In Vitro and In Vivo Association of Transforming Growth Factor-β1 with Hepatic Fibrosis

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Abstract. Despite extensive efforts, little progress has been made in identifying the factors that induce hepatic fibrosis. Transforming growth factor-β (TGF-β) has been shown to enhance collagen production, therefore its role in hepatic fibrosis was investigated. Treatment of cultured hepatic cells with TGF-β1 increased type I procollagen mRNA levels 13-fold due to post-transcriptional gene regulation. When two animal models of hepatic fibrosis, murine schistosomiasis and CCl_4-treated rats, were examined, they both exhibited increased levels of TGF-β1 gene expression at times that somewhat preceded the increase in collagen synthesis. In contrast, in murine schistosomiasis, mRNA levels of tumor necrosis factor and interleukin-1 peaked early in the fibrogenic process. Immunohistochemical analysis showed TGF-β1 to be present in normal mouse liver and to be markedly increased in mice infected with schistosomiasis. TGF-β1 appeared in the hepatic parenchyma, primarily in hepatocytes. These findings strongly suggest a role for TGF-β1 in a pathophysiological state.

TRANSFORMING growth factor-β (TGF-β)[1] was first named for its ability to induce a reversible phenotypic transformation of cells in culture (27, 31, 44), but many other properties of this cytokine have been subsequently described (35, 46).[2] Many cell types including lymphocytes, platelets, and macrophages synthesize this polypeptide (12, 33), and essentially all cells have a specific, high affinity TGF-β receptor (19, 30, 48). This cytokine has been implicated as having a major role in the production of extracellular matrix proteins. TGF-β has been shown to induce collagen formation in rodent (24), chicken (25), and human fibroblasts (40) and in rat myoblasts (24), and this increase in collagen synthesis has been shown to be associated with enhanced steady-state procollagen mRNA levels (24, 40). TGF-β is also known to stimulate the production of other matrix components such as fibronectin and proteoglycans (16, 24, 25, 40). When administered to mice, TGF-β increases the formation of collagen in granulation tissue (41) and hastens wound healing (32).

The extremely low concentration at which TGF-β exerts effects on hepatocytes in culture (21), and its apparent regulatory function in matrix formation, have led us to investigate this cytokine for a possible role in a pathophysiological condition, hepatic fibrogenesis. The cirrhotic liver is marked by a disordered and increased deposition of several matrix proteins. However, the increase in collagen content is particularly significant in the pathophysiology of this disorder (9), with type I collagen predominating in advanced cirrhosis (42). The mechanism of this increased collagen synthesis is unknown. Suggestions have been made that cytokines released by macrophages or lymphocytes may be important in inducing hepatic fibrosis (17, 38, 39, 51), but it has not been determined which factor(s) may be primarily responsible. In this study, we have attempted to delineate the role of TGF-β1 in hepatic fibrogenesis by examining the following: the effects of this protein on collagen synthesis by hepatic cells in vitro; the level of gene regulation at which TGF-β1 affects collagen gene expression; and finally, whether TGF-β1 gene expression and protein synthesis are stimulated in two animal models of hepatic fibrogenesis, murine schistosomiasis and CCl_4-induced hepatotoxicity in rats.

Materials and Methods

Cell Culture and In Vitro TGF-β1 Treatment

Male Sprague-Dawley rats were maintained under 12-h light/dark cycles and allowed food and water ad libitum. Single cell suspensions of hepatocytes were obtained from liver perfusions of rats weighing between 200 and

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1. *Abbreviations used in this paper*: IL-1, interleukin-1; TGF-β, transforming growth factor-β; TNF, tumor necrosis factor.

2. It is our assumption that most studies mentioned in this manuscript have actually investigated TGF-β1, however we make this distinction only in our own investigation.

275 g using the procedure of Berry and Friend (2), and the perfusion mixture of Leffert et al. (28). The cells were cultured on plastic dishes in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and a mixture of hormones, growth factors, and trace elements to produce a hormonally defined medium as previously described (15). For the first 4 h after isolation, the cells were also maintained in 10% fetal bovine serum (Hyclone Laboratories, Logan, UT). Half of the cells were treated during the second 24 h of culture with varying concentrations of TGF-β1 (Collagen Corp., Palo Alto, CA). The biological activity of TGF-β1 was maintained by dissolving it in a 0.01 N HCl solution with 1 mg/ml bovine serum albumin. Equal amounts of 0.01 N HCl and bovine serum albumin were added to control cells. Both control and treated cells received 50 μg/ml ascorbic acid during this second 24-h period. At the end of the 24 h of treatment, the cells were harvested for RNA extraction or nuclear run-on assays. The cells were cultured for only 48 h in a hormonally defined medium to limit hepatocyte dedifferentiation and mesenchymal cell overgrowth (15), and during this period >90% of the cells were hepatocytes.

Animal Models

CF1 female mice were infected subcutaneously with 50 cercariae of the Puerto Rican strain of *Schistosoma mansoni* (received from the Department of Geographic Medicine, Case Western Reserve Medical School, Cleveland, OH), and littermate controls were provided by the same source. Animals were killed by cervical dislocation at 5-8 wk after infection. Portions of liver were used for RNA extraction, nuclear run-on assays, or immunohistochemistry. Histologic sections of liver from the dead mice were evaluated by hematoxylin and eosin, and trichrome stains.

Male Sprague-Dawley rats weighing 60–80 g were treated with CCl_4. They received intraperitoneal injections of increasing concentrations of CCl_4 three times a week for ≤8 wk as previously described (36). These animals were killed at weekly intervals from 5 to 8 wk after the start of the injections. As with the schistosoma-infected mice, portions of liver were then used for RNA extraction or histologic examination.

RNA Extraction and Northern Blot Hybridization

Cultured hepatic cells (10) or animal livers (54) were used for RNA extractions using a modification of the Chirgwin procedure (5). The cell pellets or liver specimens were homogenized in 3.5 ml of a 4 M guanidine thiocyanate solution, and after a low speed centrifugation to remove cellular debris, the RNA was pelleted through a cesium chloride gradient. After a series of ethanol precipitations, total RNA content was calculated by A_{260} spectrophotometry. For examination of the presence of some of the low abundance mRNAs, poly(A^+) RNA was prepared by oligo(dT) columns (Collaborative Research, Inc., Waltham, MA).

Steady-state mRNA levels were determined by Northern blot hybridization using samples of 10–20 μg of total RNA or 5 μg of poly(A^+) RNA (11). Filters were probed with the following cDNA clones: rat α2(I) collagen (20), rat albumin (55), chicken β-actin (7), human TGF-β1 (12), human tumor necrosis factor (TNF) (53), and mouse interleukin-1 (IL-1) (29). The hybridized filters were washed under stringent conditions, exposed to x-ray film, and the developed film scanned by densitometry.

Transcriptional Rate Analysis

Nuclear run-on assays were performed on the cultured cells and livers using methods previously described (10, 54). Control or treated cells were pooled from five to six 100-mm dishes per condition and cell nuclei were isolated as described by Jefferson et al. (26). Liver nuclei from control and schistosoma-infected animals were isolated by centrifugation through 2.1 M sucrose as described by Clayton and Darnell (6). Nuclei from either cells or liver were labeled for 15 min with high specific activity [^{32}P]UTP (6), and the labeled RNA transcripts were isolated and hybridized with at least a tenfold excess of the cDNA probes that were bound to a nitrocellulose membrane (23). Clones of pBR322 and a mouse cDNA of arginine transfer RNA (provided by Dr. J. Darnell, Jr., Rockefeller University, New York) were used as controls. After hybridization, the filters were washed extensively, digested with RNase A, exposed to x-ray film, and densitometric scanning was performed.

Immunohistochemical Staining

Liver tissue was fixed in formalin, embedded in paraffin, and 5-μm sections were cut. TGF-β1 was localized in sections following a protocol similar to that described by Heine et al. (22), and using the avidin-biotin peroxidase kit from Vector Laboratories, Inc. (Burlingame, CA). After deparaffinization, blocking of endogenous peroxidase, and permeabilization in hyaluronidase, the sections were blocked with 1.5% goat serum, 1% BSA, and 1% ovalbumin for 30 min at room temperature. Sections were then incubated overnight at 4°C with an IgG fraction (prepared by protein A-Sepharose chromatography) of anti-LC(1-30) at a concentration of 25 μg/ml. This rabbit polyclonal antibody was raised to a peptide corresponding to the amino-terminal 30 amino acids of TGF-β1. Controls included replacing the primary antibody with nonimmune rabbit IgG or antisera depleted by incubation with TGF-β1-Sepharose resin (22). After washing of the sections and incubation with biotinylated goat anti-rabbit IgG and avidin-peroxidase as directed by the manufacturer, staining was visualized by incubation in 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and hydrogen peroxide. Sections were counterstained with 1% methyl green.

Results

Hepatic cells, which were predominantly hepatocytes, were obtained from rat liver perfusions and treated with various concentrations of recombinant TGF-β1 for 24 h. The cells were then harvested for RNA extraction, and mRNA steady-state levels were analyzed by Northern blot hybridization using the pro α2(I) collagen, albumin, and β-actin cDNA clones. The mRNA content for pro α2(I) collagen was markedly elevated in TGF-β1-treated cells (Fig. 1). In contrast, TGF-β1 treatment did not significantly alter steady-state mRNA levels for another secreted protein albumin, while mRNA levels for the constitutive protein β-actin were only slightly elevated (Fig. 1). Densitometry scanning results of Northern blots from four such experiments showed that TGF-β1 treatment increased type I procollagen mRNA levels by over 13-fold while having no significant effect on albumin or β-actin steady-state levels.

Given this data, and the demonstration that TGF-β stimulates extracellular matrix synthesis in a variety of mesenchymal cells, we examined the possible role of this protein in an animal model of fibrogenesis; i.e., murine schistosomiasis. Hepatic schistosomiasis, one of the world's leading causes of chronic liver disease in man, results from the deposition of parasite eggs in the liver sinusoids where a resultant inflammatory response leads to granuloma formation and fibrosis. This fibrotic reaction involves the excessive deposition of new connective tissue matrix of which collagen is the major component (14). Previously, we have shown that this increase in hepatic collagen content is accompanied by elevated levels of types I and IV procollagen mRNA (54).

CF1 mice were subcutaneously infected with *S. mansoni*, and the animals were then killed weekly from 5 (the time of earliest inflammation and granuloma formation) to 8 wk. 8 wk was chosen as a final endpoint because it has been shown to be the time of maximal collagen synthesis in this model (47). Portions of the livers were then used for histologic examination, RNA extraction, nuclear run-on assays, and immunohistochemical staining. Review of histologic sections taken sequentially from 5 to 8 wk after infection shows an increase in the size of granulomas, along with larger deposits of collagen. This increase in fibrosis is mirrored by the Northern blot analysis of pro α2(I) collagen mRNA levels which begin to rise at 5 to 6 wk, and peak at 8 wk after infection (Fig. 2). Total RNA was also hybridized with a human TGF-β1 cDNA clone. While TGF-β1 mRNA was detected by Northern blots in small amounts in normal livers from litter-

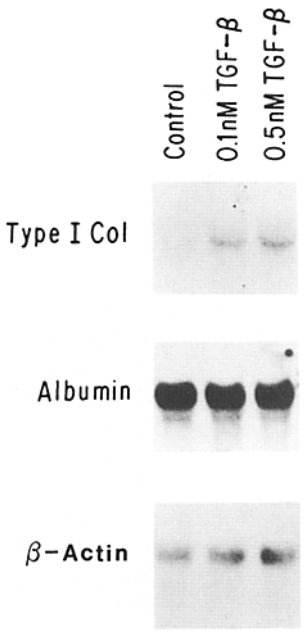

Figure 1. Autoradiograms of Northern blot hybridizations of total RNA from cultures of primary hepatic cells untreated or treated with TGF-β1. Single cell suspensions of hepatic cells were prepared and the cells were cultured for 24 h in RPMI 1640 supplemented with antibiotics, 10% fetal bovine serum, and a mixture of hormones, growth factors, and trace elements to produce a hormonally defined medium. The cells were then grown for 24 h in hormonally defined medium without serum, but with 50 μg/ml of ascorbic acid alone, or in addition to varying concentrations of TGF-β1. Total RNA was then extracted from the cells and subjected to Northern blot hybridization analysis as described in Materials and Methods. Shown are autoradiograms of total RNA from untreated control cells and from cells treated with 0.1 and 0.5 nM TGF-β1, and then hybridized with pro α2(I) collagen (*Type I Col*), albumin, and β-actin cDNA probes.

mate controls, there was a significant increase in TGF-β1 mRNA content in the fibrotic livers from the schistosoma-infected animals (Fig. 2). Moreover, this increase in TGF-β1 steady-state mRNA levels approximately paralleled, and somewhat preceded, the increase in collagen synthesis and type I procollagen mRNA levels. Densitometry scanning of Northern blots from three separate experiments revealed a greater than ninefold increase in TGF-β1 mRNA levels in the livers of the mice at 8 wk after infection with *S. mansoni* as compared to controls. To determine the level of gene expression responsible for this change in TGF-β1 mRNA steady-state content, transcriptional rate analysis was performed on portions of liver from control and 8-wk infected mice. TGF-β1 gene transcription at 8 wk after infection was found to be increased 11-fold in three sets of nuclear run-on assays (Fig. 3).

The RNA samples extracted from the livers at 5–8 wk after infection were evaluated for the expression of three other genes. These samples were probed for two other cytokines thought to be important in inflammation and collagen synthesis: TNF and IL-1 (4, 13, 18, 45). In addition, albumin gene expression was analyzed. Unlike TGF-β1, the induction of gene expression for these two cytokines did not follow the pattern seen with type I collagen. Little if any TNF or IL-1 mRNA was present on Northern blots when the total liver RNA samples were probed with cDNAs from these cytokines. However, when poly(A$^+$) RNA was probed with the TNF and IL-1 cDNAs, a distinctive and comparable pattern emerged. Neither cytokine mRNA could be found in normal liver, but detectable mRNA levels appeared with the initiation of the fibrotic response at 5–6 wk, peaked at 6–7 wk, and then declined at 8 wk (Fig. 2). In contrast, albumin mRNA content was greatest in the normal liver and declined with the progression of fibrosis (Fig. 2).

A hepatotoxic model of fibrogenesis, chronic CCl_4-induced fibrosis in rats, was also used to investigate the role of TGF-β1 in vivo. Sprague–Dawley rats were treated three times a week with intraperitoneal injections of CCl_4, and then killed at 5–8 wk after the start of these injections for histologic examination and RNA isolation. Again, the sequential increase in fibrosis was reflected in a rise in pro α2 (I) collagen mRNA levels which peaked at week 8. When total RNA was hybridized with the TGF-β1 cDNA clone, the TGF-β1 mRNA levels rose in concert with those for type I collagen (Fig. 4).

The presence of TGF-β1 in normal mouse liver, and schistosoma-infected mouse liver, was also demonstrated by means of immunohistochemical staining using an antibody raised to a peptide corresponding to the first 30 amino acids of TGF-β1. This antibody is believed to stain TGF-β1 at sites of synthesis (Flanders, K. C., N. L. Thompson, D. S. Cissel, L. R. Ellingsworth, M. B. Sporn, and A. B. Roberts, manuscript submitted for publication). Normal mouse liver demonstrated light, positive staining (Fig. 5 *A*), which was not seen using normal rabbit IgG. Positive staining was also not present when the TGF-β1-specific antibody was removed from the IgG fraction by incubation with a Sepharose resin coupled with TGF-β1 before its use. Mouse liver 8 wk after infection with *Schistosoma mansoni* showed a marked increase in staining, with the reactivity occurring in the hepatic parenchyma, primarily in hepatocytes (Fig. 5 *B*).

Discussion

The liver is composed of a variety of cells organized in a

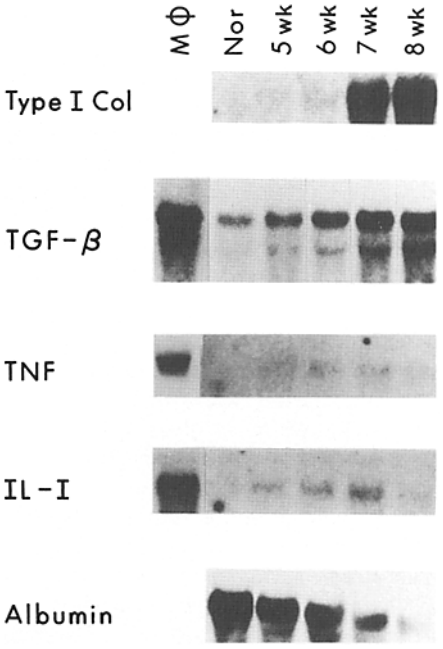

Figure 2. Autoradiogram of Northern blots with RNA extracted from a macrophage cell line (*Mϕ*) from livers of control mice (*Nor*) or from mice infected with *S. mansoni* and killed after a specified number of weeks of infection (*5–8 wk*). Poly(A$^+$) RNA was prepared from the total RNA samples for use in hybridization studies for TNF and IL-1. The RNA was denatured, electrophoresed, and hybridized with pro α 2(I) collagen (*Type I Col*), TGF-β1, TNF, IL-1, and albumin cDNA probes as described in Materials and Methods.

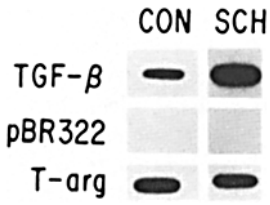

Figure 3. Autoradiogram of a nuclear run-on assay showing the transcriptional rate of TGF-β1 in the liver of a pair of normal CF-1 mice (*Con*), and littermates 8 wk after infection with *S. mansoni* (*SCH*). The nuclei were isolated and the RNA transcripts labeled as previously described. The RNA transcripts were hybridized with TGF-β1, plasmid pBR322, and mouse transfer RNA (*T-arg*).

scaffolding of extracellular matrix. Multiple factors may be involved in maintaining the proper equilibrium between matrix deposition and remodeling. During chronic hepatic injury there is a shift in this homeostatic system so that there is a relative overabundance of the fibrogenic factors, resulting in hepatic fibrosis and eventual cirrhosis. These investigations examined the specific role of TGF-β in hepatic systems because this cytokine has been shown to stimulate collagen synthesis in other in vitro and in vivo models.

TGF-β1 increased type I procollagen mRNA levels in primary cultures of hepatic cells >13-fold. An analysis of transcriptional rates of our cultured hepatic cells by use of the nuclear run-on assay showed no difference in collagen gene transcription with TGF-β1 treatment (data not shown). This finding of posttranscriptional regulation of type I collagen is consistent with the results of Raghow et al. (40), who did not find a transcriptional level of regulation when collagen synthesis was evaluated using nuclear run-on assays in TGF-β–treated fibroblasts. However, deCrombrugghe and co-workers (43) have suggested that a binding site for nuclear factor I mediates TGF-β transcriptional control of the mouse α2(I) collagen gene. Their studies used the chloramphenicol acetyltransferase transient expression assay. Thus, the mechanism by which TGF-β enhances procollagen mRNA content in vitro is not entirely clear, and other recent investigations have shown that the degree of cell confluence is a variable, which may explain these differing results (37).

In two in vivo models of hepatic fibrogenesis we demonstrated a marked increase in TGF-β1 gene transcription and mRNA levels during the period of maximal collagen synthesis. The two systems are complementary since they represent two distinct forms of hepatic fibrosis caused by different etiological agents. CCl_4 administration results in fibrosis from a toxic injury to hepatocytes which involves a panlobular inflammatory response resulting in a prominent, but not extensive, increase in collagen synthesis. In contrast, hepatic murine schistosomiasis is the result of an immune response to a foreign antigen (the schistosoma egg), and is characterized histologically by multiple focal granulomas consisting of eosinophils, mesenchymal cells, and macrophages as well as a marked increase in collagen synthesis (up to 20 times control levels [14]). Thus, in two very disparate models of hepatic fibrosis, TGF-β1 gene expression was associated with the increase in collagen synthesis. Although TGF-β1 treatment increased procollagen mRNA levels in cultured hepatic cells within 24 h, there was approximately a 1-wk delay in the rise in procollagen mRNA in relation to the increase in TGF-β1 in our in vivo models. We can only speculate about the possible causes of this lag period. The delay may have been largely artifactual due to the fact that the livers were only analyzed at weekly intervals. Or it may reflect the increased complexity of the in vivo situation. For example, this lag may be due to the need in vivo to activate TGF-β1 from its inactive to active form (52).

Immunohistochemical analysis substantiated our findings at the RNA level: low levels of immunoreactive TGF-β1 were present in normal liver, and increased staining was clearly observed in the hepatic parenchyma in schistosoma-infected livers. This positive staining occurred predominantly in hepatocytes, and to a lesser extent in endothelial and Kupffer cells. Although our immunohistochemical data may partially represent TGF-β1 being processed or metabolized in the liver (8), the increased staining in the schistosoma-infected animals with an antibody believed to stain TGF-β1 at sites of synthesis, suggests that hepatocytes are capable of synthesizing TGF-β1. This finding is in contrast to that of Braun et al. (3), who found that in a regenerating liver after partial hepatectomy, TGF-β1 mRNA was present in endothelial cells but not hepatocytes isolated by liver perfusion. In situ hybridization could be used to further delineate the cell type(s) synthesizing TGF-β1 in normal and fibrotic liver.

These findings as well as the work of others, lead to a theoretical scheme for the hepatic fibrogenic process. The liver is injured by a toxin (such as CCl_4 or ethanol), or an immune-mediated response (schistosomiasis), stimulating monocytes/macrophages to release a variety of effector molecules such as TNF, IL-1, and TGF-β1. Our results suggest that TNF and IL-1 synthesis may be enhanced during an early stage of fibrosis. Since TNF and IL-1 have been shown to enhance each other's production in vitro (34), it is not surprising to see them stimulated in unison in vivo. Release of these two cytokines could initiate a complex set of responses that enhance fibrogenesis. TNF and IL-1 are potent mitogens for fibroblasts and other mesenchymal cells (13, 49), as well as stimulators of the inflammatory response. The stimulation of the inflammatory response by these proteins in the face of continued liver damage may perpetuate the cycle of tissue injury and repair. In addition, their mitogenic effects on mes-

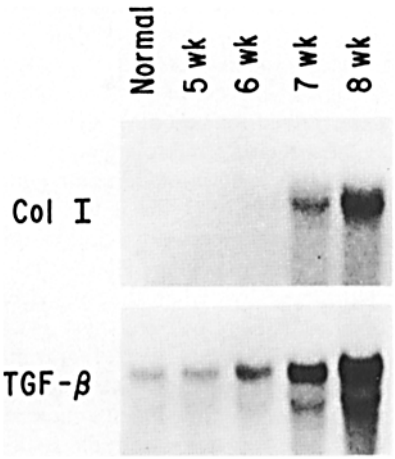

Figure 4. Autoradiogram of Northern blots with RNA isolated from the livers of control rats (*Normal*) or rats treated with CCl_4. After a specific number of weeks of CCl_4 treatment (*5–8 wk*), total RNA was extracted and hybridized with pro α2(I) collagen (*Col I*) and TGF-β1 cDNA probes.

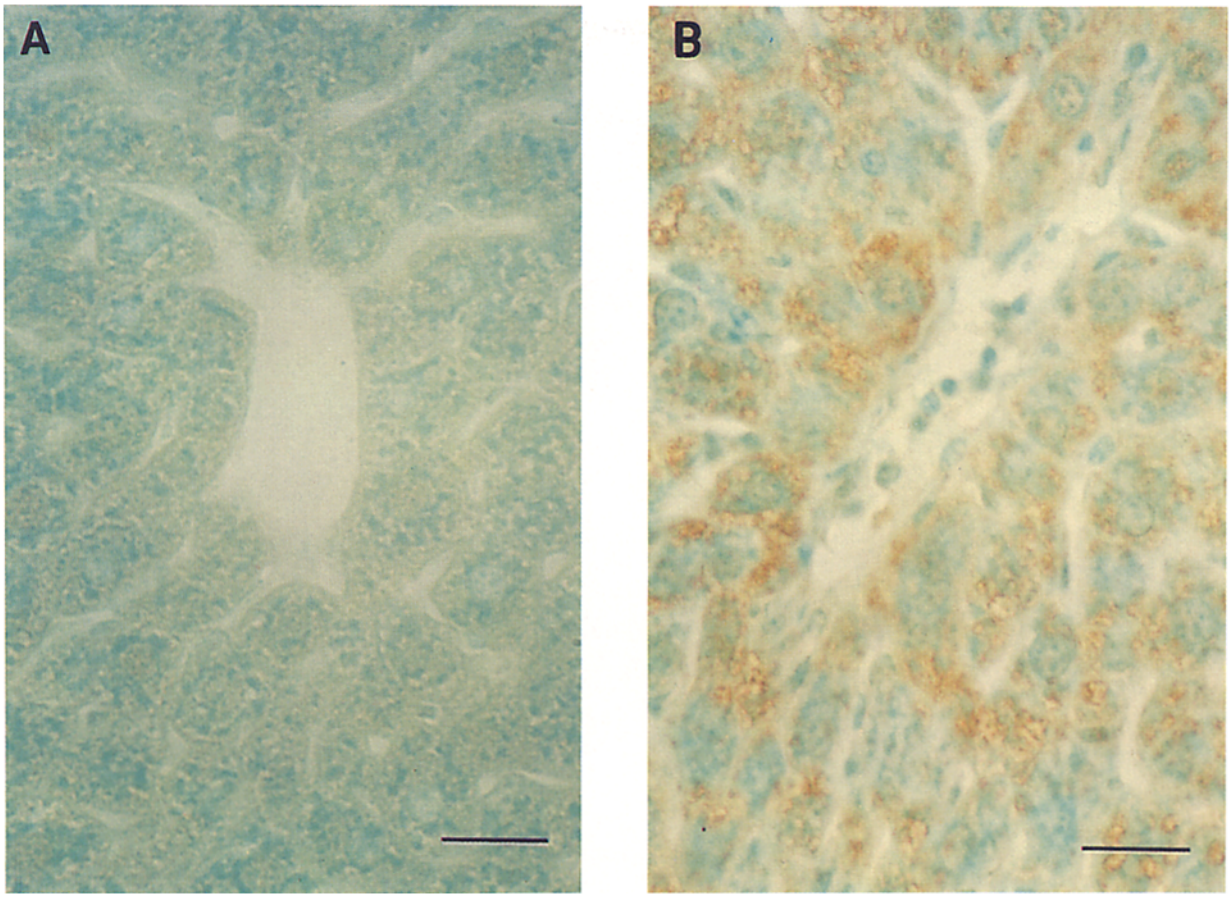

Figure 5. Immunohistochemical staining of TGF-β1 in livers from a control mouse (*A*), and a mouse after 8 wk of infection with schistosomiasis (*B*). The control mouse shows very light, positive staining in hepatocytes of the centrilobular area. Hepatocytes from a similar area of liver from a schistosoma-infected mouse have strong cytoplasmic staining. Bars, 25 μm.

enchymal cells enhance the recruitment of these cells for the matrix overproduction that eventually leads to fibrosis and cirrhosis. IL-1 can also stimulate the synthesis of TGF-β1 (50), as does macrophage activation (1). Thus, increasing levels of TGF-β1 are present in the later stages of fibrogenesis when the increased matrix deposition takes place. At this late stage in the process, (7–8 wk in our models) the synthesis of TNF and IL-1 decline and the effects of TGF-β1 on matrix synthesis become paramount.

These data do not of course preclude the possibility that other cytokines may be involved in hepatic fibrogenesis. In fact, a more complex temporal sequence or cascade of several cytokines, including some not evaluated in this study, may well be instrumental in the development of hepatic fibrosis. However, the combined findings that TGF-β1 markedly stimulates the synthesis of extracellular matrix proteins in hepatic and mesenchymal cells, and that the gene expression and production of this cytokine is increased in parallel with collagen gene expression and synthesis in two model systems of fibrogenesis, are highly suggestive that TGF-β1 may be a mediator of the hepatic fibrogenic reaction. To our knowledge, this is the first time that this regulatory molecule has been directly associated with a disease state.

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