identify the precise mechanisms responsible for this effect of PTF. The results of pulse-chase experiments described in the present paper clearly confirm that PTF treatment is able to increase the degradation of newly synthesized collagen in

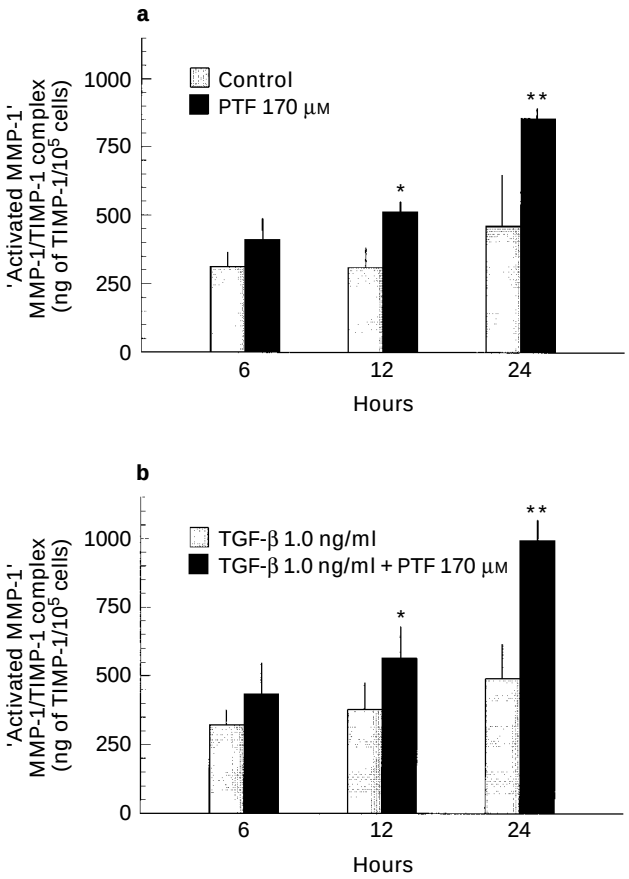

Figure 7 Effect of pentoxifylline (PTF) on 'activated MMP-1' levels in basal conditions and after stimulation with TGF-β_1 in human HSCs. Confluent cells were incubated in serum-free/insulin-free (SFIF) medium for 48 h. After extensive washing cells were incubated in fresh SFIF medium with the addition of PTF alone or TGF-β_1 (1.0 ng ml^{-1}) with or without PTF co-incubation for the indicated time points. Analysis was performed with a specific ELISA (see Methods for details). Data, expressed as nanograms of TIMP-1/10^5 cells, are mean values$\pm$s.d. of two experiments performed in quadruplicate. *$P<0.01$ vs condition without PTF at the indicated time point; **$P<0.001$ vs condition without PTF at the indicated time point.

response to well-established stimulus such as incubation with TGF-β_1. The demonstration that this effect occurs predominantly, if not exclusively, in the extracellular compartment suggests a possible effect of PTF on the expression and function of the system regulating collagen degradation following its secretion. The matrix metalloproteinases are a family of zinc-dependent enzymes responsible for degrading the connective tissue at physiological pH (Matrisian, 1992). Interstitial collagenase digests native fibrillar collagens I, II and III, whereas gelatinase (also known as MMP-II) digests denatured collagens I and III and native collagen IV. In general, activity of MMPs is regulated by three main mechanisms (Murphy *et al*., 1990): regulation of metalloproteinase gene expression, activation of proenzymes and inhibition of active forms by the specific tissue inhibitor of metalloproteinases, such as TIMP-1. Altered degradation of ECM has been implicated in the pathogenesis of hepatic as well as other organ-specific fibrosis. Overall, the available data suggest an enhanced TIMP-1 expression relative to that of MMP-1 in both experimental liver fibrosis and fibrotic human liver (Milani *et al*., 1994; Benyon *et al*., 1996; Iredale *et al*., 1996). In addition, parallel *in vitro* studies performed in cultured rat or human HSCs have provided important insights in the balance between MMP-1 and TIMP-1 actions at the cellular level (Emonard *et al*., 1990; Li *et al*., 1992; Iredale *et al*., 1992, 1996). Taken together these studies suggest that a clear switch in gene expression occurs from early cultures, in which MMP-1 mRNA is expressed but TIMP-1 mRNA is undetectable, through to full cellular activation to the myofibroblast-like phenotype where this pattern is reversed and TIMP-1 and procollagen type I mRNA expression predominates. Accordingly, analysis of collagenase and TIMP activities released into the cell culture supernatants revealed that only TIMP could be detected (Iredale *et al*., 1996). It is therefore conceivable that HSCs in their activated state are tuned to express their maximal fibrogenic potential by releasing large amounts of collagen type I, whose degradation is in turn reduced by an imbalance in the activity of the MMP-1/TIMP-1 system.

In agreement with these concepts, the results here reported suggest that activated human HSCs in basal culture conditions express MMP-1 mRNA at a low level of abundance, whereas the expression of TIMP-1 mRNA appears markedly evident. Although TGF-β_1 has been shown to downregulate MMP-1 mRNA in other mesenchymal cells (Woessner, 1991; Alvares *et al*., 1995), no regulation by this growth factor was detectable in the cell lines and in the experimental conditions employed in the present study. Several lines of evidence could help explaining these findings. It has been demonstrated that activated human HSCs express TGF-β_1 mRNA (Casini *et al*., 1993) and secrete a latent form of this growth factor (Marra *et al*., 1996). In addition, activation of HSCs is accompanied by a progressive increase in the expression of TGF-β_1 receptor types that correlates with responsiveness to TGF-β_1 (Friedman *et al*., 1994). It is therefore possible that, at least in the two HSC lines employed in this study, possible autocrine effects of TGF-β_1 were sufficient to induce a maximal downregulation of MMP-1 mRNA levels. Alternatively or in addition, downregulation of MMP-1 gene expression could represent a constitutive feature of the process of HSC activation, independently of the putative autocrine effects of TGF-β_1.

TGF-β_1 was able to induce a time-dependent upregulation of TIMP-1 mRNA expression. This observation, although original for HSCs, is in agreement with previous investigations performed in human fibroblasts (Overall *et al*., 1989) and further support the key role of this growth factor in hepatic fibrogenic disorders. In these experiments, incubation with PTF induced a significant reduction of both basal and stimulated TIMP-1 mRNA expression. These results, although limited at the level of mRNA abundance, indicate that PTF may affect degradation of newly synthesized collagen type I by affecting the limiting step in the degradation system, i.e. the amount of TIMP-1 available for inhibiting the activity of MMP-1. This possibility is further supported by the experiments investigating the levels of activated MMP-1 present in cell supernatants. Indeed, incubation with PTF increased activated MMP-1 levels in cell supernatants in both basal and stimulated conditions. These results, in agreement with the previous observation that PTF induces an evident and time-dependent degradation of newly synthesized collagen in the extracellular compartment, suggest that treatment of human HSCs with PTF is effective in increasing the activity of MMP-1, possibly by altering the equilibrium existing between MMP-1 itself and the available specific inhibitor.

Taken together the results of the present study suggest that treatment of human HSCs with PTF may affect the deposition of newly synthesized type I collagen by acting at several levels: mRNA abundance, cell-associated and extracellular degradation, the latter appearing as the most relevant action. Several lines of evidence indirectly suggest that all these effects could be related to the main pharmacological action of PTF, i.e. inhibition of cellular phosphodiesterases, followed by increased intracellular cAMP levels. Early studies have indicated that agents such as prostaglandin (PG)E_1 and cholera toxin, able to rapidly increase intracellular cAMP levels, regulate the amount of collagen produced by fibroblasts, at least in part, by modulating the level of intracellular degradation (Baum *et al*., 1980). More recent studies have shown that in cell types actively synthesizing ECM increased intracellular cAMP levels induced by forskolin, cAMP analogues or PGE_2 are associated

with a reduced expression of mRNAs encoding for fibrillar collagens (i.e. procollagen type I and III), whereas the expression of other ECM components such as procollagen type IV and fibronectin is not affected (Yamamoto *et al*., 1994; Zahner *et al*., 1994). These *in vitro* findings are supported by the evidence of a reduced collagen type I mRNA expression and accumulation following treatment with PGE_1 analogues in an experimental model of hepatic fibrogenesis (Beno *et al*., 1992).

It should be stressed, however, that all the studies so far performed have limited their attention to either the synthesis or the cell-associated degradation of procollagen type I. A central issue in the present paper is the demonstration that PTF, a compound able to increase intracellular cAMP levels, exerts a predominant effect on extracellular collagen degradation, together with the expected actions on mRNA levels and cell-associated degradation. This peculiar action appears to be consequent to the ability of inhibiting TIMP-1 expression, not yet ascribed to PTF as well as to other phosphodiesterase inhibitors. Analogously to the other effects of PTF, inhibition of TIMP-1 expression could be secondary to increased cAMP levels, as suggested by recently reported data (DiBattista *et al*., 1995).

In conclusion, the observations reported herein provide further insights in the potential anti-fibrogenic effects of PTF as well as other compounds potentially affecting phosphodiesterase activity. Indeed, although the effects of PTF were detected at high pharmacological concentrations, our observations provide additional basis for the development of similar compounds with higher bio-availability and selectivity. Along these lines it is noteworthy that selective inhibitors of cAMP phosphodiesterase isozymes types III and IV, recently developed, have been shown to be highly effective in counteracting experimental glomerulosclerosis even when employed at very low concentrations (Tsuboi *et al*., 1996). In the perspective of utilising phosphodiesterase inhibitors for the treatment of diseases characterised by chronic inflammation and fibrosis, it is worth mentioning that other pharmacological actions on PTF, i.e. inhibition of tumor necrosis factor-α (TNF-α) production by mononuclear cells (Loftis *et al*., 1997), may also contribute to the general therapeutic effectiveness of this class of compounds.

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