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 adeno virus, carrying cDNA encoding human NOS 3, was injected into the portal vein of CCL4 cirrhotic rats. Cirrhotic controls received carrier buffer, naked adeno virus, or adeno virus 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.
Technique of intraportal injection
CCI cirrhotic rats were kept under pentobarbital anaesthesia (30 mg/kg); the portal vein was temporarily clamped with a non-traumatic microsurgical clamp as close as possible to the liver hilum. A fine needle (26 gauge) was inserted in the portal vein between the clamp and the liver. The portal circulation was first rinsed with 1 ml of 100 mM Tris HCl pH 7.4. Then, 400 µl of Tris HCl buffer pH 7.4 (placebo) or 400 µl of 5 x 10^9 pfu/ml Ad5RR (empty virus), Ad5βGal, or Ad5NOS3 was slowly injected into the portal vein. This was followed by injection of 100 µl of Tris HCl buffer. The clamp was kept on the portal vein for 30 minutes to prolong adenovirus-sinusoidal cell contact. The abdomen was closed and the animal allowed to recover from anaesthesia.

In preliminary experiments, the optimal dose of recombinant adenovirus carrying the NOS 3 cDNA had been evaluated using concentrations ranging from 5 x 10^6 to 5 x 10^9 pfu/ml Ad5/ml. Low adenovirus concentrations did not lead to an increase in NOS 3 expression whereas the highest concentration provoked a mild granulomatous hepatitis (data not shown). Therefore, we used 100-fold lower concentration (5 x 10^6 pfu/ml) of adenovirus for further experiments.

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 pentobarbital and the liver, kidneys, and lungs were removed for histology. Blood was taken from the jugular vein, carotid artery, and a branch of the mesenteric artery. 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 (Mercktest 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 (Cappell Labs, Cochranville, 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 K2PO4.H2O pH 7.2 and 20 mM Chaps (Sigma). Protein concentration was quantitated according to Bradford. Equal amounts of protein (30 µg) from each sample were separated by 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. 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 obtained from 10 different high power fields is given semiquantitatively as no reactivity (0), weak reactivity (1), moderately strong (2), and strong reactivity (3).
NaH₂PO₄·2H₂O; 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 placebo treated cirrhotic rats. Livers of cirrhotic rats five days after AdβGal transfection: NOS 3 immunoreactivity is similar as that of placebo treated cirrhotic rats. (D) Cirrhotic rats five days after AdNOS3 transfection: NOS 3 expression is enhanced in sinusoidal lining cells compared with that in placebo treated livers. (E) Enzymatic staining for galactosidase in a lac Z transfected cirrhotic rat (×250).

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 AdNOS3 (93.7 (6.6)%; n=5) but not significantly following injection of AdRR (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 AdNOS3 transfected rats (n=9) (p=0.04) (fig 4). Transfection with AdβGal 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 between placebo versus Ad5NOS3 transfected carbon tetrachloride cirrhotic rats on day 4 (d4) or day 5 (d5) following transfection with Ad5NOS3 or Ad5RR (empty virus) in cirrhotic rats, and following placebo injection or transfection with Ad5NOS3 or Ad5Gal in cirrhotic rats. Ad5RR, empty adenovirus; 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.

**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, constitutonal endothelial NOS 3, and not the inducible NOS isoform, is the major intrahepatic enzyme catalysing NO formation. The activity of hepatic NOS has been reported to be decreased in CCl4 cirrhotic rat livers and often in human end stage cirrhosis. Decreased NOS activity can be due to decreased amounts of protein and/or decreased enzyme activity. Several factors may influence the activity of hepatic NOS 3: 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 3 activity.

The present study showed that NOS 3 immunostaining was reduced in liver sinusoïds 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

**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/24h)</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.

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; 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.
colleagues but are comparable with those obtained elsewhere (58 (6)% of normal values; n=40) (personal communication, Van de Castelee 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 disturbance following 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 adenovirus (10^9 pfu/ml in our study v 10^10 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 adenovirus 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 CCL, 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, adenovirus 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 adenovirus mediated gene transfer is high following intravenously administered adenoviral vectors, but liver tropism may be different in cirrhotic livers as fibrous septa and loss of endothelial fenestrae could hamper adenovirus attachment to hepatocytes but favour transmission to sinusoidal cells. Transfection with adenovirus will presumably exert only a temporary effect as the lifespan of the transgene is transient but liver tropism may be different in cirrhotic livers (fig 4).

In conclusion, the present study provides further evidence for an active component in the pathogenesis of the increased intrahepatic vascular tone in cirrhosis. CCL, 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 CCL, cirrhosis.

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Both M Van de Castelee and A Omasta contributed equally to this work.

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