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Altered liver gene expression in CCl_4-cirrhotic rats is partially normalized by insulin-like growth factor-I

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Abstract

We have previously shown that the administration of low doses of insulin-like growth factor-I (IGF-I) to CCl_4-cirrhotic rats improves liver function and reduces fibrosis. To better understand the mechanisms behind the hepatoprotective effects of IGF-I, and to identify those genes whose expression is affected in cirrhosis and after IGF-1 treatment, we have performed differential display of mRNA analysis by means of polymerase chain reaction (PCR) in livers from control and CCl_4-cirrhotic rats treated or not with IGF-I. We have identified 16 genes that were up- or down-regulated in the cirrhotic liver. IGF-I treatment partially normalized the expression of eight of these genes, including serine proteinase inhibitors such as serpin-2 and alpha-1-antichymotripsin, alpha-1-acid glycoprotein, and alpha-2u-globulin. Additionally, we show that IGF-I enhanced the regenerative activity in the cirrhotic liver, as determined by the increased expression of the proliferating cell nuclear antigen (PCNA). Finally, IGF-I treatment partially restored the expression of growth hormone receptor (GHR) and the levels of global genomic DNA methylation, which are reduced in human and experimental cirrhosis. Taken together, our observations confirm the hepatoprotective effects of IGF-I, and suggest that this action can be exerted in part through the normalization of liver gene expression, growth hormone (GH) responsiveness and global genomic DNA methylation.

Keywords: Liver injury; Gene expression; DNA methylation; Growth hormone; Insulin-like growth factor-I

1. Introduction

Insulin-like growth factor-I (IGF-I) is a polypeptide growth factor structurally related to insulin and insulin-like growth factor-II, with relevant anabolic effects on carbohydrate, protein and lipid metabolism [1]. Circulating IGF-I is mainly synthesised in the liver [2], and its production has been found to be decreased in patients with chronic liver disease as well as in experimental models of chronic liver injury

Abbreviations: IGF-I: insulin-like growth factor-I; PCR: polymerase chain reaction; DD-PCR: differential display by means of PCR; CI: cirrhotic rats; CO: control rats; CI + IGF-I: cirrhotic rats treated with IGF-I; GHR: growth hormone receptor; RT-PCR: reverse transcription-PCR; HNF-3β: hepatocyte nuclear factor-3β; PCNA: proliferating cell nuclear antigen; C/EBPβ: CCAAT/enhancer binding protein β; GH: growth hormone

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[3–6]. Impaired production of IGF-I in advanced liver disease has been suggested to contribute to malnutrition in cirrhotic patients—reviewed in [3]. In support of this notion, we have previously shown that the administration of low doses of IGF-I can correct the impaired intestinal absorption of nutrients in CCl_4-cirrhotic rats [7–9]. Furthermore, systemic administration of IGF-I to rats with chronic liver disease (CCl_4-induced cirrhosis) resulted in improved liver function and reduced collagen deposition [10]. The hepatoprotective action of IGF-I in this experimental model can be attributed in part to its ability to modulate the peroxidative liver damage elicited by CCl_4 [10]. However, the molecular mechanisms behind this in vivo effect of IGF-I are not completely known, and can be further complicated by the fact that hepatic parenchymal cells express few IGF-I receptors [11].

In order to gain more insight into the mechanisms and signals behind the hepatoprotective action of IGF-I, we have searched for genes whose expression altered in the CCl_4-cirrhotic rat liver is normalized by IGF-I treatment. Our analysis employing the technique of differential display by means of polymerase chain reaction (DD-PCR) [12], allowed us to identify 16 genes that were up- or down-regulated in the cirrhotic rat liver. IGF-I treatment partially normalized the expression of eight of these genes. In addition, this growth factor was capable of restoring global DNA methylation at CpG sites, that is reduced in the cirrhotic liver [13]. This latter effect of IGF-I, together with the nature of some of the identified genes, suggest that the hepatoprotective action of this growth factor is mediated in part at the level of gene expression.

2. Materials and methods

2.1. Materials

All chemicals were of the best quality commercially available and otherwise stated they were purchased from Sigma (St. Louis, MO, USA). Restriction enzymes were from Boehringer Mannheim (Mannheim, Germany). [^{3}H] dCTP (52 Ci/mmol) was from Amersham.

2.2. Animals and treatments

All experimental procedures were performed in conformity with our institution's guidelines for the use of laboratory animals. Cirrhosis was induced as previously described [10]. Briefly, male Wistar rats (3 weeks old, 130–150 g) were subjected to CCl_4 inhalation (Merck, Darmstadt, Germany) twice a week for 11 weeks with a progressively increasing exposure time from 1 to 5 min. From that time until the 30th week, rats were exposed to CCl_4 once a week for 3 min. During the whole period of cirrhosis induction, animals received phenobarbital (Luminal, Bayer, Leverkusen, Germany) in the drinking water (400 mg/l). Rats were housed in cages placed in a room with 12-h light-dark cycle and constant humidity and temperature (20 °C). Both, food (standard semipurified diet for rodents; B.K. Universal, Sant Vicent dels Horts, Barcelona, Spain) and water were given ad libitum. Healthy, age- and sex-matched control rats were maintained under the same conditions but receiving neither CCl_4 nor phenobarbital.

IGF-I or saline was administered the last three weeks (27th–30th) of CCl_4 exposure. Cirrhotic rats were randomly assigned to receive either vehicle (saline) (Group CI, $n = 10$) or recombinant human IGF-I (Pharmacia-Upjohn, Sweden) (20 $\mu g\,kg^{-1}$ per day in two divided doses, subcutaneously) (Group CI + IGF-I, $n = 10$) for 3 weeks. Control rats (Group CO, $n = 10$) received saline during the same period.

After 3 weeks of treatment, livers were removed, weighted and snap frozen in liquid nitrogen.

2.3. RNA isolation and DD-PCR

Total RNA was extracted from frozen liver specimens as previously described [14]. Twenty-five milligrams of total RNA were incubated for 30 min at 37 °C with 10 units of RNAse-free DNAse I (Boehringer Mannheim, Mannheim, Germany) in 10 mmol/l Tris–HCl, pH 7.5, 10 mmol/l $MgCl_2$, then samples were phenol:chloroform extracted and ethanol precipitated in the presence of 0.3 mol/l sodium acetate. RNA was redissolved in sterile nuclease-free water.

DD-PCR was performed using fluorescent tag labelled oligo (dT) anchored primers (FluoroDD, Genomyx, Beckman Instruments, Fullerton, CA)

with the Hieroglyph mRNA Profile Kit (Genomyx, Beckman) following manufacturer's instructions and as previously described [15].

Following the DD-PCR, fluorescently-labelled cDNA fragments were electrophoretically separated on 4.5% polyacrylamide gels under denaturing conditions in a Genomyx LR DNA Sequencer (Genomyx, Beckman). DD-PCR products were visualized with a Genomyx SC Fluorescent Imaging Scanner (Genomyx, Beckman). Bands of interest were excised from the gel, and the gel slices were placed directly into PCR tubes and reamplified using the Genomyx Re-Amp Kit, following manufacturer's instructions. Reamplified cDNA fragments were cloned into the plasmid vector pCR2:1-TOPO using TOPO-TA Cloning Kit (Invitrogen, Leek, The Netherlands) and sequenced in both directions using M13 reverse (−24) primer and M1 forward (−20) primer. Nucleotide sequence homology search analysis of the EMBL [16] and GenBank [17] databases were performed using the program FASTA [18].

2.4. Northern blot and RT-PCR analysis

Aliquots of 20 μg of total RNA were size-fractionated by electrophoresis in a 1% agarose gel under denaturing conditions. RNAs were then blotted and fixed to Nytran membranes (Schleicher & Schuell, Keene, NH, USA). Prehybridization and hybridization were performed as described previously [14]. Probes used were the isolated clones from DD-PCR. The rat IGF-I cDNA probe was the generous gift of Dr. Peter S. Rotwein, Washington University School of Medicine, St. Louis, MO, USA. The rat growth hormone receptor (GHR) cDNA was cloned by RT-PCR, specific primers were designed according to the published sequence [19]. Equal loading of the gels was assessed by hybridization with a probe specific for the 18S rRNA. The probes were labelled with [α-^{32}P] dCTP (3000 Ci/mmol) by random priming using the Megaprime DNA labeling system (Amersham). Specific activity was usually $\sim 5 \times 10^8$ cpm/μg of DNA. Quantitation was performed by scanning densitometry of the X-ray films.

RT-PCR was performed to measure the expression levels of hepatocyte nuclear factor-3β (HNF-3β) in total RNA samples from CO, CI and CI + IGF-I rat livers. Aliquots of 2 μg of total RNA were reverse transcribed using M-MLV Reverse Transcriptase (Gibco Life Technologies, MD, USA) and subsequently amplified by PCR using the BioTaq DNA Polymerase (Bioline, UK). The primers used were deduced from the cDNA sequence of HNF-3β [20]. The 18S rRNA was simultaneously amplified using the Alternate 18S Internal Standards primers set (Ambion, TX, USA) and served as an internal loading control. Reactions were resolved in a 2% agarose gel, stained with ethidium bromide and quantitated using the Molecular Analyst software (Bio-Rad, Hercules, CA, USA). The 100-bp DNA Ladder molecular weight standards from Gibco Life Technologies were used.

2.5. Immunoblot analysis

Frozen liver tissue samples were homogenized in RIPA buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate, 0.1 mM phenyl-methylsulfonyl fluoride, and 1 mM benzamidine). The homogenate was centrifuged for 30 min at 10,000 × *g*, and supernatants collected. Equal amounts of protein (20 μg) were subjected to 10% sodium dodecylsulfate polyacrylamide gel electrophoresis, and then electrophoretically transferred to nitrocellulose membranes as described [15]. Antibodies for the immunodetection of proliferating cell nuclear antigen (PCNA) and CCAAT/enhancer binding protein β (C/EBPβ), and a horseradish peroxidase-conjugated secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Where indicated, equal loading of gels was assessed by probing the membranes with an antibody specific for actin (Calbiochem-Novabiochem, Darmstadt, Germany). Blots were developed by enhanced chemoluminescence according to manufacturer's instructions (DuPont, Boston, MA, USA).

2.6. Global DNA methylation status

Genomic DNA was isolated from livers of CO, CI and CI + IGF-I rats as described previously [20]. The DNA methylation at CpG sites assay was carried out essentially as described [21]. This is a reverse assay, in which higher levels of [^{3}H] dCTP incorporation into *Hpa*II-digested genomic DNA indicate loss of methyl groups at the CpG dinucleotides found in the

restriction site of this methylation sensitive restriction enzyme.

2.7. Statistics

Data are expressed as means ± S.E.M. Statistical significance was estimated with Student's *t*-test. A *P*-value of <0.05 was considered significant.

3. Results

The biochemical and histological data, along with the liver function tests of the animals used in this study have been already described in our previous publication [10]. At baseline, before the onset of IGF-I treatment, groups CI and CI + IGF-I showed similar serum levels of alanine aminotransferase, aspartate aminotransferase, glucose, cholesterol, alkaline phosphatase, bilirubin, total protein and albumin, which were all of them significantly abnormal as compared to those in control rats. As previously mentioned, IGF-I treatment resulted in a significant improvement in serum albumin, total protein and clotting factors II, VII and X levels, and partially prevented the increase in liver peroxidation products and development of fibrosis [10].

It has been reported that in chronic liver disease circulating levels of IGF-I are reduced [3–6]. We have previously shown that in this experimental model of CCl_4-induced liver cirrhosis serum levels of IGF-I were significantly decreased (35% of those found in control animals) [4]. In order to evaluate if the hepatic expression of *IGF-I* gene was impaired in this experimental model, liver IGF-I steady-state mRNA levels were determined by Northern blot analysis in CO and CI animals. As shown in Fig. 1A, and in agreement with previous publications [5], after hybridization with a cDNA probe for IGF-I four transcripts of approximately 7.0, 3.9, 1.8 and 1.1–0.7 kb were detected. Quantification of the 7.0 kb transcript, and normalization of the values after hybridization with a 18S rRNA, revealed that in cirrhotic animals IGF-I expression was reduced by 50% as compared to controls (Fig. 1B). In the cirrhotic animals treated with IGF-I (CI + IGF-I group), the expression of *IGF-I* gene was also reduced (data not shown).

IGF-I gene transcription is regulated by the interaction of hepatocyte nuclear factor-I, C/EBPβ, and HNF-3β with its promoter and exon 1 [22–24]. Hepatocyte nuclear factor-I expression has been previously demonstrated to be down-regulated in the cirrhotic liver [25], we have tested the expression of C/EBPβ and HNF-3β in the liver of CO and CI animals. As shown in Fig. 2A, hepatic C/EBPβ protein levels were markedly reduced in the CI animals, and were not recovered after IGF-I treatment (data not shown). A similar situation was observed for HNF-3β,

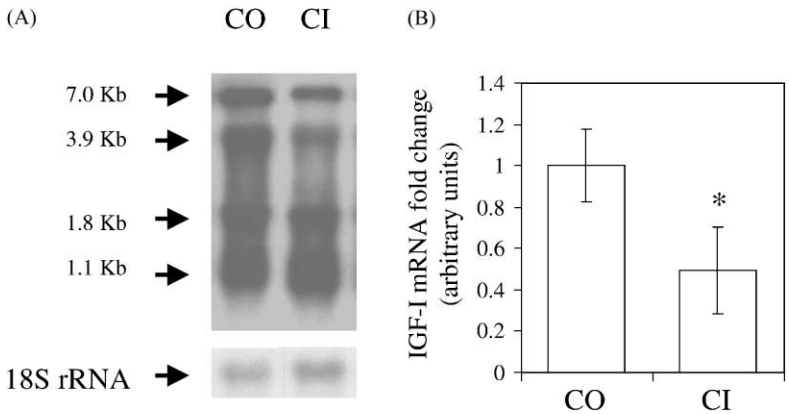

Fig. 1. Expression of IGF-I mRNA in the liver of CO (control) and CI (cirrhotic) rats. (A) Northern blot analysis of IGF-I mRNA levels in the liver of CO and CI rats. Hybridization with a cDNA probe specific for IGF-I yielded four bands of 7.0, 3.9, 1.8 and 1.1 kb. Hybridization with a probe specific for the 18S rRNA is shown to demonstrate equal loading of the gel. A representative blot is shown. (B) Quantitation of IGF-I mRNA levels in the liver of CO and CI rats. Values were normalized for loading by hybridization with a 18S rRNA probe. Values are means ± S.E.M. Asterisk (*) indicates $P < 0.05$ compared with CO value.

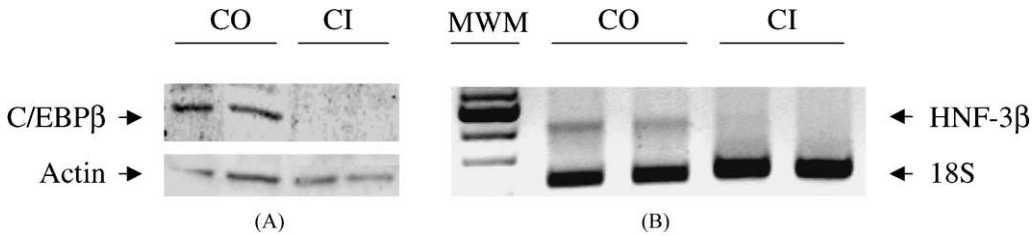

Fig. 2. Expression of C/EBPβ and HNF-3β in the liver of CO (control) and CI (cirrhotic) rats. (A) Western blot analysis of liver C/EBPβ protein levels in CO and CI animals. Equal loading of the gel was assessed by probing the membranes with an antibody specific for actin. A representative blot with two animals per group is shown. (B) HNF-3β expression in CO and CI rats was analyzed by quantitative RT-PCR. 18S ribosomal RNA was used as internal control. Representative RT-PCR reactions for two controls (CO) and two cirrhotic (CI) animals are shown. The left lane of the gel shows the molecular weight markers (MWM), the most intense band corresponds to 600 bp.

its expression was strongly reduced in the CI animals (Fig. 2B), and was not restored upon IGF-I treatment (data not shown).

In order to further characterise the biological effects of IGF-I treatment, and the hepatocyte proliferative activity in this model of cirrhosis, we assessed the expression of PCNA in the liver of CO, CI and CI+IGF-I rats. As shown in Fig. 3, PCNA levels were slightly increased in the liver of CI rats, while they were markedly enhanced in the IGF-I-treated animals. Immunohistochemical analysis of these samples indicated that PCNA expression was mainly localized in the liver parenchymal cells (data not shown).

We next performed a DD-PCR analysis to search for genes whose expression was altered in the cirrhotic liver, and to identify novel target genes through which IGF-I could mediate its hepatoprotective effects. As indicated in Section 2, after excision from the gels bands were amplified, cloned and used as probes for Northern blotting. By this procedure, we have identified 16 genes whose steady-state mRNA levels were changed in the liver of CI animals as compared to CO or CO + IGF-I rats, their identity is summarized in Table 1. The expression of 11 of these genes was down-regulated in the cirrhotic liver (clones 1.B, 2.B, 12.A4, 1.3, 2.C1, 3.7, 12.1, 2.D4, 3.2, 3.B and 6.8), four genes were induced (clones 8.A2, 9.A3, 4.A2 and 7.1) while the expression one gene (clone 4.2) was not affected (Table 1). IGF-I treatment partially recovered the expression of 5 of the 11 genes down-regulated in the cirrhotic animals (clones 1.B, 12.A4, 3.B, 3.2 and 2.D4) (Table 1), in addition the expression of three out of the four genes induced in the CI liver was also normalized in part by IGF-I administration (clones 7.1, 4.A2 and 9.A3) (Table 1). The expression of clone 4.2 was not altered in the CI liver, however, it was significantly induced in the IGF-I-treated animals (Table 1).

GHR expression has been shown to be reduced in the liver of cirrhotic patients [26]. We have measured the expression of GHR in our experimental model of cirrhosis by Northern blotting. In agreement with the situation found in humans, GHR mRNA levels were

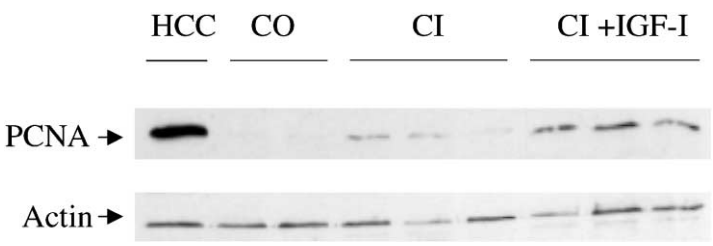

Fig. 3. Expression of PCNA in the liver of control (CO), cirrhotic (CI) and IGF-I-treated cirrhotic rats (CI + IGF-I), as determined by Western blotting. A sample of diethylnitrosamine-induced rat hepatocarcinoma tissue (HCC) was included as positive control. The image shows a representative blot with two CO, three CI and three CI + IGF-I liver samples. Equal loading of the gel was assessed by probing the membranes with an antibody specific for actin.

Table 1
Identity of the 16 clones isolated by DD-PCR screening performed in the livers of control, cirrhotic (CI) and IGF-I-treated cirrhotic rats (CI + IGF-I). Indicated is also the level of expression (expressed as %) in relation to levels found in control animals (100%)[a]

Clone	Identity/accession number	CI expression levels (% vs. CO ± S.E.M.)	CI + IGF-I expression levels (% vs. CO ± S.E.M.)
6.8	*Methionine adenosyltransferase*/**X15734**	59.3 ± 13.5%*	43.2 ± 24.6%
3.7	*Betaine-homocysteine methyltransferase*/**AF038870**	43 ± 10%*	34 ± 8%
1.3	*Apolipoprotein B100*/**X04714**	48 ± 20*	30 ± 7%
2.C1	*Apolipoprotein C2*/**Z15090**	22 ± 6%*	13 ± 3.4%
3.2	*Zn-alpha-2-glycoprotein*/**D21059**	20 ± 5.6%*	56 ± 24%**
4.A2	*Carboxylesterase precursor*/**AB010570**	1040 ± 333%*	555 ± 111%**
2.B	EST/**AI101029**	0.26 ± 0.2%*	6 ± 5%
4.2	EST/**AA945168**	73 ± 20%	190 ± 70%**
8.A2	EST/**AI030354**	796 ± 217%*	666 ± 183%
7.1	*Alpha-1-acid-glycoprotein*/**J00696**	707 ± 200%*	309 ± 126%**
9.A3	*Chemokine 6Ckine*/**AF171085**	100 ± 46.9%†	10 ± 4.1%††
12.1	*Serine protease inhibitor 1*/**M15917**	21 ± 7%*	32 ± 10.5%
2.D4	*Serine protease inhibitor 2*/**X16358**	32 ± 10%*	77 ± 21.5%**
3.B	*Contrapsin-like protease inhibitor related protein*/**D00752**	20.3 ± 9.2%*	96.7 ± 26.8%**
12.A4	*Alpha-1-antichymotrypsin precursor*/**AI583183**	20 ± 8%*	125 ± 43%**
1.B	*Alpha-2u-globulin*/**M24109**	4 ± 2.3%*	43 ± 19%**

[a] In the case of clone 9.A3 levels found in CI rats were arbitrarily given the value of 100%.
* $P < 0.05$ with respect control values.
** $P < 0.05$ with respect to CI values.
† $P < 0.01$ with respect to control values.
†† $P < 0.01$ with respect to CI values.

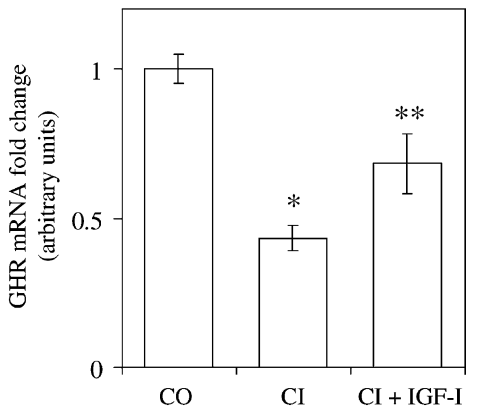

Fig. 4. GHR expression in the liver of control (CO), cirrhotic (CI) and IGF-I-treated cirrhotic rats (CI + IGF-I) rats. GHR mRNA levels were measured in the liver of CO, CI and CI+IGF-I animals by Northern blotting. Blots were hybridized with a cDNA probe for the GHR and with a probe for the 18S rRNA to assess for equal loading of the gels. One asterisk (*) indicates $P < 0.05$ with respect to CO values, two asterisks (**) indicate $P < 0.05$ with respect to CI values. Data are means ± S.E.M.

significantly decreased in the cirrhotic rat liver (Fig. 4). Interestingly, IGF-I treatment resulted in the partial recovery of GHR expression (Fig. 4).

Global DNA hypomethylation has been reported to occur in the cirrhotic human liver and in other mod-

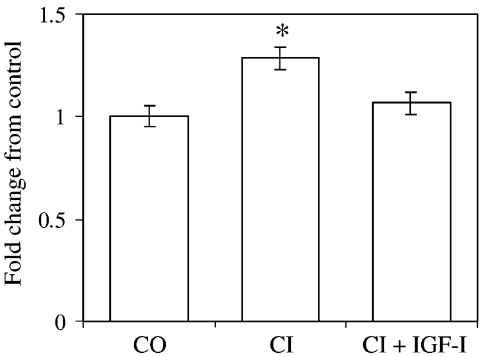

Fig. 5. IGF-I treatment restores the global levels of genomic DNA methylation which are reduced in cirrhotic rats. CO, DNA isolated from control rats; CI, DNA isolated form cirrhotic rats; CI+IGF-I, DNA isolated from IGF-I-treated cirrhotic rats. Asterisk (*) indicates $P < 0.05$ with respect to CO values.

els of CCl_4-induced cirrhosis in rats [13]. Changes in this covalent modification of DNA can participate in the alterations in gene expression observed in chronic liver damage. We have tested the level of overall methylation at CpG dinucleotides in the DNA from CI animals as compared with CO rats. As shown in Fig. 5, and in agreement with previous reports [13], we observe a statistically significant reduction in the level of overall DNA methylation in the cirrhotic livers. Interestingly, DNA methylation levels were recovered in the IGF-I-treated rats (Fig. 5).

4. Discussion

The growth hormone (GH)/IGF-I axis is markedly altered in human and experimental liver cirrhosis and in alcohol-induced liver disease [3–6,26–28]. Levels of circulating IGF-I are decreased in such condition while plasma GH is increased, however, there is a reduction in high affinity GH receptors in the liver parenchyma which leads to GH resistance in the liver [3]. These alterations are likely to play a role in the metabolic derangements associated with chronic liver disease. In this regard, we have previously shown that treatment with low doses of IGF-I is able to improve the nutritional status, intestinal absorption, osteopenia, hypogonadism and the impaired liver function in rats with CCl_4-induced cirrhosis [4,7–10]. However, the mechanism of action of IGF-I regarding the improvement of liver function is poorly understood. In the present study, we have attempted to gain further insight into the mechanisms behind the hepatoprotective action of this growth factor.

As previously mentioned, circulating IGF-I levels are reduced in cirrhosis. We have previously shown that this is also the case in our experimental model of chronic CCl_4-induced liver damage [4]. In agreement with this observation, now we demonstrate that *IGF-I* gene expression is impaired in the liver of CI animals. *IGF-I* gene transcription is under the control of multiple liver-enriched transcription factors, in particular Hepatocyte nuclear factor-I, C/EBPβ and HNF-3β play a key role in IGF-I expression in the liver [22–24]. Hepatocyte nuclear factor-I gene expression is known to be reduced in the cirrhotic liver [25]; now we observe that C/EBPβ and HNF-3β levels are also diminished in the livers of CI animals. Impaired expression of these transcription factors in the diseased liver probably compromises the synthesis of IGF-I mRNA. These observations, together with the reduced number of GH receptors in the liver, may contribute to understand the impairment in IGF-I production in cirrhosis.

Hepatocyte proliferation is an essential process in the regenerative response observed in chronic liver injury. This has been shown to occur in experimental and human cirrhosis, where a positive correlation has been found between the magnitude of this response and the prognosis of the disease [29]. The proliferative activity of hepatocytes is commonly assayed by determining the expression of PCNA, the auxiliary protein of DNA polymerase-δ [30]. In agreement with previous reports, we have observed that the expression of PCNA was very low in the liver of control animals and that it was increased in cirrhosis. Interestingly, IGF-I treatment of cirrhotic animals further increased the amount of this protein in the parenchymal cells. This observation suggests that the hepatoprotective action of IGF-I could be mediated in part by enhancing the endogenous regenerative response, aimed at the restoration of functional liver mass.

The main objective of this work was the identification of genes whose expression was altered in the cirrhotic liver, and to assess the effect of in vivo IGF-I treatment on their expression. This search was carried out using the technique of DD-PCR [12,15], using liver samples from control, cirrhotic and IGF-I-treated cirrhotic rats. We have identified 16 genes whose mRNA levels were either reduced or increased in the liver of cirrhotic rats. IGF-I treatment restored, to different degree, the expression of eight of these genes. This observation, together with the enhancement of PCNA expression, indicates that IGF-I is able to modulate gene expression in the diseased liver. “Although the contribution of non-parenchymal liver cells cannot be excluded, clone 9.A3 is an example, most of the identified genes are normally expressed, or can be induced, in the hepatocyte”.

These 16 genes belonged to different categories according to the function of the proteins they coded for. There were metabolic genes involved in the metabolism of methionine, such as methionine adenosyltransferase [31] and betaine-homocysteine methyltransferase [32], and in lipid and lipoprotein metabolism such as the apolipoproteins B100 [33]

and C2 [34], and Zn-alpha-2 glycoprotein [35]. The expression of these genes was reduced in cirrhosis, confirming our previous observations in the case of the methionine metabolizing genes [36], and in agreement with the profound alterations observed in lipoprotein metabolism in chronic liver disease [37]. Of the five metabolic genes whose expression was found to be reduced in the cirrhotic liver, IGF-I treatment partially normalized the expression of Zn-alpha-2 glycoprotein. This is a poorly characterized secretory protein with lipid-mobilizing properties, which in rats is mainly expressed in the liver although it is widely distributed in human tissues [35,38]. Other metabolic gene whose expression was strongly induced in the cirrhotic liver was the phenobarbital-inducible form of the carboxylesterases, a large family of proteins involved in drug metabolism [39]. This response was consistent with the protocol used in this work for the induction of cirrhosis, in which phenobarbital is continuously administered in drinking water. The observed partial reduction in the expression of this xenobiotic-metabolizing gene by IGF-I could be attributed in part to the more efficacious metabolism and elimination of the barbituric due to the increased hepatic metabolic potential achieved by IGF-I treatment.

We isolated three cDNA clones (clones 2.B, 4.2 and 8.A2) that had no significant homology to any published gene. There, however, existed two rat and one mouse cDNA-expressed sequence tags that were nearly identical to clones 4.2, 2.B and 8.A2, respectively. The expression of clone 2.B was completely lost in cirrhosis and IGF-I treatment was not able to recover it. Conversely clone 8.A2 expression was strongly induced and remained so in the IGF-I-treated animals. The expression of clone 4.2 was not affected in the cirrhotic liver, while IGF-I treatment induced its expression by two-fold. These observations, together with the strong induction of the inflammatory/acute-phase protein alpha-1-acid glycoprotein [40] in the cirrhotic rat, are interesting because they further illustrate the fact that overall transcriptional activity of the hepatocyte is not impaired in this model of chronic liver damage, and that IGF-I treatment exerts specific effects at the level of gene transcription in this organ.

The marked increase in alpha-1-acid glycoprotein gene expression in the cirrhotic animals is probably the combination of the stress response elicited by CCl_4, the direct effect of phenobarbital on its promoter [40] and the down-regulation of GH receptors on the damaged hepatocytes, since this hormone is a major negative regulator of alpha-1-acid glycoprotein gene transcription [40]. IGF-I administration partially counteracted the induction of this gene, suggesting that this growth factor is capable of modulating the hepatic inflammatory response elicited in this experimental model of liver damage. In line with this idea is the fact that IGF-I almost totally suppressed the strong induction in the expression of the chemokine 6Ckine observed in the cirrhotic liver. Indeed, clone 9.A3 was nearly identical to the mouse chemokine 6Ckine, a highly efficacious chemoattractant for lymphocytes [41], of which the rat homologue has not been cloned yet. This chemokine, highly expressed in high endothelial venules of lymph nodes and Peyer's patches, is expressed to a very low extent in the endothelium of small lymphatic vessels in the liver [41]. Now, we describe its strong induction in chronic liver damage and its modulation by IGF-I.

Another group of genes whose expression was impaired in cirrhosis belonged to the large family of secretory protease inhibitors. Among them, we have identified: (i) the serine protease inhibitors 1 and 2, two genes positively regulated by GH and whose transcription is inhibited during inflammation [42,43]; (ii) contrapsin-like protease inhibitor, a trypsin inhibitor which is also negatively regulated by inflammation [44] and (iii) alpha-1-antichymotrypsin, an acute phase protein induced during inflammation with serine protease inhibiting activity, whose expression is, however, reduced in our model of cirrhosis [45]. IGF-I treatment restored the expression of all these protease inhibitors with the exception of the serine protease inhibitor 1. Although the actual roles of the plasma protease inhibitors are not fully established, it is worth mentioning that alpha-1-antichymotrypsin has been recently described as a serum-borne anti-apoptotic factor for vascular smooth muscle cells [46]. If such activity is also exerted on hepatocytes, its positive regulation by IGF-I could contribute to the hepatoprotective action of this growth factor.

One last gene identified in our analysis coded for the highly abundant and male-specific liver protein alpha-2u-globulin [47]. This gene, whose expression was dramatically reduced in the cirrhotic liver, is the subject of a complex hormonal regulation. Among other factors its expression is positively regulated by

GH and androgen [47]. Its impaired expression in cirrhosis, thus could be explained in part by the GH resistance that develops in this condition, and by the fact that hypogonadism with low testosterone levels is a frequent complication of cirrhosis, that also occurs in our experimental model [4]. Since we have previously shown that IGF-I treatment reverts testicular atrophy and improves free and total testosterone levels in this same model of experimental cirrhosis [4], this effect could be behind the partial restoration of alpha-2u-globulin expression by IGF-I.

Although the beneficial effects of IGF-I treatment in liver cirrhosis cannot be directly ascribed to the improvement in the expression of one of the identified genes in particular, the fact that the expression of several members of the protease inhibitors family was partially restored could be of significance regarding the therapeutic action of this growth factor.

As previously mentioned, in patients with liver cirrhosis the GH/IGF-I axis is altered. The circulating levels of GH are markedly increased; however, the expression of the GHR in the liver is impaired, making this organ less sensitive to the hormone [26]. In our experimental model, we also observe a reduction in GHR mRNA levels in the liver of cirrhotic rats. Interestingly, IGF-I treatment partially restored the expression of GHR. Improved responses of the liver parenchyma to GH could be behind the effects of IGF-I treatment. This is supported by the fact that some genes identified in our DD-PCR study are regulated by GH. Such are the cases of alpha-2u-globulin and the serine protease inhibitor 2, which are down-regulated in cirrhosis and their expression is partially recovered in the IGF-I-treated animals. The opposite situation is also observed in the case of alpha-1-acid glycoprotein, a gene negatively regulated by GH [40], that is induced in cirrhosis and partially repressed in response to IGF-I. Nevertheless, the partial restoration of GHR levels is not sufficient to recover the expression of other GH-responding genes, such as the serine protease inhibitor 1 or IGF-I. As for IGF-I, this may be explained by the marked impairment in the expression of C/EBPβ and HNF-3β transcription factors, which are essential for *IGF-I* gene expression [23,24], and are not recovered in the CI + IGF-I animals.

These observations bring up the issue of the molecular signals and pathways through which IGF-I exerts its effects on the diseased liver. Its is known that normal hepatocytes express few IGF-I receptors, and that growth of normal liver is not affected by circulating IGF-I [11,48,49]. In agreement with this, we have not detected the expression of IGF-I receptors in total liver homogenates of CO, CI nor healthy control rats treated with IGF-I, when analysed by Western blotting (data not shown). However, regenerating rat hepatocytes have been described to possess such receptors [48], thus it is possible that IGF-I could act on the parenchymal cell fraction that proliferates in the injured liver. In addition, IGF-I has been shown to stimulate the production of hepatocyte growth factor, a potent mitogen and hepatoprotective agent, by cultured hepatic stellate cells [50]. Taken together our observations suggest that the combined actions of direct IGF-I effects on the hepatocyte, together with endocrine (GH, androgen) and paracrine (hepatocyte growth factor) factors are likely to be behind the beneficial effects of IGF-I on liver function.

In the present work, we have provided evidence showing that IGF-I treatment of cirrhotic rats can modulate the expression of certain genes, partially restoring the normal gene expression profile found in healthy animals. In addition, the ability of this growth factor to revert the global hypomethylation of genomic DNA observed in cirrhosis suggests that IGF-I could, either directly or indirectly, have a broad impact on gene expression in the diseased liver, given the central role played by DNA methylation in the control of gene activity.

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