In vivo gene transfer of endothelial nitric oxide synthase decreases portal pressure in anaesthetised carbon tetrachloride cirrhotic rats

M Van de Casteele, A Omasta, S Janssens, T Roskams, V Desmet, F Nevens, J Fevery

Background: Portal hypertension in cirrhosis results from enhanced intrahepatic resistance to an augmented inflow. The former is partly due to an imbalance between intrahepatic vasoconstriction and vasodilatation. Enhanced endothelin-1 and decreased activity of hepatic constitutive endothelial nitric oxide synthase (NOS 3) was reported in carbon tetrachloride (CCl4) cirrhotic rat liver.

Aims: To study whether an increase in hepatic NOS 3 could be obtained in the CCl4 cirrhotic rat liver by in vivo cDNA transfer and to investigate a possible effect on portal pressure.

Methods: Hepatic NOS 3 immunohistochemistry and western blotting were used to measure the amount of NOS 3 protein. Recombinant adenovirus, carrying cDNA encoding human NOS 3, was injected into the portal vein of CCl4 cirrhotic rats. Cirrhotic controls received carrier buffer, naked adenovirus, or adenovirus carrying the lac Z gene.

Results: NOS 3 immunoreactivity and amount of protein (western blotting) were significantly decreased in CCl4 cirrhotic livers. Following cDNA transfer, NOS 3 expression and the amount of protein were partially restored. Portal pressure was 11.4 (1.6) mm Hg in untreated cirrhotic (n=9) and 11.8 (0.6) in lac Z transfected (n=4) cirrhotic rats but was reduced to 7.8 (1.0) mm Hg (n=9) five days after NOS 3 cDNA transfer. No changes were observed in systemic haemodynamics, in liver tests or urinary nitrates, or in NOS 3 expression in lung or kidney, indicating a highly selective transfer.

Conclusions: NOS 3 cDNA transfer to cirrhotic rat liver is feasible and the increase in hepatic NOS 3 leads to a marked decrease in portal hypertension without systemic effects. These data indicate a major haemodynamic role of intrahepatic NOS 3 in the pathogenesis of portal hypertension in CCl4 cirrhosis.

Portal hypertension is a major complication of cirrhosis. The elevated portal pressure results from enhanced sinusoidal resistance to the augmented inflow of portal venous blood. The latter is secondary to extrahepatic arteriolar vasodilatation characterised by a diminished reactivity to endogenous vasoconstrictors, including noradrenaline and angiotensin II, presumably as a result of the enhanced presence of vasodilator substances. Recent data point to an important role of nitric oxide (NO), produced in the endothelium of the aorta and mesenteric arteries by the constitutive endothelial nitric oxide synthase (NOS 3) in the pathogenesis of this peripheral vasodilatation. Enhanced hepatic vascular resistance due to fibrotic tissue seems poorly susceptible to therapeutic intervention. However, studies in the isolated perfused cirrhotic rat liver as well as in cirrhotic patients demonstrated that enhanced intrahepatic vascular resistance is, at least in part, provoked by contractile elements and as such amendable. Activated hepatic stellate cells seem especially involved in this process as they transform into myofibroblasts that can constrict the sinusoids. Endothelin-1 and NO are considered the most important vasoconstrictor and vasodilator agents, respectively, that act on stellate cells. Normal portal vein pressure seems to result from a balance between intrahepatic NO production by endothelial cells and vasoconstrictor agents such as endothelin-1. In the carbon tetrachloride (CCl4) cirrhotic rat model, preliminary results have shown that NOS 3 immunoreactivity as well as liver NOS activity was reduced. Furthermore, endothelial cells isolated from CCl4 cirrhotic livers were shown to have reduced NOS activity. The enhanced portal vein pressure in cirrhosis could thus result from insufficient production of NO to counteract the enhanced endothelin-1. If this is correct, an increase in locally produced NO could lead to a reduction in portal hypertension.

To study these pathophysiological events, we measured hepatic NOS 3 in CCl4 cirrhotic rat liver and investigated whether NOS 3 could be enhanced in the liver by in vivo cDNA transfer and if this cDNA transfer had an effect on portal pressure.

MATERIALS AND METHODS

CCL4, cirrhosis

Cirrhosis was induced by CCl4 given by gavage over 12 weeks to inbred male Wistar rats of 200–250 g (Animal House, University of Leuven, Belgium) receiving phenobarbital in their drinking water, as outlined previously. Treatment of the animals was approved by the local committee for animal experimentation of the Catholic University of Leuven.

In vivo cDNA transfection in cirrhotic rats

Cirrhotic rats were randomly assigned to receive, by intraportal injection, recombinant E1 deleted adenovirus serotype 5 carrying human NOS 3 cDNA (Ad5NOS3) (n=9). Generation by homologous recombination of this adenovirus, its purification, as well as the feasibility of in vivo cDNA transfer have been reported previously.

Other cirrhotic rats served as controls and received:

Abbreviations: Ad5, recombinant adenovirus serotype 5; β-Gal, β-galactosidase; CCI4, carbon tetrachloride; NO, nitric oxide; NOS 3, constitutive endothelial nitric oxide synthase (NOS isoform 3).
CCl4 cirrhotic rats were kept under pentobarbital anaesthesia.

Technique of intraportal injection

received empty virus Ad5RR.

added cDNA (so-called empty virus Ad5RR) (n=3).

(c) recombinant E1 deleted adenovirus serotype 5 without

the histological effect of adenoviral transfection;

allows assessment of the distribution of cDNA transfer and

immunohistochemistry.

Normal rats (n=9) served as another control group; five underwent Ad5NOS3 cDNA transfer and the other four received empty virus Ad5RR.

Haemodynamic and biochemical measurements

Haemodynamic measurements were carried out in a non-blinded way on day 0 and day 5 in normal rats, transfected with Ad5NOS3 (n=5) or empty virus (n=4), and on day 5 in placebo cirrhotic rats (n=9), in Ad5βGal transfected cirrhotic rats (n=4), and in Ad5NOS3 transfected cirrhotic rats (n=9).

Twenty four hour urine was collected on day 4 for measurement of urinary creatinine, sodium, and nitrates. The jugular vein, carotid artery, and a branch of the mesenteric vein or portal vein were cannulated with polyethylene 10 catheters under mild pentobarbital anaesthesia. All pressures were obtained by a pressure transducer (Servocorder SR 6255n; Watanabe, Japan). Later, animals were killed with an overdose of sodium pentobarbitol and the liver, kidneys, and lungs were removed for histology. Blood was taken from the abdominal aorta. Biochemical analysis was carried out by automated laboratory procedures (BM Hitachi 911; Boehringer Mannheim, Germany). Serum bile acids and urinary nitrate concentrations were measured with photometric colour tests (Merckotest Bile Acids and Spectroquant Nitrate, respectively; Merck, Darmstadt, Germany).

Immunohistochemistry

Liver, kidney, and lung specimens from all cirrhotic rats and from 10 healthy control rats of the same age were studied. Specimens were fixed in B5-fixative for light microscopic examination. Random biopsies were snap frozen in liquid nitrogen cooled isopentane, stored at −70°C, and used for immunohistochemistry.

NO synthase 3 immunohistochemistry

A three step indirect immunoperoxidase method was used on frozen sections. A mouse monoclonal antibody specific for human NOS 3 was obtained from Transduction Labs (Lexington, Kentucky, USA) and used at a dilution of 1:200. Incubation of the primary antibody was followed by peroxidase conjugated rabbit antimouse and peroxidase conjugated swine antirabbit immunoglobulins. All secondary and tertiary antisera were from Dakopatts (Copenhagen, Denmark) and diluted in phosphate buffered saline, pH 7.2, containing 10% normal human serum. All incubations were carried out for 30 minutes at room temperature and followed by a wash in three changes of phosphate buffered saline, pH 7.2, for 15 minutes. The reaction product was revealed by 3-amino-9-ethylcarbazole and hydrogen peroxide. We performed counterstaining with Mayer's haematoxylin. Negative controls consisted of replacement of the primary antibody by non-immune mouse ascites (Cappel Labs, Cochraneville, Pennsylvania, USA). NOS 3 immunoreactivity in liver endothelial cells was semiquantitatively scored as no reactivity (0), weak reactivity (1), moderately strong (2), and strong reactivity (3) (fig 1). For semiquantitative immunohistochemical scoring of NOS 3, 10 different high power fields were viewed each time and were scored blindly and independently by two pathologists and a mean taken as the final score.

Galactosidase enzyme histochemistry

For the β-D-galactosidase reaction, cryostat sections were incubated for 12 hours with an incubation medium consisting of 3 mg of 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (Sigma, St Louis, Missouri, USA), 0.3 ml N,N-dimethylformamide (Merck, Darmstadt, Germany), 7 ml of 0.1 M citric acid phosphate, pH 5, and 0.5 ml of 1.65% K-ferrocyanide, followed by a wash in distilled water and post-fixation in 4% paraformaldehyde. The slides were counterstained with Nuclear Fast Red.

Immunoblotting (western blotting)

Samples of fresh liver tissue were homogenised at a concentration of 0.25 g wet weight of liver/ml in Chaps buffer, containing 40% glycerol; 0.2 M K,PO4 ,H2O pH 7.2 and 20 mM Chaps (Sigma). Protein concentration was quantitated according to Bradford.27 Equal amounts of protein (30 μg) from each sample were separated by 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis.28 The separated proteins underwent electrophoretic transfer onto nitrocellulose Protran membranes (Schleicher and Schuell, Dassel, Germany) for 1.5 hours. The proteins on the membrane were blocked in blotto-Tween solution (5% skimmed milk; 0.05% Tween 20) at 4°C overnight. Incubation with primary antibodies (mouse monoclonal anti-NOS 3 IgG 1:1000; Transduction Labs) was performed at room temperature for two hours, followed by washing with phosphate buffered saline-Tween buffer (137 mM NaCl; 38 mM NaHPO4 , .12 H2O; 1.8 mM

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NaH₂PO₄ · 2 H₂O; 0.05% Tween 20) and incubation with a secondary peroxidase conjugated rabbit antimouse Ig 1:1000 (Dako, Glostrup, Denmark), and visualised using enhanced chemiluminescence detection (ECL, Amersham, Rainham, UK).

Statistics
All data are expressed as mean (SEM). Results from transfected and non-transfected rats were compared using an unpaired Student’s t test.

RESULTS
NOS 3 histochemistry
The mean semiquantitative scores, obtained by two independent pathologists unaware of the treatment of the rats, are given in fig 1. Livers of normal rats (fig 2) demonstrated NOS 3 immunoreactivity in arterial and venous endothelium in the portal tracts. Bile duct epithelium was negative. In liver parenchyma, NOS 3 immunoreactivity was observed in sinusoidal and hepatic vein endothelium but hepatocytes were negative. In specimens of CCl4 cirrhotic rats treated with buffer (placebo group), the intensity of the NOS 3 immunoreactivity in sinusoidal endothelial cells was markedly reduced (fig 2) compared with normal livers but not with that of the vascular endothelium in the portal tracts. Livers of cirrhotic rats transfected with Ad5βGal (fig 2) or with empty virus had a similar intensity of sinusoidal NOS 3 immunoreactivity as placebo rats. β-Gal staining confirmed intrahepatic lac Z transfection in sinusoidal lining cells and in rare hepatocytes (fig 2). Cirrhotic rats transfected with Ad5NOS3 showed enhanced NOS 3 immunoreactivity in the sinusoidal lining cells (fig 2) compared with placebo treated or lac Z transfected animals but NOS 3 expression was still below that of non-cirrhotic rats. NOS 3 staining in endothelial cells in the lungs and kidneys was not different in the various groups (data not shown).

Immunoblotting (western blots)
The amount of immunoreactive hepatic NOS 3 protein identified in western blots was always lower in CCl4 cirrhotic than in normal control rats (fig 3). Livers from seven CCl4 rats yielded values obtained by densitometry of 66.7 (6.4)% of those of seven healthy controls. The amounts of NOS 3 protein were increased following transfection with Ad5NOS3 (93.7 (6.6)%; n=5) but not significantly following injection of Ad5RR (72 (5.2)%; n=5).

Haemodynamic and biochemical data following NOS 3 cDNA transfer
On day 5, portal vein pressure was 6.4 (0.3) mm Hg in normal rats transfected with empty virus (n=4) and significantly lower in AdNOS3 transfected animals (3.9 (0.3) mm Hg; n=5) (p=0.04). In cirrhotic rats, portal vein pressure was 11.4 (1.6) mm Hg in placebo treated rats (n=9) and only 7.8 (1.0) mm Hg in Ad5NOS3 transfected rats (n=9) (p=0.04) (fig 4). Transfection with Ad5βGal did not have an effect on portal pressure as it was 11.8 (0.6) mm Hg (n=4) in this group.
Arterial and central venous pressures remained unaltered by Ad5NOS3 gene transfer and were 101 (6) and 102 (16) mm Hg and -1.5 (2.0) and -1.6 (0.7) mm Hg in the nine Ad5NOS3 transfected and nine placebo treated cirrhotic rats, respectively. The 24 hour urinary nitrate excretion was not different transfected and nine placebo treated cirrhotic rats, respectively. The 24 hour urinary nitrate excretion was not different.

**DISCUSSION**

Sinusoidal hypertension in cirrhosis results from enhanced resistance due to hepatocytic and fibrotic alterations and to active vasoconstriction counterbalanced by vasodilating substances. In normal and cirrhotic liver, both endothelin-1 and NO, major vasoconstrictor and vasodilator substances, respectively, were shown to be important determinants of portal pressure. Reduced production of NO by endothelial cells from the CCl4 cirrhotic rat liver may be an important factor causing enhanced sinusoidal vasoconstriction. Under normal conditions, as well as in end stage CCl4 cirrhosis, constitutional endothelial NOS, and not the inducible NOS isoform, is the major intrahepatic enzyme catalysing NO formation. The activity of hepatic NO has been reported to be decreased in CCl4 cirrhotic rat livers and often in human end stage cirrhosis. Decreased NO activity can be due to decreased amounts of protein and/or decreased enzyme activity. Several factors may influence the activity of hepatic NOS: the availability of the substrate L-arginine or possible recycling of L-citrulline back to L-arginine in endothelial cells, or inhibitory actions of caveolin-1 on NOS activity.

The present study showed that NOS 3 immunostaining was reduced in liver sinusoids of CCl4 cirrhotic rats to a similar extent as that observed in human viral and alcoholic cirrhosis. NOS 3 immunostaining was homogeneous throughout the liver both in normal and CCl4 cirrhotic rat livers in contrast with the situation in human cirrhotic livers. Our semiquantitative scoring system of NOS 3, counting 10 different high power fields, minimised the impact of eventual immunohistochemical heterogeneity and demonstrated a clear cut decrease in CCl4 cirrhotic livers (figs 1, 2). This scoring allowed detection of enhanced NOS 3 expression, after NOS 3 cDNA transfection into CCl4 cirrhotic rat livers with adenovirus as carrier. These results were corroborated by western blot analysis (fig 3), demonstrating a significant decrease in NOS 3 protein in CCl4 cirrhotic rats, which was increased by gene transfer. The lower NOS 3 protein values of CCl4 cirrhotic rats differ from those reported by Shah and

**Figure 3** Four separate representative western immunoblots of constitutive endothelial nitric oxide synthase (NOS 3) are given as examples, comparing normal (N) (lanes 1 and 5) with carbon tetrachloride cirrhotic non-transfected rats (C) (lanes 2, 4, and 6), animals transfected with Ad5NOS3 (NOS) (lanes 3, 7, 8, and 10), and those who received the empty virus Ad5RR (RR) (lanes 9 and 11).

**Figure 4** Portal vein pressures (with mean values), obtained on day 5 following transfection with Ad5NOS3 or Ad5RR (empty virus) in normal rats, and following placebo injection or transfection with Ad5NOS3 or Ad5Gal in cirrhotic rats. Ad5RR, empty adenovirus; Ad5Gal, adenovirus encoding lac Z reporter gene; controls, injection of placebo; Ad5NOS3, adenovirus encoding NOS 3 cDNA. *p=0.04 compared with normal rats treated with Ad5RR; †p=0.04 compared with cirrhotic placebo treated rats.

**Table 1** Biochemical data (mean (SEM)) comparing placebo treated and Ad5NOS3 transfected carbon tetrachloride cirrhotic rats on day 4 (d4) or day 5 (d5)

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=9)</th>
<th>Ad5NOS3 transfected (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake d4 (g/24 h)</td>
<td>20 (1)</td>
<td>19 (1)</td>
</tr>
<tr>
<td>Fluid intake d4 (ml/24 h)</td>
<td>31 (2)</td>
<td>34 (2)</td>
</tr>
<tr>
<td>Diuresis d4 (ml/24 h)</td>
<td>13 (1)</td>
<td>13 (2)</td>
</tr>
<tr>
<td>Natriuresis d4 (mmol/24 h)</td>
<td>1.3 (0.1)</td>
<td>1.1 (0.1)</td>
</tr>
<tr>
<td>Urinary nitrates d4 (mmol/24 h)</td>
<td>48 (6)</td>
<td>44 (16)</td>
</tr>
<tr>
<td>Creatinine clearance d4 (ml/min)</td>
<td>1.34 (0.11)</td>
<td>1.21 (0.10)</td>
</tr>
<tr>
<td>Liver weight d5 (g)</td>
<td>14.8 (0.5)</td>
<td>15.5 (0.8)</td>
</tr>
<tr>
<td>Spleen weight d5 (g)</td>
<td>1.5 (0.3)</td>
<td>1.4 (0.3)</td>
</tr>
<tr>
<td>Serum values on d5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na (NV 132-145 mmol/l)</td>
<td>137 (7)</td>
<td>152 (6)</td>
</tr>
<tr>
<td>AP (NV 90-260 U/l)</td>
<td>251 (62)</td>
<td>368 (76)</td>
</tr>
<tr>
<td>AST (NV 5-37 U/l)</td>
<td>125 (28)</td>
<td>168 (38)</td>
</tr>
<tr>
<td>ALT (NV 5-40 U/l)</td>
<td>76 (24)</td>
<td>74 (17)</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>0.43 (0.12)</td>
<td>0.51 (0.10)</td>
</tr>
<tr>
<td>Bile acids (µmol/l)</td>
<td>160 (52)</td>
<td>155 (55)</td>
</tr>
</tbody>
</table>

NV, normal values.
AP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
colleagues but are comparable with those obtained elsewhere (58 (6)% of normal values; n=40) (personal communication, Van de Casteele and Reichen). The exact reason for this difference is not clear but may be due to a different immunoblotting procedure as these investigators used an immunoprecipitation step and resuspension prior to protein electrophoresis, or possibly to differences in the severity of cirrhosis.

Importantly, the increase in hepatic NOS 3 following transfection was paralleled by a 32–38% reduction in portal vein pressure both in normal and cirrhotic rats compared with placebo treated, Ad5RR transfected, or Ad5βGal transfected rats (fig 4). Our results are in agreement with the recent studies by Yu and colleagues who observed a decrease in portal pressure and our previous studies did not detect a major effect of this inflammation. Admittedly, the haemodynamic measurements and Ad5NOS3 transfected groups, it was shown that intraportal gene transfer of neuronal NOS 1 into both bile duct ligated and CCL, cirrhotic rats, and with that of Shah and colleagues transfecting eNOS. As they injected the gene via the femoral vein, mainly hepatocytes were transfected in addition to sinusoidal lining cells. By using the portal route and by injecting a lower amount of adenosine (10³ pfu/ml in our study v 10⁴ in the studies of Yu and colleagues and Shah and colleagues) we tried to avoid transfection of hepatocytes as NOS in hepatocytes may interact with sole activation of sinusoid lining cells. In both control groups, haemodynamic data remained unaltered. Arguments in favour of organ selectivity of intraportal NOS 3 cDNA transfer to the liver were obtained from the lack of alterations in 24 hour nitrate excretion in urine (table 1), from the unchanged arterial and central venous pressures, and from the unaltered NOS 3 immunoreactivity in the kidneys and lungs of transfected rats. In both Ad5βGal and Ad5NOS3 transfected groups, it was shown that intraportal adenosine injection at the present dose did not provoke inflammation. Admittedly, the haemodynamic measurements were carried out under general anaesthesia with barbiturates but our previous studies did not detect a major effect of this type of anaesthesia on portal pressure, and the conditions were identical for transfected and non-transfected animals. We only measured portal vein pressure; a possible effect of NOS 3 cDNA transfer on effective total hepatic blood flow in CCI, cirrhotic rats has still to be investigated. The decrease of 38% induced by Ad5NOS3 transfection in our normal rats confirms that NOS 3 plays a role in maintaining normal portal vein pressure, possibly in part by counterbalancing the effect of endothelin-1 as administration of bosentan slightly decreased portal pressure.

In recent years, adenosine mediated gene transfer has been studied as a new strategy to treat hereditary infectious and malignant diseases of the liver. The in vivo susceptibility of normal rat hepatocytes for adenosine mediated gene transfer is high following intravenously administered adenosine vectors but liver tropism may be different in cirrhotic livers as fibrous septa and loss of endothelial fenestrae could hamper adenosine attachment to hepatocytes but favour transfection to sinusoidal cells.

Transfection with adenosine will presumably exert only a temporary effect as the lifespan of the transgene is transient and because vasoconstrictor agents such as endothelin-1 could arise as part of a counterregulatory mechanism. Such an increase in the production of endothelin is supported by early enhancement of preproendothelin mRNA observed in endothelial cells and more so in stellate cells following bile duct ligation. Further studies are thus necessary to obtain prolongation of the enhancement of NOS 3 protein and activity.

In isolated normal and CCI, cirrhotic rat livers, injection of NO donating drugs such as nitroprusside or glyceryl trinitrate counteract noradrenaline induced vasoconstriction and diminish portal pressure but in the in vivo situation the effect on the systemic arterial system prevails, and even intraportal injection leads primarily to arterial hypertension. This condition differs from the one whereby NO is locally produced by endothelial cells under the action of NOS 3.

In conclusion, the present study provides further evidence for an active component in the pathogenesis of the increased intrahepatic vascular tone in cirrhosis. CCI, rat livers have a decrease in NOS 3 protein, and intraportal cDNA transfer of NOS 3 using adenovirus as vector was feasible in vivo in these rats. The decrease in portal pressure observed following NOS 3 transfection indicates a major haemodynamic role for NOS 3 in CCI, cirrhosis.

ACKNOWLEDGEMENTS

The studies were supported by a grant from the Fund for Scientific Research (FWO No 6.0111.98—F Nevens) and by a fellowship from the committee for Eastern Europe (University of Leuven) given to A Omasta. M Van de Casteele is a Postdoctoral Fellow of FWO. We would like to thank J Van Pelt for advice and Glaxo—Wellcome for a grant in aid. A preliminary communication of the work was presented at the AASLD Meeting, 1996.

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Gut 2002 51: 440-445
doi: 10.1136/gut.51.3.440

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