Halofuginone to Prevent and Treat Thioacetamide-Induced Liver Fibrosis in Rats

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Hepatic fibrosis is associated with activation of hepatic stellate cells (HSC), the major source of the extracellular matrix (ECM) proteins. The predominant ECM protein synthesized by the HSC is collagen type I. We evaluated the effect of halofuginone—an inhibitor of collagen synthesis—on thioacetamide (TAA)-induced liver fibrosis in rats. In the control rats the HSC did not express smooth muscle actin, collagen type I gene, or tissue inhibitor of metalloproteinases-2 (TIMP-2), suggesting that they were in their quiescent state. When treated with TAA, the liver displayed large fibrous septa, which were populated by smooth muscle actin–positive cells expressing high levels of the collagen $\alpha_1(I)$ gene and containing high levels of TIMP-2, all of which are characteristic of advanced fibrosis. Halofuginone given orally before fibrosis induction prevented the activation of most of the stellate cells and the remaining cells expressed low levels of collagen $\alpha_1(I)$ gene, resulting in low levels of collagen. The level of TIMP-2 was almost the same as in the control rats. When given to rats with established fibrosis, halofuginone caused almost complete resolution of the fibrotic condition. The levels of collagen, collagen $\alpha_1(I)$ gene expression, TIMP-2 content, and smooth muscle actin–positive cells were as in the control rats. Halofuginone inhibited the proliferation of other cell types of the fibrotic liver in vivo and inhibited collagen production and collagen $\alpha_1(I)$ gene expression in the SV40-immortalized rat HSC-T6 cells in vitro. These results suggest that halofuginone may become an effective and novel mode of therapy in the treatment of liver fibrosis. (Hepatology 2001;33:379-386.)

Abbreviations: ECM, extracellular matrix; HSC, stellate cells; TIMP-2, tissue inhibitor of metalloproteinases-2; cGVHD, chronic graft-versus-host disease; TSK, tight skin; TAA, thioacetamide; PCNA, proliferation cell nuclear antigen; PBS, phosphate-buffered saline.

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Hepatic fibrosis represents the response of the liver to diverse chronic insults such as parasitic disease, chronic viral infection (hepatitis B and C), immunologic attack (autoimmune hepatitis), hereditary metal overload, toxic damage, etc. Because of the worldwide prevalence of these insults, liver fibrosis is common and is associated with significant morbidity and mortality. The elucidation of the cellular and molecular mechanisms responsible for the development and progression of the liver fibrosis has provided a sound basis for development of pharmacologic strategies able to modulate the cause of the disease.2 In experimental models of fibrosis of various types, the increased deposition of type I collagen results primarily from increased transcription of the type I collagen genes.3-5 In hepatic fibrosis, increases in the gene expression of other types of collagens such as III and IV,5,6 and other matrix proteins7,8 have also been reported. In addition to being caused by increased ECM synthesis, liver fibrosis may also result from relative imbalance between production and degradation of matrix proteins. It has been shown that activated stellate cells constitute the source of various collagens and tissue inhibitors of metalloproteinases (TIMPs), which are necessary for the ECM remodeling.5,9-11 The lack of a specific inhibitor(s) of any component of the ECM in general and of collagen type I in particular, limits the success of prevention and treatment of hepatic fibrosis, although numerous agents have been tried.12,13-15 Halofuginone is a well-known inhibitor of collagen type I synthesis,23 which has been reported to inhibit the gene expression of collagen type $\alpha_1(I)$ but not of type $\alpha_2(I)$, type II,26 type III,27 or type X (M. Pines, unpublished data). In culture, halofuginone has been found to attenuate collagen $\alpha_1(I)$ gene expression and collagen production by murine, avian, and human skin fibroblasts derived from either scleroderma or chronic graft-versus-host disease (cGVHD) patients.28 In animal models in which fibrosis was induced, halofuginone prevented the increase in collagen synthesis and collagen $\alpha_1(I)$
gene expression. These models included mice afflicted with cGVHD, and rats with pulmonary fibrosis after bleomycin treatment, and rats developing adhesions after surgery in tendons, the abdomen, and uterine horns. In liver, halofuginone prevented collagen type I gene expression in dimethyl nitrosamine-induced cirrhosis in rats. In addition to its ability to prevent fibrosis by inhibiting the collagen α(I) gene expression, halofuginone treatment caused an attenuation of the collagen already deposited in skin. This may be possible by altering the balance between synthesis and degradation of collagen type I as shown in tight skin (Tsk+) mice and in a cGVHD patient.

The goal of the present study was to evaluate the possibility of using halofuginone both to prevent and to treat hepatic fibrosis. Our results show that by inhibiting collagen synthesis, halofuginone can affect stellate cell activation, accelerate collagen degradation, and cause resolution of the fibrotic lesion, and could thus become a novel therapy for liver fibrosis.

MATERIALS AND METHODS

Materials. A 1,600-bp rat collagen α(I) probe was a generous gift from B.E. Kream, University of Connecticut, Farmington, CT. Halofuginone bromhydrate was obtained from Collgard Biopharmaceuticals Ltd (Tel Aviv, Israel). Thioacetamide (TAA) was obtained from Sigma Chemicals Co. (St. Louis, MO). Smooth muscle actin monoclonal antibodies, used at 1:200 dilution, and proliferation cell nuclear antigen (PCNA) were from Dako A/S (Glostrup, Denmark). TIMP-2 polyclonal antibodies, corresponding to the carboxyl terminus of human TIMP-2, used at 1:50 dilution, were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). As a second antibody, the Histomouse SP kit was used (Zymed Laboratories Inc, South San Francisco, CA).

Animals. Male Wistar rats (200-250 g) were kept in the animal breeding house of the Wolfson Medical Center and fed Purina rodent chow ad libitum. All animals received humane care during the study, under a protocol that was in accordance with institutional guidelines. Cirrhosis was induced in rats by intraperitonealadministration of TAA 200 mg/kg twice weekly for 12 weeks, as described previously. Such long-term administration of TAA results in characteristic lesions of micronodular cirrhosis in the liver. Halofuginone was given in the diet at a concentration of 5 ppm, which had previously been found to be effective in preventing abdominal and uterine horn adhesions formation in rats. Hydroxyproline was analyzed by an amino acid analyzer after hydrolysis.

Preparation of Sections, In Situ Hybridization, and Immunohistochemistry. Liver samples were collected into phosphate-buffered saline (PBS) and fixed overnight in 4% paraformaldehyde in PBS at 4°C. Serial 5-μm sections were prepared after the samples had been dehydrated in graded ethanol solutions, cleared in chloroform, and embedded in Paraplast. Differential staining of collagenous and noncollagenous proteins was performed with 0.1% sirius red, with 0.1% fast green as nuclear antigen (PCNA) were from Dako A/S (Glostrup, Denmark). TIMP-2 polyclonal antibodies, corresponding to the carboxyl terminus of human TIMP-2, used at 1:50 dilution, were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). As a second antibody, the Histomouse SP kit was used (Zymed Laboratories Inc, South San Francisco, CA).

RESULTS

Prevention of TAA-Induced Liver Fibrosis by Halofuginone. Rats were treated with halofuginone alone (control group, n = 4), with TAA (n = 6), or with a combination of the two (n = 6). No changes in body weight were observed in any of the rats regardless of the treatment. Spleen weight increased by 11% in the TAA-treated rats compared with the control rats (1135 ± 250 mg compared with 1016 ± 152 mg). No increase in spleen weight was observed in rats that received a combination of TAA and halofuginone (960 ± 65 mg). All rats were sacrificed 12 weeks after initiation of TAA treatment, and the livers were assayed for hydroxyproline content by an amino acid analyzer (Fig. 1). TAA caused a 4-fold increase in liver hydroxyproline content compared with the control group. Halofuginone attenuated the TAA-induced increase in liver hydroxyproline levels and only a 2-fold increase in the hydroxyproline levels was observed (Fig. 1). These results were confirmed by histologic examination of the liver section. The

![Fig. 1](image-url)
liver sections of the control rats were devoid of ECM in general (hematoxylin-eosin staining) and of collagen in particular (sirius red staining). When smooth muscle actin antibodies were used, only endothelial cells surrounding blood vessels, but not stellate cells, were detected, suggesting that the latter were in their quiescent state. No cells expressing the collagen \( \alpha 1(I) \) gene or synthesizing TIMP-2 were detected by \textit{in situ} hybridization or immunohistochemistry, respectively (Fig. 2). When treated with TAA the livers exhibited a marked increase in ECM content and displayed bundles of collagen surrounding the lobules that resulted in large fibrous septa and distorted tissue architecture. These septa were populated by smooth muscle actin–positive cells expressing high levels of the collagen \( \alpha 1(I) \) gene and containing high levels of TIMP-2, all of which are characteristic of advanced fibrosis. Halofuginone given orally prevented the activation of most of the stellate cells and only traces of smooth muscle–positive cells were detected. The remaining stellate cells expressed low levels of collagen \( \alpha 1(I) \) gene, which resulted in low levels of collagen. The level of TIMP-2 was almost the same as in the control livers.

Treatment of TAA-Induced Liver Fibrosis by Halofuginone. The possibility that halofuginone may reverse advanced liver fibrosis in rats was evaluated. All rats (\( n = 22 \)) were treated with TAA and fed a control diet. After 12 weeks, the rats were divided into 3 groups, of which the first (\( n = 6 \)) were sacrificed on the same day to evaluate the severity of the fibrosis. The remaining 2 groups were fed either the control diet or a diet containing 5 ppm of halofuginone for an additional 8 weeks. At the end of the experiment, liver samples were taken for hydroxyproline analysis (Fig. 3) and for histologic evaluation (Fig. 4). High levels of hydroxyproline were detected in the livers of rats treated with TAA, and these remained unchanged in rats fed for an additional 8 weeks with the control diet. A marked reduction in the hydroxyproline level was observed in the livers of the rats fed halofuginone (Fig. 3).
inhibition of the expression of collagen \( \alpha 1(1) \) gene (Fig. 6B). More than an hour of treatment was required for any significant inhibition of the collagen \( \alpha 1(1) \) gene expression.

**DISCUSSION**

Hepatic fibrosis/cirrhosis is characterized by excessive production of ECM proteins, of which collagen type I is the major one. The collagen is synthesized primarily by the HSC that are activated in the fibrotic liver and that exhibit a myofibroblast-like phenotype. In addition, activated stellate cells synthesize TIMPs (Figs. 2 and 4), which inhibit collagenase activity, thus causing further increase in collagen deposition.\(^3\)\(^{-4}\) Thus, pharmacologic intervention in liver fibrosis should be targeted to inhibition of stellate cell activation, inhibition of ECM synthesis, or stimulation of matrix protein degradation. Current therapies for arresting or reversing cirrhosis are largely ineffective and are not targeted to specific elements or steps in the fibrogenic cascade.\(^2\)\(^{4}\) For example, glucocorticosteroids, which have been shown to inhibit collagen synthesis in culture\(^3\)\(^5\) and in animal models,\(^4\)\(^6\) rarely suppressed fibrogenesis or prevented progression to cirrhosis. In addition, long-term administration of glucocorticoids may cause serious adverse side effects that prevent their use as a general treatment of liver fibrosis. D-penicillamine, which blocks intra- and interchain crosslinking in the newly formed collagen molecules, was found to be ineffective for preventing the progression of hepatic fibrosis and was associated with a high incidence of serious side effects.\(^2\)\(^{4}\) Halofuginone was found to be an inhibitor of collagen \( \alpha 1(1) \) gene expression in various animal models in which excessive collagen synthesis is the hallmark of the disease.\(^2\)\(^{5}\) Clinical safety is a major concern when using an inhibitor of collagen synthesis. We showed that the effect of halofuginone on collagen synthesis was transient, and on halofuginone removal, collagen gene expression in vivo\(^2\)\(^8\) and in a cGvHD patient in vivo\(^3\)\(^5\) returned to control levels. The reduction in the collagen \( \alpha 1(1) \) gene expression in rats,\(^3\)\(^2\) rabbits,\(^3\)\(^7\) and in chickens raised on halofuginone-containing diets for 6 weeks from hatching\(^3\)\(^5\) was achieved at concentrations at which no side effects were apparent. Moreover, in all our experiments involving surgery, such as during abdominal or gynecologic adhesion formations\(^3\)\(^2\)\(^3\)\(^3\) no abnormal wound healing was observed in the halofuginone-treated animals. Using the wound-tumor model in which C6 glioma spheroids were implanted in surgical incisions, acceleration in wound repair was observed in the halofuginone-treated animals.\(^4\)\(^8\) All of these observations suggest that halofuginone may become a safe therapy for various fibrotic disorders.

In liver, halofuginone was found to prevent fibrosis induced by dimethylnitrosamine\(^4\)\(^4\) or TAA (Figs. 1 and 2). In addition to the known effects on collagen \( \alpha 1(1) \) gene expression and tissue collagen content, halofuginone treatment caused reduction in the number of activated stellate cells and in TIMP-2 synthesis (Fig. 2). Cell-matrix interactions play an important role in stellate cell activation; collagen type I promotes the entry of stellate cells into S-phase,\(^3\)\(^7\) and loss of normal matrix and its replacement by collagen type I promote stellate cell activation.\(^8\) The three-dimensional structure of the ECM components has been found to regulate the morphology, proliferation, and function of the hepatic stellate cells via integrins,\(^9\)\(^9\) which are known collagen receptors. Furthermore, the function of hepatocytes that are involved in stellate cell activation and recruitment\(^3\)\(^0\) may be perturbed because of an altered cell matrix interaction.\(^3\)\(^1\) Thus, the re-
duction in stellate cell activation may be secondary to the reduction in liver collagen content, which results, on one hand, from the inhibition of the collagen $\alpha_1(I)$ gene expression (Fig. 6) and, on the other hand, from the increase in collagen degradation caused by reduced TIMP-2 synthesis. Different fibrogenic cell populations may be involved in fibrosis induced by different agents. For example, an increase in Kupffer cell proliferation was observed in CCl$_4$-induced fibrosis.\textsuperscript{41} It is interesting to note that pentoxifylline, another antifibrotic agent, which has been found to affect collagen content in liver by increased collagen degradation rather than by reduced collagen synthesis,\textsuperscript{72} also causes reduction in fibrogenic cell proliferation\textsuperscript{44} as does halofuginone (Fig. 5). Using a double staining technique on skin biopsy specimens of the Tsk+ mice—immunostaining with PCNA antibodies and in situ hybridization with the collagen $\alpha_1(I)$ probe—we showed that halofuginone inhibited cell proliferation only in a subset of fibroblasts that are collagen-producing cells (Pines et al. submitted). These results again suggest that the decrease in collagen $\alpha_1(I)$ gene expression by halofuginone is the primary event.

It has been suggested that the inhibition of the collagen $\alpha_1(I)$ gene expression by halofuginone requires the synthesis of a new protein(s), probably transcription factors.\textsuperscript{28} The fact that more than an hour is required to show inhibition in the collagen $\alpha_1(I)$ gene expression by halofuginone is the primary event. Almost complete resolution of the fibrotic lesion was observed after the additional 8 weeks on the halofuginone-containing diet. No spontaneous resolution of the fibrotic lesion was observed.
regulated in concert and whether the putative transcription factor would simultaneously inhibit the collagen \( \alpha_1(I) \) and TIMP-2 genes. Cell types in various tissues other than stellate cells clearly need additional regulatory regions of the upstream promoter sequence and/or the first intron, which emphasizes the complex cell-specific transcription factors that are likely to exist. The inhibition of collagen synthesis by halofuginone is not cell-type specific, which suggests a more general mechanism of inhibition. Thus, in addition to HSC (Fig. 6), halofuginone may inhibit collagen synthesis and deposition by other cell types that contribute to the fibrotic lesion.\(^{56}\)

Advanced fibrosis and cirrhosis are generally considered to be irreversible conditions, even after removal of the injurious agent. However, there is evidence that recovery, with remodeling of excess ECM proteins is possible.\(^{57-59}\) In TAA-induced liver fibrosis, no spontaneous resolution was observed. High numbers of HSC, high levels of collagen synthesis (Fig. 4), and high numbers of PCNA-positive cells (Fig. 5) were observed 8 weeks after cessation of the insult. Halofuginone given orally caused almost complete resolution of existing fibrosis (Figs. 3 and 4). These results are in agreement with our previous observations that prolonged halofuginone treatment may resolve existing fibrosis in the TSK mice\(^{29}\) and in a cGvHD patient.\(^{35}\) Halofuginone treatment probably perturbs the balance between synthesis and degradation of collagen. Halofuginone inhibits the collagen \( \alpha_1(I) \) gene expression (Fig. 6) and, by inhibiting the synthesis of the metalloproteinase inhibitors, it probably augmented collagenase activity, causing collagen degradation and resolution of the fibrotic lesion.

In summary, up-regulation of collagen synthesis appears to have a critical role in the pathophysiology of liver fibrosis. By affecting various checkpoints in the development of hepatic fibrosis, halofuginone shows promise of becoming a novel antifibrotic agent for prevention and treatment of liver fibrosis.
REFERENCES