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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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***Keywords*: Smooth muscle actin; TIMP-2; Atlas microarrays; IGFBP-3**

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

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reported. Liver fibrosis may also result from imbalance between production and degradation of matrix proteins. Activated HSC constitutes the source of various collagenases and tissue inhibitors of metalloproteanases (TIMPs) required for ECM remodeling [17,18]. Liver fibrosis and cirrhosis are dynamic processes that can progress and regress over time [19–21], a process that requires cellular cross-talk between various liver cell types [22].

Halofuginone, an inhibitor of collagen type I synthesis [23,24], has been found to inhibit the gene expression of collagen type α1(I) but not of type II [25] or type III [26]. In culture, halofuginone attenuated collagen α1(I) gene expression by murine, avian and human skin fibroblasts derived from scleroderma 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 α1(I) gene expression, thus demonstrating human clinical efficacy [35]. In the liver, halofuginone prevented HSC activation and abolished the increase in collagen α1(I) gene expression and collagen deposition in rats treated with dimethylnitrosamine 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 (αSMA) 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 by uridine [α-S^{35}]triphosphate. Cell lines used were 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 [44] and plated on fibronectin-coated six well plates at a density of 1.5×10^6 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-^{32}P]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 denaturating conditions on 1.2% agarose/formaldehyde gels, transferred into Nytran N nylon membranes and hybridized with ^{32}P-labeled cDNA probe overnight at 68 °C. The probes were generated by RT-PCR amplification with the following primers pairs:

Rat IGFBP-3: 5′-CAGAGCACAGACACCCAGAA-3′ and 5′-AAAT-CAAGAAGGCAGAGGGC-3′; human IGFBP-1: 5′-GCACAGGAGA-CATCAGGAGA-3′ and 5′-GCAACATCACCACAGGTAGC-3′; rat IGFBP-1: 5′-CCACCACTTCCGCTACTATCT-3′ and 5′-GCTGTTC-CTCTGTCATCTCTGG-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 Priism 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

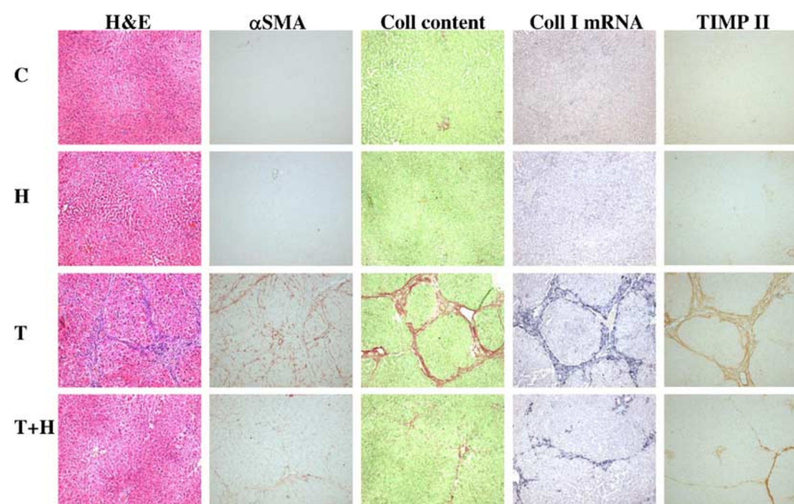
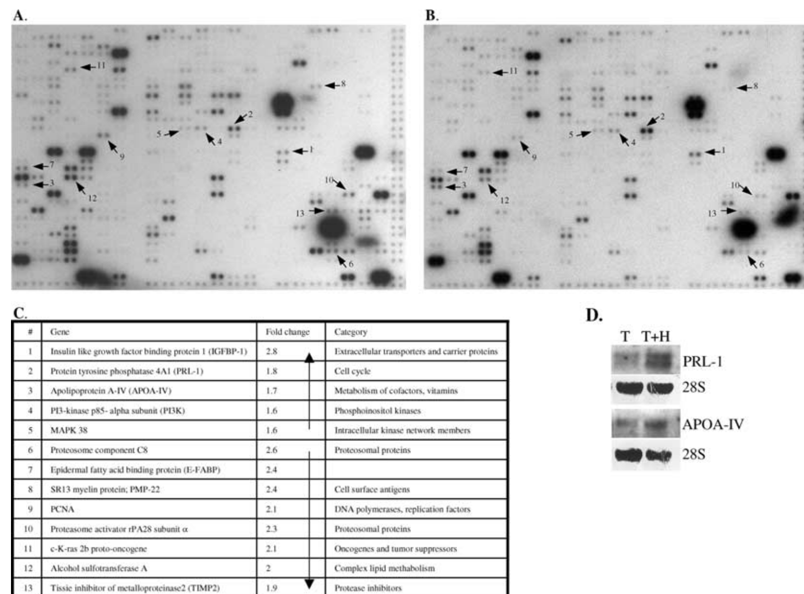


Fig. 1. Histological analysis of liver sections. Liver samples were taken from control rats, rats treated with halofuginone (H, 5 ppm in the diet), TAA (T, 200 mg/kg twice weekly) or a combination of both for 4 weeks. The sections were stained with hematoxylin and eosin (H&E), and with sirius red for collagen. Stellate cells and TIMP-2 were detected by immunohistochemistry. Collagen α1(I) gene expression was evaluated by in situ hybridization. Note the high levels of alpha smooth-muscle actin (αSMA)-positive stellate cells that express the collagen α1(I) gene and synthesize collagen and tissue inhibitors of metalloproteanases-2 (TIMP-2) after TAA treatment. A marked resolution of the fibrotic lesion was observed with halofuginone. [This figure appears in colour on the web.]



#	Gene	Fold change	Category
1	Insulin like growth factor binding protein 1 (IGFBP-1)	2.8	Extracellular transporters and carrier proteins
2	Protein tyrosine phosphatase 4A1 (PRL-1)	1.8	Cell cycle
3	Apolipoprotein A-IV (APOA-IV)	1.7	Metabolism of cofactors, vitamins
4	PI3-kinase p85- alpha subunit (PI3K)	1.6	Phosphoinositol kinases
5	MAPK 38	1.6	Intracellular kinase network members
6	Proteosome component C8	2.6	Proteosomal proteins
7	Epidermal fatty acid binding protein (E-FABP)	2.4	
8	SR13 myelin protein; PMP-22	2.4	Cell surface antigens
9	PCNA	2.1	DNA polymerases, replication factors
10	Proteasome activator rPA28 subunit α	2.3	Proteosomal proteins
11	c-K-ras 2b proto-oncogene	2.1	Oncogenes and tumor suppressors
12	Alcohol sulfotransferase A	2	Complex lipid methabolism
13	Tissie inhibitor of metalloproteinase2 (TIMP2)	1.9	Protease inhibitors

Fig. 2. Halofuginone and rat liver gene expression. Total RNA from liver tissue was hybridized with Atlas microarray filters. (A) Microarray analysis of liver biopsies of rats treated for 4 weeks with TAA alone (200 mg/kg twice weekly). (B) Microarray analysis of liver biopsies of rats treated for 4 weeks with TAA (200 mg/kg twice weekly) in combination with halofuginone (5 ppm in the diet). The arrows point to the differentially expressed genes. (C) List of differentially expressed genes. (D) Total RNA was prepared from liver biopsies of rats treated with TAA (T) and in combination with halofuginone (T + H) with PRL-1 and ApoA-IV probes. Ribosomal 28S RNA was used as the directive of RNA loading.

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) resulted 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

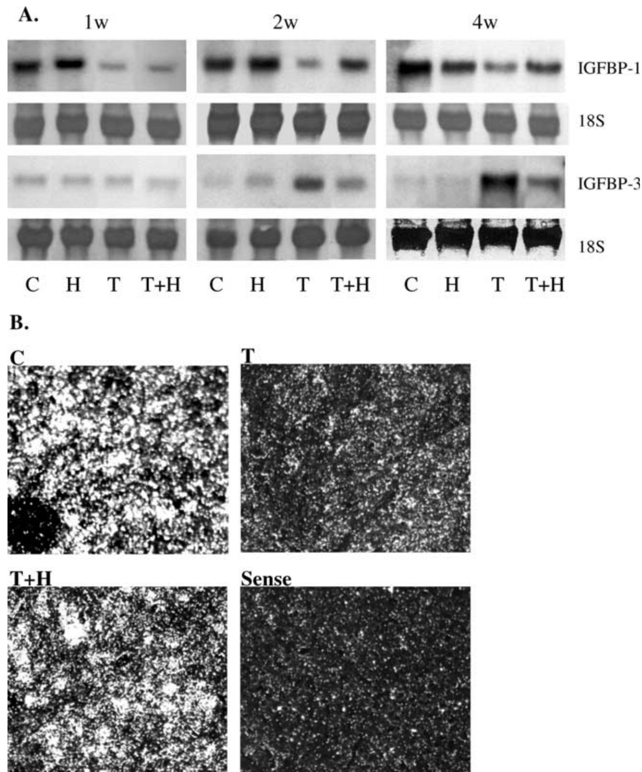


Fig. 3. Halofuginone prevented the TAA-induced down-regulation of the IGFBP-1 gene expression in vivo. IGFBP-1 gene expression was evaluated by Northern blots (A) and by in situ hybridization (B). (A) Total RNA was prepared from liver biopsies of the control rats (C), rats treated with TAA (T) and halofuginone alone (H) or in combination (T + H) after 1, 2 and 4 weeks and hybridized with the IGFBP-1 or IGFBP-3 probes. Ribosomal 18S RNA was used as the directive of RNA loading. (B) Section of livers after 4 weeks of treatment were hybridized with the IGFBP-1 probe. Dark-field photomicrographs showing hybridization of antisense IGFBP-1 probe to liver sections of control rats (C), rats treated with TAA (T) and rats treated with a combination of TAA and halofuginone (T + H). Hybridization with sense IGFBP-1 probe was used as a negative control.

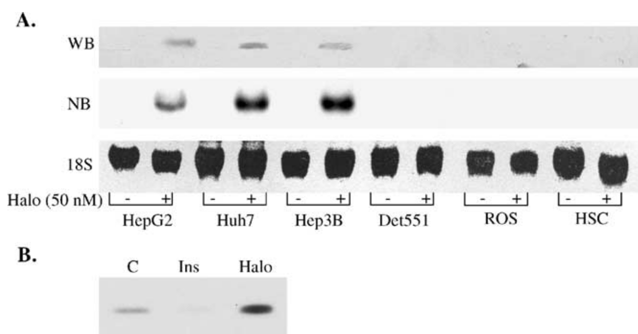


Fig. 4. Effect of halofuginone on IGFBP-1 synthesis in various cell types. (A) HepG2, Huh-7, Hep3B, Det551, HSC and ROS cells were incubated with and without 50 nM halofuginone in a serum-free medium. The IGFBP-1 gene expression was analyzed by Northern blotting (NB) and the presence of IGFBP-1 in the condition medium was evaluated by Western blotting (WB). Note that only hepatocytes synthesized IGFBP-1 in response to halofuginone. (B) Rat primary hepatocytes were incubated with insulin (Ins, 100 nM) or halofuginone (Halo, 1 nM) for 24 h and IGFBP-1 was detected by Western blot.

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 condition 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). Halofugnone 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

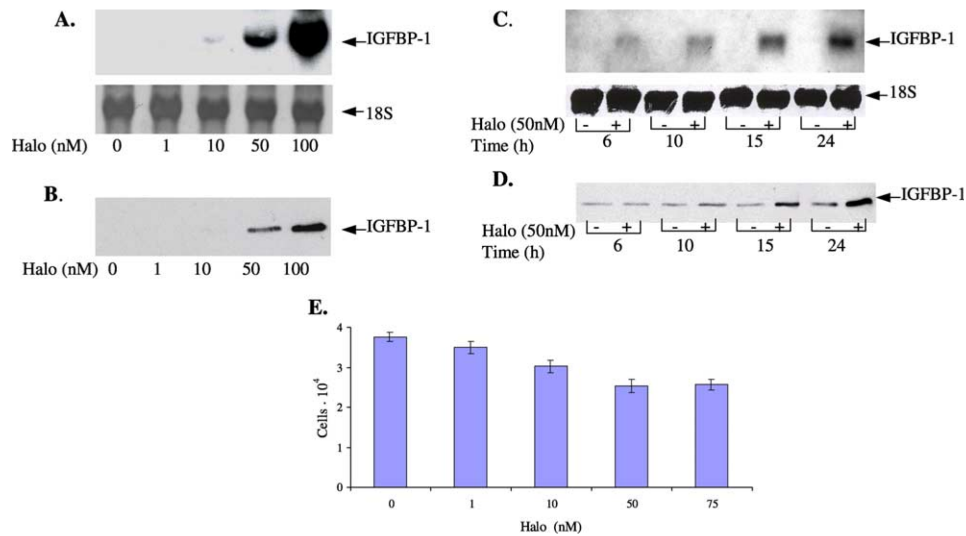


Fig. 5. Effect of halofuginone on IGFBP-1 synthesis and cell proliferation: dose and time response. HepG2 cells were incubated with various concentrations of halofuginone for 24 h and the level of IGFBP-1 gene expression was analyzed by Northern blotting (A) and the content of IGFBP-1 in the condition medium was evaluated by Western blotting (B). (C) and (D) represent the levels of IGFBP-1 gene expression and of IGFBP-1 in the condition medium and in response to 50 nM halofuginone after various intervals, respectively. (E) Cells were incubated for 24 h with various concentrations of halofuginone. The results are represented as the mean cell number ± SE of six replicates. [This figure appears in colour on the web.]

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 $\alpha 1$(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

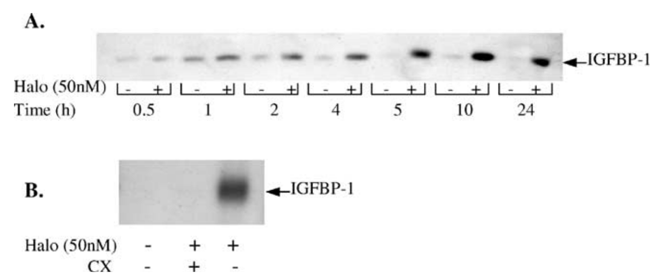


Fig. 6. (A) Following serum starvation, HepG2 cells were incubated with 50 nM halofuginone for the indicated time after which the medium was replaced with fresh medium without halofuginone. The level of IGFBP-1 was evaluated by Western blotting 24 h after the beginning of the experiment. (B) HepG2 cells were incubated for 24 h with and without 10 μg/ml cyclohexamide (CX) and 50 nM halofuginone. Expression of IGFBP-1 was analyzed by Northern blotting.

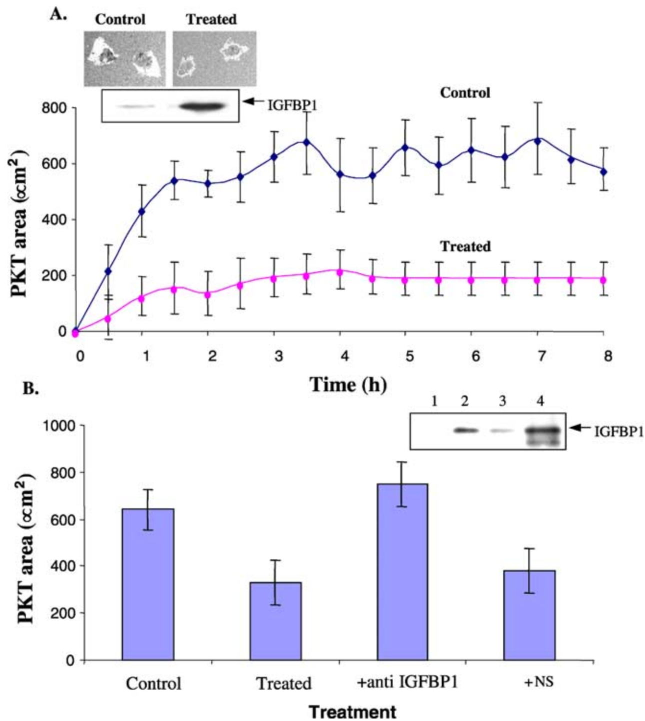


Fig. 7. IGFBP-1 caused inhibition of stellate cell motility. (A) Hepatocytes (HepG2) conditioned medium after halofuginone treatment contained higher levels of IGFBP-1 compare to the control (Insert; lane 1, no halofuginone; lane 2, + halofuginone). When added to stellate cells (HSC-T6), inhibition in cell motility was observed. Each time point represents the mean track area of 3–5 cells ± SE. (B) Hepatocytes (HepG2) conditioned medium after halofuginone treatment was immunoprecipitated with anti IGFBP-1 antibodies or with normal goat serum and incubated with the stellate cells for motility evaluation. The media were added for 8 h to the stellate cells. Each column represents the mean track area of 10–20 cells ± SE. The level of IGFBP-1 in the media before and after the immunoprecipitation is described in the insert. Lane 1, no halofuginone treatment; lane 2, after halofuginone treatment; lane 3, medium after immunoprecipitation with anti IGFBP-1 antibodies; lane 4, IGFBP-1 in the precipitate after treatment with the anti IGFBP-1 antibodies. [This figure appears in colour on the web.]

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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References

[1] George J, Roulot D, Koteliansky VE, Bissell DM. In vivo inhibition of rat stellate cells activation by soluble transforming growth factor β type II receptor: a potential new therapy for hepatic fibrosis. Proc Natl Acad Sci USA 1999;96:12719–12724.

[2] Pinzani M. Liver fibrosis. Springer Semin Immunopathol 1999;21: 475–490.

[3] Friedman SL. The cellular basis of hepatic fibrosis. Mechanism and treatment strategies. N Engl J Med 1993;328:1828–1835.

[4] Li D, Friedman SL. Liver fibrogenesis and the role of hepatic stellate cells: new insights and prospects for therapy. J Gastroenterol Hepatol 1999;14:618–633.

[5] Hautekeete ML, Geerts A. The hepatic stellate (Ito) cell: its role in human liver disease. Virchows Arch 1997;430:195–207.

[6] Reeves HL, Friedman SL. Activation of hepatic stellate cells—a key issue in liver fibrosis. Front Biosci 2002;7:d808–d826.

[7] Gressner AM. Transdifferentiation of hepatic stellate cells (Ito cells) to myofibroblasts: a key event in hepatic fibrogenesis. Kidney Int 1996;54:S39–S45.

[8] Alcolado R, Arthur MJ, Iredale JP. Pathogenesis of liver fibrosis. Clin Sci 1997;92:103–112.

[9] Aycock RS, Seyer JM. Collagen of normal and cirrhotic human liver. Connect Tissue Res 1989;23:19–31.

[10] Schuppan D. Structure of the extracellular matrix in normal and fibrotic liver: collagens and glycoproteins. Semin Liver Dis 1990;10: 1–10.

[11] Brenner DA, Westwick J, Breindl M. Type I collagen gene regulation and molecular pathogenesis of cirrhosis. Am J Physiol 1993;264: G589–G595.

[12] Panduro A, Shalaby F, Biempica L, Shafritz DA. Changes in albumin, α-fetoprotein and collagen gene transcription in CCl4-induced hepatic fibrosis. Hepatology 1988;8:259–266.

[13] Ala-Kokko L, Pihlajaniemi T, Myers JC, Kivirikko KI, Savolainen ER. Gene expression of type I, III, and IV collagens in hepatic fibrosis induced by dimethylnitrosamine in the rat. Biochem J 1987;244: 75–79.

[14] George J, Chandrakasan G. Molecular characteristics of dimethylnitrosamine induced fibrotic liver collagen. Biochim Biophys Acta 1996;1292:215–222.

[15] Ramadori G, Knittel T, Odenthal M, Schwogler S, Neubauer K, Meyer zum Buschenfelde KH. Synthesis of cellular fibronectin by rat liver fat-storing (Ito) cells: regulation by cytokines. Gastroenterology 1992;103:1313–1321.

[16] Burt AD. Cellular and molecular aspects of hepatic fibrosis. J Pathol 1993;170:105–114.

[17] Iredale JP, Benyon RC, Arthur MJ, Ferris WF, Alcolado R, Winwood P, et al. Tissue inhibitor of metalloproteinase-1 messenger RNA expression is enhanced relative to interstitial collagenase messenger RNA in experimental liver injury and fibrosis. Hepatology 1996;24: 176–184.

[18] Arthur MJ, Mann DA, Iredale JP. Tissue inhibitors of metalloproteinases, hepatic stellate cells and liver fibrosis. J Gastroenterol Hepatol 1998;13:S33–S38.

[19] Iredale JP, Benyon RC, Pickering J, McCullen M, Northrop M, Pawley S, et al. Mechanism of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. J Clin Invest 1998;102: 538–549.

[20] Murphy FR, Issa R, Zhou X, Ratnarajah S, Nagase H, Arthur MJ, et al. Inhibition of apoptosis of activated hepatic stellate cells by tissue inhibitor of metalloproteinase-1 is mediated via effects on matrix matalloproteinase inhibition: implications for reversibility of liver fibrosis. J Biol Chem 2002;277:11069–11076.
[21] Iredale JP. Hepatic stellate cell behavior during resolution of liver injury. Semin Liver Dis 2001;21:427–436.
[22] Kmiec Z. Cooperation of liver cells in health and disease. Adv Anat Embryol Cell Biol 2001;161:1–151.
[23] Pines M, Nagler A. Halofuginone: a novel anti-fibrotic therapy. Gen Pharmacol 1998;30:445–450.
[24] Pines M, Vlodavsky I, Nagler A. Halofuginone: from veterinary use to human therapy. Drug Dev Res 2000;50:371–378.
[25] Granot I, Hurwitz S, Halevy O, Pines M. Halofuginone: an inhibitor of collagen type I synthesis. Biochim Biophys Acta 1993;1156: 107–112.
[26] Choi ET, Callow AD, Sehgal NL, Brown DM, Ryan US. Halofuginone, a specific collagen type I inhibitor, reduces anastomotic intima hyperplasia. Arch Surg 1995;130:257–261.
[27] Halevy O, Nagler A, Levi-Schaffer F, Genina O, Pines M. Inhibition of collagen type I synthesis by skin fibroblasts of graft versus host disease and scleroderma patients: effect of halofuginone. Biochem Pharmacol 1996;52:1057–1063.
[28] Levi-Schaffer F, Nagler A, Slavin S, Knopov V, Pines M. Inhibition of collagen synthesis and changes in skin morphology in murine graft versus host disease and tight skin mice: effect of halofuginone. J Invest Dermatol 1996;106:84–88.
[29] Pines M, Domb A, Ohana M, Inbar J, Genina O, Alexiev R, Nagler A. Reduction in dermal fibrosis in the tight-skin (Tsk) mouse after local application of halofuginone. Biochem Pharmacol 2001;62: 1221–1227.
[30] Nagler A, Firman N, Feferman R, Cotev S, Pines M, Shoshan S. Reduction in pulmonary fibrosis in vivo by halofuginone. Am J Respir Crit Care Med 1996;154:1082–1086.
[31] Nyska M, Nyska A, Rivlin E, Porat S, Pines M, Shoshan S, Nagler A. Topically applied halofuginone, an inhibitor of collagen type I transcription, reduces peritendinous fibrous adhesions following surgery. Connect Tissue Res 1996;34:97–103.
[32] Nagler A, Rivkind AI, Raphael J, Levi-Schaffer F, Genina O, Lavelin I, Pines M. Halofuginone—an inhibitor of collagen type I synthesis—prevents postoperation abdominal adhesions formation. Ann Surg 1998;227:575–582.
[33] Nagler A, Genina O, Lavelin I, Ohana M, Pines M. Halofuginone, an inhibitor of collagen type I synthesis, prevents formation of postoperative adhesions formation in the rat uterine horn model. Am J Obstet Gynecol 1999;180:558–563.
[34] Nagler A, Gofrit O, Ohana M, Pode D, Genina O, Pines M. The effect of halofuginone, an inhibitor of collagen type I synthesis, on urethral stricture formation: in vivo and in vitro study in a rat model. J Urol 2000;164:1776–1780.
[35] Nagler A, Pines M. Topical treatment of cutaneous chronic graft versus host disease (cGvHD) with halofuginone: a novel inhibitor of collagen type I synthesis. Transplantation 1999;68: 1806–1809.
[36] Pines M, Knopov V, Genina O, Lavelin I, Nagler A. Halofuginone, a specific inhibitor of collagen type I synthesis, prevents dimethylnitrosamine-induced liver cirrhosis. J Hepatol 1997;26:391–398.
[37] Bruck R, Genina O, Aeed H, Alexiev R, Nagler A, Pines M. Halofuginone to prevent and treat thioacetamide-induced liver fibrosis in rats. Hepatology 2001;33:379–386.
[38] Spira G, Mawasi N, Paizi M, Anbinder N, Genina O, Alexiev R, Pines M. Halofuginone, a collagen type I inhibitor improves liver regeneration in cirrhotic rats. J Hepatol 2002;37:331–339.
[39] McGaha TL, Phelps RG, Spiera H, Bona C. Halofuginone, an inhibitor of type-I collagen synthesis and skin sclerosis, blocks transforming-growth-factor-β-mediated Smad3 activation in fibroblasts. J Invest Dermatol 2002;118:461–470.
[40] Huang Q, Dunn RT, Jayadev S, DiSorbo O, Pack FD, Farr SB, et al. Assessment of cisplatin-induced nephrotoxicity by microarray technology. Toxicol Sci 2001;63:196–207.
[41] Kristensen DB, Kawada N, Imamura K, Miyamoto Y, Tateno C, Seki S, et al. Proteome analysis of rat hepatic stellate cells. Hepatology 2000;32:268–277.
[42] Ormarsdottir S, Ljunggren O, Mallmin H, Olofsson H, Blum WF, Loof L. Circulating levels of insulin-like growth factors and their binding proteins in patients with chronic liver disease: lack of correlation with bone mineral density. Liver 2001;21:123–128.
[43] Moller S, Becker U, Juul A, Skakkebaek NE, Christensen E. Prognostic value of insulin-like growth factor I and its binding protein in patients with alcohol-induced liver disease. EMALD group. Hepatology 1996;23:1703–1708.
[44] Libal-Weksler Y, Gotlibovitz O, Stark AH, Madar Z. Diet and diabetic state modify glycogen synthase activity and expression in rat hepatocytes. J Nutr Biochem 2001;12:458–464.
[45] Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, et al. Histological grading and staging of chronic hepatitis. J Hepatol 1995; 22:696–699.
[46] Robertson DG, Marino EM, Thule PM, Seneviratne CK, Murphy LJ. Insulin and glucocorticoids regulate IGFBP-1 expression via a common promoter region. Biochem Biophys Res Commun 1994; 200:226–232.
[47] Lee KS, Buck M, Houglum K, Chojkier M. Activation of hepatic stellate cells by TGF α and collagen type I is mediated by oxidative stress through c-myb expression. J Clin Invest 1995;96:2461–2468.
[48] Friedman SL, Roll FJ, Boyles J, Arenson DM, Bissell D. Maintenance of differentiated phenotype of cultured rat hepatic lipocytes by basement membrane matrix. J Biol Chem 1989;264:10756–10762.
[49] Kossakowska AE, Edwards DR, Lee SS, Urbanski LS, Stabbler AL, Zhang C, et al. Altered balance between matrix metalloproteinases and their inhibitors in experimental biliary fibrosis. Am J Pathol 1998; 153:1895–1902.
[50] Donaghy AJ, Delhanty PJ, Ho KK, Williams R, Baxter RC. Regulation of growth hormone receptor/binding protein, insulin-like growth factor ternary complex system in human cirrhosis. J Hepatol 2002;36:751–758.
[51] Donaghy AJ, Ross R, Gimson A, Hughes SC, Holly J, Williams R. Growth hormone, insulin-like growth factor 1 and insulin-like growth factor binding proteins 1 and 3 in chronic liver disease. Hepatology 1995;21:680–688.
[52] Arany E, Afford S, Strain AJ, Winwood PJ, Arthur MJ, Hill DJ. Differential cellular synthesis of insulin-like growth factor binding protein-1 (IGFBP-1) and IGFBP-3 within human liver. J Clin Endocrinol Metab 1994;79:1871–1876.
[53] Zimmermann EM, Li L, Hoyt EC, Pucilowska JB, Lichtman S, Lund PK. Cell-specific localization of insulin-like growth factor binding protein mRNAs in rat liver. Am J Physiol Gastrointest Liver Physiol 2000;278:G447–G457.
[54] Holt RIG, Baker AJ, Jones JS, Crossey PA, Stone NM, Preedy VR, Miell JP. Differential effects of malnutrition, bile duct ligation and galactose-amine injection in young rats on serum levels and gene expression of IGF-binding proteins. J Endocrinol 1996;149: 465–472.
[55] Moller S, Juul A, Becker U, Flyvbjerg A, Skakkebaek NE, Henriksen JH. Concentrations, release and disposal of insulin-like growth factor (IGF)-binding proteins (IGFBP), IGF-I, and growth hormone in different vascular beds in patients with cirrhosis. J Clin Endocrinol Metab 1995;80:1148–1157.
[56] Ross RJ, Chew SL, D'Souza LL, Yateman M, Rodrigez-Arno J, Gimson A, et al. Expression of IGF-I and IGF-binding protein genes in cirrhotic liver. J Endocrinol 1996;149:209–216.
[57] Leu JI, Crissey MA, Leu JP, Ciliberto G, Taub R. Interleukin-6-induced STAT3 and AP-1 amplify hepatocyte nuclear factor 1-mediated transactivation of hepatic genes, an adaptive response to liver injury. Mol Cell Biol 2001;21:414–424.

[58] Peng Y, Du K, Ramirez S, Diamond RH, Taub R. Mitogenic up-regulation of PRL-1 protein-tyrosine phosphatase gene by Egr-1. EGR-1 activation is an early event in liver regeneration. J Biol Chem 1999;274:4513–4520.

[59] McGaha TL, Kodera T, Spiera H, Stan AC, Pines M, Bona CA. Halofuginone inhibition of COL1A2 promoter activity via a c-Jun-dependent mechanism. Arthritis Rheum 2002;46:2748–2761.

[60] Cichy SB, Uddin S, Danilkovich A, Guo S, Klippel A, Unterman TG. Protein kinase B/Akt mediates effects of insulin on hepatic insulin-like growth factor binding protein-1 gene expression through a conserved insulin response sequence. J Biol Chem 1998;273: 6482–6487.

[61] Svegliati-Baroni G, Ridolfi F, Di Sario A, Casini A, Marucci L, Gaggioti G, et al. Insulin and insulin-growth factor 1 stimulate proliferation and type I collagen accumulation by human hepatic stellate cells: differential effects on signal transduction pathways. Hepatology 1999;29:1743–1751.

[62] Fan S, Ma YX, Wang JA, Yuan RQ, Meng Q, Cao Y, et al. The cytokine hepatocyte growth factor/scatter factor inhibits apoptosis and enhances DNA repair by a common mechanism involving signaling through phosphatidyl inositol 3′ kinase. Oncogene 2000;19: 2212–2223.

[63] Weir E, Chen Q, DeFrances MC, Bell A, Taub R, Zarnegar R. Rapid induction of mRNAs for liver regeneration factor and insulin-like growth factor binding protein-1 in primary cultures of rat hepatocytes by hepatocyte growth factor and epidermal growth factor. Hepatology 1994;20:955–960.

[64] Sato M, Kakubari M, Kawamura M, Sugimoto J, Matsumoto K, Ishii T. The decrease in total collagen fibers in the liver by hepatocyte growth factor after formation of cirrhosis induced by thioacetamide. Biochem Pharmacol 2000;59:681–690.

[65] Gosiewska A, Wilson S, Kwon D, Peterkofsky B. Evidence for an in vivo role of insulin-like growth factor binding protein 1 and 2 as inhibitors of collagen gene expression in vitamin C-deficient and fasted guinea pigs. Endocrinology 1994;134:1329–1339.

[66] Leu JI, Crissey MS, Craig LE, Taub R. Impaired hepatocyte DNA synthetic response posthepatectomy in insulin-like growth factor binding protein 1-deficient mice with defects in C/EBPβ and mitogen-activated protein kinase/extracellular signal-regulated kinase regulation. Mol Cell Biol 2003;23:1251–1259.

[67] Leu JI, Crissey MS, Taub R. Massive hepatic apoptosis associated with TGF-β1 activation after Fas ligand treatment of IGF binding protein1-deficient mice. J Clin Invest 2003;111:129–139.

[68] Gleeson LM, Chakraborty C, McKinnon T, Lala PK. Insulin-like growth factor-binding protein 1 stimulates human trophoblast migration by signaling through α5β1 integrin via mitogen-activated protein kinase pathway. J Clin Endocrinol Metab 2001;86: 2484–2493.

[69] Gockerman A, Prevette T, Jones I, Clemmons DR. Insulin-like growth factor (IGF)-binding proteins inhibit the smooth muscle cell migration responses to IGF-I and IGF-II. Endocrinology 1995;136: 4173–4186.

[70] Zhang X, Yee D. Insulin-like growth factor binding protein-1 (IGFBP-1) inhibits breast cancer cell motility. Cancer Res 2002;62: 4369–4375.

[71] Yang C, Zeisberg M, Mosterman B, Sudhakar A, Yerramalla U, Holthaus K, et al. Liver fibrosis: insight into migration of hepatic stellate cells in response to extracellular matrix and growth factors. Gastroenterology 2003;124:147–159.