Halofuginone, an inhibitor of collagen synthesis by rat stellate cells, stimulates insulin-like growth factor binding protein-1 synthesis by hepatocytes

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Background/Aims: Halofuginone, an inhibitor of collagen synthesis, prevented and caused resolution of established hepatic fibrosis. A genomic approach in vivo was used to search for additional genes responsible for halofuginone mode of action.

Methods: Fibrosis was induced in rats by thioacetamide (TAA) and evaluated by collagen type I gene expression and the levels of collagen, tissue inhibitors of metalloproteinases-2 and smooth-muscle actin. Halofuginone was given in the diet. cDNA from liver biopsies was hybridized on Atlas arrays comprising of 588 genes. The results were confirmed by Northern blots and in situ hybridization.

Results: Insulin-like growth factor binding protein-1 (IGFBP-1) was one of the 13 genes differentially expressed in the fibrotic liver after halofuginone treatment. After 2 and 4 weeks, halofuginone prevented the TAA-induced down-regulation of IGFBP-1 gene expression. Halofuginone also prevented the TAA-dependent changes in IGFBP-3 gene expression. Halofuginone affected IGFBP-1 synthesis in rat hepatocytes and cells of hepatocyte origin and caused time- and dose-dependent increases in the IGFBP-1 gene expression and synthesis by HepG2 cells. The IGFBP-1 secreted by HepG2-inhibited stellate cell motility.

Conclusions: Halofuginone is an anti-fibrotic drug that inhibits collagen synthesis by stellate cells and preventing alteration in the synthesis of IGFBPs by hepatic cells.

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1. Introduction

Fibrosis represents the response of the liver to diverse chronic insults such as chronic viral infection, alcohol, immunological attack, hereditary metal overload, parasitic diseases, and toxic damage. Because of the worldwide prevalence of these insults, liver fibrosis is common and ultimately culminates in cirrhosis that is associated with significant morbidity and mortality. Hepatic fibrosis, regardless of the cause, is characterized by an increase in extracellular matrix (ECM) constituents produced especially by stellate cells (HSC) [1–4]. HSC are usually quiescent but upon activation they differentiate into myofibroblast-like cells with high proliferative and migratory capacity [5–12]. The predominant ECM protein synthesized in fibrosis is collagen type I although increase of other collagens and matrix proteins [13–16] have been
developed adhesions at various sites [31–34]. Topical transient attenuation of collagen 2. Materials and methods

have IGF-independent effects. Liver cells in vitro were not always changed during [28,29], rats with pulmonary fibrosis [30], and rats that included mice afflicted with cGvHD and tight skin mice prevented the increase in collagen synthesis. These models expressed collagen type I synthesis [23,24], has been found to inhibit the gene expression of collagen type α(1)I but not of type II [25] or type III [26]. In culture, halofuginone attenuated collagen α(1)I gene expression by murine, avian and human skin fibroblasts derived from sclerodema and chronic graft-versus-host disease (cGvHD) patients [27]. In animal models in which excess collagen is the hallmark of the disease, halofuginone prevented the increase in collagen synthesis. These models included mice afflicted with cGvHD and tight skin mice [28,29], rats with pulmonary fibrosis [30], and rats that developed adhesions at various sites [31–34]. Topical treatment of a cGvHD patient with halofuginone caused a transient attenuation of collagen α(1)I gene expression, thus demonstrating human clinical efficacy [35]. In the liver, halofuginone prevented HSC activation and abolished the increase in collagen α(1)I gene expression and collagen deposition in rats treated with dimethylsulfoxamine or thioacetamide (TAA) [36,37]. Given to rats with established fibrosis, halofuginone caused almost complete resolution of the fibrotic condition [37]. In addition, halofuginone markedly improved the capacity of cirrhotic liver to regenerate after partial hepatectomy [38]. Halofuginone affects collagen biosynthesis probably by blocking transforming-growth-factor-β-mediated Smad3 activation [39]. In the present study we applied Atlas microarray technique to identify genes involved in the halofuginone-dependent prevention of liver fibrosis. We used TAA-induced liver fibrosis in rats in vivo since genes regulated in liver cells in vitro were not always changed during physiological activation in vivo [40,41] probably due to the lack of cellular heterogeneity [22]. One of the genes up-regulated by halofuginone was identified as IGF binding protein-1 (IGFBP-1) known to be an early gene activated during liver regeneration. The IGFs, their binding proteins and receptors play an essential role in normal liver physiology and in disease states [42,43]. In addition to modulation of IGF/IGF receptor interactions, IGFBPs may have IGF-independent effects.

2. Materials and methods

2.1. Materials

Halofuginone bromhydrate was from Collgard Biopharmaceuticals Ltd (Tel Aviv, Israel); TAA was from Sigma (St Louis, MO, USA). Alpha smooth-muscle actin (α-SMA) monoclonal antibodies (1:200 dilution) were from Dako A/S (Glostrup, Denmark). TIMP-2 polyclonal antibodies (1:50 dilution) and the Histomouse SP kit (second antibodies) were from Zymed Laboratories, Inc. (South San Francisco, CA, USA). IGFBP-1 and IGFBP-3 polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (CA, USA). Atlas rat cDNA arrays consisting of 588 rat fragments organized into broad functional groups including housekeeping and negative control cDNAs spotted in duplicate dots were from Clontech (Palo Alto, CA, USA; www.clontech.com/atlas/genelists/index.html).

2.2. Animals, histology and cells

Male Wistar rats (200–250 g) were fed ad libitum and received humane care under institutional guidelines. Liver fibrosis was induced by intraperitoneal administration of TAA (200 mg/kg twice weekly) for 1, 2 and 4 weeks. Halofuginone (5 ppm) was given in the diet [32,33,37]. Preparation of sections, in situ hybridization and immunohistochemistry were performed as previously described [37]. IGFBP-1 probe was labeled with uridine [α-35S]triphosphate. Cell lines were used human hepatocellular carcinoma HepG2, Hep3B and Huh-7, human fibroblasts Detroit 551, rat osteosarcoma ROS 17/2.8 and SV40-immortalized rat HSC-T6 (generously provided by Dr S.L. Friedman). Cells were grown in DMEM with 10% FCS, and the medium was replaced by serum-free DMEM after overnight plating. Following serum starvation (18 h), the medium was replaced with the fresh medium with or without halofuginone. Rat primary hepatocytes were prepared as described [64] and plated on fibronectin-coated six well plates at a density of 1.5 x 10⁶ cells/well in DMEM with 10% FCS. Cells after 18 h of seeding were serum-starved for 6 h and treated with 1 nM halofuginone or 100 nM insulin for additional 24 h. Conditioned medium was collected and cells were scraped directly into TRI reagent for total RNA purification. For proliferation evaluation, cells were plated in 24 well plates in DMEM with 10% FCS and direct estimation of cell number was made using cell counter.

2.3. RNA purification and Atlas rat cDNA arrays hybridization

Total RNA from liver tissue (5 μg comprising identical amounts of RNA from three rats) was isolated with TRI reagent, treated with DNaseI and reverse transcribed in the presence of [α-32P]dATP (3000 Ci/mmol) using MMLV reverse transcriptase (50 U/μl) for 25 min at 48 °C. Array membranes were pre-hybridized in ExpressHyb solution at 68 °C for 1 h, and hybridized with labeled cDNA probes overnight at 68 °C. The second row from bottom represents the housekeeping genes. The cDNA microarrays images were analyzed by Atlasimage 1.01 software (Clontech, USA). The background was calculated by default external background that takes into consideration the background signals and the blank space. The signal threshold was based on the background and the signal intensity was normalized globally by means of the sum method.

2.4. Immunoprecipitation, Western, and Northern blots and probes

HepG2 conditioned medium was incubated with goat anti-IGFBP-1 or normal goat serum (1:100 dilution) overnight at 4 °C. The immune complexes were precipitated by incubation with protein A-Sepharose for 2 h at 4 °C followed by centrifugation at 13,000 rev./min for 5 min. The presence of IGFBP-1 protein in the supernatant and pellet was analyzed by Western blot. For Western blots, conditioned medium (45 μl) was electrophoresed on 12.5% SDS-PAGE, transferred into nitrocellulose membranes and probed with anti-IGFBP-1. For Northern blots, 10 μg of total RNA were resolved under denaturing conditions on 1.2% agarose/formaldehyde gels, transferred into Nytran N nylon membranes and hybridized with 32P-labeled cDNA probe overnight at 68 °C. The probes were generated by RT-PCR amplification with the following primers pairs:

Rat IGFBP-3: 5′-CAGACGACACAGACCGAGCAGA-3′ and 5′-AAAT-CAAGAGGACAGGACCAGCC-5′; human IGFBP-1: 5′-GCAACGAGCA- CAGACTACACCCACAGGTAGC-3′; rat IGFBP-1: 5′-CCACACTTCCGCTACTATCT-3′ and 5′-GCTTGTC-CTTGTGATCCGG-3′.
2.5. Cell motility assay

Motility was evaluated by HitKit (Cellomics, Inc., Pittsburgh, PA, USA). HSC were plated on a lawn of microscopic beads. As the cells move, they phagocytose and push aside the beads, clearing tracks behind them. The track area, visualized by phase contrast microscopy, is proportional to the magnitude of cell movement. Time-lapse movies were acquired at 30 min intervals using DeltaVision digital microscopy system and processed using the Prism software. The results are presented as the average ± SE of phagokinetic tracks in square micrometer after cell area subtraction.

3. Results

3.1. Effect of halofuginone on TAA-induced liver fibrosis

Liver sections of the control rats were devoid of ECM in general (H&E staining) and of collagen in particular (sirius red staining). No HSC were detected (αSMA immunohistochemistry) suggesting that they were in their quiescent state. No cells expressing the collagen α1(I) gene or synthesizing TIMP-2 were detected (Fig. 1). No changes in the above parameters were observed in rats treated with halofuginone alone. When treated for 4 weeks with TAA, the livers exhibited a marked increase in ECM content, and displayed bundles of collagen that surrounded the lobules and resulted in large fibrous septa and distorted tissue architecture. These septa were populated by αSMA-positive cells expressing high levels of the collagen α1(I) gene and containing high levels of TIMP-2, all of which are characteristic of advanced fibrosis. These sections were diagnosed as grade 5–6 according to the Ishak staging system [45]. Halofuginone prevented the activation of most of the HSC and only traces of αSMA-positive cells were detected. The remaining HSC expressed low levels of collagen α1(I) gene that resulted in low levels of collagen. The level of TIMP-2 was also reduced compared with that in the TAA-treated rats. RNA from the sections that had been diagnosed as grade 1–2 according to Ishak, was used for the Atlas microarrays.

3.2. Halofuginone-dependent gene expression

cDNA array hybridization analysis was used to identify genes that are expressed differently in TAA-treated liver biopsies (Fig. 2A) compared with those treated with TAA and halofuginone (Fig. 2B). A few differentially expressed genes were identified (Fig. 2C). Some were up-regulated by halofuginone (IGFBP-1; PRL-1 and apolipoprotein A-IV) while others were down-regulated (E-FABP, proteasome activator 28α, peripheral myelin protein 22, alcohol sulfotransferase and TIMP-2). In an effort to validate the Atlas microarray results, two of the genes—PRL-1 and Apolipoprotein A-IV—were analyzed by Northern blotting and the results confirmed the Atlas microarray findings (Fig. 2D). Reduction in TIMP-2 content after halofuginone treatment was also demonstrated (Fig. 1). Because of the well-documented involvement of the IGF-1/IGFBP axis in liver fibrosis and regeneration, we focused our attention on the IGFBP-1 gene. The effect of halofuginone on the IGFBP-1 gene expression was confirmed by Northern blot analysis (Fig. 3A). After 1 week of TAA treatment, a reduction in the IGFBP-1 gene expression was observed without any effect of halofuginone treatment. In contrast, after 2 and 4 weeks of treatment, halofuginone prevented the TAA-induced down-regulation expression of the IGFBP-1.
gene. A slight effect of halofuginone alone on the level of IGFBP-1 mRNA was observed (Fig. 3A). To determine if IGFBP-1 was the only member of the family affected by halofuginone, Northern blot analysis with IGFBP-3 probe of the same liver biopsies was performed. No changes in the IGFBP-3 mRNA levels were found in any of the groups after 1 week of treatment. After 2 and 4 weeks, TAA caused an increase in the IGFBP-3 level that was partially prevented by halofuginone. Halofuginone alone had no effect on the IGFBP-3 mRNA levels at any time-points examined. The effect of halofuginone was further confirmed by in situ hybridization (Fig. 3B). High levels of expression of the IGFBP-1 were observed in the control livers. TAA treatment caused a decrease in the expression of the IGFBP-1 gene that was prevented by halofuginone.

3.3. Effect of halofuginone on IGFBP-1 synthesis

Rat primary hepatocytes, HepG2, Hep3B, Huh-7 and HSC were used to identify the source of the halofuginone-dependent synthesis of IGFBP-1. In addition, cell-lines derived from other tissues (fibroblasts and osteoblasts) were used as well. Only cells of the hepatocyte origin demonstrated increased IGFBP-1 gene expression and synthesis in response to halofuginone (Fig. 4A). In rat primary hepatocytes, insulin caused reduction in IGFBP-1 synthesis in agreement with other studies [46] while halofuginone, at concentration as low as 1 nM, increased the synthesis of IGFBP-1 (Fig. 4B). In HepG2, no expression of the IGFBP-1 gene was detected without halofuginone (Fig. 5A).

Halofuginone, at concentrations of 10 nM, increased IGFBP-1 gene expression and a further increase was observed at higher concentrations. Without halofuginone, very low (in some cases undetectable) levels of IGFBP-1 were detected in the conditioned medium of HepG2 cells (Fig. 5B). An increase in the level of IGFBP-1 was observed starting at 50 nM of halofuginone. Increased IGFBP-1 gene expression was observed as early as 6 h after halofuginone treatment (Fig. 5C) resulting in an increase in the IGFBP-1 content in the conditioned media after 10–15 h (Fig. 5D). A significant reduction in cell proliferation was observed after 24 h of incubation of HepG2 cells with halofuginone at concentrations that affect IGFBP-1 synthesis (Fig. 5E). The presence of halofuginone throughout the incubation period was not essential and 1 h of incubation with halofuginone was sufficient to ensure the detection of an increase in IGFBP-1 secretion 23 h later. This level of expression increased with increasing incubation time with halofuginone (Fig. 6A). During this period, de novo protein synthesis was required to demonstrate any effect of halofuginone on IGFBP-1 gene expression, since incubation with cyclohexamide annulled the halofuginone-dependent increase in the IGFBP-1 gene expression (Fig. 6B).

3.4. Stellate cells motility

HepG2 cells were incubated with 50 nM halofuginone for 11 h after which the medium was removed, the cells washed twice with DMEM to remove any traces of halofuginone and incubated with a fresh medium for
additional 13 h. After halofuginone removal the cells continued to secrete IGFBP-1 and at the end of the incubation period the conditioned medium contained high levels of IGFBP-1 compared to the untreated cells (Fig. 7A). When added to HSC, the medium containing IGFBP-1 caused a significant inhibition in cell motility. Immunoprecipitation of IGFBP-1 from the conditioned medium abolished the inhibitory effect on HSC motility while no such effect was observed when normal serum was used (Fig. 7B).

4. Discussion

Hepatic fibrosis/cirrhosis is characterized by excessive production of ECM by activated HSC due to collagen synthesis and inhibition of collagen degradation [47–49]. Thus, pharmacological intervention to treat liver fibrosis should, at least in part, aim to inhibit HSC activation, to inhibit ECM synthesis and/or to stimulate matrix protein degradation. To reverse cirrhosis, inhibition of collagen synthesis by activated HSC and normal functionality of hepatocytes and other cell types is essential. Halofuginone, that has been found to prevent liver fibrosis [36,37], caused resolution of established fibrosis [37], and accelerated cirrhotic liver regeneration [38], also prevented TAA-dependent alteration in the expressions of the IGFBP-1 and IGFBP-3 genes (Fig. 3). In a first attempt to identify genes responsible for halofuginone action in vivo, we compared gene pattern of livers with Ishak grade 5–6 with those with grade 1–2 after halofuginone treatment (Fig. 2A and B). Of the 588 genes of the array, 13 were differentially expressed (Fig. 2C). We focused our attention on IGFBP-1, because of the involvement of the IGF-1 axis in liver physiology in health and disease [42,43,50,51]. In fibrosis/cirrhosis major alterations in the GH/IGF-I axis were observed including local changes in the expression of the genes encoding different members of the IGFBP family [50,52,53] and changes in the plasma levels of IGF-I and its binding proteins [51]. In liver fibrosis, a poor correlation between the expression of the IGFBP genes and their plasma concentrations has been observed [54], which may reflect an alteration in their clearance [55]. Two weeks of halofuginone treatment was required to prevent the TAA-induced down-regulation of the IGFBP-1 gene expression (Fig. 3). Halofuginone affected IGFBP-1 synthesis exclusively by cells of the hepatocytes origin (Fig. 4) consistent with the notion of hepatocytes being the major source of IGFBP-1 in the liver [53]. The primary cultures were more sensitive to halofuginone than the hepatocellular carcinoma cell lines (Figs. 4 and 5). The main reduction in the IGFBP-1 gene expression was observed in the fibrotic tissue in agreement with the human data [56].

Two of the immediate–early genes involved in maintaining hepatic metabolism in the remnant liver following partial hepatectomy, IGFBP-1 and protein tyrosine
phosphatase 4A1 (PRL-1) [57,58], were up-regulated by halofuginone (Fig. 2). In the regenerated liver, IGFBP-1 is regulated by interleukin-6 via hepatocyte nuclear factor 1 and induced factors STAT3 and activator protein-1 (AP-1, c-Fos/c-Jun) [57]. The inhibitory effect of halofuginone on collagen type I synthesis was also c-Jun dependent [59], raising the possibility that the same pathway is involved in halofuginone-dependent increase in the synthesis of IGFBP-1. Cyclohexamide annulled both the halofuginone-dependent activation of IGFBP-1 synthesis (Fig. 6) and the inhibition of collagen α(I) gene expression [27] suggesting that de novo protein synthesis is prerequisite for halofuginone signal transduction. Phosphatidylinositol 3-kinase (PI3K) has been implicated in regulation of the IGFBP-1 gene in hepatocytes [60] and of the collagen type I gene in HSC [61]. Interestingly, the p85 α-subunit of the PI3K was up-regulated by halofuginone (Fig. 2C). MAP kinase p38 was also up-regulated by halofuginone, suggesting involvement of more than one pathway. It is interesting to note that hepatocyte growth factor which signals through PI3K and accelerated liver regeneration after partial hepatectomy [62,63], decreased collagen synthesis in cirrhosis and induced IGFBP-1 gene expression [64,65]. IGFBP-1 has been implicated in inhibition of collagen type I gene expression directly [65] and to inhibit the IGF-I-dependent collagen type I synthesis by HSC [61]. IGFBP-1 regulates mitogenic signal pathways [66] and functions as a critical hepatic survival factor in the liver by reducing the level of pro-apoptotic signals [67]. Additional characteristic of IGFBP-1 is its ability to affect cell motility [68–70]. The IGFBP-1 secreted by the HepG2 after halofuginone treatment inhibited HSC motility (Fig. 7). HSC motility is dependent on collagen type I [71] thus in vivo halofuginone may inhibit HSC motility directly by inhibiting collagen type I production [36–38] and by stimulating IGFBP-1 synthesis by hepatocytes causing a further inhibition in HSC motility. This is of major importance since migration capacity is part of the ‘activated’ phenotype of HSC.

IGFBP-3 synthesized by Kupffer and endothelial cells is the most abundant circulating IGFBP in adult mammalian species [53]. IGF-I, IGFBP-3 and the acid labile subunit form a ternary complex that prolongs the plasma half-life of IGF-I and limits the amounts of free, biologically active IGF-I in circulation. IGF-I also circulates bound to other IGFBPs, but their physiological significance is less well established. Halofuginone treatment prevented the TAA-dependent increase in IGFBP-3.
gene expression (Fig. 3), but it is still to be established whether the alteration in the expression of the IGFBP-1 and IGFBP-3 genes reflects the normal physiological functional status of the liver or whether they have a specific role in the healing process.

In summary, we have demonstrated that halofuginone can be used as an anti-fibrotic/anti-cirrhotic therapy that acts by inhibiting collagen synthesis by HSC and by preventing the fibrosis-induced alteration in IGFBPs synthesis by hepatocytes and other hepatic cells. The secreted IGFBP-1 inhibited HSC motility further reducing the fibrogenic condition.

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