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BASIC STUDIES

Enhanced actions of insulin-like growth factor-I and interferon-α co-administration in experimental cirrhosis

Federico Tutau[1,2], Carlos Rodríguez-Ortigosa[3], Juan Enrique Puche[1,2], Nerea Juanarena[3], Iñigo Monreal[3], María García Fernández[1], Encarna Clavijo[1], Alberto Castilla[4,5] and Inma Castilla - Cortázar[1,2]

1 Department of Physiology, School of Medicine, University of Málaga, Málaga, Spain
2 Department of Medical Physiology, School of Medicine, University USP-CEU, Madrid, Spain
3 Department of Internal Medicine, Liver Unit, CIMA, University of Navarra, Pamplona, Spain
4 Department of Internal Medicine, Hospital Sierrallana, Torrelavega, Spain
5 School of Medicine, Cantabria, Spain

Keywords
fibrogenesis – hepatocyte growth factor (HGF) – hepatoprotection – liver cirrhosis – pregnane X receptor (PXR) – transforming growth factor-β (TGF-β)

Abbreviations:
AU, arbitrary units of fluorescence; HGF, hepatocyte growth factor; IFN-α, interferon-α; IGF-I, insulin-like growth factor-I; PXR, pregnane X receptor;α-SMA, α-smooth muscle actin; TGF-β, transforming growth factor-β; TIMPs, tissular inhibitor of metalloproteinases.

Correspondence
Inma Castilla de Cortázar Larrea, Department of Medical Physiology. School of Medicine, University USP-CEU, Campus Montepríncipe, 28660, Boadilla del Monte, Madrid, Spain
Tel: +34 686 652 710
Fax: +34 91 359 44 79
e-mails: iccortazar@uma.es, iccortazar@ceu.es

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Abstract
Background: Cirrhosis is a diffuse process of hepatic fibrosis and regenerative nodule formation. The liver is the major source of circulating insulin-like growth factor-I (IGF-I) whose plasma levels are diminished in cirrhosis. IGF-I supplementation has been shown to induce beneficial effects in cirrhosis, including antifibrogenic and hepatoprotective effects. On other hand, interferon-α (IFN-α) therapy seems to suppress the progression of hepatic fibrosis. *Aims:* The aim of this study was to investigate the effect of the co-administration of IGF-I+IFN-α to *Wistar* rats with CCl_4-induced cirrhosis, exploring liver function tests, hepatic lipid peroxidation and histopathology. *Methods:* The mechanisms underlying the effects of these agents were studied by reverse transcription-polymerase chain reaction, determining the expression of some factors [hepatocyte growth factor (HGF), transforming growth factor-β (TGF-β), α-smooth muscle actin, collagen, tissular inhibitor of metalloproteinases-1 and pregnane X receptor (PXR)] involved in fibrogenesis, fibrolysis and/or hepatoprotection. *Results:* Both IGF-I and IFN-α exerted significant effects on fibrogenesis. IGF-I significantly increased serum albumin and HGF whereas IFN-α-therapy did not. The inhibition of TGF-β expression was only observed by the effect of IFN-α-therapy. In addition, only the co-administration of IGF-I and IFN-α was able to increase the PXR. The combined therapy with both factors improved liver function tests, hepatic lipid peroxidation and reduced fibrosis, inducing a relevant histological improvement, reducing fibrosis and recovering hepatic architecture. *Conclusion:* The co-administration IGF-I+IFN enhanced all the beneficial effects observed with each factor separately, showing an additive action on histopathology and PXR expression, which is involved in the inhibition of fibrogenesis.

Cirrhosis is a diffuse process of hepatic fibrosis and regenerative nodule formation. Its pathogenesis is not fully understood. One of the main goals of liver disease therapy is to develop effective liver fibrosis therapies.

In cirrhosis, the reduction of receptors for growth hormone (GH) in hepatocytes and the diminished synthesizing ability for the liver parenchyma cause a progressive decline in serum insulin-like growth factor-I (IGF-I) levels (1). IGF-I is an anabolic hormone and its possible therapeutic use in cirrhosis is supported by studies in cirrhotic rats, demonstrating that IGF-I replacement therapy: increases food intake, nitrogen balance and food efficiency (2); enhances intestinal absorption of glucose and amino acids (3–5); reverts hypogonadism (6, 7) and the somatostatinergic tone (8); increases bone mass (9); improves liver function tests, the hepatic expression of GH receptor and decreases liver fibrogenesis (10–12); and diminishes oxidative liver damage and increases antioxidant defences (10, 12, 13). IGF-I therapy has recently been tested in cirrhotic patients, inducing clinical benefits such as improvements in serum albumin levels and resting energy expenditure (14).

On the other hand, interferon-α (IFN-α), owing to its antiviral properties (15, 16), has been used to treat chronic viral hepatitis. Moreover, IFN-α is now known to reduce liver fibrosis, inhibiting collagen production and reducing the activity of the tissular inhibitor of metalloproteinases (TIMPs). Several studies have reported antifibrogenic properties of IFN-α as decreasing fibrogenesis and enhancing fibrolysis (15–21).

Because IGF-I and IFN-α seem to exert hepatoprotection and antifibrogenic effects by different mechanisms, it is conceivable that the co-administration of both agents could induce stronger beneficial actions on the cirrhotic liver than each factor separately.

The aims of this study were (1) to evaluate the effect of a combined therapy with IGF-I and IFN-α in rats with

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CCl_4-induced cirrhosis assessing liver function tests, oxidative liver damage and hepatic histopathology and (2) to explore various mechanisms determining, by reverse transcription-polymerase chain reaction (RT-PCR), the expression of several factors such as hepatocyte growth factor (HGF), transforming growth factor-β (TGF-β), α-smooth muscle actin (α-SMA), collagen, TIMP-1 and the pregnane X receptor (PXR) involved in fibrogenesis, fibrolysis and/or hepatoprotection and regeneration.

Pregnane X receptor is a recently described nuclear receptor involved in regulating drug-and corticosteroid-inducible expression of cytochrome P450 (22). The PXR has been proposed as an effective target for antifibrotic therapy (23).

The experimental design was supported by our previous studies (2–13) including five groups of animals: healthy controls (CO); untreated cirrhotic rats (CI); cirrhotic rats treated with IGF-I (2 μg × 100 g/bw/day, for 3 weeks, CI+IGF-I); cirrhotic rats treated with IFN-α (3200 U × 100 g/bw, 3 day/week, for 3 weeks, CI+IFN); and cirrhotic rats treated with IGF-I and IFN-α at the same doses (CI+IGF+IFN).

Materials and methods

Induction of liver cirrhosis

All experimental procedures were performed in conformity with *The Guiding Principles for Research Involving Animals*. In male *Wistar* rats (3 ± 1 weeks old, 110–120 g), liver cirrhosis was induced by inhalation of CCl_4 (Merck, Darmstadt, Germany) twice a week for 12 weeks, with phenobarbital (Luminal; Bayer, Leverkusen, Germany) added to drinking water (400 mg/L) as reported previously (2–5). The time of exposure to CCl_4 was increased, starting with an initial exposure of 1 min twice/week to a maximum of 5 min twice/week. During the treatment period (weeks 13, 14 and 15), cirrhotic animals received doses of 2 min/week of CCl_4 inhalation. Healthy control rats, which did not receive phenobarbital or CCl_4, were studied in parallel.

Study design

The study period (period of administration of saline or growth factors) was initiated 21 days before completing the exposure to CCl_4 (weeks 13, 14 and 15). The study period (period of administration of saline or growth factors) was initiated 3 days after stopping CCl_4 administration (doses of 5 min twice/week) (day 0). Cirrhotic rats were randomly assigned to receive either saline (group CI, $n = 10$), recombinant human IGF-I (Chiron Company, CA, USA), (rhIGF-I; 2 μgIGF-I/100 g body wt/day in two divided doses) (group CI+IGF, $n = 8$), IFN-α (IFN-α for rat) 3.200 IU/100 g body wt three times a week) (group CI+IFN, $n = 8$) or both IGF-I and IFN-α (group CI+IGF+IFN-α, $n = 10$) subcutaneously for 21 days.

Animals were sacrificed by decapitation 24 h after receiving the last dose (day 22). Biochemical parameters were determined on days 0 and 22. Blood samples were taken from the retro-ocular venous plexus with capillary tubes (70 mm; Laboroptik, Marienfeld, Germany), divided into aliquots and stored at − 20 °C until used. The livers and spleens were weighed, and a tissue sample from the left major liver lobe was processed (fixed in Bouin' solution) for histological examination. Tissue specimens were immediately frozen by immersion in liquid N_2 and stored at − 80 °C until assaying. All animals included in the groups receiving CCl_4 had altered liver function test results at baseline (day 0), and liver biopsy specimens from the CI group on day 22 showed established cirrhosis.

Analytical methods

Liver function tests were determined in serum by routine laboratory methods using a Hitachi 747 autoanalyzer (Boehringer Mannheim, Mannheim, Germany).

Malondialdehyde (MDA) was used as an index of lipid peroxidation in liver homogenates and was measured after heating samples at 45 °C for 60 min in acid medium. It was quantified by the method reported previously (10).

Serum IGF-I levels were assessed by RIA with a γ-counter (cDPC Gmbyt CR, Madrid, Spain), using a specific antibody proportioned by Nichols Institute (San Juan Capistrano, CA, USA).

Histological degree of fibrosis

In liver sections stained with Masson's trichrome, semiquantitative assessment of fibrosis, cellular liver damage was blindly performed using a numerical score of fibrosis based on the number, length and thickness of fibrous septa. The length of the septa (examined at × 80 magnification) was assessed as follows: 1 point, minimal grade fibrosis that can be observed in normal livers; 4 points, septa confluent between portal tracts and between portal tracts and central veins; and 2 or 3 points, intermediate lengths of septa observed. The width of the fibrous septa was calculated at × 150 magnification scoring 4 points when the mean value of the thickness of 9 septa (three periportal, three perivenous and three perinodular), measured in four different fields, oscillated around 90–125 μm; score 3, 70–50 μm; and score 2, ~40–30 μm. The number of septa was scored as 4 points when there were numerous septa extending into the nodules, thus dissecting a small number of hepatocytes forming micronodules; 2–3 points when septa penetrating into nodules were less numerous surrounding larger nodules; and 1 point when there was no formation of micronodules inside macronodules. Evident tissular damage and steatosis were scored with 1 point for each one. Four fields from each preparation were evaluated twice by two different observers, receiving a maximum of 14 points each time. The arithmetical mean of the two punctuations was taken as the final score.

RNA isolation and quantitative polymerase chain reaction

Real-time RT-PCR was performed to quantify the expression of the mRNAs of TGF-β, procollagen α1(I) (Col-α1), HGF, TIMP-1, α-SMA, PXR and β-actin as control (Table 1). Total RNA extraction was carried out using Tri reagent (Sigma, St. Louis, MO, USA). Two micrograms of RNA were treated with DNaseI (Invitrogen, Barcelona, Spain), before reverse transcription with M-MLV Reverse Transcriptase (Invitrogen) in the presence of RNAseOUT (Invitrogen). Primers were designed to specifically amplify cDNA, and PCR products were sequenced to assess specificity. 1/20 of the PCR reaction was used to perform real-time PCR in an iCycler (BioRad, Hercules, CA, USA) and with the IQ SYBR Green Supermix (BioRad). mRNA levels were normalized according to β-actin quantification in the same sample. Specificity was confirmed by analysing the final PCR products using melting curves and electrophoresis. Quantification was represented as expression units (i.e. 2^(CT) × 10 000,

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Table 1. Oligonucleotide sequences used in reverse transcription-polymerase chain reactions

TGF-β	Sense	5′-CGG CAG CTG TAC ATT GAC-3′
	Antisense	5′-TCA GCT GCA CTT GCA GGA GC-3′
Col-α1	Sense	5′-CAG ATT GAG AAC ATC CGC AG-3′
	Antisense	5′-TCG CTT CCA TAC TCG AAC TG-3′
HGF	Sense	5′-GCA GAA GGA CAG AAG AAG AG-3′
	Antisense	5′-CCA GTA GCA TCG TTT TCT CG-3′
TIMP1	Sense	5′-TCC CCA GAA ATC ATC GAG AC-3′
	Antisense	5′-TCA GAT TAT GCC AGG GAA CC-3′
α-SMA	Sense	5′-ACT GGG ACG ACA TGG AAA AG-3′
	Antisense	5′-CAT CTC CAG AGT CCA GCA CA-3′
PXR	Sense	5′-TTC AAG GGC GTC ATC AAC T-3′
	Antisense	5′-CAG GGA GAT CTG GTC CTC AA-3′
β-actin	Sense	5′-CGC GTC CAC CCG CGA G-3′
	Antisense	5′-CCT GGT GCC TAG GGC G-3′

TGF-β, transforming growth factor-β; Col-α1, procollagen α1; HGF, hepatocyte growth factor; TIMP-1, tissue inhibitor of metalloproteinase 1; α-sma, α-smooth muscle actin; PXR, pregnane X receptor.

where *CT* is the difference in the number of cycles between β-actin expression – as control – and that of the measured gene), as described elsewhere (24).

Statistical analysis

Data are expressed as mean ± SEM. To assess the homogeneity among the five groups of rats, a Kruskall–Wallis test was used, followed by multiple *post hoc* comparisons using Mann–Whitney *U*-tests with Bonferroni's adjustment. Student's *t*-test was used to assess the differences between before and after treatment in each animal. Any *P*-value of < 0.05 was considered to be statistically significant. Calculations were performed with SPSSWIN v.10.0. program (SPSS Inc., Chicago, IL, USA).

Results

On day 0, before starting the treatments, rats from the four groups of cirrhotic animals presented significantly altered values of serum albumin, total bilirubin, total cholesterol, aspartate aminotransferase (AST) and alanine aminotransferase (ALT). No differences between cirrhotic groups were found before starting treatments. Table 2 summarizes analytical data before and after treatments.

Liver function tests

Serum total proteins

In this series, the untreated cirrhotic group (CI) showed no significant reduction of serum total proteins (see Table 2 and Fig. 1a). However, an anabolic effect of IGF-I therapy was also observed in this series in agreement with previously reported results (10), when data were compared before and after treatment (see Table 2 and Fig. 1b).

Serum albumin levels

On day 22, cirrhotic animals showed a reduction of serum albumin levels as compared with healthy controls (see Table 2 and Fig. 1c). Although no significant differences were found in cirrhotic groups, the anabolic effect of IGF-I on this parameter was observed when data before and after treatment were compared (see Fig. 1d). IGF-I therapy induced an increase of serum albumin levels in agreement with reported data (10, 14). This effect was not statistically significant in cirrhotic animals treated only with IFN-α.

Cholestasis parameters

Serum bilirubin levels

On day 22, untreated cirrhotic animals showed elevated levels of bilirubin as compared with controls ($P < 0.001$ vs CO, Table 2). All treatments reduced this parameter of cholestasis ($P < 0.05$ vs CI).

Serum cholesterol levels

Untreated cirrhotic groups (CI group and all groups of cirrhotic rats before treatments) showed high serum levels of cholesterol as compared with controls ($P < 0.01$ vs CO). IGF-I therapy resulted in a reduction of this marker of cholestasis ($P =$ NS vs CO) and IFN-α did not induce significant changes ($P < 0.05$ vs CO). The improvement of this parameter was also evident in the group treated with the co-administration of IGF+IFN ($P =$ NS vs CO and $P < 0.01$ vs CI).

Serum transaminases

Aspartate aminotransferase

Untreated cirrhotic animals (CI) presented a significant increment of AST compared with controls (Table 2). Both groups treated with IGF-I showed a reduction of AST serum levels but they did not reach statistical significance. Only cirrhotic animals treated with IFN-α normalized AST serum levels.

Although the treatment with IFN-α had the clearest effect, when all the groups were studied comparing data before and after treatment, the co-administration of IGF-I+IFN showed a reduction in all animals of this group ($P < 0.01$ between data before and after treatment in the IGF-I+IFN group; $P < 0.05$ for the CI+IFN and CI+IGF groups).

Alanine aminotransferase

Untreated cirrhotic animals showed elevated ALT serum levels compared with controls (Table 2, $P < 0.05$ vs CO). The treatment with IFN-α did not change this parameter. However, treatments with IGF-I and IGF-I+IFN-α normalized this cytolysis marker ($P =$ NS vs CO, $P < 0.05$ vs CI and CI+IFN groups). A similar result was observed between the series of animals treated with IGF-I and IGF-I+IFN-α, when data from before and after treatment were compared.

Lipid peroxidation marker in liver homogenates (malondialdehyde)

The hepatic levels of the lipid peroxidation products (estimated as nanomoles of MDA per gram of tissue) were increased in the cirrhotic rats as compared with healthy controls $P < 0.001$). This finding is in accordance with the known toxic effect of CCl_4, which causes oxidative damage in the liver.

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Table 2. Analytical data before (day 0) and after (day 22) treatment in the five experimental groups

	Healthy controls (CO)	Untreated cirrhotic (CI)	CI+IGF-I	CI+IFN	CI+IGF-I+IFN
Total bilirubin					
Day 0	0.06 ± 0.01	$0.64 \pm 0.02^{***}$	$0.68 \pm 0.19^{***}$	$0.65 \pm 0.10^{***}$	$0.67 \pm 0.16^{***}$
Day 22	0.1 ± 0.01	$0.48 \pm 0.18^{***}$	$0.26 \pm 0.1^{\#}$	$0.13 \pm 0.02^{££\#}$	$0.14 \pm 0.03^{£\#\#}$
Total cholesterol					
Day 0	49.33 ± 3.19	$62.13 \pm 5.79^{**}$	$63.38 \pm 5.67^{**}$	$89.67 \pm 4.18^{**}$	$64.22 \pm 4.37^{**}$
Day 22	49.55 ± 3.6	$69.72 \pm 4.5^{**}$	$47.63 \pm 3.40^{\#}$	$67.33 \pm 3.54^{*}$	$48.44 \pm 3.89^{\&\&\#\#}$
AST					
Day 0	76.22 ± 4.67	$200.63 \pm 41.69^{***}$	$205.25 \pm 36.81^{***}$	$200.83 \pm 23.88^{***}$	$191.11 \pm 24.29^{***}$
Day 22	80.22 ± 6.83	$163.25 \pm 42.75^{*}$	$123.75 \pm 21.09^{*\#}$	$92.16 \pm 15.85^{\#}$	$104.77 \pm 7.24^{*\#\#}$
ALT					
Day 0	34.78 ± 0.86	$104.88 \pm 6.62^{***}$	$108.88 \pm 7.71^{***}$	$103.17 \pm 10.49^{***}$	$105.22 \pm 6.53^{***}$
Day 22	34.55 ± 1.30	$57.63 \pm 9.36^{*\$}$	$46.38 \pm 7.23^{\#}$	$60.16 \pm 6.76^{**\$\$\#}$	$39.00 \pm 3.40^{\#\#}$
Total proteins					
Day 0	6.63 ± 0.12	6.64 ± 0.26	6.64 ± 0.26	6.63 ± 0.22	6.58 ± 0.17
Day 22	$6.91 \pm 0.12^{\#}$	6.61 ± 0.18	$6.94 \pm 0.19^{\&\#}$	6.73 ± 0.10	$6.84 \pm 0.13^{\#\#}$
Albumin					
Day 0	3.57 ± 0.05	3.11 ± 0.16	3.08 ± 0.13	3.12 ± 0.18	3.03 ± 0.13
Day 22	3.64 ± 0.06	3.15 ± 0.14	$3.25 \pm 0.11^{\#}$	3.23 ± 0.07	$3.31 \pm 0.06^{\#\#}$

$X \pm$ SEM.
$^{*}P < 0.05$.
$^{**}P < 0.01$.
$^{***}P < 0.001$ vs CO.
$^{£}P < 0.05$.
$^{££}P < 0.01$ vs CI.
$^{\&}P < 0.05$.
$^{\&\&}P < 0.01$.
$^{\&\&\&}P < 0.001$ vs CI+IFN.
$^{\$}P < 0.05$.
$^{\$\$}P < 0.01$ vs CI+IGF+IFN.
$^{\#}P < 0.05$.
$^{\#\#}P < 0.01$ day 0 vs day 22.
ALT, alanine aminotransferase; AST, aspartate aminotransferase.

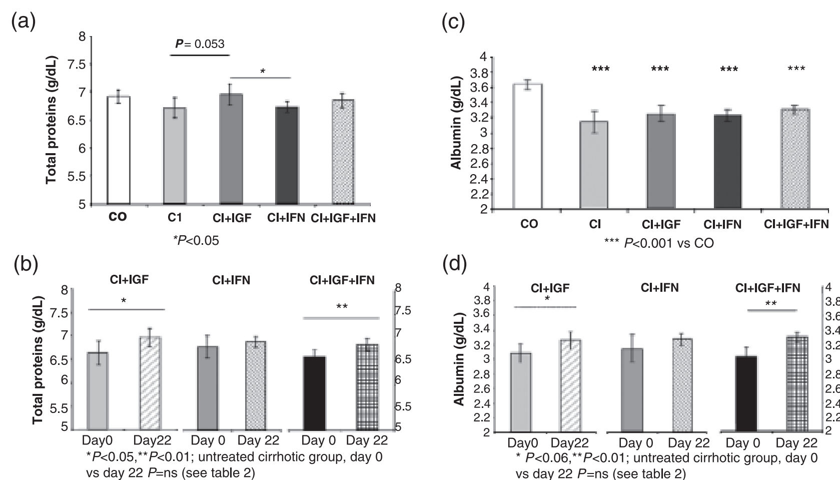

Fig. 1. Liver function tests: albumin and total proteins in the five experimental groups on day 22.

The treatment with IGF-I significantly reduced the lipid peroxidation products observed in cirrhotic rats ($P < 0.01$) and vs CI+IGF+IFN ($P < 0.05$), in agreement with previous findings (11, 14). This effect was observed in both groups treated with IGF-I (CI+ IGF and CI+IGF-I+IFN). The group treated only with IFN showed a reduction of this marker of lipid

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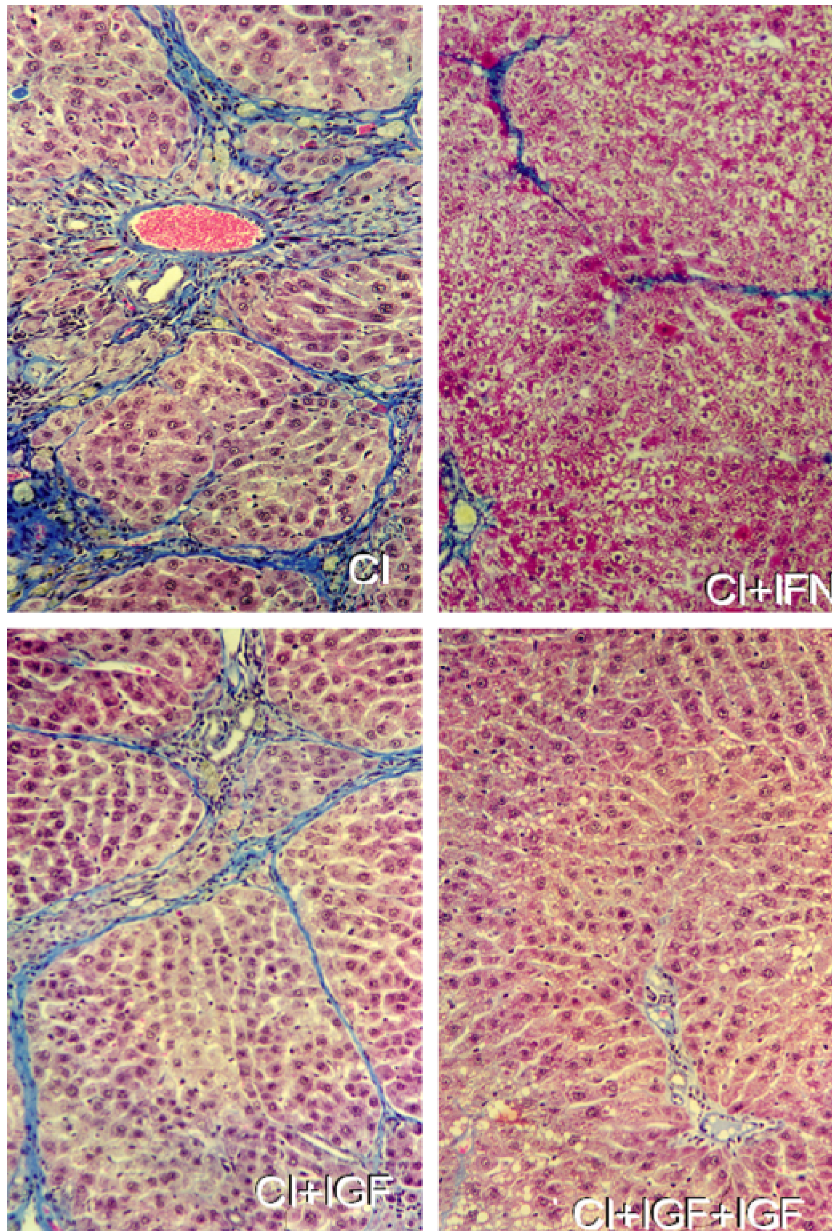

Fig. 2. Histopathological comparative study including the four cirrhotic groups (4 μm sections; Masson's trichrome stain). An evident reduction of fibrosis was found in cirrhotic groups with the three treatments as compared with the untreated cirrhotic group (CI). IFN-α therapy reduced fibrosis (see upper-right) but extensive areas of parenchyma appear to be damaged. However, IGF-I therapy resulted in a clear improvement of liver histology (see alignment of hepatocytes) in both treated groups. The co-administration of the two factors induced a more effective antifibrogenic effect (CI+IGF+IFN). IGF, insulin-like growth factor; IFN, interferon.

peroxidation, although it did not reach statistical significance (CI+IFN = 76.32 ± 7.69 vs CI *P* = NS).

Anatomopathological findings: histopathological score of fibrosis

The histological study proved the establishment of liver cirrhosis (see Fig. 2 upper-left picture). In liver sections stained with Masson's trichrome, semiquantitative assessment of fibrosis and liver damage was blindly performed using a numerical scoring system described previously (see 'Materials and methods'). Figure 3 summarizes the results of the histological score and Figures 2 and 4 show the histopathological findings in liver biopsy from rats included in the four cirrhotic groups.

The histological score showed a significant reduction of fibrosis in the three treated cirrhotic groups as compared with the untreated cirrhotic group (Fig. 3). The greatest reduction of fibrosis was observed in animals treated with the

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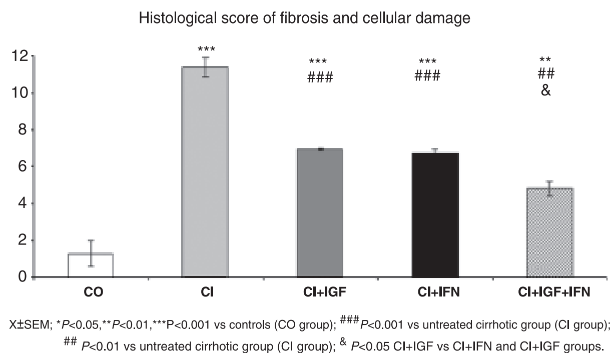

Fig. 3. Histological score of fibrosis and cellular damage. The semiquantitative score of fibrosis and cellular damage showed a significant reduction in cirrhotic animals treated with the combined therapy. Both factors separately reduced fibrosis as compared with untreated cirrhotic rats.

co-administration of IGF-I+IFN (see Fig. 2 bottom-right and 4). The histological score was significantly lower in the CI+IGF+IFN group than in the CI+IGF, CI+IFN and CI groups. Both IFN-α and IGF-I treatments separately induced an evident reduction of collagen deposition as compared with untreated cirrhotic animals (CI group).

Interferon-α therapy reduced fibrosis notably (see Fig. 2 upper-right and Fig. 3) but it did not produce a clear hepatoprotection (from a histological point of view) because extensive areas of parenchyma appeared to be damaged (see Fig. 2 upper-right).

Insulin-like growth factor-I therapy and IFN-α therapy reduced fibrosis. However, IGF-I therapy resulted in a recovery of the hepatic architecture (see Fig. 2 bottom-left) with a clear improvement of liver histology (see alignment of hepatocytes) in both treated groups (CI+IGF and CI+IGF+IFN). This effect was more evident in animals treated with both factors (CI+IGF+IFN).

Therefore, the co-administration of the two factors induced more effective antifibrogenic and hepatoprotective effects (CI+IGF+IFN) (see Fig. 2 bottom-right and Fig. 4).

Serum levels of insulin-like growth factor-I

Untreated cirrhotic animals showed a significant reduction in the IGF-I circulating levels as compared with healthy controls (ng/ml, CI = 524.50 ± 35.94 vs CO = 1031.67 ± 21.97, $P < 0.05$) as well as the cirrhotic group treated with IFN-α (CI+IFN = 557.21 ± 42.72).

As expected, IGF-I therapy resulted in a significant improvement of IGF-I serum levels in both treated groups as compared with the CI group (ng/ml, CI+IGF = 945.83 ± 64.84) and the CI group treated with IGF-I and IFN-α (CI+ IGF-I+IFN = 976.67 ± 25.39, $P < 0.05$).

Study of mechanisms related to fibrogenesis, fibrolysis and hepatoprotection or regeneration

Figure 5 shows that both therapies were able to reduce collagen and α-SMA expression as well as the expression of TIMP-1, which, under normal conditions, inhibits metalloproteinase activity leading to increased fibrolysis.

Insulin-like growth factor-I therapy was associated with an increase of HGF expression, a factor involved in mechanisms of hepatoprotection and liver regeneration (see Fig. 6a). However, IFN therapy induced a significant reduction of TGF-β, which is the main factor involved in collagen expression by myofibroblasts (see Fig. 6b). Only the combined therapy significantly increased the expression of the PXR, a nuclear receptor involved in the inhibition of fibrogenesis (22, 23) (see Fig. 6c).

Discussion

This study analysed the effect of the simultaneous administration of IGF-I and IFN-α on liver damage and function in rats with CCl_4-induced cirrhosis and compared the effects with those observed when each agent was administered alone. These results confirm that low doses of IGF-I induce anabolic, hepatoprotective and antifibrogenic effects (10, 12–14) because this factor increased albumin, reduced cholestasis and hepatic levels of lipid peroxidation and improved histopathology. On the other hand, IFN-α therapy decreased hepatic fibrosis, total bilirubin and AST serum levels in agreement with other studies (15–21). Interestingly, the combined therapy with both factors enhanced all the beneficial effects observed with each factor in isolation, proving an additive beneficial action of IGF-I and IFN-α in this experimental model.

Compared with healthy rats, untreated cirrhotic rats showed moderately altered liver function tests, increased hepatic lipid peroxidation and fibrosis and elevated collagen, α-SMA, TIMP-1 and TGF-β expressions in agreement with previous studies (12, 20, 21). In addition, a reduction of HGF and PXR was observed in untreated cirrhotic rats. The PXR reduction is in agreement with other studies (23). However, in acute liver damage and after several doses of CCl_4, an increase of HGF expression has been reported (25).

All these findings allow a better characterization of this widely used model of experimental cirrhosis in an early stage (no ascites presented at the time of this study and only moderate deficiency in liver function tests was observed in this series).

The major finding in this work is the recognition that the co-administration of IGF-I and IFN-α is more effective than each factor alone in improving liver fibrosis and function in this experimental model of cirrhosis. The mechanisms of this action are not completely understood but data in this paper provide some insights.

First, IFN-α therapy has been used to treat chronic viral hepatitis, owing to its antiviral properties. However, several studies have demonstrated antifibrogenic properties of IFN-α by both decreasing fibrogenesis and enhancing fibrolysis (15–21). IFN-α exerts antifibrogenic effects by inhibiting TGF-β1 expression (16, 17, 20), decreasing hepatic stellate cell activation and stimulating its apoptosis (20). IFNs are known to prevent collagen deposition in the liver by mechanisms involving mRNA procollagen regulation (17, 21). Moreover, IFN-α seems to induce fibrolysis, increasing the plasminogen activator activity and decreasing the activity of the TIMPs (19). Accordingly, in the present study, IFN-α administration was associated with reduced expression of TGF-β, collagen and α-SMA, showing a lower transformation of ITO cells into myofibroblasts. In addition, the expression of TIMP-1 was also reduced. All these findings indicate that the antifibrogenic and fibrolytic properties of IFN-α are exerted not only in cirrhosis induced by chronic viral infection but also in cirrhosis induced by toxic agents such as CCl_4.

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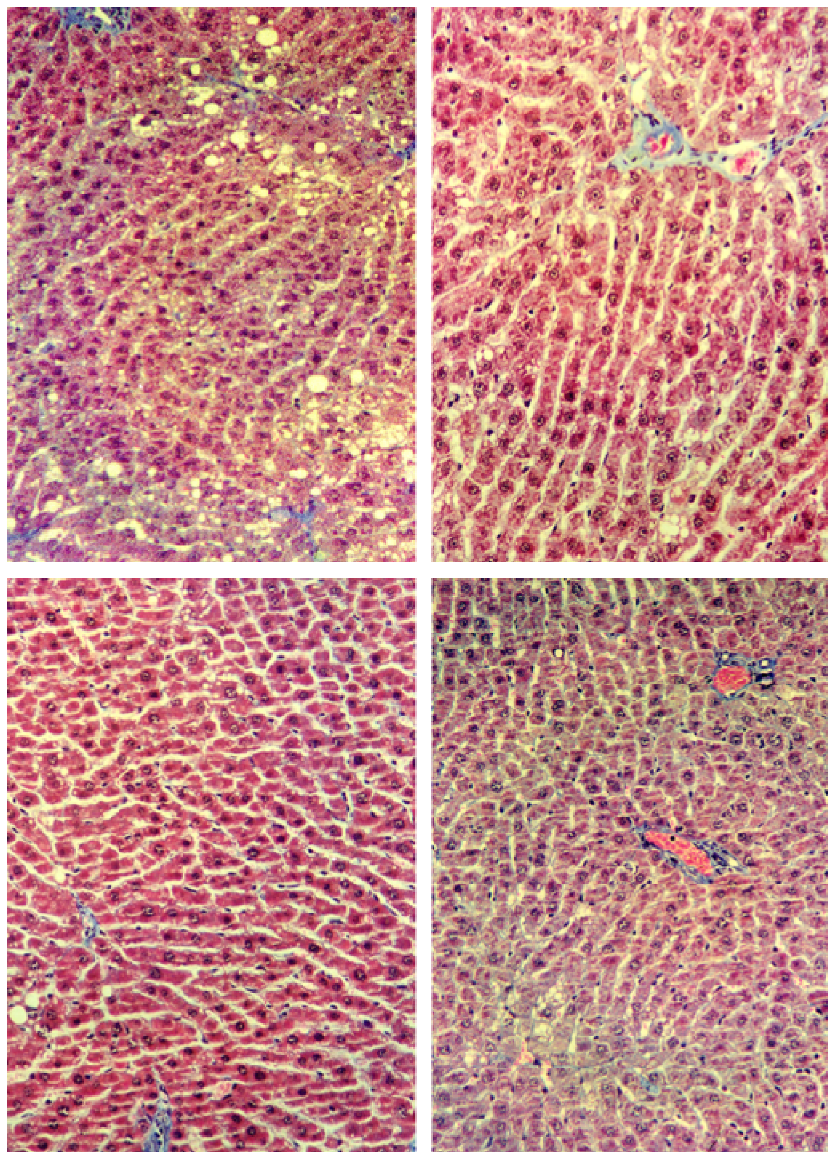

Fig. 4. Histopathological findings in cirrhotic animals which received the combined therapy of IGF-I and IFN-α (CI+IGF+IFN group) (4 μm sections; Masson's trichrome stain from four animals of the CI+IGF+IFN group). A notable diminution of collagen deposition and a notable recovering of normal hepatic architecture with steatosis were found in cirrhotic rats treated with the combined therapy. CI, cirrhotic group; IGF, insulin-like growth factor; IFN, interferon.

Second, we have already published some of the mechanisms underlying the beneficial effect of IGF-I on the cirrhotic liver (10–13). Low doses of IGF-I induced a hepatoprotective effect with several antioxidant and anti-inflammatory actions, reducing hepatic lipid peroxidation, myeloperoxidase activity, hepatic iron and copper content (13), increasing antioxidant enzyme activities (10) and protecting mitochondrial function (10). IGF-I therapy reduced collagen I and III mRNA expression, hydroxyproline content and prolyl hydroxylase activity in cirrhotic rats (12). IGF-I has been described as proapoptotic to hepatic stellate cells (26). In addition, we have described an IGF-I-induced partial normalization of the alterations observed in the hepatic gene expression with global genomic DNA methylation in these animals (11). In the present study, IGF therapy was associated with a reduced expression of collagen, α-SMA and TIMP-1. Furthermore, IGF-I therapy, in contrast to IFN therapy, did not reduce TGF-β expression but did induce an increase of HGF expression in agreement with the hepatoprotective actions described in previous studies in this experimental model of cirrhosis (27, 28).

Third, the co-administration of IGF+IFN improved the same parameters and enhanced the beneficial effects observed with each factor separately, but only the combined therapy increased the PXR expression. PXR is the nuclear receptor transcriptor factor that mediates the induction of CYP450 3A genes in response to the binding of several drugs and

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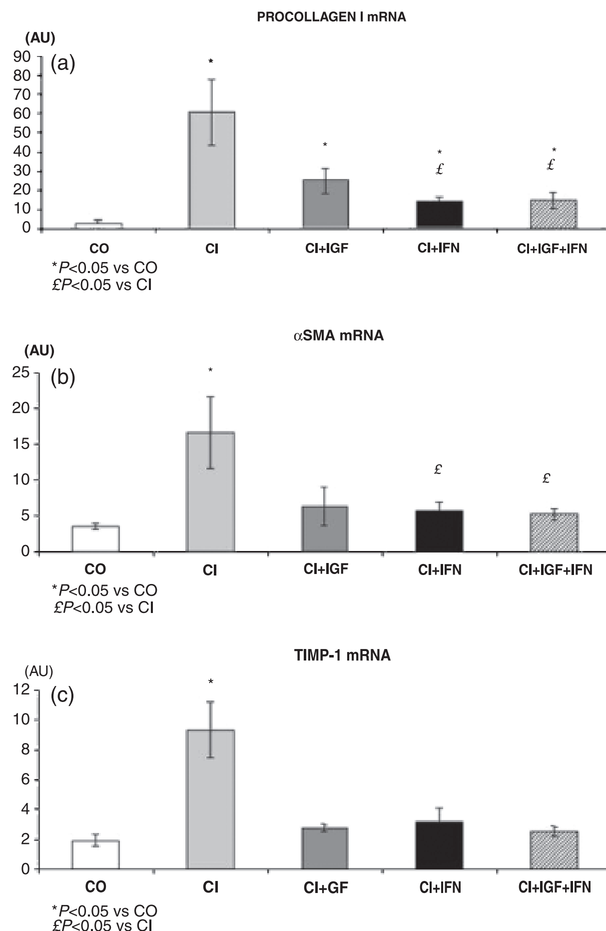

Fig. 5. Reverse transcription-polymerase chain reaction for collagen, α-SMA and TIMP-1: Significant reduction of the expression of the three genes in the treated cirrhotic group (IGF-therapy, IFN therapy and the co-administration of both factors) as compared with untreated cirrhotic rats. α-SMA, α-smooth muscle actin; IGF, insulin-like growth factor; IFN, interferon; TIMP, tissular inhibitor of metalloproteinases.

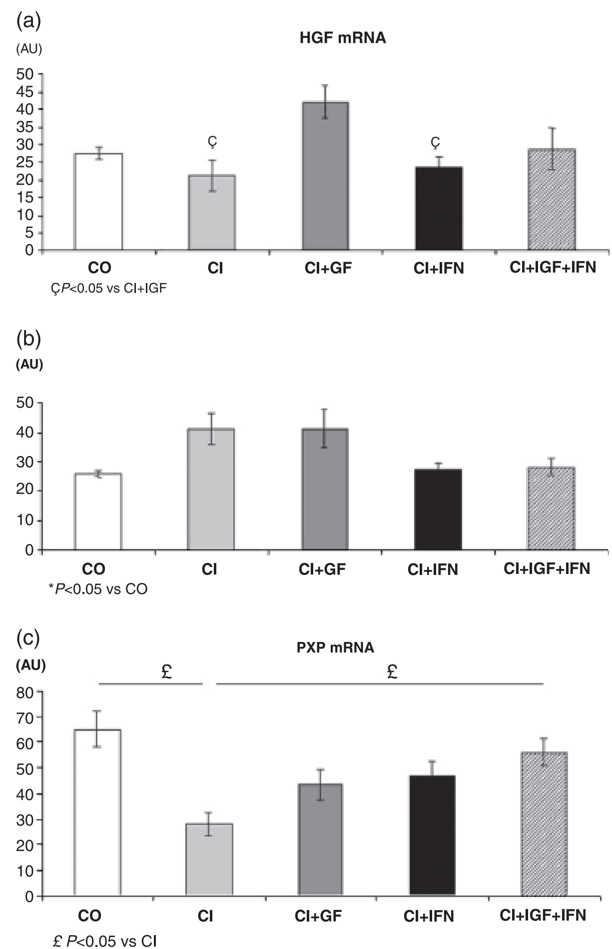

Fig. 6. Reverse transcription-polymerase chain reaction for hepatocyte growth factor (HGF), transforming growth factor-β (TGF-β) and pregnane X receptor activators (PXR): only IFN therapy was able to reduce the expression of TGF-β whereas IGF-I therapy increased the expression of HGF. Only a significant increase of the expression of PXR was found in the cirrhotic group (CI) treated with the co-administration ($P < 0.05$ vs untreated cirrhotic rats, CI; P = NS vs CO). CO, control; IGF, insulin-like growth factor; IFN, interferon.

endogenous compounds such as bile acids and pregnane steroids (22, 23). PXR activation inhibits liver fibrosis in the absence of attenuation of the severity of the damaging agent (23). PXR activators inhibit TGF-β expression and hepatic stellate cell proliferation and transdifferentiation, reducing fibrogenesis (23). It has been suggested that PXR is a potential target for the drug treatment of liver fibrosis (22).

In our study, both cirrhotic groups treated with IGF-I (CI+IGF and CI+IGF+IFN) presented a marked improvement in the alterations of liver structure, which IFN-α administration was unable to induce when it was administered alone (see Figs 2 and 4).

Taken together, all these data show that the main activities of IGF-I seem to be anabolic and hepatoprotective, with antioxidant actions leading to improved liver function tests and reduced fibrosis (see Fig. 2 bottom-left) (2–14). The antioxidant actions could explain the antifibrogenic effects, because oxidative damage is relevant in the development of fibrosis (10, 12). However, IFN-α appears to have potent antifibrogenic and fibrolytic properties (15–21), and secondarily, it exerts hepatoprotective activities. These mechanisms are summarized in Figure 7. On the other hand, Figure 8 summarizes the mechanisms involved in the improvement of liver regeneration with the co-administration of IGF-I and IFN. An additive action of these factors could explain hepatocyte proliferation by increasing HGF and reducing TGF-β-expressions. HGF is a well-known activator of liver regeneration (27, 28) and TGF-β inhibits the cellular cycle in the G1 step (28–30).

The IGF-I+IFN-α co-administration enhanced the hepatoprotective and antifibrogenic effects reported with only IGF-I therapy. Both therapies reduced fibrogenesis (diminishing collagen and α-SMA expressions) and increased fibrolysis (inhibiting TIMP-1 expression). Only IGF-I therapy was associated with an increase of HGF and albumin plasma levels, whereas IFN therapy was able to decrease TGF-β expression. In conclusion, these results provide an experimental basis for further studies aiming at exploring the potential of the

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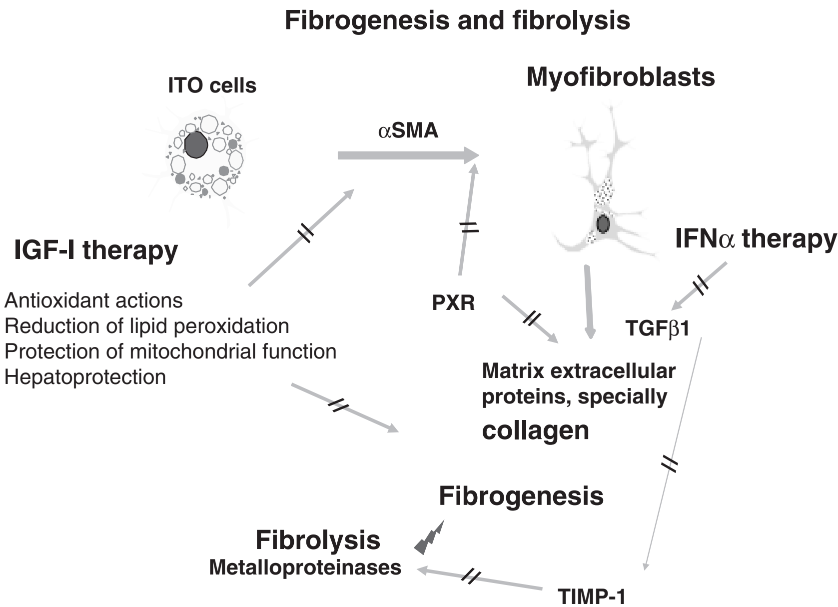

Fig. 7. Mechanisms of IGF-I and IFN-α actions: By different pathways, IGF-I and IFN-α induce antifibrogenic effects. IFN-α exerts a direct antifibrogenic action, decreasing the TGF-β expression. The antifibrogenic activity of IGF-I seems to be secondary to the antioxidant and the hepatoprotective activities of this hormone. IGF, insulin-like growth factor; IFN, interferon; TGF, transforming growth factor.

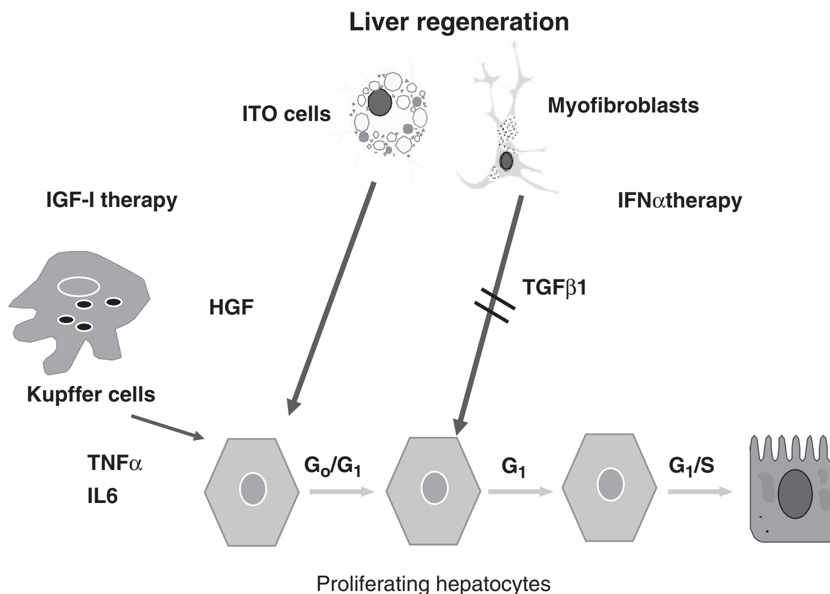

Fig. 8. Diagram of the mechanisms of hepatocyte regeneration induced by IGF-I and by IFN-α: IGF-I induces the proliferation of hepatocytes by increasing the expression of HGF. However, IFN-α therapy contributes to regeneration by decreasing the expression of TGF-β which inhibits the cellular cycle in G1. HGF, hepatocyte growth factor; IGF, insulin-like growth factor; IFN, interferon; TGF, transforming growth factor.

combined therapy with IGF-I and IFN-α in the treatment of human cirrhosis.

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The authors declare that they have no competing interest.

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