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Effect of Pentoxifylline on the Fibrogenic Functions of Cultured Rat Liver Fat-Storing Cells and Myofibroblasts

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ABSTRACT. The effects of pentoxifylline (PTX) an analogue of the methylxanthine theobromine, on basic fibrogenic reactions of cultured fat-storing cells and myofibroblasts (MFB), the cell types most important for the excessive production of extracellular matrix components in fibrosing liver, were studied. The proliferation of MFB (i.e. activated, transdifferentiated fat-storing cells) was more dose–dependently inhibited by pentoxifylline than that of unactivated fat-storing cells (ED_{50} 50 μg/mL). In addition, PTX retarded the transdifferentiation of fat-storing cells into smooth muscle alpha-actin positive MFB, a 50% reduction in actin-positive cells being reached with concentrations of 0.5 mg PTX/mL medium. The transdifferentiation-associated decrease in retinyl palmitate of cultured fat-storing cells was delayed by PTX. The synthesis of [^{35}S] sulfate-labeled glycosaminoglycans (GAG) and total and cellular fibronectin was not significantly reduced by treatment of MFB with PTX up to 1.0 mg/mL. It is concluded that PTX reduces the transdifferentiation of fat-storing cells to MFB and the proliferation of MFB, but leaves the synthesis of extracellular matrix components GAG and fibronectin unaffected. The effect of PTX on the former reactions might account for the reported antifibrogenic properties of this drug in experimental hepatic fibrogenesis. BIOCHEM PHARMACOL 51;5:577–584, 1996.

KEY WORDS. fat-storing cells; liver fibrogenesis; pentoxifylline; inhibition; matrix synthesis

Liver fibrosis is a complex multicellular process initiated by liver cell injury leading to fibroproliferation and a disturbed turnover of ECM† components [1–3]. An excessive accumulation of nearly all ECM constituents, a disproportionate increase in the various collagenous and noncollagenous matrix molecules, and a preponderant deposition of newly formed matrix in Disse space (perisinusoidal fibrosis) are hallmarks of human and experimental liver fibrosis subsequently leading to a loss of the typical anatomical structure of the tissue. Liver FSC (also called perisinusoidal lipocytes, hepatic stellate cells, vitamin-A-storing cells or Ito cells [4]) are the main (precursor) cell type for the excessive production of ECM material in diseased liver [2, 5, 6]. In chronically injured and inflamed liver tissue FSC are "activated" to proliferate and transdifferentiate phenotypically into MFB [7]. The transdifferentiation (also called "transformation") is attested to by morphological changes (e.g. loss of vitamin A-loaded lipid droplets, hypertrophy of rough endoplasmic reticulum, expression of smooth muscle alpha-actin [8], and expression of cell surface receptors for various cytokines and growth factors [9]). During this process, the biochemical functions of the cells change dramatically, leading to an exaggerated production, secretion, and extracellular deposition of ECM components, including collagens, proteoglycans, glycosaminoglycans (hyaluronan), and structural glycoproteins such as fibronectin and laminin [10–12]. MFB are the main source of ECM components in liver fibrosis. Therapeutic strategies for liver fibrosis are focused on FSC as the target cell type [3]. Drugs that exert an antiproliferative effect, reduce FSC transdifferentiation to MFB, and/or inhibit the synthesis of ECM components are of particular therapeutic interest [13, 14].

PTX, a methylxanthine derivative, has been found to have inhibitory effects on proliferation of human skin fibroblasts and their synthesis of ECM components [15–17]. We hypothesized that, with regard to the pathobiochemical mechanisms of liver fibrogenesis, PTX might exert beneficial effects on the course of the disease by affecting some of the fibrogenic reactions of FSC and MFB, respectively. Negligible side effects and toxic properties [18] suggest that PTX might be a potentially useful drug for the treatment of fibrosis. In fact, it has been shown that PTX reduces liver fibrosis in a swine model [19] and, very recently, in a rat model of biliary cirrhosis [20], but the mechanism of its action is still unknown.

Under cell culture conditions, FSC transdifferentiate spontaneously into MFB and show very similar properties to those found in fibrotic liver disease [21–23]. Therefore, cultured FSC

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† *Abbreviations:* CHAPS, 3-[(3-cholamidopropyl)dimethyl ammonio]-1 propanesulfonate; DMEM, Dulbecco's modified Eagles medium; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; FSC, fat-storing cells; GAG, glycosaminoglycans; MFB, myofibroblasts; NEM, n-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; PTX, pentoxifylline; TNF, tumor necrosis factor

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are a valuable tool for testing drugs that could be relevant in the treatment of liver fibrosis as well as for the analysis of their mechanism of action. Until now, the effects of PTX on fibroproliferation have only been investigated in nonhepatic cell cultures (e.g. in cultured dermal fibroblasts [15–17] but not in FSC or MFB. In our study, we focused on the effects of PTX on important partial reactions of FSC activation in culture (i.e. proliferation, transdifferentiation, and synthesis of ECM components).

MATERIALS AND METHODS

Isolation and Culture of Fat-storing Cells and Myofibroblasts

The cells were isolated from the intact livers of 1-year-old male Sprague-Dawley rats (body weight 500–700 g, Lippische Versuchstierzucht, Extertal, Germany); they had free access to a standard laboratory chow diet containing 15,000 I.U. vitamin A/kg and tap water. Nonparenchymal liver cells were isolated by the pronase-collagenase method [24] with slight modifications as described elsewhere [25]. FSC were purified from the nonparenchymal cell suspension by a single step density-gradient centrifugation with Nycodenz (Nyegaard Co. AS, Oslo, Norway), which was reported in detail previously [25]. The cells were identified by their typical light and electron microscopic appearance, immunofluorescent stainings for desmin and vimentin [26], vitamin A-specific autofluorescence [25] and, negatively, by their inability to phagocytose latex beads (LB11 polystyrene beads, diameter 1.1 μm), to stain for peroxidase, and to express Fc receptors [27]. The mean purity of freshly isolated cells was 89% and mean cell viability checked by trypan blue exclusion was 91%. The cells were seeded at a density of 0.4×10^6 cells/10 cm^2 on 6-well culture plates (Greiner, Nürtingen, Germany) and maintained in 2 mL of DMEM containing 4 mM L-glutamine, penicillin (100 IU/mL), streptomycin (100 μg/mL), and 10% (v/v) fetal calf serum (FCS) (all from Boehringer GmbH, Mannheim, Germany). The cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Plating efficiency was at least 60%. The first medium change was carried out 16 hr after seeding, after which time the purity of FSC was higher than 97%. MFB were obtained by trypsinising and reseeding FSC cultures 10 days after their initiation.

Immunofluorescence Staining of Smooth Muscle Alpha-actin

The effect of PTX on the transdifferentiation was assessed by immunofluorescent staining of alpha-smooth muscle actin during continuous exposure of FSC to PTX for 6 days, starting 24 hr after seeding. At the end of the culture time, cells were washed twice with PBS and fixed with ethanol/acetic acid (95:5, v/v) for 15 min at 4°C. The cells were washed again and incubated for 2 hr at 37°C with appropriate dilutions of monoclonal mouse antibody to human alpha-actin isoform of smooth muscle cells (Boehringer). Then, the cells were washed with PBS and incubated for 90 min at 37°C with biotinylated rabbit antimouse-immunoglobulin followed by FITC-conjugated streptavidine (DAKO, Hamburg, Germany) demonstrating cytoskeletal proteins. Control cultures were processed without first incubation with the specific antibody. Cells with the typical alpha-actin structure were considered to be alpha-actin positive. For each group, 1000 cells were determined microscopically and the percentage of alpha-actin expressing cells was calculated.

Quantitative Measurement of Retinyl Palmitate

Retinyl palmitate was determined according to the method described by Vuilleumier *et al.* [28] with minor modifications described by Bachem *et al.* [29]. FSC were incubated with concentrations of PTX ranging from 0.01 to 1 mg/mL medium for 7 days, starting 24 hr after seeding. Retinoids were extracted 1, 3, 6, and 8 days after seeding. To determine cellular retinyl palmitate, cultures were washed twice in the dark with PBS. All the following steps were performed in the dark. Retinoids were extracted with methanol/ethylacetate 1:1 (v/v) (1 mL per culture well, 2 hr at 4°C). After centrifugation (10 min at 600 *g*) the transparent extraction fluid was used for HPLC. Retinyl palmitate was determined in 100 μL aliquots by reverse phase HPLC using an RPC18 analytical column (Waters, Milford, U.S.A.), developed at 1.3 mL/min for 30 min with methanol/ethylacetate/water 510:140:25 (v/v/v) and fluorescence detection (excitation 338 nm, emission 425 nm). The concentration of retinyl palmitate was determined by comparing the peak area of the probes with external standards of retinyl palmitate (Sigma Chemical Company, Munich, Germany) in methanol/ethylacetate.

Determination of Fat-Storing Cell and Myofibroblast Proliferation

Proliferation of the cells was measured by the incorporation of [^{3}H]thymidine into the DNA. The cells were incubated with [^{3}H]thymidine (1.0 μCi/mL medium, NEN-DuPont, Dreieich, Germany) during a labeling period of 24 hr. Radioactivity incorporated into DNA was measured as described previously [30]. Medium containing different concentrations of PTX was added starting 2 days after seeding (FSC) and 4 days after trypsinization (MFB). The cells were labeled with [^{3}H]thymidine 24 hr later. Simultaneously, the content of DNA in each well was measured fluorometrically.

Measurement of Fibronectin Concentration in Culture Medium

Total and cellular fibronectin were measured in the culture medium by a time-resolved fluorescence immunoassay using europium chelate. This method has been described in detail elsewhere [31]. The MFB were treated with different concentrations of PTX for 5 days. Two days before termination of the culture, the cells were washed twice with PBS and then kept FCS-free to avoid contamination of fibronectin by FCS;

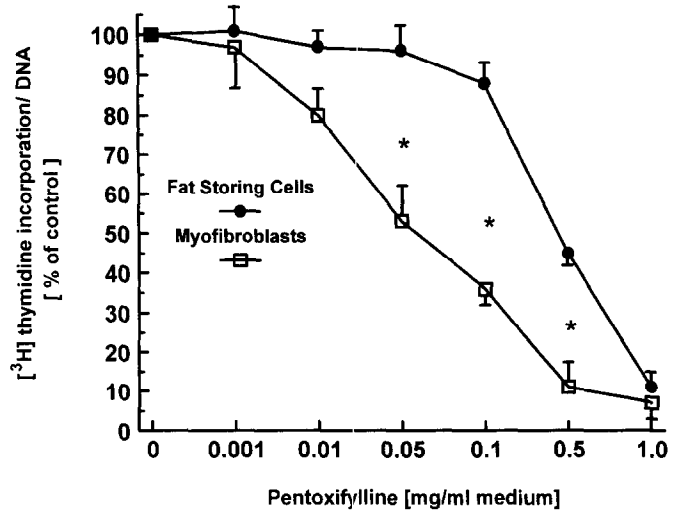

FIG. 1. **Inhibition of proliferation of FSC and transdifferentiated fat-storing cells (MFB) by PTX. Percentage of [^{3}H]thymidine incorporation/DNA during a 24-hr labeling period, exposing FSC and MFB to PTX for 48 hr starting 2 days after seeding (FSC) and 4 days after trypsinization (MFB). PTX was added in concentrations ranging from 0.001 to 1 mg/mL medium. Results are given as percentage compared to the untreated control. Values represent the mean of 3 experiments ± SD, *P < 0.01.**

cellular and total fibronectin were measured at the end of the 2-day FCS-free period. The *de novo* synthesis of fibronectin was proven by blocking protein synthesis with cycloheximide (25 μM).

TABLE 1. **Effect of PTX on the Proliferation of FSC and MFB. The Data Show [^{3}H]thymidine Incorporation [kBq/mg DNA]. PTX Was Added in Concentrations Ranging from 0.001 to 1 mg/mL Medium (n = 3, mean values ± SD, *P < 0.05).**

PTX (mg/mL)	FSC (kBq/mg DNA)	SD	MFB (kBq/mg DNA)	SD
0	1219.8	87.0	391.4	19.9
0.001	1227.8	25.3	376.2	20.2
0.01	1190.4	125.3	324.4*	19.1
0.05	1168.1	134.8	212.5*	18.9
0.1	1076.2	45.5	147.9*	7.6
0.5	546.5*	60.9	41.2*	8.3
1	141.2*	45.3	24.8*	7.4

Determination of Glycosaminoglycan Synthesis

The synthesis of sulfated GAG was determined by the incorporation of [^{35}S]sulfate (20 μCi/mL medium; NEN-DuPont) into the GAG during a labeling period of 24 hr. Proteoglycans were determined in the medium (where normally approximately 80% of newly synthesized material is found [25]) by a nonproteolytic isolation procedure [30]. The medium was removed and centrifuged immediately (6 min, 1000 g, 4°C). Cell-free medium (700 μL) was mixed with 3 mL buffer A (7 M urea, 1 mM EDTA, 1 mM PMSF, 10 mM NEM, 0.1% CHAPS, 0.13 M Tris-Cl, pH 7.5) and unlabeled GAG (hyaluronan, heparin, chondroitin-4-sulfate, chondroitin-6-sulfate; all from Sigma) were added as carrier. Then the proteo-

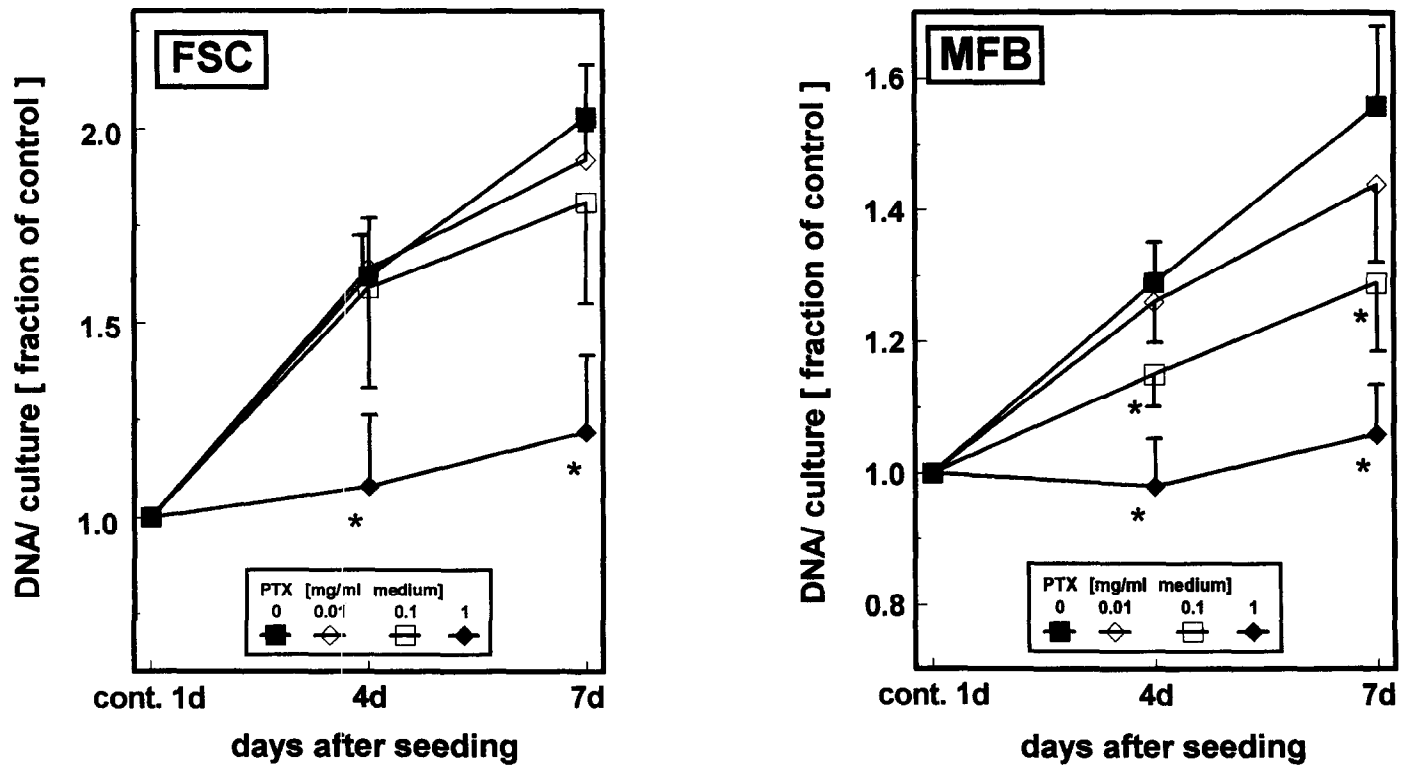

FIG. 2. **DNA content of FSC and MFB cultures continuously exposed to various concentrations of PTX in the culture medium, starting 24 hr after seeding. DNA content per culture was determined fluorometrically 1, 4, and 7 days after seeding (FSC) or passage (MFB). Mean values of 5 experiments ± SD, *P < 0.05.**

glycans were bound to a batch of DEAE-Sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with the same buffer. After the resin was washed with buffer B (=buffer A + 0.1 M NaCl, pH 7.5) total proteoglycans were eluted with buffer C (=buffer A + 0.8 M NaCl, pH 7.5). An aliquot of the effluent was counted for radioactivity and referred to the DNA content of the culture.

General Techniques

Cells were quantified by fluorometric determination of their DNA [32]. Viability was assayed by the trypan blue exclusion test and fluorochromasia with propidium iodide [33]. PTX (Sigma) dissolved in PBS was added to the culture medium, reaching final concentrations of between 0.001 and 1 mg/mL medium. In controls, the same medium without PTX was added.

Statistical Analysis

Data are presented as mean ± SD. Differences between groups were tested using analysis of variance. Differences between selected groups were tested for significance with Scheffe's multiple range test for homogeneous groups.

RESULTS

Effect of Pentoxifylline on the Proliferation of Fat-storing Cells and Myofibroblasts

The proliferation of early cultured FSC, measured by [^{3}H]thymidine incorporation in relation to the DNA content of the culture, was reduced dose-dependently by PTX. It was reduced by 50% at a concentration of 0.5 mg PTX/mL medium compared to control cultures. After transdifferentiation to MFB, the proliferation was affected by PTX more than in early cultures and also in a similar dose-dependent manner: reduction of MFB proliferation by 50% was reached at 0.05 mg PTX/mL as compared to control cell cultures (Fig. 1). Despite a lower proliferative activity compared to the FSC, MFB proved to be more susceptible to the antiproliferative action of PTX (Table 1). Fluorometric measurement of the DNA content per well during 7 culture days confirmed the results of the [^{3}H]thymidine incorporation studies. FSC were less susceptible to the antiproliferative effect of PTX than the transformed cells (Fig. 2).

The number of trypan blue positive (nonviable) cells (FSC as well as MFB) exposed to PTX up to concentrations of 1 mg PTX/mL culture medium never exceeded that in the untreated controls (approximately 5 to 10%). This result was confirmed using fluorochromasia with propidium iodide as an index of viability.

Influence of Pentoxifylline on Fat-storing Cell Transdifferentiation to Myofibroblasts

Taking into account the fraction of alpha smooth muscle-actin positive cells in the culture wells 7 days after seeding under continuous exposure to PTX for 6 days, the transdifferentiation of FSC into MFB was delayed dose-dependently by the drug. Statistically significant differences were reached with concentrations beginning at 0.1 mg PTX/mL medium (Fig. 3). A reduction of 50% in comparison to the fraction of alpha-actin positive cells in the control cultures was reached at concentrations of 0.5 mg PTX/mL medium. However, a complete inhibition of transdifferentiation was not reached despite maximal concentrations of PTX (Figs. 3 and 4).

The content of retinyl palmitate in FSC, observed during primary culture for 8 days after seeding, decreased continuously. This time course was only slightly affected by PTX. A concentration of 1 mg PTX/mL medium, after an incubation time of 8 days, delayed the loss of retinyl palmitate of the FSC, resulting in a content similar to that on days 3 to 6 in the untreated group (Fig. 5).

Effects of Pentoxifylline on the Synthesis of Extracellular Matrix Molecules

When the incorporation of [^{35}H]sulfate into the sulfated GAG was measured in relation to DNA content, concentrations of PTX up to 1 mg/mL medium had neither an inhibitory nor a stimulatory effect on the synthesis of proteoglycans (GAG) in MFB when compared to the untreated control (Fig. 6).

The synthesis of cellular fibronectin in MFB also remained unaffected by PTX. Total fibronectin synthesis was slightly, but statistically not significantly, reduced by PTX in a dose-dependent manner (Fig. 7).

DISCUSSION

Liver fibrosis is a complex process characterized by increased synthesis and deposition of ECM components. In this context,

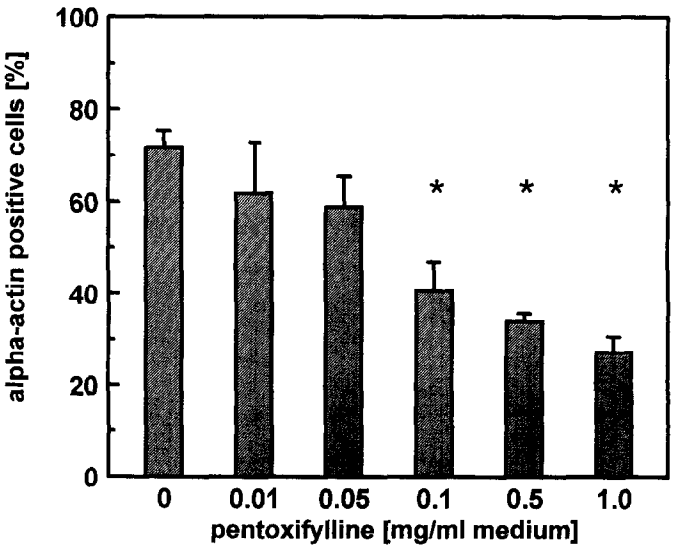

FIG. 3. Alpha-smooth muscle actin expression in FSC after continuous exposure to various concentrations of PTX for 6 days, starting 24 hr after seeding. The cells were assessed by light microscopy after immunofluorescence staining, determining the percentage of alpha-actin positive cells in each group. The results represent the mean values ± SD of 4 experiments. *$P < 0.01$.

FIG. 4. Indirect immunofluorescent staining for alpha-smooth muscle actin in FSC. The cells were exposed to various concentrations of PTX (0, 0.01, 0.1, and 1 mg PTX/mL medium) for 6 days, starting 24 hr after seeding.

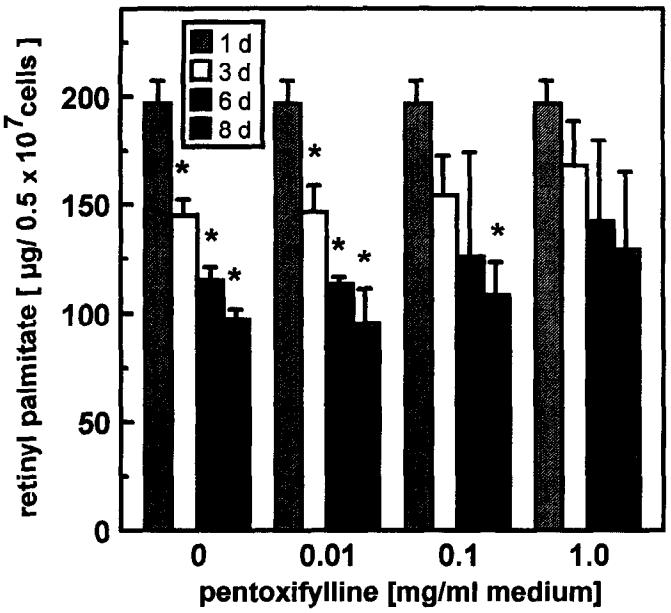

FIG. 5. Time-dependent loss of retinyl palmitate in primary cultures of FSC. The cells were incubated with different concentrations of PTX (0, 0.01, 0.1, 1 mg/mL medium) for 7 days, starting 1 day after seeding. 1, 3, 6, and 8 days after seeding, retinoids were extracted and determined by HPLC. Results represent the mean values ± SD (*$P < 0.05$) of 3 cultures from separate preparations.

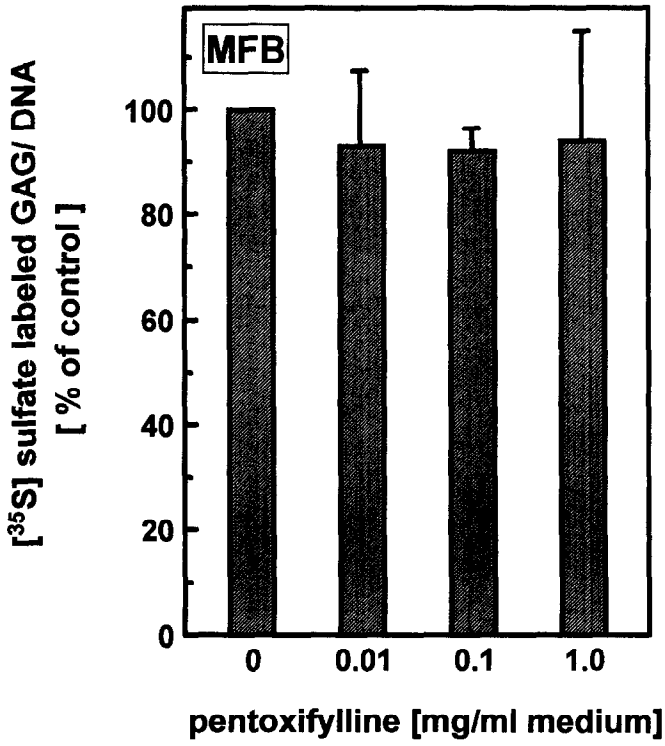

FIG. 6. Proteoglycan synthesis in MFB exposed to various concentrations of PTX for 48 hr, measuring [^{35}S]sulfate incorporation into GAG during a labeling period of 24 hr in relation to the DNA content per well. The results are given as percentage of untreated control ($n = 3$, mean values ± SD).

fibrosis was originally defined as the "presence of excess collagen deposition due to new fibre formation" and liver cirrhosis as "a diffuse process characterized by fibrosis and conversion of normal architecture into structurally abnormal nodules" [34]. The pathophysiological basis of the disease is a disturbed cooperation between parenchymal and nonparenchymal cells due to liver injury, initiating a cascade of mechanisms beginning with hepatocyte necrosis and followed by inflammation, activation of macrophages, release of fibrogenic mediators, activation of FSC, stimulated ECM synthesis, and diminished matrix degradation resulting in a net matrix accumulation [35]. FSC and their activated counterparts (MFB) play a central role in the process of fibrogenesis [7]. These cells produce the majority of the ECM components (e.g. collagens, proteoglycans, glycoproteins, and hyaluronan) [10, 11]. They have the ability to stimulate themselves *via* production of growth regulators in an autocrine way [35]. Therefore, a useful antifibrotic drug either has to influence hepatocyte damage, hinder the transdifferentiation of FSC into MFB, attenuate the proliferative activity of MFB, or reduce the synthesis capacity of matrix components.

PTX, an analog of the methylxanthine theobromine, was initially characterized as a hemorheologic agent for the treatment of peripheral vascular disease. Therapeutic benefit derives primarily from increased red blood cell deformability, reduced blood viscosity and decreased potential for platelet aggregation. Phosphodiesterase inhibition resulting in an increase in intracellular cAMP concentration, is the generally accepted mechanism of action of PTX [18]. PTX exerts anticytokine activity, inhibiting the pro-inflammatory actions of interleukin-1 and TNF alpha on neutrophil function [36]. Inhibitory effects on the proliferation and ECM synthesis of human dermal fibroblasts are described [15–17]. *In vivo*, PTX prevented the biochemical and histological changes associated with animal models of liver fibrosis [19, 20]. Until now, no investigation has been carried out focusing on the effects of PTX on FSC and MFB as the main source of ECM in liver fibrosis.

Our results demonstrate that, under cell culture conditions, exposure of FSC to PTX results in retardation of their transdifferentiation into MFB. The participation of factors stimulating transdifferentiation into MFB such as TNF alpha [29] should be the object of further studies because it is known that PTX diminishes the synthesis and effects of TNF alpha in other cell systems [36, 37]. Moreover, the proliferative activities of FSC as well as of MFB were attenuated by PTX in a dose-dependent manner. Unspecific toxic effects of PTX were excluded by trypan blue staining of the treated cells compared to control. The results might suggest that *in vivo* PTX could reduce the pool of MFB in damaged, fibrosing liver. Interestingly, MFB were approximately 10-fold more sensitive compared to FSC. Therefore, the pathogenically most relevant active cells are affected preferentially. The increasing sensitivity to PTX during the transdifferentiation of FSC might be due to qualitative and/or quantitative alterations of growth-factor receptors during phenotypic transition of these cells. Because platelet-derived growth-factor receptor expression depends strictly on transformation [38], an involvement of this growth

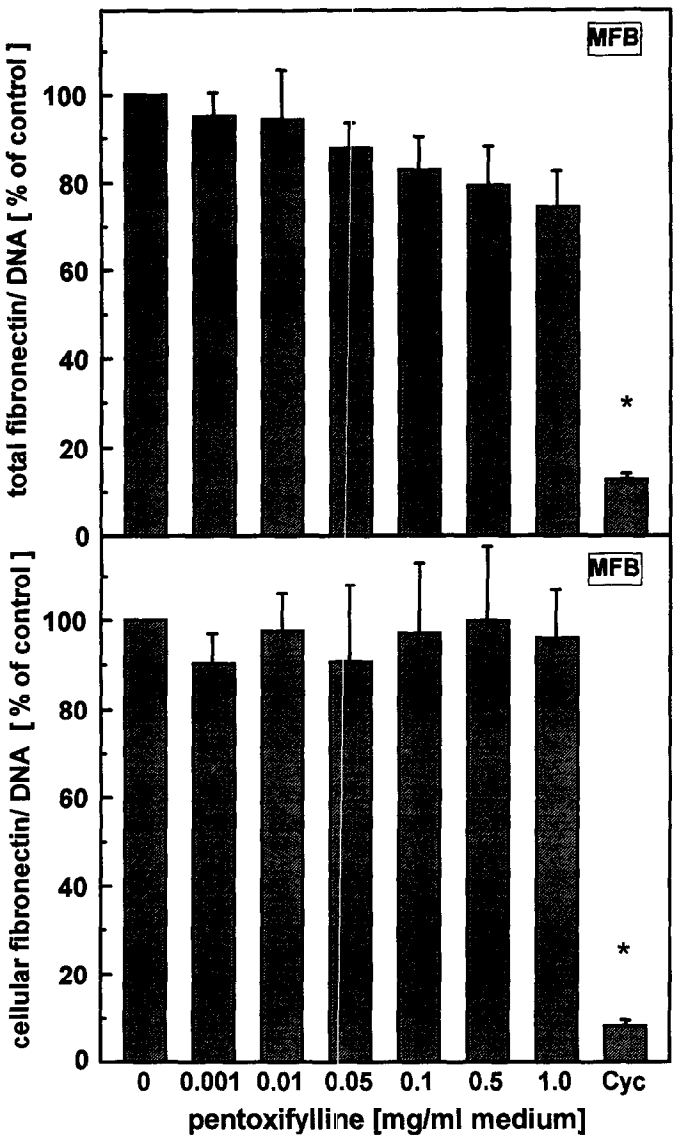

FIG. 7. Synthesis of total fibronectin/DNA and of cellular fibronectin/DNA after 5 days of exposure to various concentrations of PTX in MFB. The cells were kept FCS-free for the final 2 days. The results are given as percentage of untreated control. Fibronectin synthesis was completely blocked with cycloheximide (Cyc)(n = 3, mean values ± SD, *$P < 0.01$).

factor in PTX-induced mitotic inhibition is likely. Results obtained with fibroblasts support this hypothesis, because PTX reduced the stimulatory potential of platelet-derived growth factor on the proliferation of these cells [39].

The synthesis of ECM components such as GAG and fibronectin by MFB was not significantly inhibited despite exposure to maximal doses of PTX. This reaction distinguishes MFB from dermal fibroblasts, which reduce their synthesis of glycosaminoglycans and fibronectin in response to PTX [15, 16]. This different reaction of MFB is not surprising because dermal fibroblasts and MFB are quite different cell types, with noncomparable physiological properties. Recently, Pinzani *et al.* published preliminary results showing an inhibitory effect of PTX on collagen synthesis in human FSC [40], indicating that the various ECM components might respond differently to PTX.

The present study suggests that PTX exerts an inhibitory effect on basic pathobiochemical mechanisms of fibrogenesis (i.e. the transdifferentiation of FSC into MFB and the proliferation, preferentially that of MFB, the major source of ECM components). Therefore, PTX has to be regarded as a potential antifibrotic agent for the treatment of ongoing human liver fibrosis characterized by an enlarged number of myofibroblasts [41–43]. In support of this assumption, PTX was shown to inhibit liver fibrosis using a porcine model [19] and first results using a rat model of biliary fibrosis have recently been published [20]. Interpretation of these results indicates a hepatoprotective effect of PTX as well, because typical parameters of liver cell injury were less severely affected by application of PTX during exposure to noxious agents [19]. Oral administration of PTX produces serum concentrations of the unmetabolized drug of only 2–5 μg/mL [44]; intravenous administration or even organ-site-directed drug targeting may, however, achieve *in vivo* concentrations similar to inhibitory concentrations *in vitro*.

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