Role of NF-κB on liver cold ischemia-reperfusion injury

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Takashi Yoshiihiito, Raymond W. Ganster, Andrea Gambotto, Lifang Shao, Takashi Kaizu, Tong Wu, Gautam P. Yagnik, Atsunori Nakao, George Tsoufas, Takashi Ishikawa, Toyokazu Okuda, David A. Geller, and Noriko Murase. Role of NF-κB on liver cold ischemia-reperfusion injury. Am J Physiol Gastrointest Liver Physiol 283: G1175–G1184, 2002. First published July 17, 2002; 10.1152/ajpgi.00515.2001.—The role of NF-κB, the rapid-response transcription factor for multiple genes, in cold ischemia-reperfusion (I/R) injury was examined after synergistic transplantation of liver grafts. Lewis rat recipients were killed 1–48 h after reperfusion of three different liver grafts: 1) uninfected control, 2) infected ex vivo with control adenoviral vector (AdEGFP), and 3) infected ex vivo with AdIκB. In uninfected control livers, NF-κB was activated biphasically at 1–3 and 12 h after reperfusion with aspartate transaminase (AST) levels of 4,244 ± 891 IU/l. The first peak of NF-κB activation associated with an increase of mRNA for TNF-α, IL-1β, and IL-10. AdEGFP transfection resulted in similar outcomes. Interestingly, AdIκB-transfected liver grafts suffered more severe I/R injury (AST >9,000 IU/l). Transfected IκB was detected in transplanted livers as early as 6 h, and this correlated with the abrogation of the second, but not the first, peak of NF-κB activation at 12–48 h and increased apoptosis. Thus inhibition of the second wave of NF-κB activation in IκB-transfected livers resulted in an increase of liver injury, suggesting that NF-κB may have a dual role during liver I/R injury.

LIVER TRANSPLANTATION has been widely used as the therapy for patients with end-stage liver disease. However, the transplant procedure obligates cold perfusion, hypothermic storage, warm ischemia, and warm reperfusion of the liver grafts, resulting in ischemia-reperfusion (I/R) injury of the transplanted liver. Although the introduction of University of Wisconsin (UW) solution has significantly improved clinical outcome, I/R injury remains one of the major clinical problems after liver transplantation. Prolonged cold ischemic storage, as well as poor donor quality such as hepatic steatosis, increase I/R injury, resulting in dys-function or nonfunction of the transplanted liver grafts (1, 16, 37, 39, 43).

Multiple factors have been shown to contribute to I/R injury. The lack of oxygen during ischemia induces depletion of ATP followed by a deterioration of the intracellular Ca2+ and Na+ homeostasis and the activation of cytotoxic enzymes (e.g., proteases, phospholipases, endonucleases). Additionally, generation of reactive oxygen species during reperfusion is believed to contribute to direct injury of the cells in the liver. Accordingly, endothelial cell damage and disturbance of microcirculation and Kupffer cell activation initiate the complex network of proinflammatory signal-transduction cascade. Subsequent recruitment of neutrophils further amplifies tissue damage (12, 15, 25).

NF-κB, an inducible dimeric transcription factor that belongs to the Rel family of transcription factors, is a main mediator of the cellular response to a variety of extracellular stress stimuli. The targets for transcriptional activation of NF-κB include the genes for cytokines, acute phase-response proteins, immunoglobulins, and cell-adhesion molecules (30). It has been shown that NF-κB is activated during the acute phase of I/R injury after liver transplantation (7, 19). However, the role of NF-κB activation during I/R injury of the transplanted liver is not clear. As a proinflammatory transcription factor, NF-κB may trigger upregulation of cytokines (e.g., TNF-α and IL-1) and adhesion molecules (e.g., ICAM-1), thereby initiating inflammatory responses. However, NF-κB also plays a role in acute cellular stress responses such as in the protection from TNF-α-induced apoptosis (3, 44). NF-κB has been shown to be an essential survival factor in several physiological conditions including embryonal liver development and liver regeneration (4, 24). Thus NF-κB possibly induces both harmful (inflammatory) and beneficial (antiapoptotic/survival) effects during I/R injury.

Activation of NF-κB is tightly controlled by its endogenous inhibitor IκB, which complexes NF-κB in the cytoplasm. Phosphorylation and proteolytic degradation of IκB allows the release and nuclear translocation
of NF-κB, followed by transcription of many proinflammatory genes. Degradation of IκB is linked to phosphorylation of serine residues 32 and 36 located in the NH₂-terminal part of the polypeptide. A mutant form of IκBo, in which serines 32 and 36 are replaced by alanine residues (S32A/S36A), has been shown to be effectively protected from degradation and thereby prevents NF-κB activation (45).

This study was undertaken to examine the detailed activation pattern of NF-κB in I/R injury induced in rat liver grafts stored in UW solution for 18 h and subsequently transplanted in syngeneic recipients. In addition, we attempted to evaluate the role of NF-κB in this animal model by overexpressing a dominant-negative mutant form of IκBo (S32A/S36A) using the adenoviral gene-transfer technique (11, 41).

MATERIALS AND METHODS

Viral vectors. The adenovirus encoding the IκBo superrepressor gene (AdIκB) was kindly provided by D. A. Brenner (University of North Carolina) (24, 27, 42). The cDNA insert of plasmid pBC/CMV-IκBoS32A/S36A, which contains a hemagglutinin (HA)-tagged human superrepressor of NF-κB, was subcloned into the XbaI site of the pACCMV.PLPASR(+) plasmid to construct the plasmid pACCMV/IκBo, in which IκBo is driven by the cytomegalovirus (CMV) promoter/enhancer. The adenovirus encoding the enhanced green fluorescent protein gene under a CMV promoter (AdEGFP) was used as a control (17, 33, 41).

Recombinant adenoviruses were amplified in CRES cells, purified by CsCl density gradient centrifugation, then dialyzed against storage buffer containing 3% sucrose, 10 mM Tris, 150 mM NaCl, and 10 mM MgCl₂ (pH 7.8). Titers of viral particles were determined by optical densitometry, and recombinant viruses were stored at −80°C until required for use. The presence of the viral transgene product IκBo was confirmed by Western blot analysis.

Orthotopic liver transplantation and viral vector delivery to liver grafts. Inbred male Lewis rats (LeW: RTI, Harlan Sprague Dawley, Indianapolis, IN) were used for all syngeneic transplantation experiments in this study to prevent immunological interference. Rats weighing 200–300 g were maintained in a laminar-flow, specific-pathogen-free atmosphere at the University of Pittsburgh. Liver graft harvesting and orthotopic transplantation without hepatic arterial reconstitution were performed as described previously by Kamada and Calne (28). Adenoviral vector was delivered to the liver grafts by ex vivo cold infusion into the harvested liver via the portal vein and hepatic artery with clamp technique (CT) (11, 41). Briefly, the donor liver was flushed in situ with 20 ml of cold (4°C) UW solution (DuPont, Merck Pharmaceuticals, Wilmington, DE) through the infrarenal aorta. After a routine skeletonization, the isolated liver graft was slowly perfused with a total of 6 ml UW containing adenoviral vector (1 × 10⁶ pfu); after the initial flush out with 2 ml via the portal vein and 1 ml via the hepatic artery, vascular clamps were placed on the supra- and infraportal inferior vena cava, and subsequent perfusate via the portal vein (2 ml) and hepatic artery (1 ml) was infused into the liver, retaining the viral suspension in the hepatic vasculature. After visible expansion of the liver capsule was noted, the hepatic artery was ligated and the portal vein was clamped. Liver grafts were stored in UW solution at 4°C for a total preservation period of 18 h. Stored livers were transplanted into syngeneic LeW recipients after being rinsed with 3 ml of cold lactated Ringer solution via the portal vein. Chia et al. (11) and Takahashi et al. (41) previously showed that adenoviral gene delivery with CT induced 10–30% infectivity exclusive to hepatocytes using a dose of 1 × 10⁶ pfu.

Recipient rats were killed at 1, 3, 6, 12, 24, and 48 h after transplantation (n = 2–5 for each time point). Blood samples were collected from the aorta, and the liver grafts were harvested for further studies. All procedures in this experiment were performed according to the guidelines of the Council on Animal Care at the University of Pittsburgh and the National Research Council’s Guide for the Care and Use of Laboratory Animals.

Isolation of nuclear and cytoplasmic proteins. Frozen liver tissues were suspended in buffer containing 10 mM Tris (pH 7.5), 1.5 mM MgCl₂, 10 mM KCl, and 0.1% Triton X-100 and lysed by dounce homogenization. Nuclei were recovered by microcentrifugation at 7,500 rpm for 5 min. The supernatant containing cytoplasmic and membrane protein was collected and stored at −80°C for Western blot analysis. Nuclear proteins were extracted at 4°C by gently resuspending the nuclei pellet in buffer containing 20 mM Tris (pH 7.5), 20% glycerol, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, and 0.1% Triton X-100, followed by 1 h incubation at 4°C with occasional vortexing. After microcentrifugation at 13,000 rpm for 15 min at 4°C, the supernatant containing nuclear protein was collected and frozen at −80°C for EMSA. All buffers contained the following additional ingredients: 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM diithiothreitol, and 0.1 mM Na vanadate. Protein concentration was quantitated with Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Western blot analysis. To detect the transfected IκBo superrepressor expression in liver grafts, Western blot analysis was carried out using cytosolic proteins. One hundred micrograms of cytosolic proteins were homogenized by electrophoresis on 10% acrylamide sodium dodecyl surface gels and transferred to nitrocellulose membranes (Sleicher and Schuell, Keene, NH). For the blocking of nonspecific binding, 5% nonfat dry milk in PBS-Tween was added to the membrane for 2 h at room temperature. Membranes were washed in PBS-Tween and then incubated with primary rabbit polyclonal anti-HA antibody (Y-11, 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA) for 1.5–2 h. After repeat washings with PBS-Tween, membranes were incubated with secondary goat anti-rabbit antibody (1:10,000, Pierce Chemical, Rockford, IL) for 45 min. Membranes were developed with the SuperSignal detection systems (Pierce Chemical) and exposed to film.

EMSA. NF-κB DNA binding activity was measured by EMSA using nuclear extracts from graft liver tissues. The NF-κB oligonucleotide (Life Technologies, Rockwell, MD) was based on the NF-κB oligonucleotide (Life Technologies, Rockwell, MD) was based on the NF-κB sequence in the immunoglobulin light-chain enhancer. DNA probes were prepared by end labeling with [γ-3²P]dATP (DuPont, Merck Pharmaceuticals) and T4 polynucleotide kinase (Boehringer-Mannheim BioMedical Products, Mannheim, Germany) and purified in Tris EDTA buffer containing NaCl (100 mM) using G-50 resin columns (Whatman, Newmarket, MA). Typically, 5 μl (10–20 μg) of hepatic nuclear extract were incubated with ~100,000 counts/min of [³²P]-labeled oligonucleotides (~0.5 ng) for 1–2 h at room temperature in a buffer containing 10 mM Tris (pH 7.6), 10% glycerol, 1 mM EDTA, 1 mM BSA, and 0.125% Nonidet P-40. Additionally, 2–4 μg of poly(dI-dC) (Boehringer-Mannheim Biomedical Products) were added to the mixture as nonspecific competitor DNA. Protein-DNA complexes were resolved on 4% nondenaturing polyacrylamide gels in 0.4 × running buffer containing 450 mM Tris borate
and 1 μM EDTA (pH 8.0). Gels were dried after electrophoresis and subjected to autoradiography.

Antibody supershift experiments were carried out with antibodies directed against NF-κB p50 (NLS), p52 (K-27), p65 (F-6), RelB (C-19), and c-Rel (N-466; all from Santa Cruz Biotechnology). Each antibody (2 μl) was incubated with nuclear extracts in binding buffer for 15 min before the addition of labeled oligonucleotide.

RNA preparation and RNase protection assay. Total RNA was extracted from frozen samples by using TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. The concentration of RNA was determined by ultraviolet (UV) spectrophotometer at 260 nm.

RNase protection assay (RPA) using commercially available kits (all from PharMingen, San Diego, CA) was used to quantify mRNA levels for cytokines and apoptosis-related molecules. Radiolabeled antisense RNA multiple probes were synthesized using In Vitro Transcription kit and rat cytokine multiprobe template sets (rkC-1 and rAPO-1, PharMingen), which included probes for cytokines (IL-1α, -1β, -2, -3, -4, -5,-6, and -10, TNF-α and -β, and IFN-γ), apoptosis-associated molecules (caspase-1, -2, -3, -6, -7, -8, -9, bcl-xL and -2, and bax), and housekeeping genes (L32 and GAPDH). 32P-labeled probes (8.0 × 10⁵ cpm) and sample RNA (5 μg) were hybridized at 35°C for 24 h. All transcription reactions were scaled appropriately for a single experiment, and single-stranded RNAs including antisense RNA probes were digested by using RPA Kit. The protected RNA duplexes were isolated by RNA inactivation/precipitation solution (Ambion, Austin, TX) and electrophoresed in a standard sequencing gel. Dried gels were exposed to Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA) for 48 h at room temperature. The radioactivity of each band was measured by β-scan (PhosphorImager, Molecular Dynamics) and National Institutes of Health (NIH) image-analysis software. The results were normalized to GAPDH and expressed as a ratio of cytokine or apoptotic signal to GAPDH.

Liver function tests. Hepatic function and injury following rat liver transplantation was assessed by serum aspartate (8.0 kU/l) and subjected to autoradiography. AntikB antibodies directed against NF–p65 (NLS), p52 (K-27), p65 (F-6), RelB (C-19), and c-Rel (N-466; all from Santa Cruz Biotechnology). Each antibody (2 μl) was incubated with nuclear extracts in binding buffer for 15 min before the addition of labeled oligonucleotide.

DNA was performed using a 1:1 suspension of phenol and chloroform. After 1/10 volume of 3 M Na acetate (pH 5.2) was added to the extraction, the DNA was precipitated by adding an equal volume of cold isopropanol and stored overnight at −20°C. The DNA pellet was then washed twice with 75% ethanol and resuspended in buffer containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. After RNA were digested with RNase at 37°C for 2 h, samples (30 μl) were mixed with gel-loading buffer (0.05% (wt/vol) bromophenol blue, 40% (wt/vol) sucrose, 0.1 M EDTA (pH 8.0)) and electrophoresed through a 1.2% agarose gel. Gels were stained with ethidium bromide and visualized by UV transillumination. A double-stranded 123-bp DNA ladder served as a standard.

Statistical analysis. Data are represented as the mean ± SE. Comparisons between the groups at different time points after reperfusion were performed using the Student’s t-test or ANOVA using the StatView program (Abacus Concepts, Berkeley, CA). Differences were considered significant at P values <0.05.

RESULTS

NF-κB DNA binding activity following cold I/R. After the liver graft underwent 18 h of cold preservation and reperfusion, NF-κB DNA binding activity in the liver markedly rose (Fig. 1A). Sequential analysis after I/R showed that NF-κB DNA binding activity started to increase at 20 min (data not shown), significantly
increased at 1–3 h, and dropped at 6 h after reperfusion. This early peak of NF-κB binding activity at 1–3 h contained two distinct protein-DNA complexes. Subsequently, the second peak of NF-κB binding activity was observed at 12 h after reperfusion, which diminished by 24–48 h.

To identify the proteins complexed with the NF-κB oligo in this liver cold I/R model, an antibody supershift experiment was performed using antibodies specific for NF-κB subunits, p50, p52, p65, RelB, and cRel (Fig. 1B). In nuclear extracts obtained 1 h after reperfusion, the anti-p50 and -65 antibodies caused a supershift of NF-κB complexes (columns 2 and 4). The protein-DNA complex of lowest mobility (upper band) was supershifted with anti-p65 antibody, whereas the anti-p50 antibody caused a supershift of both upper and lower...
complexes, indicating that the early peak of NF-κB binding activity was composed of p50/p65 heterodimers and p50 homodimers. During the second NF-κB peak at 12 h, a single NF-κB complex was observed, and antibody for p50 but not p65 caused supershift of NF-κB complex, indicating that NF-κB p50 homodimer translocated to the nucleus at this period. The antibodies against other NF-κB proteins failed to react with the protein-DNA complexes.

mRNA for cytokines and apoptosis-associated molecules following cold I/R. Early after cold I/R injury, there was a sharp increase of mRNA for cytokines (Fig. 2A). mRNA for IL-1α, -1β, -6, and -10 and TNF-α increased 5- to 10-fold at 1–3 h after I/R, gradually decreased, and returned to baseline level by 12 h. IL-6 and -10 and TNF-α were downregulated thereafter, whereas IL-1α and -1β showed a secondary small increase 48 h after I/R. As expected, Th1-type cytokines, such as IL-2 and IFN-γ (not shown), were not involved in the early phase of I/R injury.

mRNA for apoptosis-associated molecules, such as caspase-3 and bcl-2, increased relatively late after I/R (Fig. 2B). mRNA for caspase-3 slowly increased and reached peak value at 6 h, whereas peak of antiapoptotic bcl-2 delayed to 12 h. Both showed a secondary increase at 48 h.

AdIkB expression by adenovirus-mediated gene therapy using clamp technique. We have previously shown that the back-table CT using AdLacZ and AdEGFP provides a highly efficient gene transfer to liver grafts (11, 41). AdIkB, which contains an HA-tagged human superrepressor of NF-κB (1 × 10⁹ pfu), was delivered to the liver grafts with CT, and early time course of transferred gene expression was studied by Western blot analysis using anti-HA antibody (Fig. 3). Normal liver without transplantation was negative. Transfected IkB gene product started to appear at 6 h after reperfusion, increased with time, and was maintained at a high level for 48 h after reperfusion.

Fig. 5. Effect of AdIkB gene therapy on liver function. Serum aspartate transaminase (AST) and alanine aminotransaminase (ALT) levels were sequentially measured after cold I/R injury of control and AdEGFP (1 × 10⁹ pfu)- or AdIkB (1 × 10⁹ pfu)-treated liver grafts. AdIkB infection significantly increased AST and ALT. *p < 0.05 vs. control animals.

Fig. 6. Effect of AdIkB gene therapy on liver histopathology and apoptosis (TUNEL assay). Representative liver sections at 24 h after I/R were stained with hematoxylin and eosin. A: normal liver. Control (B) and AdEGFP-treated liver graft (C) showed mild foci of necrosis with neutrophil infiltration. On the other hand, AdIkB-treated liver graft (D) had massive necrosis. TUNEL-positive cells increased in AdIkB-treated liver graft (F) compared with control liver (E). (Original magnification ×20.)
Effects of AdIκB gene therapy on NF-κB DNA binding activity. The effects of AdIκB or AdEGFP infection on NF-κB activation were evaluated by EMSA using liver nuclear extracts obtained at different time points after reperfusion (Fig. 4). The early peak of NF-κB at 1–3 h was not affected by AdIκB or AdEGFP gene transfer, and potent DNA binding activity was seen in all groups of liver grafts early after reperfusion. In contrast, NF-κB DNA binding activity was partially inhibited in AdIκB-treated liver grafts between 12 and 48 h, compared with AdEGFP-treated or control liver grafts. The inhibition of the second, but not the first, peak of NF-κB DNA binding correlated with the timing of transferred IκB protein expression (Fig. 3). Zwacka et al. (49) showed dissociation of NFκB-IκB is induced by tyrosine phosphorylation of IκB rather than proteolytic degradation of IκB in the mouse hepatic warm ischemia model. In this study, we did not directly address the relative importance of tyrosine phosphorylation, and it is possible that the early 1-h NF-κB peak rate is explained by this mechanism. However, the fact that the superrepressor AdIκB did diminish NF-κB binding activity at 12–48 h suggests that serine phosphorylation is a triggering event.

Effects of AdIκB gene therapy for liver injury. The effects of AdIκB gene transfer on the hepatic cold I/R injury were examined by measuring in the liver transaminase (serum AST and ALT) and by histopathology. Both AST and ALT in control group increased soon after reperfusion, reaching peak levels (4,244 ± 691 U/l) at 24 h with a subsequent gradual decrease (Fig. 5). Most interestingly, AST and ALT significantly increased in recipients of AdIκB-treated liver grafts, compared with control and AdEGFP-treated animals. Peak values at 24 h after reperfusion were doubled in AdIκB-infected liver recipients, and both AST and ALT were still significantly high at 48 h (P < 0.05).

The exacerbated deterioration of I/R injury in AdIκB-infected liver was confirmed by histopathological examination. The control isograft (Fig. 6B) and AdEGFP-infected liver graft (Fig. 6C) showed small scattered foci of necrosis at 24 h after reperfusion. On the other hand, liver grafts expressing mutant IκB demonstrated extensive necrosis and congestion (Fig. 6D). More significant increase of injury with AdIκB than with control and AdEGFP was observed when quantitative analysis was performed (Fig. 7).

TUNEL assay and DNA fragmentation assay were carried out to examine whether apoptosis was a relevant mechanism to worsening cold I/R injury by AdIκB gene transfer. AdIκB-treated liver graft (Fig. 6F) showed increased TUNEL-positive cells compared with control liver graft (Fig. 6E). Immunohistochemical stain of activated caspase-3 revealed a significant increase of caspase activation with AdIκB (Fig. 8). In DNA fragmentation assay (Fig. 9), normal rat liver failed to exhibit DNA laddering. DNA laddering was augmented in AdIκB-treated liver graft compared with both control and AdEGFP-treated liver grafts. These results suggest that the deterioration of cold I/R injury by AdIκB gene therapy in this study also involves an increase of hepatic apoptosis. A large number of apoptotic hepatocytes has been shown to represent a signal for primed neutrophils to extravasate and augment injury (26). Increased neutrophils and necrosis in AdIκB-treated liver may, in part, be secondarily caused by enhanced neutrophil responses.

DISCUSSION

This study shows a biphasic pattern of NF-κB activation in cold I/R injury of liver grafts. The early peak of NF-κB DNA binding was seen 0.5–3 h after reperfusion and represents the nuclear translocation of NFκB p50/p65 heterodimer and possibly p50 homodimers. NF-κB DNA binding activity subsequently diminished for a few hours, and then a second peak of nuclear DNA binding was observed at 9–12 h after reperfusion. The second peak of NF-κB activation was less vigorous than the first and was mainly composed of p50 homodimer. The timing of the early peak of NFκB activation correlated well with the acute phase of I/R injury. During the acute phase (~6 h after reperfusion), the burst of reactive oxygen intermediates (peroxide, superoxide, and hydroxyl radicals) generated by I/R induces cellular damage by means of protein oxidation/degradation, lipid peroxidation, and

![Graph of Congestion, Necrosis, and Neutrophil grades]

Fig. 7. Quantitative histopathological analysis of liver injury. Liver graft section at 48 h after I/R was graded for congestion, neutrophil infiltration, and necrosis. *P < 0.05 vs. control and AdEGFP.
DNA damage. Subsequent cellular responses to the damage include the activation of proinflammatory signal-transduction pathways, changes in the microcirculation and interactions between leukocytes, cytokine production, and induction of chemoattractants. These changes are believed to initiate the subacute phase of I/R injury that is characterized by massive neutrophil infiltration, peaking 18–24 h after reperfusion.

Early activation of NF-κB after I/R, similar to the first peak of NF-κB activation seen in this study, has been well documented in both warm (23, 40, 49) and cold (6, 7, 19) I/R injury. In an effort to examine the role of NF-κB activation after I/R, several studies attempted to inhibit early NF-κB binding activity using antioxidant/anti-NF-κB reagents (e.g., diethyldithiocarbamate) (9, 23, 31, 32), double-stranded DNA with a specific affinity for NF-κB (35), or IkB superrepressor (6). Inhibition of early NF-κB binding activity in the nucleus was found to associate with downregulation of proinflammatory cytokines (e.g., IL-1β and -6 and TNF-α) and inducible nitric oxide synthase with an amelioration of I/R-induced tissue injuries (7, 9, 23, 32, 35). These results identify the role for NF-κB in the early cascade of I/R injury: I/R releases free radicals that induce cellular damage, followed by NF-κB activation, which, in turn, initiates proinflammatory signal-transduction pathways. Thus early activation of NF-κB in I/R injury seems to have harmful (inflamma-
Fig. 9. Effect of AdIkB gene therapy on DNA fragmentation assay. At 24 h after I/R, DNA laddering of AdIkB (1 x 10^9 pfu)-treated liver graft (lane 6) was augmented compared with both control (lane 4) and AdEFGFP (1 x 10^9 pfu)-treated liver grafts (lane 5). Normal liver did not show DNA laddering (lane 3). A double-stranded 123-bp DNA ladder showed as a standard (lane 1). Lane 2 (−) contains no sample.

Definite roles of secondary NF-κB activation in I/R injury need to be further investigated; however, on the basis of several observations, some hypotheses can be illustrated. A possible discrete role of secondary NF-κB activation in this study may relate to the fact that the second peak is mainly composed with NF-κB p50 homodimer. The NF-κB transcription factor family consists of five different members: p50, p52, p65 (RelA), c-Rel, and RelB and various forms of homo- and heterodimers. The induced NF-κB prototype in many cell types is predominantly composed of the p50/p65 heterodimer subunit, which possesses strong transactivating potential as a transcription factor in the NF-κB family. As shown in hepatic warm I/R injury (23, 40, 49), the early peak of NF-κB activation after cold I/R in this study contained NF-κB complexes of p50/p60 heterodimers. In contrast, the secondary phase of NF-κB activation in this study was composed of p50 homodimers, which may downregulate NF-κB-mediated initial responses to subsequent stimuli in the regulatory step. Because p50 homodimer lacks transcription activation domains, nuclear translocation of p50 homodimer may inhibit NF-κB-mediated transcription by competing with other NF-κB complexes for access to binding sites (48, 49). Inhibition of p50 homodimers with AdIkB in this study may result in persistent transcription of proinflammatory genes. However, our preliminary data show that AdIkB treatment does not significantly alter mRNA levels of proinflammatory cytokines (data not shown), suggesting that secondary NF-κB activation may have different roles.

NF-κB has been suggested to play an essential role in cell proliferation and differentiation. NF-κB controls the expression of a number of growth-promoting cytokines. Moreover, NF-κB transcriptional activity appears to be linked to extracellular signaling that controls cell-cycle progression. Mice lacking the p65 (RelA) subunit of NF-κB as a result of targeted mutation of the relA gene die embryonically at 15–16 days of gestation with massive degeneration of liver cells caused by extensive apoptosis (4). Typically, the control of mammalian cell proliferation by extracellular signals takes place in middle to late G1 phase of the cell cycle, and several reports have described an association of NF-κB activation with the early G1 phase of the cell cycle (2, 13, 14). In the liver, a rapid NF-κB DNA binding activity is seen following partial hepatectomy, when hepatocytes progress in the cell cycle from G0 to G1 (13). Inhibition of NF-κB activation after partial hepatectomy with AdIkB leads to massive apoptosis of hepatocytes and liver dysfunction (24). Thus inhibition of NF-κB correlates with failure to progress through the normal cell cycle. It has been recently shown that NF-κB can activate the transcription of cyclin D1, which is a key promoter for G1-to-S phase progression (20, 21).

The recovery of the liver grafts from cold I/R injury requires liver regeneration to replace damaged hepatic mass. Apoptosis seen in the injured area of AdIkB-transfected liver in this study is reminiscent of that seen in the degenerating liver of NF-κB p65 knockout mice and in AdIkB-transfected liver after partial hepatectomy (4, 24). It is tempting to speculate that the inhibition of NF-κB by AdIkB results in the failure to transmit growth signals that are required for the recovery from I/R injury.

In addition to the roles on regulation of cell cycle, NF-κB may regulate apoptosis by directly controlling genes that inhibit or promote apoptosis. Apoptosis has been known to play a role in I/R injury (18, 29, 34, 47), and inhibition of apoptotic signals by administration of...
caspase-3-inhibitor (36), or by overexpression of antiapoptotic Bcl-2, ameliorates I/R injury (5). The antiapoptotic function of NF-κB is further supported by several studies demonstrating that NF-κB activity prevents the induction of apoptosis by TNF-α, ionizing radiation, and anticancer agents (3, 44, 45). Candidates of target genes include caspase-8 and -3 (22) and antiapoptotic molecules, such as cellular inhibitors of apoptosis (10, 38, 46). It is therefore considered a possibility that toxic molecules, such as cellular inhibitors of apoptosis (36), or by overexpression of antiapoptotic Bcl-2, ameliorates I/R injury in AdIκB-infected liver grafts is due, in part, to the inhibition of the antiapoptotic functions of NF-κB. Together, these findings suggest a complex balance for NF-κB in regulating proinflammatory cytokine gene expression, cell proliferation/differentiation, and apoptosis-related signaling during the reperfusion period following liver transplantation.

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REFERENCES


