LIVER DISEASE

Insulin-like growth factor I improves intestinal barrier function in cirrhotic rats

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Background and aims: In liver cirrhosis, disruption of the intestinal barrier facilitates bacterial translocation and spontaneous bacterial peritonitis. Insulin-like growth factor I (IGF-I) is an anabolic hormone synthesised by hepatocytes that displays hepatoprotective activities and trophic effects on the intestine. The aim of this study was to investigate the effect of IGF-I on intestinal barrier function in cirrhotic rats.

Methods: In rats with carbon tetrachloride induced cirrhosis, we investigated the effect of IGF-I therapy on: (a) portal pressure; (b) intestinal histology and permeability to endotoxin and bacteria; (c) intestinal expression of cyclooxygenase 2 (COX-2) and tumour necrosis factor α (TNF- α), two factors that influence in a positive and negative manner, respectively, the integrity of the intestinal barrier; (d) intestinal permeability to ^3H -mannitol in rats with bile duct ligation (BDL); and (e) transepithelial electrical resistance (TER) of polarised monolayers of rat small intestine epithelial cells.

Results: IGF-I therapy reduced liver collagen expression and portal pressure in cirrhotic rats, induced improvement in intestinal histology, and caused a reduction in bacterial translocation and endotoxaemia. These changes were associated with diminished TNF- α expression and elevated COX-2 levels in the intestine. IGF-I reduced intestinal permeability in BDL rats and enhanced barrier function of the monolayers of epithelial intestinal cells where lipopolysaccharide (LPS) caused a decrease in TER that was reversed by IGF-I. This effect of IGF-I was associated with upregulation of COX-2 in LPS treated enterocytes.

Conclusions: IGF-I enhances intestinal barrier function and reduces endotoxaemia and bacterial translocation in cirrhotic rats. IGF-I therapy might be useful in the prevention of spontaneous bacterial peritonitis in liver cirrhosis.

In liver cirrhosis, portal hypertension, malnutrition, and poor liver function cause trophic changes in the intestine and disruption of intestinal barrier function (IBF) by mechanisms not fully understood.^{1,2} As a result, there is increased permeability of the intestinal wall to enteric bacteria leading to endotoxaemia and septic complications, such as spontaneous bacterial peritonitis (SBP).³ Bacterial translocation across the bowel wall induces tumour necrosis factor α (TNF- α) production which in turn decreases IBF and increases bacterial translocation.^{4–5} Endotoxaemia resulting from bacterial translocation may adversely affect liver function and may contribute to the systemic haemodynamic derangement of liver cirrhosis.⁶ Therefore, restoring the integrity of the intestinal barrier is an important goal in the management of liver cirrhosis.

Insulin-like growth factor I (IGF-I) is produced in different tissues following growth hormone (GH) stimulation, with the liver being the major source of the circulating hormone.^{7,8} IGF-I exerts potent anabolic and trophic effects in many tissues acting in an endocrine, paracrine, and autocrine manner.⁹ Levels of IGF-I are markedly decreased in liver cirrhosis,¹⁰ and previous data from our laboratory have shown that IGF-I therapy reduces liver fibrosis,¹¹ improves liver function,¹² increases intestinal absorption of nutrients,^{13–15} and corrects osteopenia¹⁶ and hypogonadism in experimental liver cirrhosis.¹⁷

It has been reported that IGF-I enhances the growth of the intestinal mucosa after intestinal resection or severe burns^{18,19} and stimulates proliferation of different cell lines derived from the intestinal epithelium.²⁰ As the intestine is particularly rich in IGF-I receptors,²¹ we have postulated that IGF-I

deficiency might contribute to derangement of IBF in liver cirrhosis. In the present work, we investigated whether IGF-I could improve intestinal histology and reduce bacterial translocation and endotoxaemia in cirrhotic rats. Moreover, we analysed whether a short course of IGF-I therapy could decrease intestinal permeability in rats with bile duct ligation (BDL) (another model of disrupted IBF),²² and whether IGF-I could enhance the barrier function of monolayers of isolated rat intestinal epithelial cells. As it has been demonstrated that TNF- α disrupts the integrity of the intestinal barrier and cyclooxygenase 2 (COX-2) derived prostaglandins exert a protective effect on IBF,^{23,24} we analysed the influence of IGF-I on intestinal expression of TNF- α and COX-2. Our findings indicate a potential therapeutic value of this hormone in the prevention of bacterial translocation in liver cirrhosis.²⁵

METHODS

Animal models

The study was conducted in agreement with the guidelines for animal research, according to the Guide for the Care and Use of Laboratory Animals, and was approved by the Ethical

Abbreviations: BDL, bile duct ligation; Col α 1, procollagen α 1(I); GH, growth hormone; IBF, intestinal barrier function; IGF-I, insulin-like growth factor type I; PHE, portal hypertensive enteropathy; SBP, spontaneous bacterial peritonitis; TER, transepithelial electrical resistance; COX-2, cyclooxygenase 2; TNF- α , tumour necrosis factor α ; LPS, lipopolysaccharide; CCl₄, carbon tetrachloride; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; RT-PCR, reverse transcription-polymerase chain reaction

and Research Committee of the Germans Trias i Pujol University Hospital.

Induction of cirrhosis by carbon tetrachloride administration

Male Sprague-Dawley rats weighing 100–110 g were caged individually at a constant room temperature of 21°C and a 17/7 hour light/dark cycle. Animals were fed 20–25 g/day of standard rat chow (A04; Panlab SA, Barcelona, Spain) and received 1.5 mmol/l phenobarbital (Luminal; Bayer, Leverkusen, Germany) in drinking water. Cirrhosis was induced in 30 rats by gavage of carbon tetrachloride (CCl₄) using an orogastric feeding tube (Popper and Sons; New Hyde Park, New York, USA) without general anaesthesia, as described previously,²⁶ to complete eight weeks of CCl₄ administration. After eight weeks of CCl₄ administration, animals (15 in each group) were randomised to subcutaneous treatment with recombinant human IGF-I (Chiron Corporation, Emeryville, California, USA) (2 µg/100g body weight/in two divided doses) (CI-IGF group) or vehicle (CI group) for 21 days. Ten healthy rats receiving vehicle were analysed in parallel.

On days 0 and 21 after the beginning of treatment, animals were weighted and plasma was collected and stored at –80°C until use. At the end of the treatment period, animals underwent laparotomy for measurement of portal pressure, as well as collection of blood and tissue samples, and then the animals were sacrificed. A tissue sample from the left major liver lobe and one from the ileum, previously washed with phosphate buffered saline (BioMérieux SA, Marcy l'Etoile, France), was processed (fixed in Bouins solution) for histological examination. Tissue specimens were immediately frozen by immersion in liquid nitrogen and stored at –80°C. Blood samples were used for determination of biochemical parameters and levels of IGF-I.

Bile duct ligation (BDL) model

Three groups of rats weighing 200–220 g were anaesthetised by isoflurane inhalation (n = 3, each). After a 3 cm abdominal midline incision, the common bile duct was isolated, double ligated with a 3–0 silk suture, and transected between ligatures. In sham operated rats (n = 3), the same operation was performed but neither ligatures nor bile duct section were carried out. The day after surgery, treatments were started: one group of BDL rats received subcutaneous administration of recombinant human IGF-I (Chiron Corporation) (2 µg/100 g body weight/in two divided doses) over nine days (BDL-IGF-I group) whereas sham operated and control BDL rats received two subcutaneous injections of equivalent volumes of vehicle. At day 10 after surgery, *in vivo* intestinal permeability was determined as described below.

Cell culture

Rat small intestine epithelial cells (IEC–6), which are known to possess IGF-I receptors,²⁷ were purchased from American Type Culture Collection (Rockville, Maryland, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), together with 100 mg streptomycin and 60 mg penicillin per litre of growth medium and used for the experiments as monolayers at 37°C in 5% CO₂.

Analytical methods

Liver function tests were determined by routine laboratory methods using an Autoanalyser DAX-48 (Toshiba Corporation, Nasu Works, Shimuishigami, Japan). Plasma IGF-I levels were measured by enzyme immunoassay (DSL-10-2900 Active Rat IGF-I; DSL, Webster, Texas, USA) and results are expressed in ng/ml.

Assessment of histopathological changes

Liver histopathological changes were assessed in haematoxylin-eosin stained sections, and Masson's trichrome stainings were used to verify the degree of fibrosis. In the ileum, assessment of histopathological alterations was based on evaluation of villous atrophy, submucosal oedema, and ileal lymphangiectasias. The degree of villous atrophy was scored as follows: 1, absent; 2, mild; 3, moderate; and 4, severe.

Portal pressure determination

At laparotomy, portal pressure was measured by direct intraportal insertion of a small polyethylene catheter (Abbocath-T 20G, Ø 1.1 mm, L 32 mm; Abbott, Sligo, Ireland), connected to a Lectromed High Gain Preamplifier type 5240 (Lectromed, Letchworth, UK). Results are expressed as mm Hg.

Assessment of bacterial translocation

At the end of the treatment period, animals underwent laparotomy in an unfasted state. The operation was performed under general anaesthesia (subcutaneous ketamine, atropine, and diazepam) and strict aseptic conditions. At laparotomy, potential sources of secondary peritonitis were carefully sought. Samples for culture were harvested in the following order: portal venous blood, systemic venous blood (inferior vena cava) (2 ml of each), and three mesenteric lymph nodes (particularly those draining lymph from the ileum and caecum). A section of liver and ileum were obtained for histology.

Blood samples were cultured by immediate inoculation into blood culture bottles (FAN; Organon Teknika Corp, Durham, North Carolina, USA). Mesenteric lymph nodes were washed free of blood with sterile saline solutions, and immediately homogenised in brain-heart medium. Aliquots were also plated on blood agar and MacConkey agar plates for qualitative and quantitative studies.

Plasma endotoxin levels

Endotoxin quantification was carried out with a Kinetic-colorimetric Limulus Amebocyte Lysate test (Endosafe Endochrome-K; Charles River, Lyon, France) in samples of cirrhotic rats and healthy animals. Briefly, plasma samples were collected in pyrogen-free tubes, diluted 1/10 with sterile normal saline, heated at a temperature of 75°C for five minutes in a water bath, and then diluted again five times with sterile normal saline to obtain a final dilution of 1:50 (vol:vol). Aliquots (100 µl) were transferred under aseptic conditions to a 100 well microplate. Then, the Limulus amoebocyte lysis reagent (100 µl) was added to each well. The kinetics of cytolysis were determined to permit calculation of endotoxin concentration (EU/ml).²⁸

Western blotting

COX-2 expression was determined in intestinal and IEC-6 cell protein lysates that were obtained as follows. Intestinal tissue (5 mg) of each sample were homogenised in a lysis buffer (50 mmol/l Tris-HCl, 0.1 mM EGTA, 0.1 mM EDTA, 2 µmol/l leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride, 1% (v/v) Nonidet P-40, 0.1% sodium dodecyl sulphate, and 0.1% deoxycholic acid; pH = 7.5) for one hour at 4°C. Lysate was centrifuged for 10 minutes at 3000 g and supernatant was ultracentrifuged at 100 000 g for one hour. Samples were purified with Streptococcus G protein and sepharose, and protein quantification of supernatant samples was performed using the Bradford assay.

IEC–6 cells were grown in 60 mm dishes to 50–70% confluence and then switched to DMEM medium (Gibco-BRL, Invitrogen, Barcelona, Spain) without serum. Cells were homogenised according to the following protocol: cells were

washed twice with phosphate buffered saline buffer and resuspended in 1 ml of lysis buffer (phosphate buffered saline pH 7.4, 0.1% Nonidet P40, complete protease inhibitor cocktail, and 50 mM NaF). Lysates were centrifuged (15 000 *g*, 30 minutes, 4°C) and the supernatant (crude extract) was quantified for total protein content using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, California, USA).

Protein lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 12% polyacrylamide gel (for resolutive zone) and 30% polyacrylamide (for carrier zone), and proteins were electroblotted on nitrocellulose membranes (Protran, Schleicher and Schuell, Dassel, Germany) over 45 minutes at 120 volts. Membranes were washed in Tris buffered saline and blocked with 5% milk in TTBS (Tris-HCl 20 mM, NaCl 140 mM and Tween 20 0.05%) and incubated with first antibody for one hour and then with second peroxidase conjugated antibody for one hour. Antibodies were from Santa Cruz Biotechnology (Santa Cruz, California, USA). Equal loading was demonstrated with an antiactin polyclonal antibody (Calbiochem, Bad Soden, Germany).

RNA isolation and quantitative PCR

Real time reverse transcription-polymerase chain reaction (RT-PCR) was performed to quantify expression of the mRNAs of procollagen $\alpha 1(I)$ (Col $\alpha 1$), TNF- α and β -actin (table 1). For real-time quantitative RT-PCR, reverse transcription of total liver RNA to cDNA was performed at 42°C for one hour using random primers (Boehringer Ingelheim, Ingelheim, Germany) and RTase (Gibco). During PCR amplification, cDNA was incubated with a mix containing SYBR Green I, a dye specific for double stranded DNA (LightCycler-FastStart DNA Master SYBR Green I; Roche Diagnostics, Barcelona, Spain). Amplification conditions using a thermocycler (LightCycler; Roche Molecular Biochemicals, Barcelona, Spain) were one step denaturation for 10 minutes at 95°C, followed by multiple cycles of denaturation for 15 seconds at 95°C, annealing for five seconds at 61°C, and extension for 13 seconds at 72°C. The number of cycles was 35, 40, and 35, for Col $\alpha 1$, TNF- α , and β -actin, respectively. Specification of each PCR product was established based on its melting temperatures (LightCycler software version 3.39; Roche Molecular Biochemicals), and appropriate size was verified on agarose gels. To prepare standards, PCR generated fragments were isolated from agarose gels, purified (GeneClean II; Q-Biogene, Irvine, California, USA), cloned into the pGEM T-Easy vector (Promega, Madison, Wisconsin, USA) and sequenced (ABI PRISM 310 Genetic Analyzer; Perkin Elmer, Wellesley, Massachusetts, USA). Standard curves were generated from serial dilutions of control linearised plasmids to obtain relative quantitation. Quantification was represented as expression units (that is, $2^{CT*10000}$, where CT is the difference in number of cycles between β -actin expression and that of the measured gene).

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

Col $\alpha 1(I)$	Sense	5'-GCA AAG AGT AGT CTA CAT GTC TAG-3'
	Antisense	5'-CCT ACA TCT TCT GAG TTT GG-3'
TNF- α	Sense	5'-GAG TGA CAA GCC TGT AGC CC-3'
	Antisense	5'-CCC TTC TCC AGC TGG AAG AC-3'
β -actin	Sense	5'-GTG ACG AGG CCC AGA GCA AGA G-3'
	Antisense	5'-AGG GGC CGG ACT CAT CGT-3'

Measurement of intestinal permeability in vivo

Intestinal permeability was evaluated in a BDL model by measuring intestinal absorption of ^3H -mannitol (Perkin Elmer, Boston, USA). Briefly, 10 days after BDL or sham operation, an abdominal incision was performed to expose a jejunal loop of approximately 15 cm long. Both ends of the loop were cannulated to recirculate (2 ml/min) a modified Krebs-Henseleit buffer (122 mM NaCl, 3.5 mM KCl, 25 mM NaHCO_3 , 1.2 mM KH_2PO_4 (pH 7.5)) with 1 Ci/ml of ^3H -mannitol and 1 mM of cold mannitol for 30 minutes. Samples of perfusion medium were collected every 10 minutes for counting radioactivity in a Beckman LS 6500 scintillation counter (Beckman, Fullerton, USA). At the end of the experiment the intestinal loop was extracted, weighted, and dried in a drying oven at 65°C. Intestinal permeability was estimated as the difference between total radioactivity present in the recirculation medium before and after the experiment, divided by the dry weight of the intestine.

Measurement of transepithelial electrical resistance

Transepithelial electrical resistance (TER) was used to monitor the integrity of the barrier function of confluent polarised monolayers of IEC-6. A higher TER reflects stronger tight junctions between cells and decreased monolayer permeability. Transwells (6.5 mm diameter, 0.4 μm pore size; Costar, Cambridge, Massachusetts, USA) were seeded with 10×10^6 epithelial cells per well in DMEM and 10% FBS and placed in 24 multiwell plates. Confluence of IEC-6 was usually reached within seven days. Twenty hours before stimulation, cells were equilibrated with fresh medium with an apical and basolateral volume of 2 ml in each chamber. IGF-I (100 ng/ml) and lipopolysaccharide (LPS 0.4 $\mu\text{g}/\text{ml}$) were added to basolateral and apical chambers, respectively. Each experiment was performed in triplicate. TER measurements were made using a Millicell Electrical Resistance System (Millipore, Bedford, Massachusetts, USA). To calculate the TER of each cell monolayer, the mean value of transwells without cells was subtracted from the monolayer measurements and corrected for the area of the transwell (0.33 cm^2).

Statistical analysis

Results are expressed as mean (SEM). Comparisons of quantitative variables among groups were made using one way ANOVA or its corresponding non-parametric (Kruskal-Wallis) test, as required. Post hoc comparisons were performed with the Duncan or Mann-Whitney non-parametric tests. The χ^2 test was used for comparisons of percentages. Intergroup variations of quantitative and qualitative variables were assessed by the paired Student *t* test and McNemar's test, respectively. A *p* value <0.05 was considered to be significant.

RESULTS

IGF-I, liver damage, and portal pressure

At baseline, plasma IGF-I concentration was similar in the three groups of animals (1283.5 (89.1); 1387.2 (97.7), and 1188.5 (179.6) ng/ml in CI-IGF, CI, and normal rats, respectively; NS). At the end of the experimental period, administration of human IGF-I caused, as expected, a decrease in levels of endogenous IGF-I in treated rats resulting from feedback inhibition of GH synthesis by exogenously administered hormone (571.09 (47.7) v 1285.23 (130.6) ng/ml in CI-IGF and CI, respectively; *p*<0.05).

In agreement with our previous observations,²⁹ CI-IGF animals showed a rate of weight gain significantly higher than that observed in the CI group (22.9 (1.5)% and 14.85

(1.1)%, respectively; $p < 0.01$). Also, levels of Col α 1 mRNA were significantly decreased in CI-IGF rats compared with CI animals (3814 (523); 7420 (1697), and 2089 (637) expression units in CI-IGF, CI, and normal rats, respectively; $p < 0.05$). Reduced hepatic fibrogenesis in CI-IGF rats was consistent with our previous data^{12,29} and was associated with lower inflammatory activity in the liver, as reflected by decreased TNF- α mRNA levels in this group compared with CI animals (92.4 (10.5), 158.6 (28.8), and 93.0 (15.6) expression units in CI-IGF, CI, and normal rats, respectively; $p < 0.05$). The value of portal pressure was 5.2 (0.4) mm Hg in normal rats and reached values of 11.6 (0.6) mm Hg in untreated cirrhotic animals. In CI-IGF rats, portal pressure was significantly lower (6.4 (0.7) mm Hg; $p < 0.01$) than in CI rats.

Ileal histopathology and expression of TNF- α and COX-2 in the intestine

In cirrhotic rats, ileal structure was altered by the presence of villous atrophy, lymphangiectasias, and submucosal oedema. These changes most probably reflect the intestinal disturbance associated with liver cirrhosis and portal hypertension and not any injurious effect of CCl₄ on the intestine as the toxin was stopped three weeks before sacrificing the animals. The degree of villous atrophy was slightly but significantly lower in CI-IGF rats than in the CI group (2.13 (0.35) v 2.60 (0.50); $p < 0.01$). Submucosal oedema was not found in CI-IGF rats while it was noticeable in CI animals. Lymphangiectasias were also less prominent in the CI-IGF group than in untreated cirrhotic rats (fig 1). The improvement in ileal histopathology observed in CI-IGF rats was associated with significant changes in TNF- α and COX-2 expression in the intestinal wall. As shown in fig 2A, TNF- α mRNA was intensely upregulated in untreated cirrhotic rats compared with normal animals while CI-IGF animals showed values comparable with healthy controls. Interestingly, in contrast with TNF- α , levels of COX-2 protein were markedly reduced in the CI group compared with healthy animals. COX-2 increased to values comparable to normal in cirrhotic rats that received IGF-I therapy (fig 2B).

Bacterial translocation and plasma endotoxin levels

Bacterial translocation to mesenteric lymph nodes was not detected in healthy animals while it occurred in 10 of 15 CI animals (66.6%). In rats from the CI-IGF group, bacterial translocation was found in only four of 15 rats (26.6%). The difference between CI and CI-IGF was statistically significant ($p < 0.001$) (fig 3). The organisms isolated from mesenteric lymph nodes were all aerobes of enteric origin. *Escherichia coli* was the most frequent Gram negative bacteria that was translocated (table 2).

The lower prevalence of bacterial translocation in CI-IGF rats was associated with reduced plasma endotoxin levels in this group compared with group CI (0.949 (0.020) v 1.102 (0.083) EU/ml; $p < 0.05$). Plasma endotoxin values in CI-IGF rats were comparable with those found in healthy controls (0.934 (0.026) EU/ml) (fig 3).

Figure 2 (A) Real time quantitative reverse transcription-polymerase chain reaction analysis of tumour necrosis factor α (TNF- α) in the ileum of rats from the three experimental groups: healthy rats (HR, n=8), cirrhotic rats treated with vehicle (CI, n=10), and cirrhotic rats treated with insulin-like growth factor type I (CI-IGF-I, n=8). Bars represent mean (SEM). Statistical significant values are shown. (B) Representative image from western blot analysis of cyclooxygenase 2 (COX-2) protein expression in the ileum of rats from the three experimental groups (HR, CI, and CI+IGF).

Figure 3 Percentage of bacterial translocation and plasma endotoxin levels in healthy rats (n=8), cirrhotic rats treated with vehicle (n=15), and cirrhotic rats treated with insulin-like growth factor type I (IGF-I, n=15). Endotoxin levels are expressed as mean (SEM) whereas bacterial translocation is represented as the percentage of animals in which bacterial translocation to lymph nodes was detected. * $p < 0.05$ versus cirrhotic rats treated with IGF-I; *** $p < 0.001$ versus the other groups.

Figure 1 Ileal histology in the three experimental groups of animals. HR, healthy rats; CI, cirrhotic rats who received placebo; and CI-IGF, cirrhotic rats treated with insulin-like growth factor type I. Arrows show lymphangiectasias and submucosal oedema, which were more prominent in the CI group.

Table 2 Bacterial species isolated from mesenteric lymph nodes of cirrhotic rats with bacterial translocation

	Bacterial species	No of positive cases
CI	<i>Escherichia coli</i>	10
CI-IGF	<i>Escherichia coli</i>	2
	<i>Enterococcus</i> sp	1
	<i>Streptococcus viridans</i>	1

CI, cirrhotic rats treated with vehicle (n = 15); CI-IGF, cirrhotic rats treated with insulin-like growth factor type I (n = 15).

Effect of IGF-I on intestinal permeability in vivo

To further analyse the effect of IGF-I on IBF in vivo, we measured intestinal permeability to ^3H -mannitol in BDL rats treated with either IGF-I or vehicle for nine days. Figure 4 shows that BDL produces a significant increase in intestinal permeability to ^3H -mannitol compared with the sham operated group. In contrast, in BDL rats treated with IGF-I, intestinal permeability was significantly lower than in non-treated BDL rats, and similar to that observed in sham operated rats (fig 4).

Effect of LPS and IGF-I on transepithelial electrical resistance and COX-2 expression in monolayers of rat enterocytes

We then determined whether enhancement of IBF by IGF-I could involve a direct effect of this hormone on the intestinal epithelial cell layer. To this aim rat small intestine epithelial IEC-6 cells were cultured as confluent polarised monolayers in transwell bicameral chambers and TER was measured as a reflection of monolayer integrity. A higher TER indicates stronger tight junctions between cells and decreased monolayer permeability. As LPS has been implicated in disruption of epithelial barrier integrity in different pathological conditions,^{30–33} including liver cirrhosis,³⁴ we examined whether IGF-I might counteract the effect of LPS on barrier function of IEC-6 monolayers.

After incubation of cells with apical LPS for 24 hours, we observed that TER decreased by approximately 50% with respect to control values ($p < 0.05$) (fig 5A). In contrast,

Figure 4 Intestinal permeability to ^3H -mannitol in the intestine of sham operated rats, bile duct ligated rats (control BDL), and bile duct ligated rats treated with insulin-like growth factor type I (BDL-IGF-I). An abdominal incision was performed in animals to expose a jejunal loop of approximately 15 cm long. Both ends of the loop were cannulated to recirculate (2 ml/min) medium containing 1 $\mu\text{Ci}/\text{ml}$ of ^3H -mannitol and 1 mM of cold mannitol for 30 minutes. Values represent the difference between total radioactivity present in the recirculation medium before and at the end of the experiment, divided by the dry weight of the intestine (see methods). Bars show mean (SEM) of three individual experiments in each group. * $p < 0.05$ versus the other groups.

incubation with basolateral IGF-I for the same period of time caused an approximate 50% increase in TER ($p < 0.05$). Interestingly, cells that were simultaneously incubated with IGF-I (basolateral) and LPS (apical) showed values of TER slightly above control, indicating that IGF-I was able to completely reverse the disruption of epithelial barrier function induced by LPS.

As COX-2 displays strong protective effects on the gut epithelial barrier,^{24 35 36} we tested whether LPS and/or IGF-I might influence COX-2 protein levels in cultured IEC-6 cells. We found that incubation of IEC-6 cells for six hours with IGF-I or LPS alone induced little change in COX-2 expression while the combination of IGF-I and LPS resulted in noticeable COX-2 upregulation, suggesting that this may be a mechanism by which IGF-I may counteract the deleterious effect of LPS on epithelial barrier function (fig 5B).

DISCUSSION

We have previously shown that IGF-I displays anabolic and hepatoprotective effects in rats with experimental liver cirrhosis.^{12–15 37} In agreement with these data, in our present work we found a greater increase in weight gain and reduced TNF- α and Col α 1 expression in liver tissue in cirrhotic rats that received IGF-I treatment. Moreover, CI-IGF animals exhibited a significant decrease in portal pressure compared with control cirrhotic rats, probably because of attenuated fibrogenesis and reduced hepatic production of proinflammatory mediators that may increase intrahepatic vascular resistances.^{38 39}

This study showed that intestinal histopathological changes are improved by administration of IGF-I to cirrhotic rats. Thus partial villous atrophy, submucosal oedema and lymphangiectasias present in CI animals were partially prevented by therapy with IGF-I. In parallel with these findings, we observed that bacterial translocation and

Figure 5 (A) Transepithelial electrical resistance in control rat intestinal epithelial cells (IEC-6), and after treatment with insulin-like growth factor type I (IGF-I), lipopolysaccharide (LPS), or LPS plus IGF-I. Bars represent mean (SEM) of at least three individual experiments. * $p < 0.05$ versus control cells; † $p < 0.05$ versus LPS treated cells. (B) Western blot analysis of cyclooxygenase 2 (COX-2) protein expression by IEC-6 cells treated as mentioned in (A). Actin expression is shown as a protein loading control.

endotoxaemia were significantly reduced in animals that received IGF-I treatment. These favourable effects of IGF-I on the intestine can be explained in part by reduction of portal pressure observed in CI-IGF rats but also by direct protection of the integrity of the mucosal barrier.

Here we showed that altered IBF in liver cirrhosis is associated with upregulation of TNF- α in the intestinal wall. It seems possible that enhanced TNF- α expression in the gut of cirrhotic rats may result from increased intestinal leakiness, allowing penetration of bacteria and endotoxin into the gut wall. In this context, the reduction in intestinal TNF- α levels observed in CI-IGF rats might reflect improvement of IBF. However, TNF- α is similarly downregulated in the liver of these animals and we also found that IGF-I attenuates TNF- α production in response to endotoxin in a macrophage cell line (data not shown). These observations suggest that IGF-I may display a direct anti-inflammatory activity in the gut that contributes to the protection of IBF. In this respect it has been shown that increased production of TNF- α in the intestine is a critical event leading to loss of IBF and bacterial translocation,^{4 5 40} and that anti-TNF- α therapies may prevent bacterial translocation and SBP.⁴¹

Interestingly, we observed that intestinal expression of COX-2, a mediator of the synthesis of protective prostaglandins in the gut, was reduced in CI animals. To our knowledge, the role of defective COX-2 expression in the disruption of intestinal barrier in liver cirrhosis has not been investigated previously. Our data suggest that impaired COX-2 expression may contribute to the derangement of IBF in cirrhotic animals as IGF-I treatment leads to improvement in IBF in parallel with recovery of intestinal COX-2 expression. Enhancement of COX-2 expression in the intestine is in agreement with the reported ability of IGF-I to upregulate this enzyme and prostaglandin synthesis in colon cancer cells.⁴²

To see whether the benefit of IGF-I on IBF of cirrhotic rats was due not only to a reduction in portal pressure but also to a trophic effect on the intestine, we analysed whether our IGF-I therapeutic regimen given for a short period of time could influence intestinal permeability in a more acute model of impaired intestinal barrier, as is the case of rats with BDL.²² By measuring intestinal permeability to ³H-mannitol in BDL rats, we demonstrated that IGF-I given for a few days markedly improved IBF, suggesting a direct protective effect of the hormone on the intestinal wall. This concept is in agreement with data in the literature showing that IGF-I given for only five days to rats with severe burns increased mucosal DNA and protein content and reduced the incidence of bacterial translocation to the mesenteric lymph node from 89% in the placebo group to 30% in the treated group.¹⁹ Thus it appears that the improvement in IBF observed in cirrhotic rats treated with IGF-I may depend on both the reduction in portal pressure and direct trophic effects on the gut wall.

In order to investigate whether IGF-I might act directly on enterocytes to increase IBF, we performed in vitro studies with cells of the rat small intestine (IEC-6 cells). We found that IGF-I not only increased the barrier function of the epithelial monolayer (as assessed by TER), but was also able to revert the deleterious effect of LPS on TER. Moreover, we found that cultured IEC-6 cells increased production of COX-2 on LPS exposure when IGF-I was added to the incubation medium, indicating that this hormone was able to enhance defensive mechanisms in intestinal epithelial cells when challenged with bacterial endotoxin. These findings are relevant to the pathophysiology of liver cirrhosis as LPS has been shown to be implicated in the disruption of epithelial barrier integrity in this condition.³⁴

IGF-I has been proposed as a promising therapy for cirrhotic patients on the basis of preclinical data showing

that this hormone displays hepatoprotective and antifibrogenic activities in vivo.^{11 12} Data from a recent, double blind, randomised, clinical trial of IGF-I versus placebo in patients with alcoholic cirrhosis showed that this therapy is well tolerated and was associated with a significant increase in albumin levels during the treatment period.⁴³ However, in this trial, few patients with advanced cirrhosis were included and the effect of the therapy on bacterial translocation was not addressed.

In cirrhotic patients bacterial translocation has severe pathophysiological consequences. In addition to SBP and other septic complications leading to renal and multiorgan dysfunction, bacterial invasion may alter platelet function and may contribute to an increase in portal pressure by promoting splanchnic vasodilation in the bowel and by inducing the synthesis of endothelin and proinflammatory mediators in the liver, thus increasing the risk of variceal bleeding.^{42 44} IGF-I might therefore be an attractive therapy not only because of its hepatoprotective properties but also because of its trophic effect on the intestine that defends against bacterial translocation.

In conclusion, our data indicate that IGF-I therapy prevents the development of portal congestive enteropathy and the occurrence of bacterial translocation in experimental cirrhosis. Clinical trials are needed to determine whether this hormone might favourably influence the natural history of liver cirrhosis and reduce the risk of SBP and other septic complications in cirrhotic patients.

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