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Insulin-Like Growth Factor (IGF)-Binding Protein-1 Is Highly Induced during Acute Carbon Tetrachloride Liver Injury and Potentiates the IGF-I-Stimulated Activation of Rat Hepatic Stellate Cells

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Hepatic stellate cells (HSC) play a pivotal role in hepatic tissue repair and fibrogenesis. IGF-I has been considered a mitogenic signal for activation and proliferation of HSC *in vitro*. In the present study IGF-I and IGF-binding protein (IGFBP) gene expression was studied in a model of acute liver injury induced by a single intragastric dose of carbon tetrachloride (CCl_4) in adult rats. Northern blot analysis revealed a marked increase in IGFBP-1 mRNA levels, with a maximum between 3 and 9 h after CCl_4 application, whereas steady state mRNA levels of IGF-I were only moderately altered. *In situ* hybridization experiments demonstrated that this increase in IGFBP-1 mRNA was due to a strong expression of IGFBP-1 in the perivenous region 6–12 h after CCl_4 application, extending to the midzonal region of the acinus within 24–48 h. Consequently, a prominent immunostaining for IGFBP-1 was observed in perivenous areas, with a maximum 24–48 h after intoxication. Preincubation of early cultured HSC with a nonphosphorylated IGFBP-1 from human amniotic fluid resulted in a 3.4-fold increase in IGF-I-induced DNA synthesis. The mitogenic effect of IGF-I was also potentiated when HSC were cocultivated with IGFBP-1-overexpressing BHK-21 cells compared with nontransfected cells. These data suggest that IGFBP-1 released during the early steps of liver tissue damage and repair may interact with HSC and potentiate the sensitivity of IGF-I to mitogenic signals. (*Endocrinology* 145: 3463–3472, 2004)

IGF-I AND IGF-II, their receptors, and their binding proteins constitute a family of cellular modulators that play essential roles in the regulation of metabolism, growth, and development. The insulin-like and cell-proliferative effects of the IGFs are mediated by the IGF-I receptor and the insulin receptor (1), whereas the IGF-II/mannose 6-phosphate receptor is involved in transport of lysosomal enzymes as well as in binding and internalization of IGF-II (2). The biological actions of the IGFs are modulated by specific IGF-binding proteins (IGFBPs), which not only regulate the bioavailability of the growth factors, but can also inhibit or enhance the actions of IGFs on cells (3–6). To date, six distinct high affinity IGFBPs have been identified that differ in molecular mass, binding properties for IGFs, and posttranslational modifications, such as phosphorylation and glycosylation (6). The abundance of IGFBPs can be regulated at the level of gene expression as well as by limited proteolytic processing (7, 8).

In adult rats the liver is considered the main source of both circulating IGFs (9, 10) and distinct IGFBPs (11). Several studies in rats have demonstrated that biosynthesis of individual components of the IGF axis is located in different liver cells (12–16). Thus, IGFBP-1 and -4 are synthesized by hepatocytes, whereas IGFBP-3 is secreted from nonparenchymal cells, *e.g.* Kupffer cells, endothelial cells, and hepatic stellate cells (HSC), but not by hepatocytes. On the other hand, IGF-I is predominantly released from hepatocytes (17–20).

Several lines of evidence indicate that HSC (also termed fat-storing cells, Ito cells, and perisinusoidal or parasinusoidal lipocytes) are one of the principal effectors involved in the repair process after liver tissue damage as well as in fibrogenesis. In response to both acute and chronic liver injuries, HSC proliferate and undergo phenotypic transformation from a quiescent, vitamin-A rich phenotype (early cultivated HSC) to a myofibroblast-like phenotype (activated HSC) characterized by increased proliferation, motility, contractility, and synthesis of extracellular matrix components (21–23). Recent studies have demonstrated that due to a high level of IGF-I receptor expression, early cultivated HSC are highly susceptible to the mitogenic activity of IGF-I, whereas activated HSC become less sensitive to IGF-I through down-regulation of the IGF-I receptor (18, 24). Indeed, *in vitro* proliferation of early cultivated HSC is stimulated to a higher magnitude by IGF-I than that of activated HSC (18, 25), indicating a role for IGF-I in the initiation of HSC proliferation in early steps of liver tissue repair and fibrogenesis. The role of IGFBPs in these processes is still unclear. In the present study, acute liver injury was induced by a single intragastric dose of carbon tetrachloride (CCl_4) in adult

Abbreviations: BrdU, 5-Bromo-2′-deoxyuridine; CM, conditioned medium; h, human; HSC, hepatic stellate cell; IGFBP, IGF-binding protein; MMP, matrix metalloprotease.

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rats, resulting in necrosis of perivenous hepatocytes within 24 and 48 h, followed by a resolution of the necrotic material and an almost complete normalization of liver histology 96 h after intoxication (26). In this study we report an accumulation of IGFBP-1 in perivenous areas of the liver acinus in response to CCl_4 administration. Treatment of HSC on d 2–3 of culture with human IGFBP-1 (hIGFBP-1) purified from amniotic fluid as well as coculture with hIGFBP-1-overexpressing cells significantly stimulated the proliferative effects of IGF-I. These data indicate a role for IGF-I/IGFBP-1 as a mitogenic signal for the initiation of HSC proliferation during acute liver injury.

Materials and Methods

Materials

Sodium [^{125}I] (carrier free) (specific activity, 16.85 mCi/μg iodine) and [^{35}S]UTP (specific activity, 1000 Ci/mmol) were purchased from Amersham-Buchler (Braunschweig, Germany) and [^{32}P]deoxy-CTP (specific activity, 3000 Ci/mmol) was obtained from Hartmann Analytic (Braunschweig, Germany). Recombinant human IGF-I was a gift from Dr. Märki (Ciba-Geigy AG, Basel, Switzerland). IGF-I was iodinated by the chloramine-T method to a specific activity of about 50–70 μCi/μg. hIGFBP-1 prepared from human amniotic fluid was purchased from Merck Biosciences GmbH (Bad Soden, Germany). Chemicals were reagent grade and obtained from commercial sources as indicated: fetal calf serum, DMEM (Flow Laboratories, Bonn, Germany), BSA and antibiotics (Serva Feinbiochemica GmbH & Co., Heidelberg, Germany), pronase E (Merck & Co., Darmstadt, Germany), Nycodenz (Nyegaard, Oslo, Norway), enhanced chemiluminescence detection kit (Amersham-Buchler), ^{T7}Quick prime kit (Pharmacia Biotech, Uppsala, Sweden), nick translation kit (Life Technologies, Inc., Gaithersburg, MD), alkaline phosphatase from calf intestine, and 5-bromo-2′-deoxyuridine (BrdU) labeling kit (Roche, Mannheim, Germany), and hIGFBP-1 ELISA (DSL, Sinsheim, Germany).

Antibodies

Antibodies to rat IGFBP-1 were provided by Drs. Ling and Shimasaki (Whittier Institute for Diabetes and Endocrinology, La Jolla, CA). Rabbit polyclonal antiserum against mouse fibulin-2 was provided by Dr. Timpl (Max Planck Institut für Biochemie, Martinsried, Germany). The antiserum against hIGFBP-1 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The monoclonal antibody ED2 was supplied by Serotec (Oxford, UK). The antiserum directed against human albumin and that against smooth muscle α-actin were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Rabbit IgG against human von Willebrand factor and monoclonal antibody to desmin were obtained from Dako (Hamburg, Germany). Biotinylated mouse monoclonal antibodies against rabbit and goat IgG were obtained from Dianova (Hamburg, Germany), and horseradish peroxidase-conjugated streptavidin was purchased from Zymed Laboratories (San Francisco, CA).

cDNA probes

The following cDNA probes were used for Northern blot analysis: a 407-bp fragment of rat IGFBP-1 cDNA clone pRBP-1–501 (6), a 700-bp fragment of rat IGF-I cDNA provided by Drs. Schwander and Margot (Basel, Switzerland), a 700-bp fragment of mouse albumin cDNA (27). To quantify Northern blots, an oligonucleotide (5′-AAC GAT CAG AGT AGT GGT ATT TCA CC-3′) complementary to 28S rRNA was used.

Animals

Adult male Wistar rats were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany) and kept under standard conditions with free access to food and water. All animals received human care in compliance with institutional and NIH guidelines. To induce acute liver injury, 32 adult male Wistar rats (200–250 g body weight) were given a single intragastric dose of 150 μl CCl_4, dissolved in an equal volume of olive oil, per 100 g body weight. Control animals were treated with the solvent alone. Animals were killed at intervals of 3, 6, 9, 12, 24, 48, 72, and 96 h after intoxication. At each time point, four animals were killed. Livers from two of the four rats were processed for histology, whereas sera from the other two rats were collected, and their livers were used for RNA isolation and studies by *in situ* hybridization. No differences in food intake were noted between control and treated animals.

Processing of liver tissue and immunohistochemical analyses

The method used has been described in detail previously (28). In brief, before removal of the liver under anesthesia, the portal vein was cannulated, and the liver was perfused with Ringer's solution containing 0.5% (vol/vol) procaine and 4% (wt/vol) dextran for 2 min, followed by fixation using a cocktail of Ringer's solution, 4% (wt/vol) dextran, 0.3% (vol/vol) glutaraldehyde, and 3% (vol/vol) formaldehyde freshly prepared from paraformaldehyde. Sections of about 2–3 mm thickness were dehydrated and embedded in paraffin. Liver sections were used for immunohistochemistry essentially as described previously (28). The antiserum against rat IGFBP-1 was administered in a final dilution of 1:50 (vol/vol). Antibody detection was performed with the LSAB$^+$ kit (Dako) and the DAB$^+$ kit (Dako) according to the instructions of the manufacturer. For desmin staining, sections were deparaffinized with xylol, boiled for 20 min in 1 mM EDTA buffer (pH 8.0), and digested thereafter with protease (0.5 U/ ml; Ventana, Illkirch, France). The monoclonal antibody against desmin was used in a final concentration of 1:100 (vol/vol). Bound primary antibodies were detected using the biotin streptavidin system with amplifier (IView, Ventana). Tissue sections were counterstained with hematoxylin, dehydrated, and coverslipped with Eukit (Merck & Co.).

In situ hybridization

Prehybridization, hybridization, and washing procedures including removal of nonspecifically bound probe by ribonuclease A digestion were performed for both antisense and sense strand ^{35}S-labeled RNA probes as described previously (29, 30). Sections from all time points after CCl_4 administration were processed simultaneously using the same batches of probes and reagents. After exposing the slides for 2–6 wk, autoradiographic detection was performed as described in detail previously (29).

Tissue extraction

Protein extraction was essentially performed as described previously (31). In brief, approximately 20 mg liver tissue were placed in 1.5-ml plastic tubes and mixed with 0.4 ml 20 mmol/liter Tris-HCl buffer, pH 7.4, containing 2% Triton X-100 and 0.1 ml Laemmli buffer containing 2% SDS. The tissue homogenates prepared by an Ultraturrax (Janke und Kunkel, Staufen, Germany) were boiled for 5 min and then incubated overnight at 4 C. Homogenates were centrifuged for 5 min at 10,000 $\times$ *g*, and supernatants were stored at −70 C until further analysis.

Isolation and culture of liver cells

Rat liver HSC were isolated and purified as described previously (32). Cells were plated onto 24-well Falcon plates (BD Biosciences, Heidelberg, Germany) at a density of 2×10^5 cells/well in 1 ml culture medium. Cells were cultured in DMEM supplemented with 10% (vol/vol) fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% (vol/vol) L-glutamine. Culture medium was replaced 2 d after plating and then every other day. Cells were kept in culture at 37 C in a 5% CO_2 atmosphere and 100% humidity. The purity of the HSC was assessed on d 2 and 7 of culture by immunofluorescence microscopy for the presence of desmin, smooth muscle α-actin, von Willebrand factor, ED2, and albumin as stated previously (32). HSC cultures showed less than 1% contamination with Kupffer cells, whereas endothelial cells and hepatocytes were not detectable. A significant contamination of HSC with rat myofibroblasts (<5%) was excluded by immunostaining with antibodies against fibulin-2, which has recently been identified as a specific marker for rat myofibroblasts (33, 34). Isolation and cultivation of rat hepatocytes were performed essentially as previously described (35).

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Cell culture and transfection of BHK-21 cells

The human hepatoma (HepG2) cells were obtained from American Type Culture Collection (Manassas, VA). HepG2 cells were maintained in DMEM supplemented with 10% (vol/vol) fetal calf serum and antibiotics. The hIGFBP-1 cDNA clone p19, consisting of the entire coding region (36), was digested with *Eco*RI, and a 1.4-kb fragment was subcloned in the expression vector pBHE. Transfection of BHK-21 cells using the calcium phosphate technique was performed essentially as previously described (37). Selection was performed with 0.5 mg/ml G418. Stable colonies were isolated, and screening for IGFBP-1 was performed by ligand and immunoblotting of conditioned medium (CM) as described below. For coculture experiments, HSC were grown on 96-well plates (Nunc, Wiesbaden, Germany) separated from transfected and nontransfected BHK cells by a 0.2-μm pore size Anopore membrane (Nunc). IGFBP-1 was quantified by an ELISA according to the manufacturer's instructions.

[^{125}I]IGF-I ligand blot and immunoblot of CM

CM were analyzed for the presence of IGFBPs by [^{125}I]IGF-I ligand blot according to the method described by Hossenlopp *et al.* (38) with slight modifications (35, 39). Identification of IGFBPs by immunoblotting was performed essentially as previously described (35).

Dephosphorylation of IGFBP-1

CM from HepG2 cells, IGFBP-1-overexpressing BHK cells, and rat hepatocytes were dialyzed overnight against 50 mM Tris-HCl and 0.1 mM EDTA (pH 8.5). CM equivalent to 0.3 ml or 200 ng hIGFBP-1 from human amniotic fluid were digested with 40 U alkaline phosphatase at 37 C for 90 min, followed by nondenaturing PAGE in the absence of sodium dodecyl sulfate (40, 41).

Isolation of total RNA and Northern blot analysis

Total RNA was isolated from rat liver as described recently (18, 36). For IGFBP-1, total RNA was evaluated using digoxigenin-labeled antisense RNAs generated by *in vitro* transcription with RNA polymerase (Roche) and the T3 promoter of pBluescript SK$^+$ plasmid as described previously (35). Fragments of the rat IGF-I plasmid (700 bp) were [^{32}P]deoxy-CTP labeled by means of random priming, and mouse albumin cDNA was labeled by nick translation. Prehybridization, hybridization, and washing of membranes were essentially performed as previously described (18, 42).

Determination of DNA synthesis

DNA synthesis of HSC was assessed by incorporation of BrdU as previously described (18). In brief, HSC on d 2–3 of culture were labeled for 24 h with 10 μmol/liter BrdU in the presence or absence of IGF-I and/or hIGFBP-1 at the indicated concentrations. Incorporated BrdU was measured with an ELISA kit (Roche) according to the instructions of the supplier. IGF-I-stimulated HSC was expressed as a percentage of the control value, which was set 100%.

Statistical analysis

All experiments were replicated, and representative autoradiograms of at least three independent experiments are shown. Autoradiographs of ligand and Northern blots were scanned using a laser densitometer (Epson GT-8000, Biometra, Göttingen, Germany). The relative densities of the bands were expressed as a percentage of the control. The BrdU assays were performed in duplicate or triplicate using at least three separate HSC isolations. The mean ± SD are indicated. A paired t test was used to evaluate differences between the sample of interest and its respective control. For statistical analysis of time-course experiments, multiple measurement ANOVA, followed by Newman-Keuls posttest analysis, were performed. $P < 0.05$ was considered statistically significant.

Results

Expression of IGF-I and IGFBP-1 after CCl_4 administration

We examined the steady state mRNA levels of IGF-I and of IGFBP-1, the most abundant components of the IGF axis secreted from hepatocytes under physiological conditions, in livers of control rats and rats at various times after treatment with a single dose of CCl_4. When total RNA isolated from rat liver was probed for Northern blot analysis, the IGF-I cDNA hybridized with a prominent transcript at 7.5 kb and minor bands at 4, 2, and 1.5–1.0 kb (Fig. 1). Control animals demonstrated constant steady state mRNA levels for IGF-I throughout the time period studied. Densitometric evaluation of the 7.5-kb IGF-I mRNA species revealed that 3 h after CCl_4 intoxication, the IGF-I mRNA level was slightly, but significantly, increased compared with control values. After a transient decrease 12–48 h after CCl_4 intoxication, IGF-I mRNA transcripts were increased again and were slightly elevated 72–96 h after CCl_4 application compared with the respective control animals. A similar biphasic expression pattern was observed for albumin-specific transcripts that showed an early increase 3 and 6 h after CCl_4 intoxication, a decrease 24–48 h after intoxication, and again an increase in specific transcripts in the recovery phase (Fig. 1) (43).

Northern blot analysis for IGFBP-1 revealed transcripts of 1.5 kb that were slightly elevated at 9, 24, and 96 h of the experimental period studied in control rats. These individual variations of hepatic IGFBP-1 expression are presumably caused by nutritional effects (44). However, in all experiments the steady state IGFBP-1 mRNA levels were significantly increased 3–12 h after CCl_4 treatment, with a maximum at 6 h (Fig. 2). Densitometric evaluation of three independent experiments revealed that this increase was approximately 12-fold compared with control livers.

Levels of hepatic IGFBPs after CCl_4 administration

The abundance of IGFBPs extracted from rat livers was investigated by ligand blot analysis (Fig. 3). In liver extracts from control rats and CCl_4-treated animals, IGFBP species in the molecular range of 24, 30, and 41–45 kDa were detected, which have been identified as IGFBP-4, -1, and -3, respectively (18, 35). As the livers were perfused with Ringer's solution before removal, IGFBPs extracted from these liver tissues represent intracellular and extracellular matrix-associated/bound IGFBPs rather than secreted IGFBPs, which might explain the low levels of IGFBPs detected in liver extracts. In control rats, levels of hepatic IGFBPs, specifically IGFBP-1, remained unchanged throughout the observation period (data not shown). After CCl_4 intoxication, hepatic IGFBP-1 levels significantly increased and reached maximum values at 12 h (2.5 ± 0.2-fold increase compared with control animals), followed by a decrease between 24 and 72 h, reaching normal levels 96 h after intoxication. A similar time course was observed for IGFBP-3. For IGFBP-4, no significant change in protein levels was detectable throughout the observation period.

In situ hybridization

Figure 4 summarizes the results of the *in situ* hybridization study investigating the expression of IGFBP-1 mRNA in nor-

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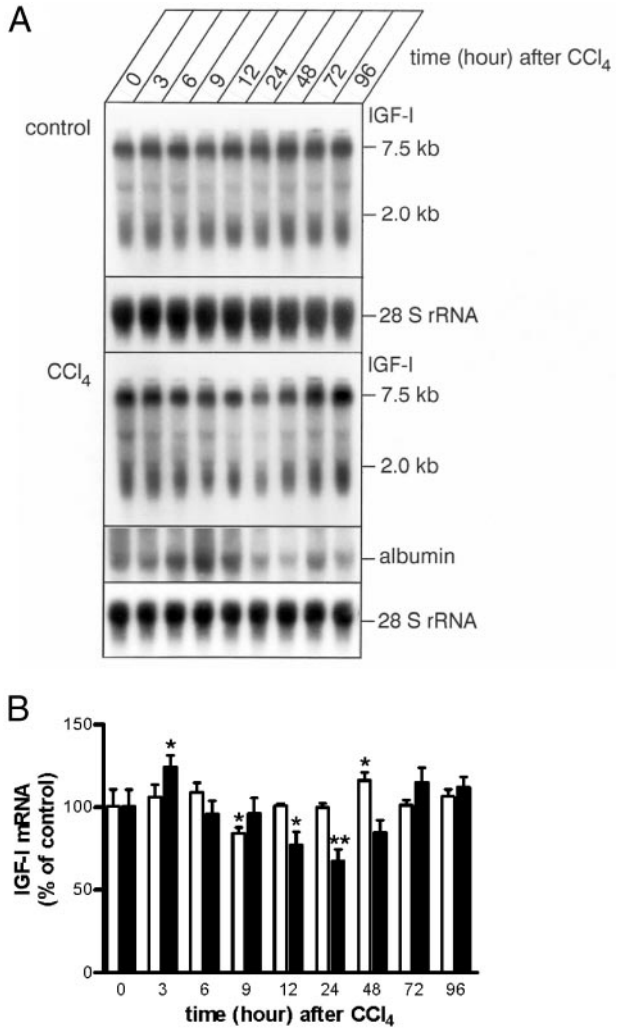

FIG. 1. Biphasic IGF-I mRNA expression in rat liver after CCl_4 treatment. A, Total RNA was isolated from control animals (*upper panel*) and from animals treated with CCl_4 (*lower panel*) at various time after CCl_4 intoxication, separated by 2% agarose gel electrophoresis, and transferred to nylon membranes. Membranes were hybridized using a labeled cDNA specific for rat IGF-I or albumin as indicated. The sizes of the hybridization bands (in kilobases) are indicated on the *right*. Equal loading of RNA (10 μg/lane) was shown after stripping and rehybridization of the membranes with an oligonucleotide complementary to 28S rRNA (*lowest panel*). B, Densitometric analysis of the 7.5-kb IGF-I mRNA species in livers from controls (□) and from animals treated with CCl_4 (■). The relative densities of bands were expressed as the percent increase or decrease in IGF-I mRNA compared with the respective mRNA level at 0 h (n = 3). Statistically significant differences are indicated ($P < 0.001$ for IGF-I after CCl_4 by ANOVA; $P = 0.002$ for IGF-I in controls by ANOVA, posttest Newman-Keuls analysis: *, $P < 0.05$; **, $P < 0.01$, for individual values compared with the respective control).

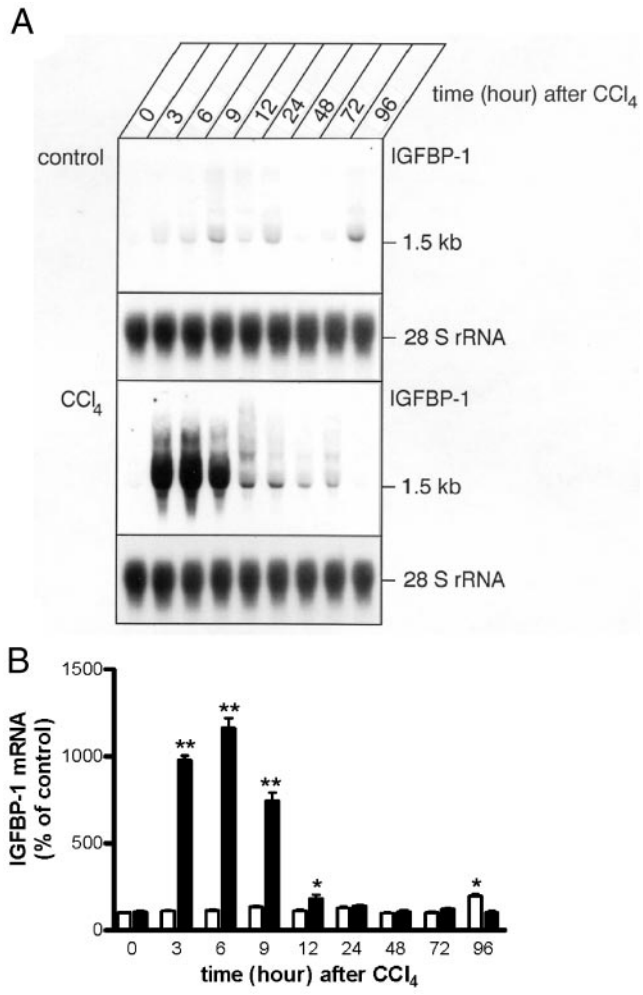

FIG. 2. Time course of IGFBP-1 mRNA expression in rat liver after CCl_4 treatment. A, Total RNA was isolated from controls (*upper panel*) and from animals treated with CCl_4 (*middle panel*) at different time points after CCl_4 treatment and analyzed by Northern blotting. Membranes were hybridized using a digoxigenin-labeled antisense RNA specific for rat IGFBP-1, yielding a specific hybridization band of 1.5 kb. Equal loading of RNA (5 μg/lane) was demonstrated by rehybridization of the membranes with an oligonucleotide complementary to 28S rRNA (*lower panel*). B, Densitometric analysis of IGFBP-1 mRNA expression in livers from controls (□) and from animals treated with CCl_4 (■). The relative densities of bands were expressed as the percent increase in IGFBP-1 mRNA compared with the respective mRNA level at 0 h (n = 3). Statistically significant differences are indicated ($P < 0.0001$ for IGFBP-1 after CCl_4 by ANOVA, post-test Newman-Keuls analysis: *, $P < 0.01$; **, $P < 0.001$, for individual values compared with the respective control).

mal and acutely damaged livers. Six to 12 h after CCl_4 administration, *in situ* hybridization for IGFBP-1 revealed a strong expression in the perivenous region (zone 3 of the liver acinus; Fig. 4, A and B). Twenty-four to 48 h after CCl_4 administration, the IGFBP-1-specific mRNA signal appeared in the midzonal (zone 2) and periportal (zone 1) regions of the acinus (Fig. 4C). Seventy-two and 96 h after intoxication, the IGFBP-1 transcript level returned to normal (data not shown). In control liver, transcripts for IGFBP-1 were homogeneously distributed over parenchymal cells (Fig. 4D). *In situ* hybridization with a sense IGFBP-1 riboprobe was negative (Fig. 4E), indicating that the hybridization signal observed with the antisense riboprobe was specific for IGFBP-1.

Immunohistochemical analysis of IGFBP-1 expression in normal and acutely damaged rat liver

Administration of a single dose of CCl_4 resulted in steatosis and necrosis of perivenous hepatocytes (zones 2 and

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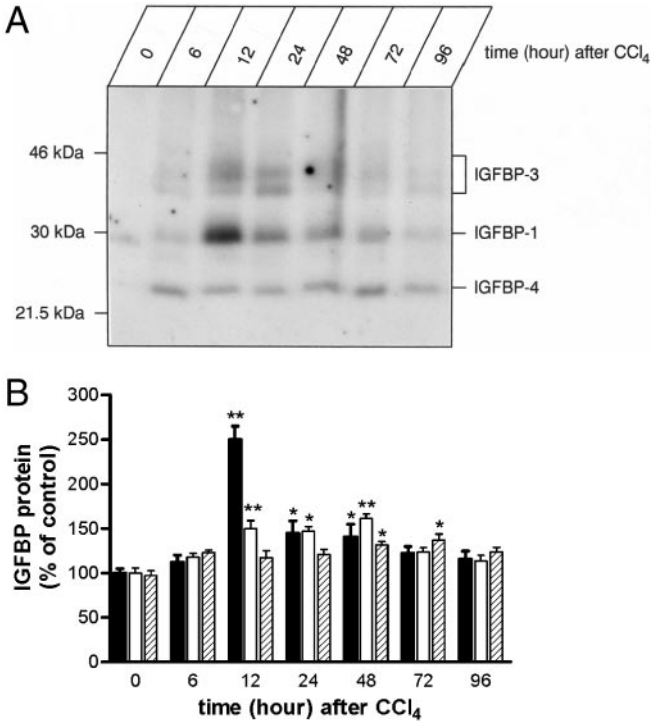

FIG. 3. Analysis of IGFBPs in rat livers after CCl_4 intoxication. A, Proteins were extracted from total liver of control and CCl_4-treated rats at the indicated times as described in *Materials and Methods*. One hundred and fifty micrograms of protein per lane were separated by SDS-PAGE under nonreducing conditions and transferred to nitrocellulose membranes, followed by ligand blotted using [^{125}I]IGF-I as a tracer. The positions of the molecular mass standards in kilodaltons are indicated. The identities of the IGFBPs were analyzed by Western blotting, indicated at the *right*. B, Densitometric analysis of IGFBP-1 (■), IGFBP-3 (□), and IGFBP-4 (▨) proteins. The relative densities of bands were expressed as the percent increase in IGFBPs compared with the respective protein level at 0 h (n = 3). Statistically significant differences are indicated ($P < 0.001$ for IGFBP-1 by ANOVA; $P < 0.001$ for IGFBP-3 by ANOVA; $P = 0.0062$ for IGFBP-4 by ANOVA, posttest Newman-Keuls analysis: *, $P < 0.01$, **, $P < 0.001$, for individual values compared with the respective control).

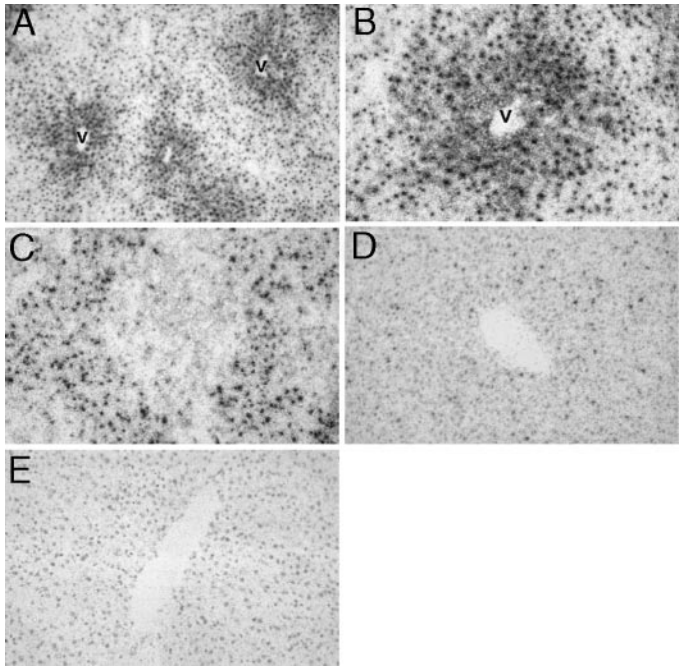

FIG. 4. *In situ* hybridization of IGFBP-1 mRNA in rat liver after CCl_4-intoxication. *In situ* hybridization with ^{35}S-labeled IGFBP-1 antisense RNA probe was carried out in rat liver sections 6 h (A), 12 h (B), and 24 h (C) after a single administration of CCl_4. Liver slices from control animals killed 12 h after treatment with the solvent alone were hybridized in parallel (D). At 6 and 12 h after CCl_4 treatment, zone 3 cells expressed high levels of IGFBP-1 mRNA transcripts. At 24 h the signal had spread to zone 2 areas and displayed reduced intensity. *In situ* hybridization with IGFBP-1 sense RNA probe is shown in E. V, Terminal hepatic venule. Exposure time, 6 wk. Original magnification, ×12 (A) and ×24 (B–E).

3 of the liver acinus) within 2 d. Twenty-four hours after CCl_4 administration, hepatocellular swelling and occasional acidophilic bodies, surrounded by a small number of inflammatory cells, were observed in the perivenous area. Forty-eight hours after CCl_4 administration, the perivenous area was characterized by necrotic hepatocytes, acidophilic bodies, and a dense cellular infiltrate containing macrophages and polymorphonuclear leukocytes. After 72 h, the necrotic material tended to disappear. Ninety-six hours after CCl_4 intoxication, liver histology returned almost completely to normal (data not shown).

Six to 12 h after CCl_4 intoxication, IGFBP-1 immunoreactivity was detected around single or groups of hepatocytes undergoing acidophilic degeneration and necrosis (Fig. 5, A and B). Twenty-four and 48 h after treatment, prominent IGFBP-1 staining was found in perivenous areas of hepatocellular necrosis (zones 2 and 3), particularly around groups of necrotic hepatocytes (Fig. 5, C and D). Concomitantly with the disappearance of necrotic cell debris between 72 and 96 h after CCl_4 application, the distribution of IGFBP-1 immunoreactivity was similar to that observed in normal liver (Fig. 5, E and F), showing a homogeneous staining over parenchymal cells (Fig. 5G). For specificity control the anti-IGFBP-1 antibody was omitted. Under these conditions no immunostaining was observed (Fig. 5H).

Immunohistochemical analysis of HSC in acutely damaged rat liver

HSC are considered the principal effector cells responsible for increased deposition of extracellular matrix proteins during fibrogenesis (21–23). To study whether the increase in IGFBP-1 in perivenous regions of the acinus after acute liver injury is associated with a subsequent activation of HSC *in vivo*, liver sections of CCl_4-treated animals were incubated with antibodies specific for desmin, an intermediate filament marker for muscle cells, myofibroblasts, and activated HSC, showing higher levels of expression in HSC that in rat myofibroblasts (45). Twenty-four hours (data not shown) and 48 h (Fig. 6A) after treatment, desmin-immunoreactive cells were detected in perivenous areas of the acinus. Seventy-two hours after CCl_4 application, the number of desmin-positive cells further increased, appearing in the perivenous and midzonal region of the acinus (Fig. 6B). Thus, during acute liver injury induced by CCl_4 the increase in desmin-immunoreactive cells, most likely HSC, followed the increase in IGFBP-1 in a similar anatomical distribution.

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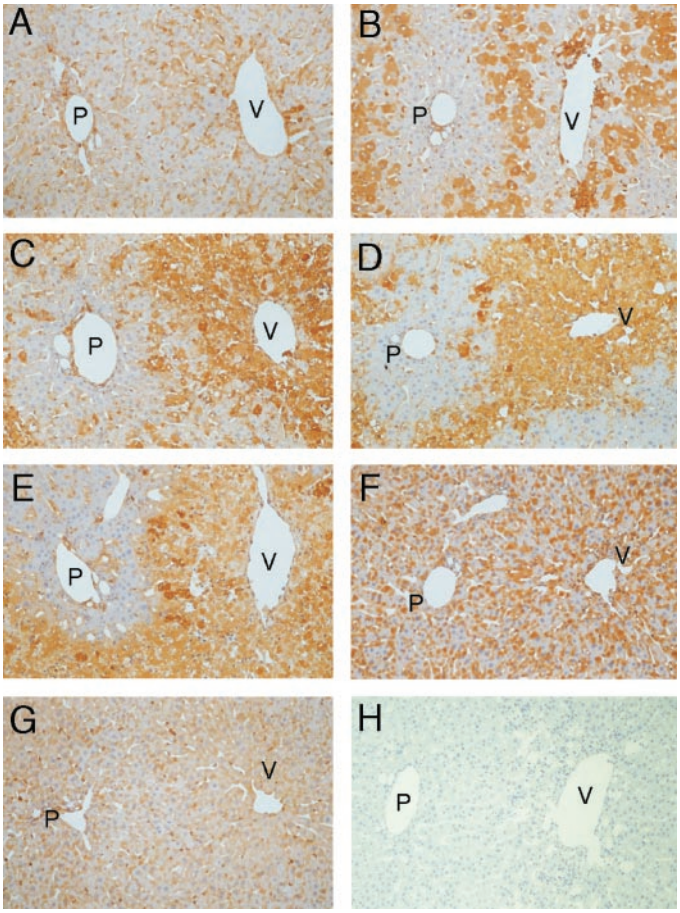

FIG. 5. Immunohistochemical localization of IGFBP-1 in rat liver after CCl_4 intoxication. Rat liver sections prepared from rats treated for 6 h (A), 12 h (B), 24 h (C), 48 h (D), 72 h (E), and 96 h (F) with a single injection of CCl_4 were processed for IGFBP-1 immunohistochemistry. G, Staining of sections from control animals treated with the solvent only (24 h). A polyclonal antiserum directed against rat IGFBP-1 (1:50) was used as the primary antibody. The immune complexes were detected by peroxidase-labeled antirabbit IgG and diaminobenzidine as substrate. H, Immunostaining was performed after omitting the first antibody. V, Terminal hepatic venule; P, portal field. Original magnification, ×30.

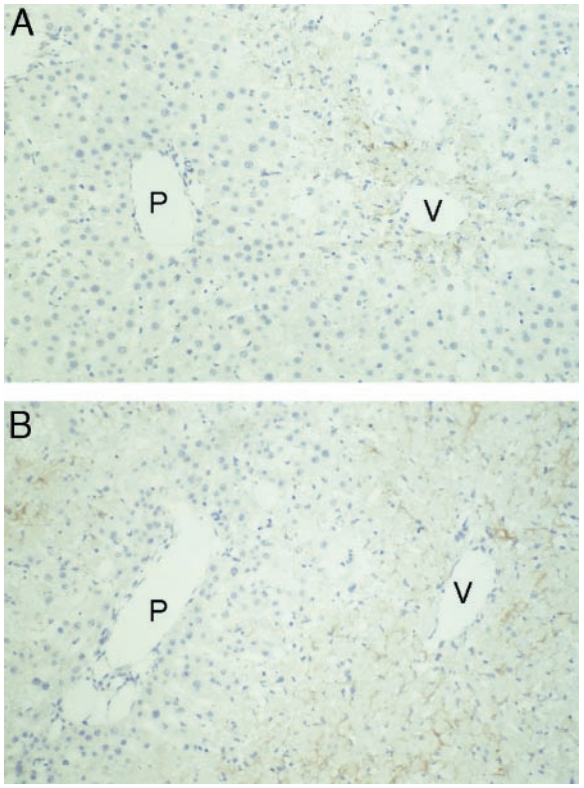

FIG. 6. Desmin expression in rat liver after CCl_4 intoxication. Rat liver sections were examined 48 h (A) and 72 h (B) after a single injection of CCl_4 stained with desmin antibodies (1:100, vol/vol). Bound primary antibodies were detected using the biotin-streptavidin system. V, Terminal hepatic venule; P, portal field. Original magnification, ×52.

Modulation of IGF-I-stimulated DNA synthesis in HSC by IGFBP-1

Because IGFBPs have been reported to either enhance or inhibit the mitogenic effects of IGF-I, the effect of IGFBP-1 on IGF-I-stimulated DNA synthesis of HSC was determined by BrdU incorporation. When HSC on d 2–3 of culture were cultivated in the presence of IGF-I for 24 h, a dose-dependent stimulation of DNA synthesis in HSC was observed, with a maximum at 100 nmol/liter. At this concentration, DNA synthesis was 3.1 ± 0.1-fold increased compared with that in untreated HSC (Fig. 7A).

When HSC on d 2–3 of culture were coincubated simultaneously with 100 nmol/liter IGF-I and increasing concentrations of hIGFBP-1 from amniotic fluid, the rate of DNA synthesis was consistently higher than that in HSC incubated with IGF-I alone (Fig. 7B). The most pronounced effect was observed at an IGFBP-1 concentration of 10 nmol/liter, resulting in a 2.1 ± 0.1-fold increase in BrdU incorporation compared with HSC stimulated with IGF-I alone (Fig. 7B). Preincubation of HSC with increasing concentrations of IGFBP-1 for 1 h before addition of 100 nmol/liter IGF-I to the IGFBP-1-containing medium resulted in additional increases in the proliferative rate compared with the effect of simultaneous addition of both IGFBP-1 and IGF-I (Fig. 7C). Thus, preincubation of HSC with 100 nmol/liter IGFBP-1, followed by addition of 100 nmol/liter IGF-I, resulted in a 3.4 ± 0.4-fold increase in DNA synthesis compared with HSC incubated with 100 nmol/liter IGF-I alone (Fig. 7C).

In a second approach, the proposed cellular interactions of HSC with IGFBP-1-secreting parenchymal cells were mimicked by cocultivation of HSC with IGFBP-1-overexpressing BHK cells. The expression of IGFBP-1 in G418 resistant BHK cells was evaluated by [^{125}I]IGF-I ligand and Western blotting. The IGFBP-1 concentrations were quantified in selected clones using an hIGFBP-1-specific ELISA. The amounts of IGFBP-1 secreted varied from 10.9 ± 1.8 to 133.5 ± 12.5 ng/ml·24 h depending on the clone (Table 1). BHK/IGFBP-1 with the highest expression (clone 9) was used for further experiments. The secretion of human IGFBP-1 into the medium is demonstrated by [^{125}I]IGF-I ligand blotting (Fig. 8A) and Western immunoblotting (Fig. 8B). Cocultivation of BHK/IGFBP-1 (clone 9) or nontransfected BHK cells separated by an Anopore membrane from HSC on d 2–3 of culture in the absence of IGF-I did not affect the DNA synthesis of HSC (data not shown). When 100 nmol/liter IGF-I were added after a 12-h preincubation of HSC

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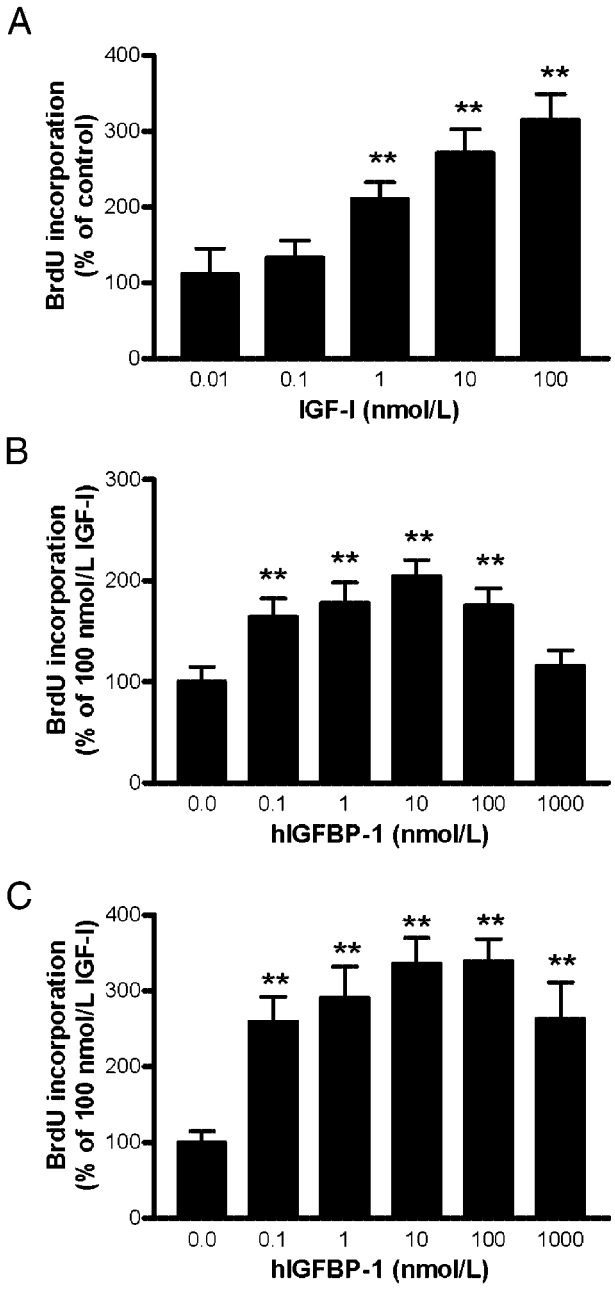

FIG. 7. Effect of hIGFBP-1 on IGF-I-stimulated DNA synthesis in HSC. A, IGF-I at concentrations ranging from of 0.1–100 nmol/liter was added to the culture medium of HSC on d 2–3 of culture, and incorporation of BrdU into DNA was determined 24 h later. IGF-I at a concentration of 100 nmol/liter was added to HSC on d 2–3 simultaneously (B) or after 1 h of preincubation of HSC with increasing concentrations of hIGFBP-1 (C). All experiments were performed in duplicate or triplicate using at least four independent HSC isolations. Results are expressed as the mean ± SD. Statistically significant differences are indicated ($P < 0.001$ in A–C, by ANOVA, post-test Newman-Keuls analysis: **, $P < 0.001$ for individual values compared with untreated HSC in A or with HSC treated with 100 nmol/liter IGF-I in B and C).

with BHK/IGFBP-1 cells, DNA synthesis of HSC was stimulated 1.8 ± 0.2-fold, whereas BrdU incorporation of HSC cocultured with nontransfected BHK cells was similar to that of HSC treated with 100 nmol/liter IGF-I alone (Fig. 8C). Taken together, preincubation of HSC with IGFBP-1 appears to have a priming effect, resulting in an increased susceptibility of HSC for the mitogenic effects of IGF-I.

TABLE 1. IGFBP-1 secretion in IGFBP-1-overexpressing BHK cells

BHK/IGFBP-1	hIGFBP-1 secretion (ng/ml·24 h)
Clone 3	21.0 ± 2.5
Clone 4	46.6 ± 9.2
Clone 9	133.5 ± 12.5
Clone 11	10.9 ± 1.8

Different clones of BHK/IGFBP-1 (1×10^6 cells grown on 3.5-cm plates) were kept under serum-free conditions for 24 h. Supernatants were collected and processed for determination of hIGFBP-1 concentration as described in *Materials and Methods*. The results of three different passages carried out in triplicate are shown as the mean ± SD.

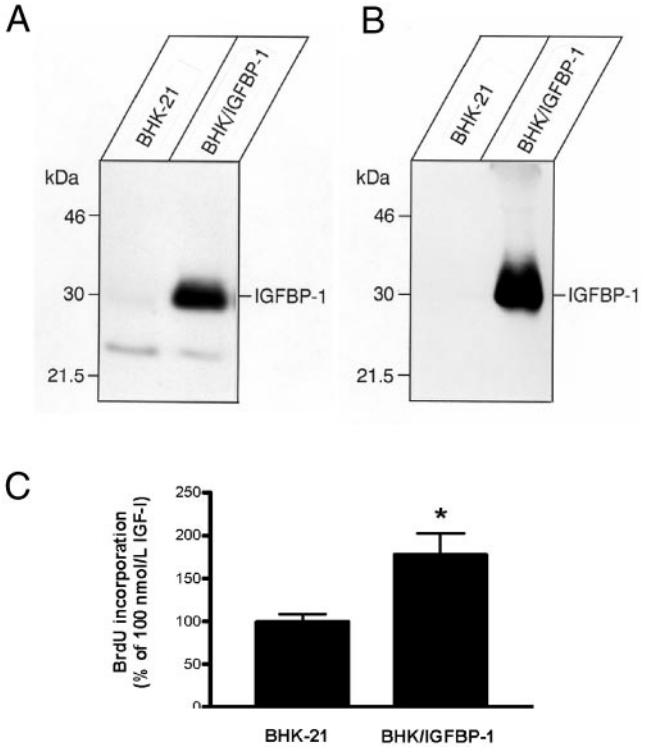

FIG. 8. Effect of cocultured BHK cells overexpressing IGFBP-1 on IGF-I-stimulated DNA synthesis in HSC on d 2–3 of culture. IGFBP-1 secreted by overexpressing BHK/IGFBP-1-cells (clone 9) is demonstrated by [^{125}I]IGF-I ligand blotting (A) and Western blotting (B) in comparison with CM of nontransfected BHK-21 cells. For determination of DNA synthesis, nontransfected BHK-21 or BHK/IGFBP-1 cells were grown on Anopore membranes separated from HSC (C). IGF-I (100 nmol/liter) was added after a 12-h preincubation of HSC with BHK cells. Incorporation of BrdU into DNA was determined 24 h later. All experiments were performed in triplicate using three independent HSC isolations. Results are expressed as the percentage of incorporation relative to HSC treated with 100 nmol/liter IGF-I in the absence of BHK cells. The mean ± SD are shown. Statistically significant differences are indicated (*, $P = 0.03$, by Student's t test).

hIGFBP-1 overexpressed in BHK cells is not phosphorylated

Because phosphorylation of IGFBP-1 has been demonstrated to influence the effects of IGFBP-1 on IGF availability (46), phosphoisoforms of the IGFBP-1 used in this study were analyzed by nondenaturing PAGE and Western immuno-

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blotting (Fig. 9). hIGFBP-1 prepared from amniotic fluid contained a nonphosphorylated isoform that was resistant to treatment with alkaline phosphatase. For comparison, IGFBP-1 secreted from the human hepatoma cell line HepG2 cells consisted of five phosphoisoforms of IGFBP-1 (Fig. 9) (40, 41). After incubation with alkaline phosphatase, two bands were detectable. IGFBP-1 secreted from rat hepatocytes in primary culture contained lesser phosphorylated isoforms that were reduced to a single band after digestion with alkaline phosphatase. Analysis of CM from BHK/IGFBP-1 demonstrated a single protein band resistant to alkaline phosphatase digestion with a more basic pI than nonphosphorylated IGFBP-1 from amniotic fluid or HepG2 medium (Fig. 9). Thus, BHK/IGFBP-1 cells secrete a nonphosphorylated form of hIGFBP-1, confirming previous findings that hIGFBP-1 transfected into mouse hepatoma (Hepa 1–6) cells is not phosphorylated (47).

Discussion

In the present study the roles of IGF-I and IGFBP-1, the most abundant components of the IGF axis secreted by hepatocytes under physiological conditions, were studied during CCl_4-induced acute liver injury. In this study we report a marked increase in IGFBP-1 mRNA levels very early after CCl_4 application, with a maximum between 3 and 9 h. The steady state mRNA levels of IGF-I were only moderately altered. *In situ* hybridization experiments demonstrated that the increased IGFBP-1 expression was due to an enhanced IGFBP-1 mRNA expression in perivenous areas of the lobule at early stages after CCl_4 intoxication. Consequently, increased IGFBP-1 immunoreactivity was detected in perivenous areas of hepatocellular damage and necrosis, reaching its maximum 24–48 h after CCl_4 treatment. At later stages, increased IGFBP-1 expression spread to midzonal areas of the liver acinus. A perivenous expression of IGFBP-1 mRNA has been reported in livers of female Sprague Dawley rats fed normal and high protein diets (48). The discrepancy with data of the present study demonstrating a homogeneous distribution of IGFBP-1 mRNA expression in the liver acinus of nontreated rats may be explained by differences in the rat strains used, the gender of the animals, as well as the composition of the normal diet used in both laboratories.

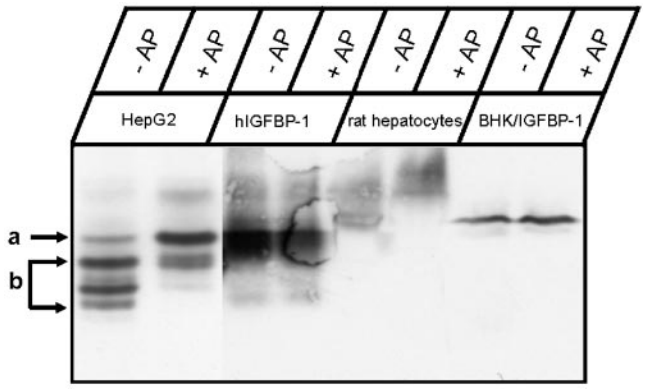

FIG. 9. Phosphorylation status of IGFBP-1. CM of HepG2 cells, rat hepatocytes, and BHK/IGFBP-1 clone 9 each equivalent to 0.3 ml or 200 ng IGFBP-1 from amniotic fluid (hIGFBP-1) were incubated in the presence (+AP) and the absence (−AP) of calf intestinal alkaline phosphatase (AP) for 90 min at 37 C. The samples were analyzed by nondenaturing PAGE and IGFBP-1 immunoblotting. The positions of nonphosphorylated (a) and phosphorylated (b) isoforms of IGFBP-1 are indicated.

The temporal cellular IGFBP-1 expression pattern after CCl_4 application is followed by an increase in desmin-immunoreactive cells, most likely HSC rather than rat liver myofibroblasts (45), in a similar anatomical distribution. To examine the physiological significance of these observations and the underlying mechanisms, the ability of IGFBP-1 to modulate IGF-I-stimulated DNA synthesis was studied in cultured HSC. These experiments were focused on HSC on d 2–3 of culture because these early cultured HSC have been reported to be highly sensitive to the mitogenic effects of IGF-I, whereas HSC become refractory to IGF-I during the process of activation (18, 24). The simultaneous addition of hIGFBP-1 and IGF-I resulted in a 2-fold higher rate of DNA synthesis compared with HSC treated with IGF-I alone. The potentiating effect was even more pronounced when HSC were preincubated with hIGFBP-1 before the addition of IGF-I. Similarly, IGF-I-stimulated DNA synthesis in HSC was potentiated when HSC were cocultivated with hIGFBP-1-overexpressing BHK cells, but not with nontransfected BHK cells.

Recent studies suggested a role of components of the IGF axis as hepatocyte-derived mitogenic signals for HSC (49, 50). This assumption is deduced from several experimental findings, such as 1) high concentrations of IGF-I in CM of hepatocytes (17–20), 2) mitogenic effects of IGF-I on human and rat HSC (18, 25, 49, 51–55), 3) the presence of high affinity IGF-I receptors on the surface of early cultured HSC (18, 24), 4) potentiation of the mitogenic effect of IGF-I by dilutions of CM from hepatocytes (49, 50), and 5) the increase in IGFBP-1 after partial hepatectomy (56–58). Because CM from primary cultures of rat hepatocytes contain IGFBP-1, -2, and -4 (18, 35), it is likely that the effect of IGF-I on HSC proliferation reported in this study is potentiated by at least one of these IGFBPs. The present data indicate that IGFBP-1 may be a potential IGFBP candidate leading to a potentiation of the mitogen-activated proliferation of HSC. However, it cannot be excluded that IGFBP-2, IGFBP-4, or a combination of these IGFBPs might also contribute to stimulate mitogenic effects of IGF-I *in vitro* and *in vivo*.

IGFBP-1 has been shown to either inhibit or potentiate the actions of IGF, which appear to be due to the existence to several IGFBP-1 phosphoisoforms (7). Highly phosphorylated IGFBP-1 species have been found in normal plasma and in CM of human endometrial stromal cells and HepG2 cells, which exhibit a higher affinity for IGF-I than the nonphosphorylated IGFBP-1 isoforms (40, 41, 46, 59). In the present study subequimolar concentrations of hIGFBP-1 from amniotic fluid have been found sufficient to enhance IGF-I-stimulated DNA synthesis in HSC. Furthermore, hIGFBP-1 secreted from overexpressing BHK cells in coculture with HSC also potentiated IGF-I-stimulated DNA synthesis, but to a lesser extent. The calculated concentrations of IGFBP-1 secreted by the BHK/IGFBP-1 clone 9 under the conditions of HSC coculture reach approximately 250 nmol/liter within 12 h of preincubation. Therefore, the differences in the extent of potentiation appear to be the result of lower local concentrations of IGFBP-1 affecting HSC due to the diffusion length and the barrier through the separating

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membranes rather than of absolute hIGFBP-1 concentrations. In addition, the BHK/IGFBP-1 cells secrete a second 24-kDa IGFBP, presumably IGFBP-4, which might display an inhibitory effect on IGF-I-stimulated DNA synthesis of HSC. Finally, molecular differences between the purified hIGFBP-1 from amniotic fluid and IGFBP-1 from overexpressing BHK cells may contribute to the different response to HSC. Nondenaturing PAGE revealed that hIGFBP-1 from amniotic fluid and hIGFBP-1 in CM from BHK/IGFBP-1 cells are nonphosphorylated, but obviously contain additional modifications affecting the pI. These data suggest that the nonphosphorylated hIGFBP-1 from amniotic fluid or from overexpressing BHK cells is responsible for the potentiation of IGF-I-stimulated DNA synthesis of HSC *in vitro*. This was confirmed by experiments showing that highly phosphorylated IGFBP-1 isoforms isolated from CM of HepG2 cells inhibit IGF-I-stimulated DNA synthesis in HSC, whereas nonphosphorylated isoforms elevate the effects of IGF-I (Scharf, J.-G., B. Kübler, and T. Braulke, unpublished results). Because IGFBP-1 secreted by primary cultures of rat hepatocytes contain weakly phosphorylated IGFBP-1 isoforms, it can be speculated that rat IGFBP-1 expressed during CCl_4-induced acute liver injury may affect HSC via similar mechanisms *in vivo*.

hIGFBP-1 can associate with the cell surface. IGFBP-1 of both species has been shown to contain an RGD sequence in the carboxyl terminus (6), facilitating the binding to $\alpha_5\beta_1$ integrin receptors on the cell surface (41). The binding of IGFBP-1 to $\alpha_5\beta_1$ integrin promotes cell migration (41) and wound healing *in vivo* (60, 61). Because no data on integrin expression in rat HSC at early stages of transformation are available, it can be speculated that binding of IGFBP-1/IGF-I complexes to $\alpha_5\beta_1$ integrins might result in continuous release and presentation of IGF-I to IGF-I receptors, thereby leading to a stimulation of IGF-I-dependent mitogenic effects by IGFBP-1. Alternatively, the IGF-I-independent binding of IGFBP-1 to a cell surface receptor, presumably the $\alpha_5\beta_1$ integrin receptor, might affect the sensitivity of HSC for IGF-I (*e.g.* by an increase in IGF-I receptor expression) or by activation of a signal transduction pathway. It has been reported that IGFBP-1 stimulates the cell migration of the human placental extravillous trophoblast by binding of its RGD domain to the $\alpha_5\beta_1$ integrin, leading to activation of focal adhesion kinase and stimulation of the MAPK pathway (62). Furthermore, cross-talks of IGF-IR signaling pathways with those of other growth factors, such as platelet-derived growth factor, TGF, fibroblast growth factor, or ILs dysregulated in response to carbon tetrachloride (63), may contribute to HSC activation during acute liver injury.

A rapid and high induction of IGFBP-1 gene expression after CCl_4 administration, as observed in the present paper, has been demonstrated after partial hepatectomy as well (56–58). Of interest, IGFBP-1-deficient mice demonstrate normal development, but have abnormal liver regeneration characterized by liver necrosis and both reduced and delayed hepatocyte DNA synthesis (64). IGFBP-1-deficient mice also displayed increased hepatic injury induced by the Fas agonist mediated by integrin receptor signaling and matrix metalloprotease-9 (MMP-9) and TGFβ activation (65). *Vice versa*, the expression of MMP-9 and TGFβ was down-regulated by IGFBP-1 treatment. It is tempting to speculate that the up-regulated expression of IGFBP-1 and the activation of HSC during acute CCl_4-induced liver injury use the same integrin/MMP-9/TGFβ signaling pathway. Taken together, these findings support the concept that IGFBP-1 plays a central role as a hepatic survival factor involved in the regulation of mitogenic pathways *in vivo*.

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