Pentoxifylline Prevents Fibrosis in an Animal Model and Inhibits Platelet-derived Growth Factor–driven Proliferation of Fibroblasts

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Liver fibrosis is a complex process characterized by two major events: fibroproliferation and increased collagen synthesis. The exact role of cytokines in the pathogenesis of hepatic fibrosis remains to be established, but platelet-derived growth factor clearly stimulates proliferation of fibroblasts and increases collagen synthesis. In *in vitro* studies, pentoxifylline, a methylxanthine, significantly reduced platelet-derived growth factor–driven proliferation of fibroblasts. Platelet-derived growth factor has also been identified as a fibroproliferative factor produced spontaneously by monocytes obtained from patients with liver disease. Long-term administration of pentoxifylline (16 mg/kg orally, 5 days/wk for 12 wk) in an animal model of liver fibrosis prevented elevations in γ-glutamyl transpeptidase and alkaline phosphatase levels and prevented the reduction in serum albumin level normally observed in this animal model of liver disease. The animal model used was a long-term, low-dose yellow phosphorus–induced model in pigs that reproducibly results in extensive fibrosis after 10 to 12 wk of treatment. Long-term administration of pentoxifylline also prevented the histological changes characteristic of fibrosis in this animal model. Collagen concentration was significantly elevated in liver sections obtained from animals receiving yellow phosphorus, compared with controls. Long-term pentoxifylline treatment resulted in significantly lower collagen concentrations in liver sections from animals receiving yellow phosphorus than in sections from animals receiving yellow phosphorus alone; this was supported by histological observation. Therefore administration of pentoxifylline prevented the biochemical and histological changes associated with an animal model of liver disease. Pentoxifylline will likely have an important therapeutic role in liver fibrosis. (HEPATOLOGY 1993;17:486-493.)

Fibrosis occurs as a result of an initial liver injury (1). In hepatic fibrosis, the Kupffer cell, hepatocyte and Ito cell communicate by way of cytokines. Cell communication is involved in the transformation of Ito cells to myofibroblasts, proliferation of fibroblasts and changes in cellular matrix components and down-regulation of drug metabolism (2, 3). In theory, if cell communication can be blocked it may be possible to halt or prevent fibrosis. The pathogeneses of liver fibrosis are diverse, including cystic fibrosis (4-7) but regardless of the type of agent producing the damage, the events leading to hepatic fibrosis may be similar and evidence suggests that the mononuclear cells play a significant role in these events (8). The number of cytokines involved in the pathogenesis of hepatic fibrosis remains to be established, but platelet-derived growth factor (PDGF) clearly stimulates fibroproliferation (9) and also stimulates synthesis of collagen by fibroblasts (10). Other cytokines, including interleukin-1 (IL-1) and transforming growth factor-β (TGF-β), have also been implicated in fibrosis (11-13). Monocyte-conditioned medium from monocytes of patients with liver disease is fibroproliferative. This fibroproliferation can be blocked by PDGF antibody (14) but not by IL-1 antibody (15). With the help of an *in vitro* fibroproliferation assay (16), we determined that exogenous PDGF is proliferative for human fibroblasts (ED_{50} = 0.8 ng/ml). In *in vitro* studies the effect of pentoxifylline was tested on PDGF-driven proliferation of human fibroblasts. Pentoxifylline is a methylxanthine reported to reduce serum-driven and IL-1–driven fibroproliferation (17). Pentoxifylline is currently indicated for chronic use in peripheral vascular disorders because of its effects on RBC deformability and blood flow (18). The antifibrotic activity of pentoxifylline was assessed in an animal model of liver fibrosis that reproducibly results in extensive fibrosis in 10 to 12 wk (19).

MATERIALS AND METHODS

In Vivo Study. Female Duroc-Hampshire pigs were administered yellow phosphorus (0.6 mg/kg/day, 5 days/wk, orally) starting at the age of 6 wk (19). The study protocol was approved by the ethics committee at this institution. This long-term, low-dose yellow phosphorus model is an adaptation of the original model by Mallory (20) designed to study the development of fibrosis in rodents. Mallory suggested that yellow phosphorus treatment produced the degenerative changes characteristic of alcoholic cirrhosis. Treatment of pigs with yellow phosphorus (0.6 mg/kg orally, 5 days/wk) repro-

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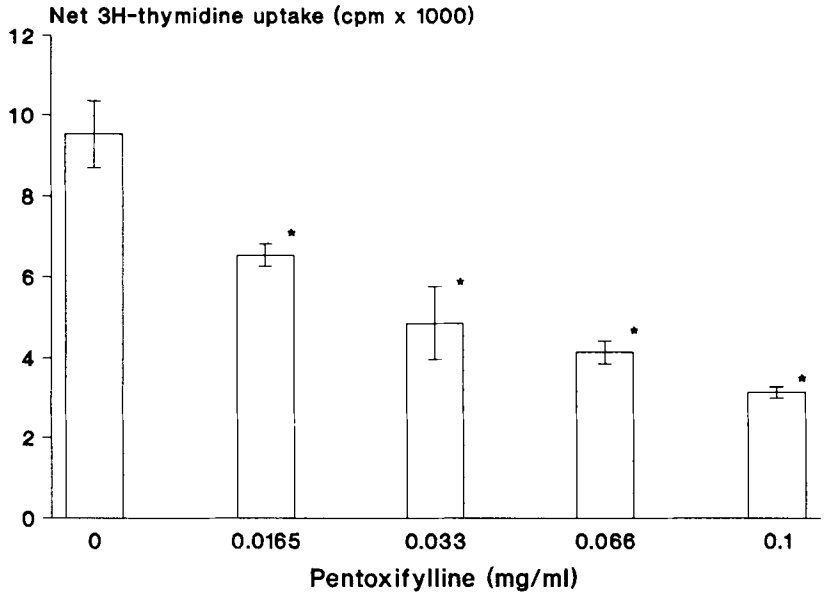

FIG. 1. Effect of pentoxifylline on PDGF-driven fibroproliferation *in vitro*. Increasing concentrations of pentoxifylline were incubated with fibroblasts exposed to 10 times the ED_{50} level for PDGF (8 ng/ml). Results expressed as net tritiated thymidine uptake by human fibroblasts. Each bar represents the mean ± S.E.M.; n = 4. *Significantly different from fibroblasts exposed to PDGF (8 ng/ml) alone; $p < 0.05$.

ducibly and significantly elevates serum biochemical liver enzymes, including alkaline phosphatase and γ-glutamyl transpeptidase and lowers serum albumin concentration by 8 wk of treatment. Lower doses of yellow phosphorus were not as effective in producing fibrosis in this time frame, and higher doses were associated with mortality. No mortality was observed with the 0.6 mg/kg dose for the 12-wk period. The animal model reproducibly results in hepatic fibrosis by 8 wk of treatment with yellow phosphorus, extensive fibrosis by 12 wk and cirrhosis by 16 wk of treatment. Changes in histological appearance or liver function as assessed on the basis of serum biochemical tests are not usually evident until after 4 wk of treatment with yellow phosphorus (19). Between the fourth and eighth weeks of treatment with yellow phosphorus, changes in collagen deposition, with early fibrosis, are evident on histological study. Elevation in γ-glutamyl transpeptidase activity also occurs at this time. Marked decreases in hepatic drug-metabolizing enzymes also occur in the animal model during development of fibrosis (19). Collagen concentration is significantly elevated in liver sections obtained from animals treated with yellow phosphorus for 10 to 12 wk (19). The time of onset and the stimulus for fibrosis is unknown in this animal model. To test the effect of long-term pentoxifylline treatment in this animal model, pigs were treated simultaneously with yellow phosphorus (0.6 mg/kg) and pentoxifylline (16 mg/kg) daily, 5 days/wk for 12 wk. The dose of pentoxifylline administered was approximately equivalent to that used in human subjects (1,200 mg) for chronic peripheral vascular disorders (18). A plasma concentration of 0.033 mg/ml would be expected after a dose of 16 mg/kg (21).

Blood samples were taken from pigs at 10 to 12 wk for assay of alkaline phosphatase activity, γ-glutamyl transpeptidase activity, ALT activity, AST activity, serum albumin levels, bilirubin levels and serum ammonia concentration. Blood levels of γ-glutamyl transpeptidase are also reported for 0, 4, 6, 8 and 12 wk in the follow-up experiment. Animals were weighed and liver biopsy specimens were obtained at the completion of the experiment. Liver sections were stained with Masson's trichrome for histological study.

Collagen concentration in liver sections was determined by staining with Fast green and Sirius red using the method of Gascon Barré (22) as originally developed by Lopez-de Leon and Rojkind (23). This method has previously been established as a good index of liver fibrosis in this animal model (20).

In Vitro Study. The fibroproliferative effect of PDGF was assessed with a modification of the tritiated thymidine incorporation method of Dohlman (24) using normal skin fibroblasts. Normal human fibroblasts were grown in Dulbecco's modified Eagle's (DBE) medium (Gibco BRL Canadian Life Technologies, Inc., Burlington, Ontario, Canada) supplemented with antimycotic antibiotic (AA; 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25 U/ml amphotericin B [Gibco/BRL] and 10% controlled process serum replacement-1 [CPSR-1; Sigma Chemical Co., St. Louis, MO]) in 5% CO_2 until confluent. When a sufficient stock of fibroblasts was obtained, the monolayers were maintained on DBE, AA and 10% CPSR-2 (Sigma Chemical Co.), a low-mitogenicity serum replacement. For assay of fibroproliferation, medium was removed by aspiration. The cells were briefly rinsed with sterile saline solution, and then the fibroblasts were removed from the flasks by the addition of trypsin-EDTA (Gibco BRL). Cells were resuspended in DBE, AA and 0.5% CPSR-2 and counted in a Neubauer-type hemocytometer. The volume used per well was 200 μl (2,000 cells). Aliquots of cell suspension (200 μl) were incubated for 24 hr at 37° C in 5% CO_2 in air on microtiter plates. The medium was removed from each well by aspiration and replaced with 200 μl DBE supplemented with PDGF (PDGF-AB chain; R&D Systems, Inc., Minneapolis, MN) with and without the inhibitor pentoxifylline (Sigma Chemical Co.). Pentoxifylline was added in varying concentrations and incubated for a further 24 hr. During the last 2 hr of incubation, 0.5 μCi of methyl [^{3}H]thymidine (Amersham Canada Ltd., Oakville, Ontario, Canada) was added to each well. To harvest the fibroblasts, we removed the medium from wells by

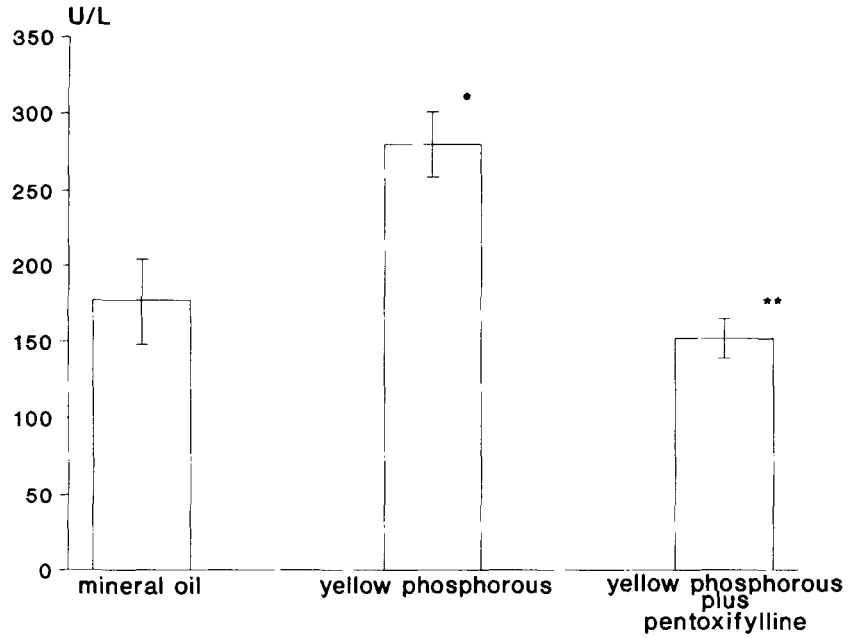

FIG. 2. Effect of pentoxifylline treatment on alkaline phosphatase activity in animals treated with yellow phosphorus to induce fibrosis. Animals were treated simultaneously with pentoxifylline (16 mg/kg) and yellow phosphorus (0.6 mg/kg daily) 5 days a week for 10 to 12 wk. Each bar represents the mean ± S.E.M.; n = 3. *Significantly different from mineral oil–treated controls; $p < 0.05$. **Significantly different from yellow phosphorus–treated animals; $p < 0.05$.

aspiration and replaced it with 100 μl trypsin-EDTA (1%) for 15 min at room temperature. This treatment with trypsin was verified as sufficient to detach all fibroblasts. The fibroblasts were then aspirated onto glass-fiber filters with a Brandel cell harvester (Xymotech Biosystems, Ontario, Canada) and washed eight to 10 times with PBS. Radioactivity contained on each filter was determined by liquid scintillation counting. PDGF was assessed in a dose-response manner to determine the ED_{50}. Then the fibroproliferation due to PDGF (8 ng/ml equivalent to 10 times ED_{50} and clearly maximal) was tested in the presence of pentoxifylline. Pentoxifylline was tested at 0.0165 mg/ml, 0.033 mg/ml, 0.066 mg/ml and 0.1 mg/ml. Fibroproliferation assays were also performed in the absence of PDGF and in the presence of pentoxifylline to determine whether the inhibitors effected baseline fibroproliferation. Cell-viability tests were also performed (trypan blue exclusion) to determine whether pentoxifylline affected cell viability. All samples were tested in quadruplicate and in the presence of CPSR-2. Repeated experiments showed the protocol to be highly reproducible.

Statistical Analysis. Unpaired Student's *t* tests were used to compare two variables. ANOVA and Student-Newman-Keul's tests were used when more than two variables were compared (25).

RESULTS

In *in vitro* studies, inhibition of fibroproliferation was tested at 10 × ED_{50} for PDGF (Fig. 1). Of several compounds tested, pentoxifylline most effectively blocked PDGF-driven fibroproliferation, with a 50% decrease in proliferative effect using 0.033 mg/ml, a concentration that could be achieved in plasma with current clinical applications of pentoxifylline.

The results of *in vivo* studies indicated that alkaline phosphatase levels were significantly elevated in animals receiving yellow phosphorus for 12 wk. Pentoxifylline treatment (16 mg/kg orally, 5 days/wk) for 12 wk prevented elevation in alkaline phosphatase activity (Fig. 2). Serum albumin levels were significantly reduced in animals receiving yellow phosphorus. Pentoxifylline treatment prevented reduction in serum albumin levels (Fig. 3). γ-Glutamyl transpeptidase activity was significantly elevated in animals receiving yellow phosphorus treatment. Pentoxifylline treatment prevented the elevation in γ-glutamyl transpeptidase level observed on treatment with yellow phosphorus alone (Fig. 4). Serum ammonia levels were elevated in the animal model of liver disease, but pentoxifylline treatment reduced serum ammonia levels to near normal (Fig. 5). Serum ALT and AST activities and bilirubin levels were not changed in animals treated with yellow phosphorus compared with controls, and simultaneous treatment with pentoxifylline did not alter this result (not shown).

Collagen levels were determined in liver sections taken from animals treated with yellow phosphorus for 10 to 12 wk or those treated with a combination of pentoxifylline and yellow phosphorus. These were compared with sections from mineral oil (vehicle)–treated controls. Collagen levels were significantly increased in liver sections taken from pigs treated with yellow phosphorus compared with sections from mineral oil (vehicle)–treated control animals. Liver sections from animals treated with yellow phosphorus plus pentoxi-

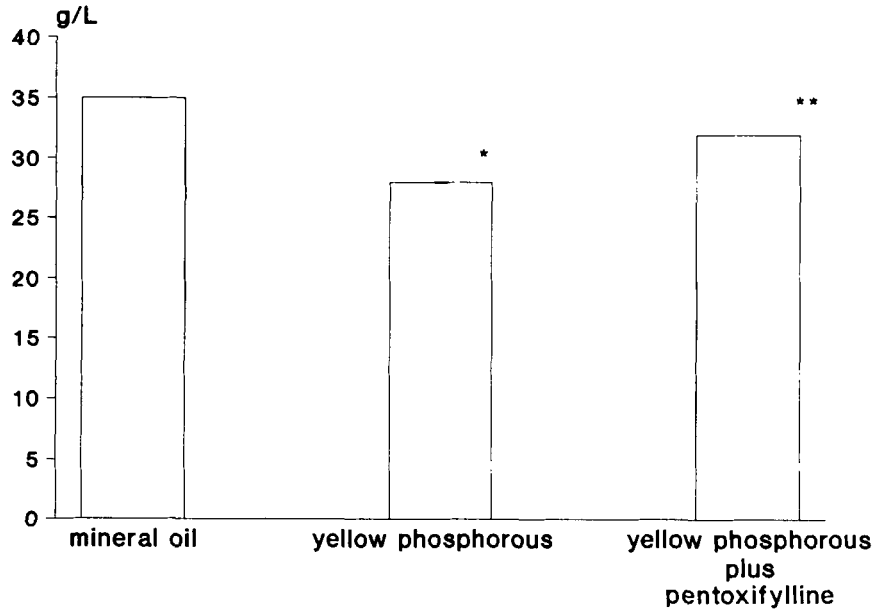

FIG. 3. Effect of pentoxifylline treatment on serum albumin level in an animal model of liver disease. Each bar represents the mean ± S.E.M.; n = 3. *Significantly different from mineral oil–treated controls; $p < 0.05$. **Significantly different from yellow phosphorus–treated animals; $p < 0.05$.

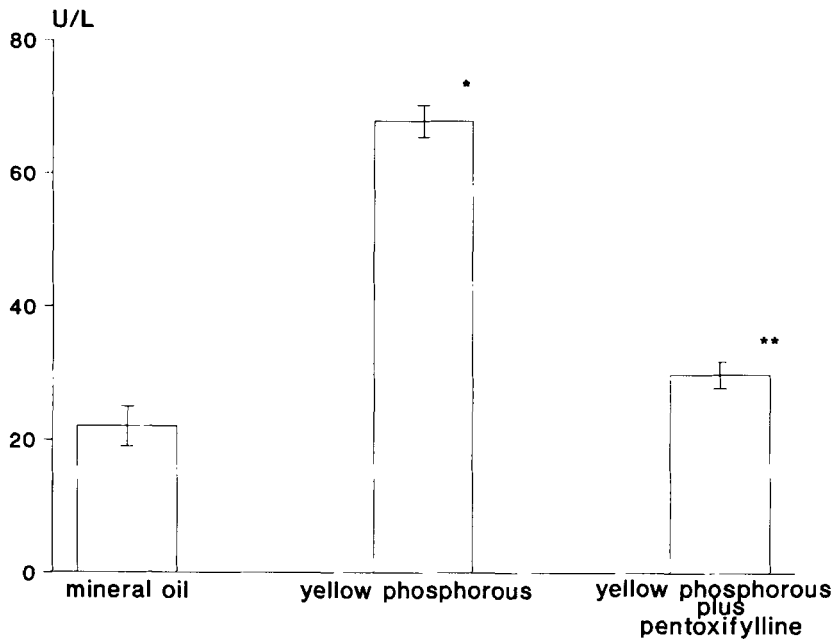

FIG. 4. Effect of pentoxifylline treatment of γ-glutamyl transpeptidase activity in animals treated with yellow phosphorus. Each bar represents the mean ± S.E.M.; n = 3. *Significantly different from mineral oil–treated controls; $p < 0.05$. **Significantly different from yellow phosphorus–treated animals; $p < 0.05$.

fylline had significantly lower collagen levels than did sections from animals treated with yellow phosphorus alone. Collagen concentrations in liver sections taken from animals treated with yellow phosphorus plus pentoxifylline were not different from those of controls (Fig. 6). The differential staining of collagenous and noncollagenous protein in liver sections with Sirius red and Fast green, respectively, is illustrated (Fig. 7A) for a control animal treated with mineral oil. Sirius red stains the fine delicate septa characteristic of normal pig liver histological appearance. Histological appearance of a pig liver exposed to yellow phosphorus (Fig. 7B) illustrates the thickened, irregular pattern of the fibrous septa. Extensive nodulation and some bridging fibrosis were apparent. A liver section taken from an animal treated with a combination of pentoxifylline and yellow

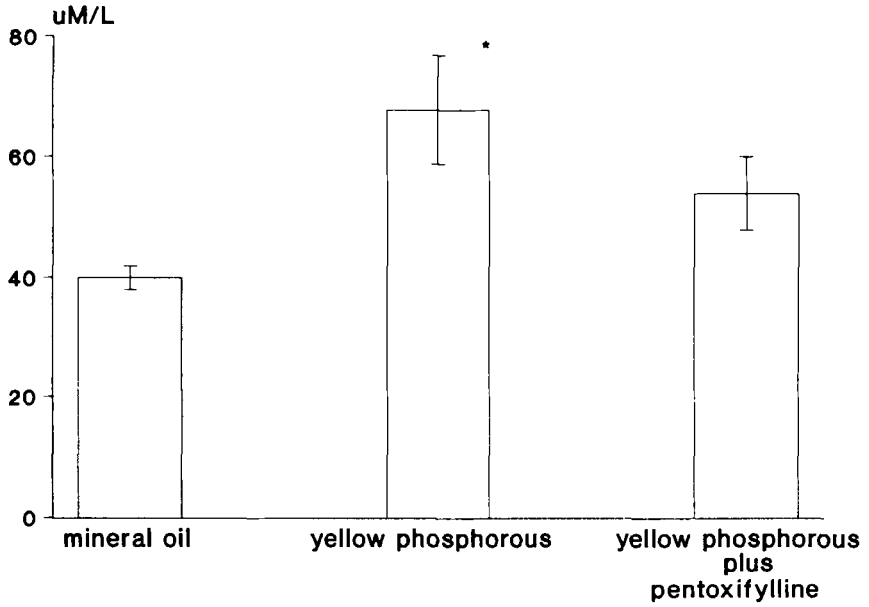

FIG. 5. Effect of pentoxifylline treatment on serum ammonia level in an animal model of liver disease. Each bar represents the mean ± S.E.M.; n = 3. *Significantly different from mineral oil–treated controls; $p < 0.05$.

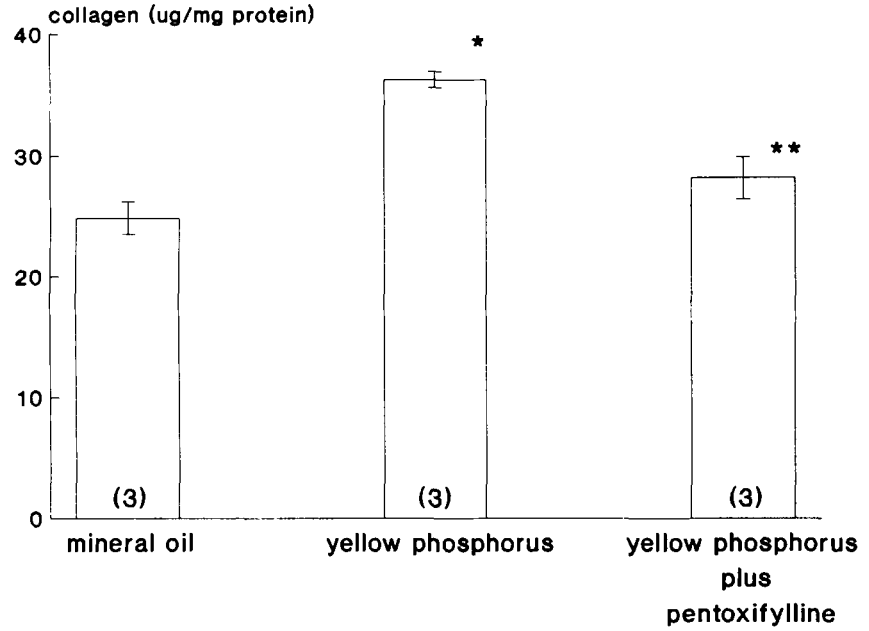

FIG. 6. Effect of pentoxifylline treatment on collagen estimation in liver section taken from pigs treated with yellow phosphorus. Each bar represents the mean ± S.E.M.; n = 3. *Significantly different compared with mineral oil–treated controls; $p < 0.05$. **Significantly different from yellow phosphorus–treated animals; $p < 0.05$.

phosphorus (Fig. 7C) was more suggestive of normal histological appearance, with delicate fibrous septa, further illustrating the protective action of pentoxifylline in this animal model of hepatic fibrosis.

In a follow-up experiment pentoxifylline was administered to animals receiving yellow phosphorus after we noted significant elevation in γ-glutamyl transpeptidase activity in this group compared with controls. After 6 wk of yellow phosphorus treatment, γ-glutamyl transpeptidase level was significantly elevated (Table 1). Pentoxifylline was started at wk 7 and continued until the end of the experiment (wk 12). The results indicate that γ-glutamyl transpeptidase level continued to increase at wk 8 in this group but that by wk 12 it was markedly decreased and not significantly different from that of controls (Table 1). Collagen levels measured at wk 12 in liver sections from this group were not significantly different from levels in liver sections from pigs treated

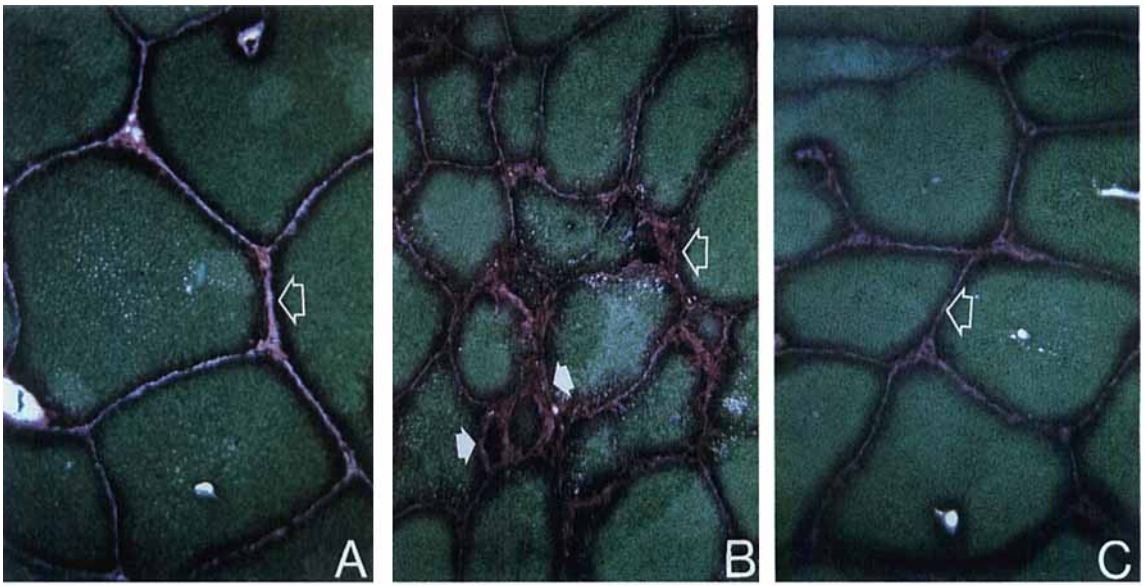

FIG. 7. Differential staining of collagenous and noncollagenous protein with Sirius red and fast green. Liver sections shown had collagen concentrations nearest the mean concentration of their treatment group. (A) This section was taken from a control animal treated with mineral oil and shows the fine, delicate septa *(open arrow)* characteristic of normal histological appearance (original magnification ×40). (B) Liver section taken from an animal treated with yellow phosphorus for 12 wk. This section illustrates the widened irregular fibrous septa *(open arrow)*. Bridging fibrosis and nodulation are also illustrated *(solid arrows)* (original magnification ×40). (C) Liver section taken from an animal treated with a combination of pentoxifylline (16 mg/kg/day) and yellow phosphorus (0.6 mg/kg/day). The delicate, fibrous septa *(open arrow)* are seen in this liver section (original magnification ×40).

TABLE 1. γ-Glutamyl transpeptidase activity in an animal model of hepatic fibrosis and the effect of pentoxifylline treatment

Time (wk)	Control	Yellow phosphorus	Yellow phosphorus + pentoxifylline	
			Wk 0	Wk 7
0	26 ± 5[a]	20 ± 3	18 ± 4	23 ± 17
4	16 ± 2	49 ± 4	27 ± 3	60 ± 16
6	27 ± 8	63 ± 1[b]	24 ± 7	41 ± 10[b]
8	17 ± 3	59 ± 8[b]	36 ± 3	85 ± 13[b]
12	22 ± 3	68 ± 2[b, c]	21 ± 4	37 ± 17

[a]Data expressed as mean ± S.E.M. for three to six animals.
[b]Significantly different ($p < 0.05$) from control or yellow phosphorus + pentoxifylline group (wk 0).
[c]Significantly different ($p < 0.05$) from yellow phosphorus + pentoxifylline group (wk 7).

simultaneously with yellow phosphorus and pentoxifylline, suggesting that protection might be achieved and fibrosis halted if pentoxifylline treatment were started after the animal had undergone 7 wk of yellow phosphorus treatment.

DISCUSSION

The *in vivo* results indicate that long-term administration of pentoxifylline (16 mg/kg) prevented the alterations in serum biochemical liver function tests (i.e., the elevation of γ-glutamyl transpeptidase and alkaline phosphatase activities and the decrease in serum albumin level) characteristically observed in animals treated with yellow phosphorus. Several other compounds, including colchicine, interferon-α and ursodeoxycholic acid, have been reported to improve serum biochemical liver function in patients with liver disease, but with little effect on liver fibrosis as seen on histological study (12, 13, 26, 27). Collagen estimation in this animal model of liver disease provides a quantitative index of fibrosis (19). Previous reports comparing this colorimetric method of collagen quantitation with morphometrical quantitation of collagen indicated excellent correlation between the two methods (22). Pentoxifylline treatment prevented the elevation in collagen content of liver sections observed in animals treated with yellow phosphorus alone. The quantitation of collagen is supported by the histological observations showing thickening of fibrous septa and extensive regenerative nodule formation in animals treated with yellow phosphorus compared with normal, delicate septa in animals receiving pentoxifylline or in controls. Recently PDGF was proposed to contribute to nodule formation (28). The extensive nodulation in this animal

model suggests a role for PDGF in its pathogenesis. The prevention of fibrosis and regenerative nodulation by pentoxifylline might therefore be related to the ability of pentoxifylline to block PDGF-driven events.

The mechanism of pentoxifylline protection in liver fibrosis is unknown. Pentoxifylline has been shown here to block PDGF-driven fibroproliferation (i.e., pentoxifylline [0.033 mg/ml] produced 50% inhibition of PDGF-driven fibroproliferation). Pentoxifylline has also been reported to reduce IL-1–driven fibroproliferation and synthetic activity (17). However, in previous studies in patients with liver disease, the fibroproliferative effect of monocyte-conditioned medium obtained from monocytes of these patients was found to be mediated by PDGF and not by IL-1 (14, 15). Recent evidence suggests that pentoxifylline acts as a reversible, competitive antagonist for the PDGF receptor (Peterson TC, et al., Unpublished data, 1992). Pentoxifylline reduces constitutive collagen synthesis, which may be mediated by inhibition of PDGF (10), IL-1 or TNF (29). Pentoxifylline (1 mg/ml) has been reported to reduce TNF-stimulated collagen synthesis (29), but a 30-fold lower concentration of pentoxifylline (0.033 mg/ml) was shown here to reduce PDGF-stimulated fibroproliferation. These results suggest that the effects mediated by PDGF are inhibited by concentrations of pentoxifylline that would be expected in this animal model of liver disease or could be achieved clinically. Inhibition of the effects mediated by TNF require higher concentrations of pentoxifylline. Together the results suggest that the inhibition of the effects of PDGF by pentoxifylline provides a possible mechanism of action for the prevention of liver fibrosis.

Human fibroblasts were used in this and previous studies (9, 14, 15) to assess proliferation. It will be necessary to reproduce these results in fibroblasts of pig origin to establish the mechanism for inhibition of proliferation by pentoxifylline in the animal model.

Evidence suggests that TGF-β may have a role in the pathogenesis of fibrosis in chronic liver disease, but recent studies illustrate that interferon-α therapy reduced the TGF-β_1 messenger RNA levels in six of eight patients but did not affect the histopathological score for liver fibrosis (13). A recent report suggests that the two growth factors TGF-β and PDGF may cooperate in their proliferative effect on fibroblasts by acting on a common intermediate or on the same receptor (30), but in our hands TGF-β antibody did not attenuate PDGF-driven fibroproliferation (data not shown). The possibility of a concerted action involving PDGF, TGF-β and cytokines such as IL-1 and TNF-α in liver fibrosis *per se* still exists. Such a possibility does not diminish the role for pentoxifylline in hepatic fibrosis; rather, reports suggest that pentoxifylline can block the synthesis of TNF-α (31), block the induction of collagen by TNF-α (29) and prevent the action of IL-1β (17). Pentoxifylline also improves peripheral blood flow and theoretically could improve sinusoidal circulation and tissue oxygenation. *In vivo*, the protective action of pentoxifylline likely is related to its effect on a combination of these factors.

Studies using pentoxifylline suggest that this drug is safe and has minimal side effects with long-term administration. Chronic administration of pentoxifylline for up to 1 yr carries a low incidence of side effects (32, 33). Most side effects are gastrointestinal disturbances (2.6%), with some cardiovascular, psychological or neurological, hepatic or dermatological effects (each occurring in less than 0.25% of patients treated [32, 33]). In the animal model studied here, pentoxifylline produced minor dermatological side effects in the form of a rash.

Long-term administration of pentoxifylline prevented both the biochemical and histological changes associated with this animal model of liver fibrosis and was without major side effect. These results suggest that pentoxifylline is a safe, effective treatment for liver fibrosis. The clinical application for a drug that can prevent fibrosis if administered at the same time as the fibrotic stimulus will be restricted to predictable hepatic fibrosis such as that which occurs in adult cystic fibrosis patients (4-7) or in patients receiving chronic methotrexate, although the relationship between chronic methotrexate treatment and fibrosis is a matter of debate.

Our follow-up results suggest that pentoxifylline prevents the biochemical and histological changes characteristic of the yellow phosphorus–induced hepatic fibrosis model, even if pentoxifylline treatment is delayed until significant elevations caused by yellow phosphorus treatment are observed in γ-glutamyl transpeptidase activity. Treatment with pentoxifylline after γ-glutamyl transpeptidase activity was elevated (wk 7) was as effective in preventing the biochemical and histological changes as treatment with pentoxifylline simultaneous with administration of the fibrotic agent. The prevention of fibrosis by pentoxifylline (when administered after the initial injury) corresponds better to clinical situations in which patients are identified after the initial injury has occurred. Further information is required to understand the temporal relationship between the onset of fibrosis and the administration of protective agent. At this stage it is clear that treatment with pentoxifylline during the induction of liver fibrosis in the animal model prevented the fibrosis normally observed. The initial results reported in this work suggest that the protective effect of pentoxifylline is manifested in both biochemical and histological parameters and could be explained as a prevention of the injury by yellow phosphorus or a stopping of the fibrotic process—both would achieve the same result. The follow-up study reports the protective effect of pentoxifylline (both biochemical and histological parameters improved) when administered after the injury is apparent (i.e., pentoxifylline was started at a time when the γ-glutamyl transpeptidase activity was elevated significantly and when changes in collagen deposition such as early fibrosis are evident in this animal model). This suggests that protection by pentoxifylline involves halting the fibrotic process. Thus this drug may have applications in other models of fibrosis; these are being tested.

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