Nuclear factor κB inactivation in the rat liver ameliorates short term total warm ischaemia/reperfusion injury

H Suetugu, Y Limuro, T Uehara, T Nishio, N Harada, M Yoshida, E Hatano, G Son, J Fujimoto, Y Yamaoka

Background: In hepatic ischaemia/reperfusion injury, activated liver macrophages (Kupffer cells) are dominantly regulated by a transcription factor, nuclear factor κB (NFκB), with respect to expression of inflammatory cytokines, acute phase response proteins, and cell adhesion molecules.

Aims: We assessed whether inactivation of NFκB in the liver could attenuate total hepatic warm ischaemia/reperfusion injury.

Methods: We studied rats with hepatic overexpression of inhibitor κBα super-repressor (IkBα SR) caused by a transgene introduced using an adenoviral vector. Hepatic ischaemia/reperfusion injury was induced under warm conditions by total occlusion of hepatoduodenal ligament structures for 20 minutes, followed by reperfusion. Controls included uninfected and control virus (AdLacZ) infected rats.

Results: IkBα SR was overexpressed in Kupffer cells as well as in hepatocytes, blocking nuclear translocation of NFκB (p65) into the nucleus after reperfusion. Gene transfection with IkBα SR, but not with LacZ, markedly attenuated ischaemia/reperfusion injury, suppressing inducible nitric oxide synthase and nitrotirosine expression in the liver. Moreover, no remarkable hepatocyte apoptosis was detected under IkBα SR overexpression.

Conclusions: Adenoviral transfer of the IkBα SR gene in the liver ameliorates short term warm ischaemia/reperfusion injury, possibly through attenuation of hepatic macrophage activation.

Warm hepatic ischaemia/reperfusion injury often occurs during certain surgical manipulations, such as when Pringle’s manoeuvre is performed to reduce blood loss during tumour resection. Relatively brief ischaemia primes cells for damage, but cell injury may occur after the ischaemic liver is reperfused. After reperfusion, reactive oxygen species (ROS) play a critical role in inducing inflammatory responses and cellular damage. Inflammation, including ongoing neutrophil recruitment and microcirculatory disturbance, further accelerates cellular damage to result in necrotic cell death.

Kupffer cells, the resident macrophages of the liver, are activated after ischaemia/reperfusion stress, and have been recognised to produce ROS, proinflammatory cytokines, chemokines, and other mediators. Pharmacological inactivation of Kupffer cells has been reported to suppress hepatic ischaemia/reperfusion injury, suggesting pathogenetic involvement of these macrophages. A transcription factor, nuclear factor κB (NFκB), has been determined to be crucial in the cascade bringing about Kupffer cell activation. ROS produced by Kupffer cells have been proposed to activate DNA binding activity of NFκB in these cells, although such activation of NFκB by ROS remains controversial. Moreover, stimulation of Kupffer cells with endotoxin, which may participate in total ischaemia/reperfusion injury of the liver, leads to increased DNA binding of NFκB through the Toll-like receptor 4, myeloid differentiation factor 88, and interleukin 1 receptor associated kinases. Thus inactivation of NFκB in Kupffer cells might be of clinical value in blocking injurious events in total hepatic ischaemia/reperfusion.

Several recent studies have proposed that apoptotic cell death may affect both hepatocytes and sinusoidal endothelial cells after either warm or cold ischaemia/reperfusion. Through release of tumour necrosis factor α (TNF-α), Kupffer cells and platelets reportedly induce apoptosis in these target cells. However, some reports have indicated that apoptosis in such cells never involves more than 2% of liver cells when strict morphological criteria for apoptosis are employed. NFκB participates importantly in promoting cell survival by regulating several antiapoptotic factors, including inhibitors of apoptosis. Inactivation of NFκB resulting from overexpression of inhibitor κBα super-repressor (IkBα SR) renders hepatocytes susceptible to various proapoptotic stimuli. Such blockade of NFκB activity in hepatocytes could lead to massive cell death after hepatic ischaemia/reperfusion if the dominant mechanism of cell death after reperfusion is apoptosis.

In the present study, we overexpressed dominant negative IkBα SR in the liver using an adenoviral vector (Ad5IkBα) capable of transfecting both hepatocytes and Kupffer cells. We examined whether the consequent blockade of NFκB DNA binding activity in the liver protected against or accelerated short term warm total ischaemia/reperfusion injury. We employed a rat model of total hepatic ischaemia/reperfusion that closely simulated a common surgical context of such injury (Pringle’s manoeuvre). We note that the model may not be suitable for study of pure hepatic ischaemia/reperfusion injury as endotoxin translocation from the congested intestine could occur during the ischaemic period.

MATERIALS AND METHODS

Recombinant adenoviruses

The recombinant adenovirus (Ad5IkBα) encoding a haemagglutinin (HA) tagged cDNA of the dominant negative forms

Abbreviations: NFκB, nuclear factor κB; IkB, inhibitor κB; IkBα SR, IkBα super-repressor; ROS, reactive oxygen species; TNF-α, tumour necrosis factor α; HA, haemagglutinin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; iNOS, inducible nitric oxide synthase; HNE, 4-hydroxy-2-nonenal; RT-PCR, reverse transcriptase-polymerase chain reaction; NPC, non-parenchymal liver cells; ssDNA, single stranded DNA

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of human IκBα (IκBα S32A/S36A) was generated as reported elsewhere. Ad5IκBα, encoding the Escherichia coli β-galactosidase gene, was used as a control adenovirus. Adenoviral stock was amplified in HEK293 cells (CRL1573; ATCC; Manassas, Virginia, USA) and purified by double caesium gradient, as described previously, and plaque titred. Seventy-two hours after infection, rats were anaesthetised by intraperitoneal injection of 0.1 μg/l Nembutal (pentobarbital sodium 50 mg/ml; Dainippon Pharmaceutical, Japan). After laparotomy, whole hepatic ischaemia was induced clamping the hepatic artery, portal vein, and bile duct for 20 minutes without any decompression of the splanchic circulation, resembling a clinical situation (Pringle’s manoeuvre). After 20 minutes, these vessels were unclamped leading to reperfusion of the liver. This model is sublethal and exhibits near normal. Liver injury induced by adenovirus should have returned to normal.

Animal protocols and hepatic ischaemia/reperfusion procedure

All animals were handled according to the method approved under the institutional guidelines outlined in the Guide for Use and Care of Laboratory Animals of Kyoto University Graduate School of Medicine. Male Sprague-Dawley rats with a starting weight of 240–255 g (7–8 weeks old) were used. Recombinant adenoviruses were administered through their tail veins in a volume of 250 μl (5×10⁹ pfu/body) with 27 G needles. No viruses were injected in uninfected control rats. Seventy-two hours after infection, rats were anaesthetised by intraperitoneal injection of 0.1 μg/l Nembutal (pentobarbital sodium 50 mg/ml; Dainippon Pharmaceutical). After laparotomy, whole hepatic ischaemia was induced clamping the hepatic artery, portal vein, and bile duct for 20 minutes without any decompression of the splanchic circulation, resembling a clinical situation (Pringle’s manoeuvre). After 20 minutes, these vessels were unclamped leading to reperfusion of the liver. This model is sublethal and exhibits less liver injury compared with that previously published. Because adenoviral infection per se possibly induces transient liver injury due to its immunogeneity, we performed the ischaemia/reperfusion procedure at 72 hours when transient liver injury induced by adenovirus should have returned to near normal. Small amounts of blood (0.4 ml) were collected from the inferior vena cava at 10 and 40 minutes after reperfusion, and liver tissues and blood samples were taken when the animals were sacrificed. At least 4 rats in each group were analysed at each time point. Serum separated from these samples was used for enzymatic measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH). Serum concentration of TNF-α in each animal was also measured by means of an ELISA kit (Genzyme, Cambridge, Massachusetts, USA). Samples of the liver were snap frozen in liquid nitrogen or mounted in Tissue Tec (Sakura Finetechnical Co., Tokyo, Japan) and stored at −80°C for immunohistochemistry. Some of the tissues were fixed in 10% buffered formalin for subsequent histological analysis (haematoxylin-eosin staining).

Histological assessment

Liver injury was assessed using liver specimens stained with haematoxylin-eosin. The extent of sinusoidal congestion, cytoplasmic vacuolisation, and liver necrosis was semiquantitatively assessed, respectively, according to a scoring criteria previously published. Namely, congestion and vacuolisation were evaluated as follows: none = 0, minimal = 1, mild = 2, moderate = 3, and severe = 4. Liver necrosis was scored as follows: none = 0, single cell necrosis = 1, up to 30% lobular necrosis = 2, up to 60% lobular necrosis = 3, and more than 60% lobular necrosis = 4. Scoring was performed in five independent high power fields on each sample, and mean values were represented. Blind analysis was performed on all samples. Infiltration of neutrophils into the liver was also estimated by means of naphthol AS-D chloroacetate esterase staining. The number of esterase positive polymorphonuclear cells was counted in 10 high power fields (×400) in each sample, and mean values were calculated.

X-gal staining analysis and immunofluorescence

Efficiency of gene transfer after adenoviral infection was assessed with X-gal staining of liver tissues from rats infected with Ad5IκBα at 72 hours. Frozen sections from the liver were evaluated for β-galactosidase activity by incubation in X-gal solution (3.3 mM K₄Fe(CN)₆·3H₂O, 3.3 mM K₃Fe(CN)₆, 1 mM MgCl₂, 0.2% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside); Roche Diagnostics, Switzerland). To assess whether liver macrophages were transfected with Ad5IκBα more accurately, immunofluorescent staining against β-galactosidase and surface antigen of macrophages was performed. Frozen liver samples were cut into cryostat sections 5 μm in thickness, and fixed in acetone for 10 minutes. After washing with phosphate buffered saline, sections were incubated with mouse antisurface antigen of macrophages monoclonal antibody (ED-1, 1: 100 dilution; Chemicon International, Temecula, California, USA) and rabbit anti-β-galactosidase polyclonal antibody (1:200; ICN Pharmaceuticals, Inc., Aurora, Ohio, USA) for 60 minutes. Slides were washed with phosphate buffered saline and then incubated for 30 minutes with FITC conjugated goat anti-mouse IgG (1:50 dilution; Southern Biotechnology Associates, Inc., Birmingham, UK) and Texas red conjugated goat antirabbit IgG (1: 50 dilution; Southern Biotechnology Associates, Inc.). Localisation of β-galactosidase and liver tissue macrophages, and Kupffer cells was analysed using a confocal laser scanning microscope LSM510 (Carl Zeiss, Jena, Germany).

Western blot analysis for IκBα and iNOS

Rats were infected with Ad5IκBα (2.5×10¹⁰ pfu/body) for 72 hours. Expression of dominant negative IκBα protein was assessed in 20 μg of whole liver homogenates using rabbit anti-IκBα antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, California, USA). Equal amounts of lysates were electrophoresed on 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After blocking, the membrane was incubated with the first antibody at 4°C overnight and then with the horseradish peroxidase linked goat antirabbit secondary antibody at 1:1000 dilution (Santa Cruz Biotechnology). Chemiluminescence was detected with an ECL kit, as previously described. Equal protein loading was confirmed by staining the gels with the Coomassie stain solution (Bio-Rad Laboratories, Hercules, California, USA). For western blot of inducible nitric oxide synthase (iNOS), rabbit anti-iNOS antibody (1:1000 dilution; sc-8310, Santa Cruz Biotechnology) was used, and subsequent procedures were performed as described above.

Immunohistochemistry for NFκB, HA tagged IκBα SR, 4-HNE, and nitrotyrosine

Paraffin embedded sections were pretreated by microwave for 20 minutes with 1% bovine serum albumin in 0.05 M Tris
HCl, pH 7.6. After blocking of endogenous peroxidase with peroxidase blocking agent, sections were incubated for 60 minutes at 37°C with goat polyclonal antibody against NFκB p65 (1:100 dilution; c-20-sc-372G; Santa Cruz Biotechnology). After being washed, sections were incubated for 60 minutes with biotin conjugated rabbit antibody against goat (1:200 dilution; AP106-b; Chemicon) labelled with peroxidase streptavidin, and examined, after being incubated with chromogen conjugated DAB substrate for 90 seconds.

For detecting the HA tagged IκBα, mouse monoclonal anti-HA antibody (5 µl/ml, clone 12CA5; Boehringer Mannheim, Indianapolis, Indiana, USA) was used as the primary antibody, and sections were stained as described above. Expression of nitrotyrosine and 4-hydroxy-2-nonenal (HNE) adducts in the liver were analysed by immuno-histochemistry using polyclonal antinitrotyrosine antibody (AB5411, 1:100; Chemicon) and monoclonal anti-4-HNE antibody (JaICA, MHN-20, 1:5; Shizuoka, Japan) as the primary antibody, respectively.

**Reverse transcriptase-polymerase chain reaction (RT-PCR) assay for NFκB**

 Messenger RNA was extracted from frozen liver tissue with a mRNA purification kit (Amersham Biosciences Corp., Piscataway, New Jersey, USA). The concentration of mRNA was ascertained by UV spectrophotometer at 260 nm and 10 µg of mRNA were used to synthesise first strand cDNA with the First Strand cDNA kit (Amersham Pharmacia). Rat TNF-α was amplified using the primer pair (each 100 µM, Bp, 5’-CAC GCT CTT CTG TCT ACT GA-3’; forward; 5’-GGA CTC CGT GAT GTC TAA GT-3’, reverse) in a 50 µl PCR reaction containing 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris HCl, pH 8.3, and 1.4 µl deoxynucleotides (10 mM each) using 3 µl cDNA template. Dilutions of cDNA were amplified for 30 cycles at 94°C for 20 seconds, 56°C for 60 seconds, and 72°C for 60 seconds. β-Actin probe was used as the integrity control, using the primers synthesised from conserved coding sequences (265 bp, 5’-TCC TAT GTG GGT GAC GAG GC-3’, forward; 5’-TAC ATG GCT GGG GTG TTG AA-3’, reverse). PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualised under UV transillumination.

**In situ detection of apoptosis**

Formalin fixed paraffin embedded liver sections (5 µm) were deparaffinised in xylene and rehydrated through graded ethanol. After blocking of endogenous peroxidase with peroxidase blocking agent, sections were incubated with protease K (20 µg/ml) for 10 minutes. Then, sections were incubated with a goat anti-single strand DNA polyclonal antibody (1:400 dilution; A4506; Dako Cytomation, Kyoto, Japan), which detects apoptotic cells in situ, for 60 minutes at room temperature. After being washed, sections were incubated for 30 minutes with biotin conjugated rabbit antibody against goat (1:200 dilution; AP106-b; Chemicon) labelled with peroxidase streptavidin, and with chromogen conjugated DAB substrate for 90 seconds. Counterstaining was performed with methyl green.

**Statistical analyses**

Data are expressed as means (SD), and the statistical significance of differences among groups was assessed by the Student’s t test or Mann-Whitney test, as appropriate. A p value less than 0.05 was regarded as statistically significant.
RESULTS
Efficiency and targets of adenoviral gene transfer in vivo
Efficiency of adenoviral gene transfer in vivo was determined by western blotting with antibodies against IkBα and immunostaining of HA tagged IkBα SR or β-galactosidase. As reported previously, western blotting using anti-IkBα antibody demonstrated high expression of HA tagged IkBα S32A/S36A only in liver homogenates from Ad5IkBα infected rats, not those from uninfected or Ad5LacZ infected rats (fig 1A). Immunohistochemistry using anti-HA antibody directed against HA tagged IkBα SR demonstrated that more than 80% of liver cells expressed IkBα SR (fig 1B, C). This efficiency was similar to that of Ad5LacZ, which was determined by X-gal staining (data not shown). More detailed morphological analysis revealed that non-parenchymal cells of the liver as well as hepatocytes were expressing IkBα SR (fig 1C, arrowheads). To further determine whether liver macrophages were infected with recombinant adenoviral vectors, immunofluorescent analysis of frozen liver tissues was carried out. Immunofluorescence against β-galactosidase and macrophage specific surface antigen revealed that approximately 60% of liver macrophages as well as about 80% of hepatocytes were immunoreactive for β-galactosidase (fig 1D–F). Only a few sinusoidal endothelial cells or hepatic stellate cells were reactive for β-galactosidase according to immunofluorescence using an antirat platelet endothelial cell adhesion molecule 1 monoclonal antibody or a rabbit antirat desmin polyclonal antibody (data not shown).

Attenuation of short term warm ischaemia/reperfusion injury in the liver by IkBα SR
In the present study, all rats in each group survived after reperfusion, possibly reflecting the short term nature of ischaemia. At 180 minutes after reperfusion, histological examination of uninfected and Ad5LacZ infected liver tissues demonstrated ballooning, and to some extent necrosis, of hepatocytes; these findings were most evident around the central veins (fig 2A, B). Inactivation of NFκB in the liver by overexpression of IkBα SR markedly attenuated these post reperfusion histological changes (fig 2C). Semiquantitative scores for sinusoidal congestion, cytoplasmic vacuolisation, and hepatocytic necrosis at three hours were 1.4 (0.5), 1.1 (0.4), and 1.2 (0.5) (mean (SD)) in uninfected rats, and 1.7 (0.7), 1.3 (0.5), and 1.3 (0.6) in Ad5LacZ infected animals, respectively; these scores were 0.4 (0.5), 0.6 (0.6), and 0.4 (0.5) (mean (SD)) in Ad5IkBα infected rats, all showing significant attenuation (p<0.05). Serum concentrations of AST, ALT, and LDH gradually increased in uninfected and Ad5LacZ infected groups and then returned to near normal values by 24 hours. No significant differences in serum concentrations of these liver enzymes were observed between these two groups (fig 2D–F). The transient increases in serum AST, ALT, and LDH were significantly attenuated in Ad5IkBα infected rats at various time points. These results indicate that inactivation of NFκB in the liver by adenoviral gene transfer of IkBα SR could ameliorate short term warm ischaemia/reperfusion injury.

Inhibition of nuclear translocation of NFκB and its DNA binding activity in the liver after I/R by transduction of IkBα SR
Immunohistochemistry for NFκB (p65) revealed that p65 had translocated to the nucleus in hepatocytes as well as in non-parenchymal liver cells (NPC) at three hours after reperfusion in uninfected or Ad5LacZ infected rats (fig 3A, B). Adenoviral gene transfer of IkBα SR markedly suppressed this nuclear translocation of p65 in hepatocytes as well as in NPC (fig 3C). The percentage of NPC in which nuclear translocation of NFκB was detected was 85% in uninfected, 87% in Ad5LacZ infected, and 15% in Ad5IkBα infected livers. Transgenes of LacZ or IkBα SR had no effect on distribution of p65 in the liver before ischaemia/reperfusion (data not shown). The gel
mobility shift assay for NFκB demonstrated that gene transfer of IκBα SR dramatically suppressed DNA binding activity of NFκB after ischaemia/reperfusion while its DNA binding activity had increased markedly at three hours after reperfusion in uninfected or Ad5IκBα infected liver (fig 3D).

We also analysed mRNA and protein expression of TNF-α and protein expression of inducible NOS (iNOS), whose genes are a target for NFκB, in the liver after reperfusion. TNF-α mRNA was barely detected in uninjured normal livers while its expression was markedly increased after reperfusion in uninfected and Ad5IκBα infected livers (fig 3E, lanes 2–4). Overexpression of IκBα SR in the liver clearly blocked this increase even after reperfusion (fig 3E, lane 5). Serum concentrations of TNF-α at three hours after reperfusion, which were determined by ELISA, were similar to those of mRNA (fig 3F). Protein expression of iNOS markedly increased after reperfusion in uninfected and Ad5IκBα infected livers while IκBα SR dramatically suppressed iNOS expression following reperfusion (fig 3F). These data suggest that transfer of IκBα SR effectively suppresses NFκB activation after reperfusion in non-parenchymal hepatic cells as well as in hepatocytes.

**Attenuation of neutrophil infiltration into the liver by IκBα SR**

Infiltration of neutrophils, which play pivotal roles in inflammatory injury, was assessed in the liver by naphthol AS-D chloroacetate esterase staining. The number of infiltrating neutrophils obviously increased after reperfusion (three hours) in uninfected and Ad5IκBα infected livers (fig 4A, B) while that in Ad5IκBβ SR infected rats was not prominent, even after reperfusion (fig 4C). This effect was confirmed by counting esterase positive polymorphonuclear cells under microscopic high power fields (fig 4D).

**Lack of attenuation by IκBα SR of lipid peroxidation after reperfusion**

As a marker of lipid peroxidation resulting from oxidative stress in the liver after reperfusion, we used immunohistochemistry to detect HNE adducts in liver tissues. HNE adducts were not demonstrated in untreated normal rat livers (fig 4E) while significant amounts of HNE adducts were detected after reperfusion in untreated, Ad5IκBα infected, and Ad5IκBβ infected livers, particularly surrounding central veins (fig 4F–H). No difference in the distribution pattern of HNE adducts in the liver was observed between these three groups. These data indicate that inactivation of NFκB in the liver did not block lipid peroxidation by oxidative stress after ischaemia/reperfusion.

**Blockade of nitrotyrosine production after reperfusion by overexpressed IκBα SR**

We assessed tyrosine nitration, an index of nitrosylation of proteins by peroxynitrite and/or other free radicals, because iNOS expression was affected after reperfusion. Interestingly, nitrotyrosine adducts were detected preferentially along the sinusoidal area but not in hepatocytes, after short term ischaemia/reperfusion in uninfected and
Ad5LacZ infected livers (fig 5A, B). This increase in nitrotyrosine expression along sinusoids was dramatically suppressed in Ad5IkBaSR infected livers, suggesting that IkBaSR attenuated ischaemia/reperfusion injury through blockade of nitric oxide production by non-parenchymal hepatic cells (fig 5C).

Suppression by IkBaSR of non-parenchymal cell apoptosis after reperfusion
As the appearance of apoptotic cells in hepatic ischaemia/reperfusion injury remains controversial, we assessed apoptosis in the liver after reperfusion in our model. Using an antibody against single stranded DNA (ssDNA), a relatively small number of apoptotic cells were detected along the sinusoidal area at three hours after reperfusion in uninfected and Ad5LacZ infected livers, while occasionally apoptosis was also detected among hepatocytes (fig 5D, E). Gene transfer of IkBaSR suppressed the appearance of ssDNA positive cells after reperfusion (fig 5F), even though over 80% of hepatocytes were transfected with Ad5IkBa according to anti-HA immunostaining (fig 1B, C). Immunohistological analysis at 12 hours after reperfusion detected ssDNA in a few non-parenchymal cells, but not in hepatocytes, even in uninfected and Ad5LacZ infected livers (data not shown). These data suggest that proapoptotic signalling is not the main cause of reperfusion injury, at least after reperfusion following short term warm ischaemia.

DISCUSSION
In pathogenetic sequences underlying hepatic ischaemia/reperfusion injury, activated liver macrophages (Kupffer cells) have been assigned critical roles.5–7 During the cascade leading to activation of Kupffer cells, DNA binding activity of NFkB, a key regulator of genes encoding inflammatory cytokines, acute phase response proteins, and cell adhesion molecules,36-37 is upregulated through several mechanisms, including pathways dependent on oxidative stress or endotoxin.16-18 In the present study, we demonstrated that inactivation of NFkB in the liver using adenoviral gene transfer of IkBaSR effectively blocked a short term warm ischaemia/reperfusion injury that was transient and sub-lethal, showing a good resemblance to liver injury observed clinically after an intraoperative manipulation, Pringle’s manoeuvre.

Immunohistochemical analysis demonstrated that the adenoviral gene transfer method used in the present study (dose 5 x 10⁹ pfu/rat) successfully delivered LacZ or IkBaSR into both parenchymal and non-parenchymal cells,
particularly Kupffer cells (fig 1B–F). Overexpressed 1kBz SR effectively blocked nuclear translocation of NFkB (p65) in both cell types after reperfusion (fig 3C), abolishing increased p65 DNA binding activity that was observed in uninfected or Ad5LacZ infected rats (fig 3D). This effect resulted in marked suppression of genes whose expression is regulated by NFkB, such as TNF-α and iNOS, even after reperfusion (fig 3E–G).

Considering that a variety of inflammatory cytokines regulated by NFkB are produced predominantly by non-parenchymal cells in the liver,11,15 our results suggest that inactivation of Kupffer cells through blocking NFkB from DNA binding is possibly the major mechanism underlying the protective effect of 1kBz SR observed in the present study.

Among potential mechanisms contributing to hepatic ischaemia/reperfusion injury,4 formation of ROS and reactive nitrogen species after reperfusion has been recognised as a critical factor.1 4 38 39 Generally, SOD scavenges superoxide to form oxygen and hydrogen peroxide, which is further detoxified via catalase to produce water and oxygen. An excessive amount of hydrogen peroxide, however, can undergo a one electron reduction with Fe (II) to form Fe (III) and the highly toxic hydroxyl radical, a very reactive species that rapidly induces lipid peroxidation.40 In the present study, inactivation of NFkB in parenchymal and non-parenchymal hepatic cells did not affect lipid peroxidation after reperfusion (fig 4E–H). This lipid peroxidation after reperfusion possibly accounts for the incomplete nature of suppression of transient increases in serum AST, ALT, and LDH in Ad5IkBz infected livers (fig 2D to F), even though they appeared nearly intact by haematoxylin-eosin staining (fig 2C).

Nitric oxide is another bioregulatory molecule produced in the liver after reperfusion; production involves upregulated expression of iNOS which has been implicated in the pathogenesis of ischaemia/reperfusion injury.5 6 While nitric oxide can directly affect cell signalling, it also forms peroxynitrite, a highly reactive nitrogen species produced by reaction with ROS that carries out nitration of tyrosine residues in proteins. In the present study, iNOS expression was blocked dramatically via NFkB inactivation (fig 3G), which should suppress nitric oxide synthesis even after reperfusion of Ad5IkBz infected livers. If nitric oxide synthesis is blocked, formation of peroxynitrite should be attenuated. This hypothesis was supported by suppressed expression of nitrotyrosine after reperfusion in these livers (fig 5C). Thus we conclude that suppression of iNOS induction by 1kBz SR in non-parenchymal cells, possibly Kupffer cells, can account for part of the beneficial effect from 1kBz SR gene transfer in the present study.

While the generally accepted mechanism of hepatic reperfusion injury is cell damage involving oncoytic necrosis,4 several recent reports have proposed that apoptotic cell death during hepatic ischaemia/reperfusion also may participate in the mechanism of the injury.21 22 NFkB is known as an antiapoptotic transcription factor in the liver23 24 and its blockade results in frequent occurrence of apoptosis of hepatocytes under proapoptotic stimuli.25 26 In the present study, we blocked NFkB activation after reperfusion in both parenchymal and non-parenchymal cells by transfer of 1kBz SR. However, we observed no remarkable appearance of apoptotic cells in Ad5IkBz infected livers even at 12 hours after reperfusion, while a few apoptotic cells, mainly non-parenchymal cells, were detected in uninfected and Ad5LacZ infected livers. Because the ssDNA staining might not be a definitive tool for detecting apoptotic cells, we cannot conclude that apoptotic cell death does not participate in the mechanism underlying reperfusion injury in short term warm total hepatic ischaemia. From our data, however, we speculate that apoptotic cell death does not play a major role in our model. Inactivation of NFkB in donor livers was found to increase histologically evident tissue injury and apoptosis after experimental liver transplantation representing cold hepatic ischaemia/reperfusion.47 Differences in animal models and duration of ischaemia may account for the disagreement between that report and our study.

In summary, we successfully attenuated hepatic reperfusion injury after short term warm total ischaemia by delivering the 1kBz SR gene to the rat liver. Because NFkB may be required for proliferation of intact hepatocytes,27 non-parenchymal cell selective as opposed to non-selective inactivation of NFkB should be more beneficial if the strategy of the present study were applied to clinical situations. Moreover, relatively short term inactivation of NFkB limited to non-parenchymal cells would be safer than adenoviral gene expression of 1kBz SR for 1–2 weeks. For example, Kupffer cell selective transfer of NFkB decoy, which binds NFkB and blocks its translocation to the nucleus,11 may come to represent a clinically important way of preventing ischaemia/reperfusion injury during hepatic surgery.

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Conflict of interest: None declared.

REFERENCES

EDITOR’S QUIZ: GI SNAPSHOT

Answer

From question on page 813

Helical computed tomography scan revealed a low density, well circumscribed cystic mass, 21 \times 22 \text{ cm}^2 in size, in the left hepatic lobe with multiple daughter cysts floating inside, compatible with a hydatid cyst. The biliary system and spleen were normal (fig 1). Results of serum IgG antibody titre to *Echinococcus granulosus* was higher than 1:2560. Likewise, a pretreatment magnetic resonance cholangiogram was performed ruling out bile leaks.

In this case, perioperative treatment with albendazole, simple endocystectomy, aspiration of the residual contents of the cyst cavity, and omentoplasty were performed. After two months the patient was asymptomatic.

Cystic hydatic disease due to infection with the metacestode of *Echinococcus granulosus* has a worldwide distribution, and the liver is the most common site (70%). Surgery is the recommended treatment for hepatic hydatid cysts. However, encouraging reports suggest that under carefully controlled conditions, percutaneous aspiration with the use of concomitant antihelminthic therapy may be a safe alternative treatment.

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