NFκB Prevents Apoptosis and Liver Dysfunction during Liver Regeneration

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Abstract

Although NFκB binding activity is induced during liver regeneration after partial hepatectomy, the physiological consequence of this induction is unknown. We have assessed the role of NFκB during liver regeneration by delivering to the liver a superrepressor of NFκB activity using an adenoviral vector expressing a mutated form of IκBα. This adenovirus (Ad5IκB) was almost exclusively expressed in the liver and inhibited NFκB DNA binding activity and transcriptional activity in cultured cells as well as in the liver in vivo. After partial hepatectomy, infection with Ad5IκB, but not a control adenovirus (Ad5LacZ), resulted in the induction of massive apoptosis and hepatocytes as demonstrated by histological staining and TUNEL analysis. In addition, infection with Ad5IκB but not Ad5LacZ decreased the mitotic index after partial hepatectomy. These two phenomena, increased apoptosis and failure to progress through the cell cycle, were associated with liver dysfunction in animals infected with the Ad5IκB but not Ad5LacZ, as demonstrated by elevated serum bilirubin and ammonia levels. Thus, the induction of NFκB during liver regeneration after partial hepatectomy appears to be a required event to prevent apoptosis and to allow for normal cell cycle progression. (*J. Clin. Invest.* 1998. 101:802–811.) Key words: adenoviruses • cell division • gene transfer • hepatectomy • IκB

Introduction

Hepatocytes of normal adult liver divide infrequently. However, immediately after partial hepatectomy (PH),[1] there is a sequential and regulated series of events as the quiescent hepatocytes progress in the cell cycle from G-0 to G-1 (1, 2). The earliest signals after PH include increased JNK and ERK activity (3–5), increased NFκB binding activity (6–8), and increased STAT-3 DNA binding activity (9, 10). The hepatocytes progress through the cell cycle and proliferation continues until the liver mass is restored.

NFκB is a ubiquitous transcription factor that is activated by a variety of cytokines and mitogens (11) and is thought to be a key regulator of genes involved in inflammation, responses to infection, and stress. The targets for transcriptional activation of NFκB include the genes for cytokines, acute phase response proteins, immunoglobulins, and cell adhesion molecules. Classic NFκB is a heterodimer of p50 (NFκB-1) and p65 (Rel-A), but proteins that constitute the NFκB family form a variety of homodimers and heterodimers (12). NFκB is retained in an inactive form in the cytoplasm through association with one of the IκB inhibitory proteins, including IκBα or IκBβ (13). After cellular stimulation, the phosphorylation, ubiquination, and subsequent proteolysis of IκBα in proteosomes enables NFκB to translocate into the nucleus (14–16), where it regulates the transcription of NFκB-responsive genes by interacting with κB binding sites (17, 18).

A superrepressor form of IκBα contains serine-to-alanine mutations at amino acids 32 and 36, which inhibit signal-induced phosphorylation and subsequent proteosome-mediated degradation of IκBα. This IκB superrepressor has been used to demonstrate that inhibition of NFκB induces apoptosis through a variety of cancer therapeutic agents and TNF-α (19, 20). Further support for an antiapoptotic role of NFκB was suggested by the embryonic death of NFκB p65 knockout mice from extensive apoptosis in the liver (21).

Thus NFκB is believed to be a pivotal transcription factor in liver regeneration as well as in liver development. However, the pathophysiological sequelae of induced NFκB DNA binding activity during liver regeneration is unknown. In this study, we assess the role of NFκB in liver regeneration after PH by expressing the IκB superrepressor in the adult rat liver in vivo.

Methods

Construction of recombinant adenovirus Ad5IκB. The recombinant replication-deficient adenovirus Ad5IκB was constructed by the methods of Graham et al. (22, 23). In brief, plasmid pRc/CMV-IκBα S32A/S36A (generous gift of Dr. Joseph Didonato, University of San Diego, San Diego, CA), which contains a hemagglutinin (HA)-tagged human superrepressor of NFκB, was subcloned into the XbaI site of the pACCMV.PLPASR (+) plasmid to construct the plasmid pACCMV/IκB, in which IκB is driven by the CMV promoter/enhancer. The plasmid DNA was prepared by the alkaline lysis method and purified by CsCl-ethidium bromide density gradient centrifugation. The recombinant adenovirus IκB was then constructed by cotransfection of the 293 embryonic human kidney cell line with the pACCMV/IκB plasmid plus the purified fragment of ClaI-digested DNA from E1-deleted adenovirus type 5 (Ad5). The presence of the mutant IκB sequence packaged into the recombinant Ad5 virus (Ad5IκB) was confirmed by PCR and by Western blotting, as described below. Ad5IκB was grown in 293 cells and purified by banding twice on CsCl gradients. Viral titers were determined by optical densitometry (particles per milliliter) and by plaque assay, and recombinant virus was then

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1. *Abbreviations used in this paper:* ALT, alanine aminotransferase; AP, alkaline phosphatase; HA, hemagglutinin; H&E, hematoxylin and eosin; pfu, plaque forming units; PH, partial hepatectomy; TNFRI, TNF receptor I.

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stored in 10% (vol/vol) glycerol at −20°C. Ad5LacZ, which contains *Escherichia coli* β-galactosidase gene, was also grown and purified as described above and used as a control virus.

Cell culture, viral infection, transfection, and reporter gene assay. Rat-1 fibroblasts were grown on 6-well culture plates (Costar Corp., Cambridge, MA) in MEM plus 10% FBS. When the fibroblasts reached 50–60% confluency, the medium was changed to MEM containing 2% FBS, and Ad5IκB or Ad5LacZ viral stock solutions were added to the medium at various multiplicities of infection (moi). After 24 h in some experiments, cells (70–80% confluent) were transfected with 0.5 μg of NFκB-Luc (a reporter plasmid containing one copy of an NFκB binding site [24]), with or without 0.1 μg of a p65 expression plasmid (a plasmid containing the NFκB p65 driven by a CMV promoter [25]) using Lipofectin reagent (GIBCO BRL, Gaithersburg, MD) per plate, and incubated in serum-free MEM. 24 h after transfection, cells were washed once with PBS, and medium was replaced with MEM containing 10% FBS. After an additional 24 h, recombinant human TNF-α (50 ng/ml) was added to the media in some wells. After a 5-h incubation, cellular extracts were prepared using enhanced luciferase assay reagents (Analytical Luminescence, San Diego, CA). Luciferase activity was measured on a Monolight 2010 for 15 s, and results were normalized for protein concentration. For NFκB electrophoretic mobility shifts assay, the transfected cells were treated with TNF-α (50 ng/ml) for 1 h and nuclear extracts were obtained as described below. For Western blot analysis, cells were collected after a 24-h infection with adenovirus, and whole cell extracts were prepared as described below.

Western blot analysis. Rat-1 fibroblasts infected with Ad5IkB or Ad5LacZ and samples of liver, spleen, kidney, lung, and heart were homogenized in lysis buffer (10 mM Hepes, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5% NP-40, and 25% glycerol) with protease inhibitors (4) at 4°C, followed by rotating the tubes for 30 min at 4°C. After centrifugation, cleared tissue lysates were collected and stored at −80°C for later analysis. Lysate containing 30 μg of protein was separated by electrophoresis on 10% acrylamide SDS gels and transferred to PVDF membranes as described previously (3). Equal loading was confirmed by Ponceau S staining. IκBα was detected using rabbit anti-IκBα antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were blocked in blocking buffer (5% nonfat dry milk in PBST) for 1 h, incubated overnight at 4°C in primary antibody, diluted 1:1,000 in blocking buffer, and then 1 h with horseradish peroxidase–conjugated goat anti–rabbit secondary antibody (Santa Cruz Biotechnology), diluted 1:1,000 in blocking buffer. Proteins were detected with ECL detection reagents (Amersham Corp., Arlington Heights, IL).

Nuclear extract preparation and electrophoretic mobility shift assay. Nuclear proteins were prepared from Rat-1 fibroblasts and the liver tissues using previously described methods (26). Protein-DNA binding reactions were carried out for 20 min on ice, using 5 μg nuclear extract, 10 pg of ^{32}P-labeled DNA probes for the NFκB consensus binding site (27), with or without 1 ng (100×) unlabeled competitor probe. Complexes were separated by electrophoresis on nondenaturing 5% acrylamide gels and assayed by autoradiography and PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). Antibodies against p65 or p50 subunit of the NFκB complex (Santa Cruz Biotechnology) were used for super-shift assays.

Adenoviral infection, PH, and BrdU labeling. Ad5IkB or Ad5LacZ was stored in 10% glycerol and was then twice dialyzed against a solution containing 10 mM Tris, pH 8.0, 1 mM $MgCl_2$, and 140 mM NaCl, for 2 h just before use. The viral solution containing 10^{10} plaque forming units (pfu) (0.5 ml) was injected into male Sprague-Dawley rats (220–240 g) via tail vein. 24 h after viral injection, two-thirds PH was performed according to the methods of Higgins and Anderson (28) under pentobarbital anesthesia. 11, 23, or 47 h after PH, animals were injected with BrdU (100 mg/kg; Sigma Chemical Co., St. Louis, MO) and killed 1 h later (n = 3–5 per time point). Blood samples were collected by puncture of abdominal aorta and serum was stored at −80°C until analysis. Samples of the liver were snap-frozen in liquid nitrogen or were fixed in 10% buffered formalin for subsequent histological analysis. Samples of spleen, kidney, lung, and heart were also collected and snap-frozen in liquid nitrogen. To assess BrdU staining at 0 h, some animals (n = 3) were injected with BrdU (100 mg/kg) at 23 h after viral injection and killed 1 h later. Control animals (n = 3) were injected with the same amount of saline, underwent two-thirds PH 24 h later, and then were killed 1 h later.

Histology and immunohistochemistry. Paraffin-embedded tissues were sectioned and stained with hematoxylin and eosin (H&E). For immunohistochemistry, sections were deparaffinized by four 5-min incubations in xylene, followed by several changes of ethanol (100, 95, 70, and 30%) and rehydration in dH_2O. DAKO Envision system™ (DAKO Corp., Carpinteria, CA) was used for BrdU and HA peptide epitope immunostaining. For BrdU assay, sections were incubated in 4 N HCl for 20 min at 37°C and washed several times with dH_2O and 1× PBS-Tween 20 (PBST; Sigma Chemical Co.). After blocking of endogenous peroxidase with peroxidase blocking agent, sections were incubated with mouse monoclonal anti-BrdU antibody (1:200 in PBS containing 5% BSA; Sigma Chemical Co.) for 10 min at 25°C. After two 3-min washes in PBST, sections were incubated with labeled polymer (peroxidase-labeled polymer conjugated to goat anti–rabbit and goat anti–mouse immunoglobulins) for 10 min at 25°C. Sections were then washed twice with PBST, incubated with 3,3-diaminobenzidine (DAB) substrate-chromogen for 8 min, washed with dH_2O, incubated with DAB enhancer (Innovex Biosciences, Richmond, CA) for 5 min, and washed with dH_2O before counterstaining with hematoxylin. BrdU-positive hepatocytes were counted in five high-power (×400) fields on each slide and the percentage of the BrdU-positive cells in total hepatocytes was determined after counting the number of hepatocytes in the same field. For detecting the HA-tagged IκB, mouse monoclonal anti-HA antibody (5 μl/ml, clone 12CA5; Boehringer Mannheim, Indianapolis, IN) was used as the primary antibody, and sections were stained as described above except for deleting the 4 N HCl incubation. Mitotic figures were also counted in the H&E-stained slides in the same way and expressed as a percentage of total hepatocytes.

In situ detection of apoptosis. Formalin-fixed and paraffin-embedded liver sections (5 μm) were deparaffinized in xylene and rehydrated through graded ethanols. The TUNEL assay was performed using the In Situ Cell Death Detection Kit, AP (Boehringer Mannheim). DNA ends were tagged in situ with dUTP using terminal deoxynucleotidyl transferase by incubating sections in a humidified chamber for 60 min at 37°C. Apoptotic cells were then detected with antifluorescein antibody conjugated with alkaline phosphatase (AP) for 30 min at 37°C. Slides were incubated with NBT/BCIP (Boehringer Mannheim) for 10 min at 25°C to visualize AP-positive cells, and then counterstained with hematoxylin. AP-positive cells were counted in five high-power fields on each slide and their percentage of total hepatocytes was determined.

Liver function tests. Blood samples were collected from each animal by aortic puncture before killing. Serum was stored at −80°C until measurement of alanine aminotransferase (ALT), bilirubin, and ammonia by automatic analysis by the UNC Division of Laboratory Animal Medicine.

RNA isolation and RT-PCR. Total RNA from liver was isolated by ultracentrifugation through a CsCl cushion (29). For RT-PCR, 1 μg of total liver RNA was reverse-transcribed using polyT oligonucleotide and MMLV reverse transcriptase (Perkin-Elmer/Applied Biosystems, Foster City, CA) in 25 μl. Rat TNF-α was amplified using primers TNF5′ (5′-CAC CAT GAG CAC GGA AAG CA-3′) and TNF3′ (5′-GCA ATG ACT CCA AAG TAG ACC-3′) (1 μM each) in a 50-μl PCR reaction containing 1.5 mM $MgCl_2$, 25 mM KCl, 10 mM Tris pH 9.2, and 1 μl deoxynucleotides (1 mM each) using 5 μl cDNA template. Dilutions of cDNA were amplified for 25, 28, 30, 33, and 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s to define optimal conditions for linearity to permit semiquantitative analysis of signal strength. The amplified PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Results

Assessment of a recombinant adenovirus expressing NFκB superrepressor. A superrepressor IκB contains serine-to-alanine mutations in residues 32 and 36, which inhibit its phosphorylation and proteosome-mediated degradation (12, 19, 20). We packaged an HA-tagged version of this κB S32A/S36A driven by the CMV promoter enhancer into the replication-deficient adenovirus Ad5 (Ad5IκB) (23, 30). Rat-1 fibroblasts were infected with this Ad5IκB adenovirus at a different moi, and then whole cell extracts were prepared for Western blot analysis. Using an anti-IκBα antibody, the HA-tagged IκB was identified by its slower mobility compared with the endogenous IκB by Western blotting, documenting the delivery of this cDNA by the adenoviral vectors (Fig. 1 *A*). The effectiveness of this Ad5IκB in blocking induction of NFκB was assessed by prior infection with Ad5IκB and then 24 h later treating the Rat-1 cells with TNF-α (50 ng/ml) for 1 h. Nuclear extracts were used in electrophoretic mobility shift assays to demonstrate the induction of NFκB DNA binding activity. Inhibition of NFκB DNA binding activity was demonstrated with Ad5IκB, but not the control adenovirus Ad5LacZ (Fig. 1 *B*). Specificity of the NFκB DNA binding interaction was demonstrated by competition with 100-fold excess cold oligonucleotide (Fig. 1 *B*). The effect of the Ad5IκB adenovirus on NFκB functional activity was assessed in a reporter gene assay in which the NFκB-Luc plasmid was transfected into Rat-1 fibroblast after infection with Ad5IκB or the controlled adenovirus Ad5LacZ. Using either p65 or TNF-α as an inducer of NFκB transcriptional activity, prior infection with Ad5IκB decreased both the control and the induced levels of the reported gene (Fig. 1, *C* and *D*). The residual stimulation of NFκB after Ad5IκB infection probably reflects the cells that were not infected by the adenovirus. Thus, Ad5IκB delivered a functional IκB superrepressor that inhibited NFκB DNA binding and transcriptional activity in cultured cells. The control adenovirus Ad5LacZ had no effect on basal and induced levels of NFκB transcriptional activity (Fig. 1, *C* and *D*), even though ~ 90% of cells were infected with Ad5LacZ at an moi of 1:50, as confirmed by β-galactosidase staining (data not shown).

In vivo infection with Ad5IκB. Rats were injected in their tail veins with 10^{10} pfu of Ad5IκB and killed 24 h later. Western blotting using anti-IκB antibody demonstrated high expression of the IκBα S32A/S36A in the liver with lower levels detected in the spleen (Fig. 2 *A*). After PH, rats infected with the control Ad5LacZ demonstrated a small increase in expression of IκBα after 2 h and persisting for 24 h, as previously reported during liver regeneration (31). On the other hand, Ad5IkB infection produced high levels of IκB at 0 h and for 12 h after PH with some decreased expression by 24 h after PH (Fig. 2 *B*). Immunohistochemistry using anti-HA antibody directed against the HA-tagged κB demonstrated that ~ 90% cells were expressing the NFκB superrepressor at 24 h after PH with localization in both the cytoplasm and nucleus (Fig.

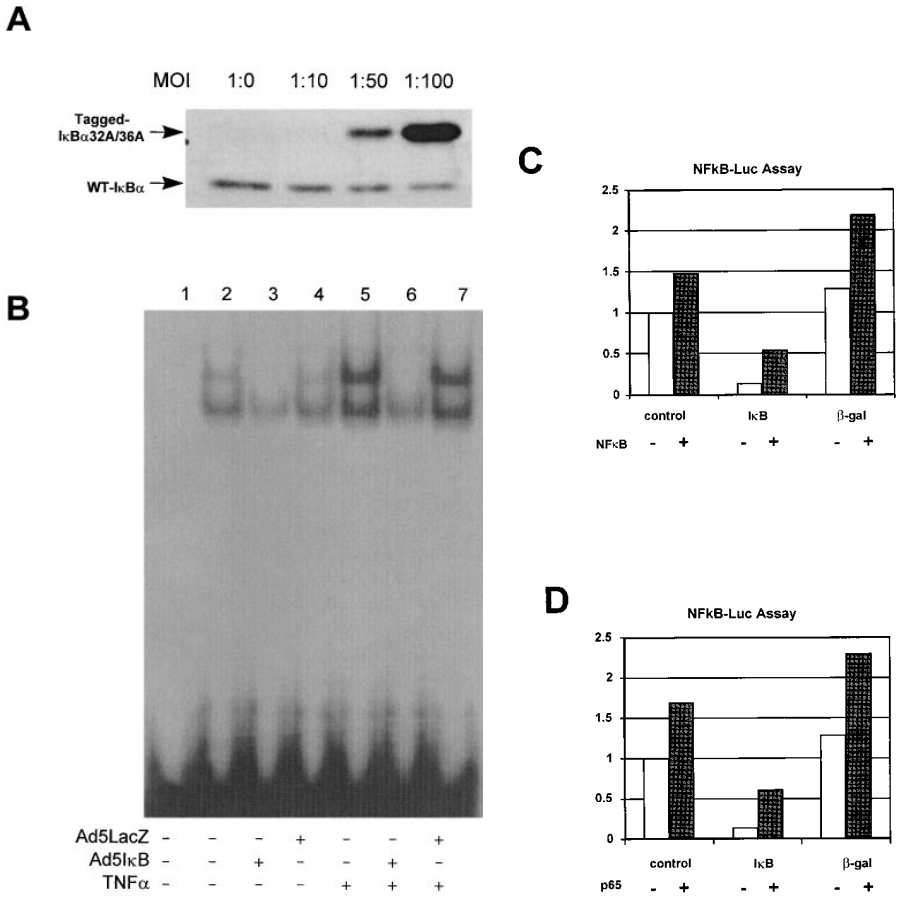

Figure 1. Expression of IκBα S32A/S36A inhibits NFκB activity in Rat-1 fibroblasts. Extracts were generated from Rat-1 fibroblasts 24 h after infection with Ad5IκB or Ad5LacZ, as described in Methods. (*A*) IκBα was detected in whole cell extracts by Western blotting using anti-IκB antibody. The HA-tagged IκBα was identified by its lower mobility compared with the endogenous IκBα. (*B*) NFκB DNA binding activity was assessed by electrophoretic mobility shift assay using an NFκB binding site as probe with nuclear extracts prepared after a 1-h incubation with vehicle (lanes *2–4*) or TNF-α (lanes *5–7*). Infection with Ad5IκB (lanes *3* and *6*) or Ad5LacZ (lanes *4* and *7*) was at an moi of 50:1. (*C* and *D*) A reporter gene assay was performed using NFκB-Luc. NFκB activity was induced by either a 5-h incubation with TNF-α (*C*) or a cotransfection with a p65 expression plasmid (*D*). Infection with Ad5IκB decreased both the basal and induced levels of the reporter gene.

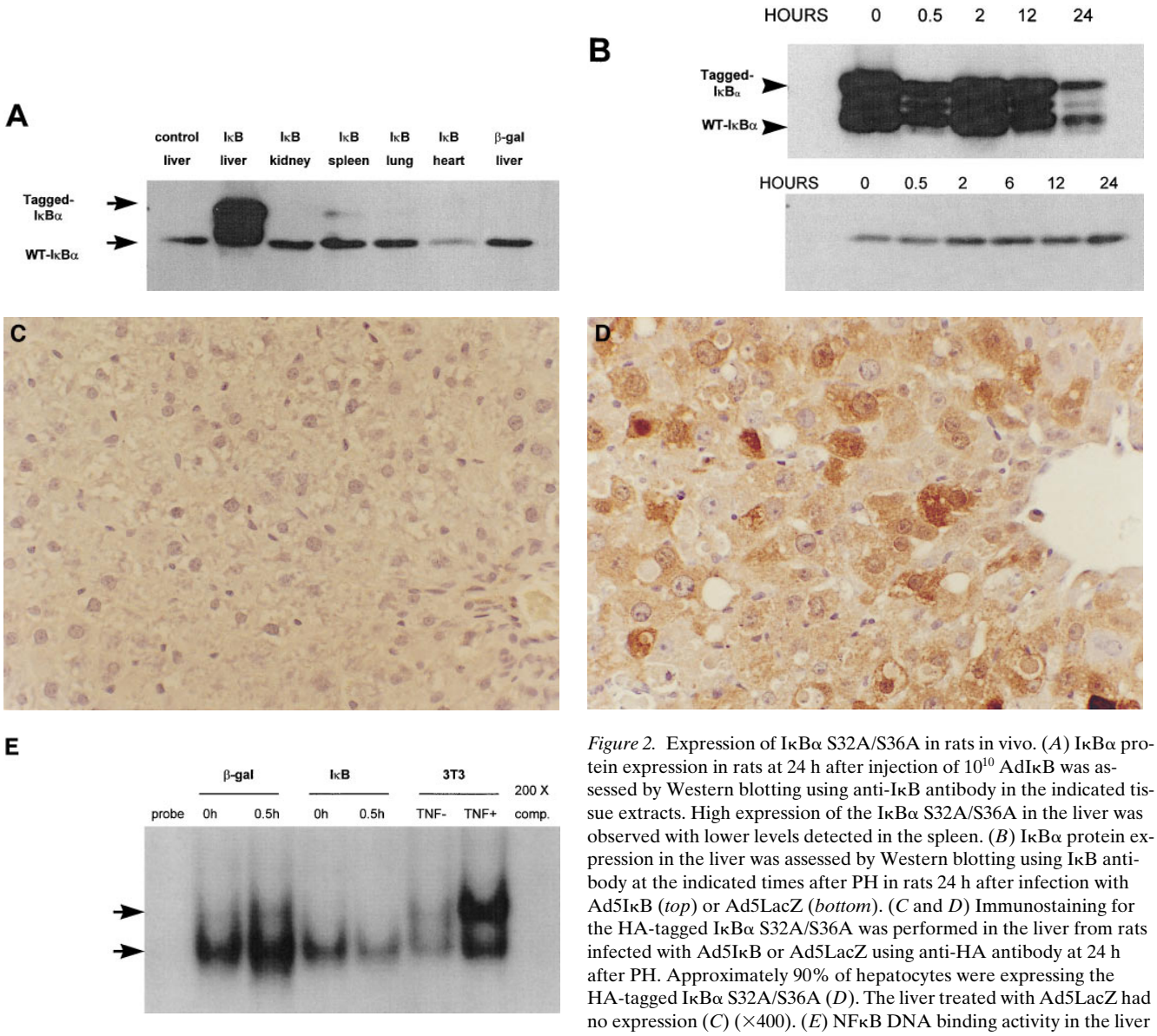

Figure 2. Expression of IκBα S32A/S36A in rats in vivo. (*A*) IκBα protein expression in rats at 24 h after injection of 10^{10} AdIκB was assessed by Western blotting using anti-IκB antibody in the indicated tissue extracts. High expression of the IκBα S32A/S36A in the liver was observed with lower levels detected in the spleen. (*B*) IκBα protein expression in the liver was assessed by Western blotting using IκB antibody at the indicated times after PH in rats 24 h after infection with Ad5IκB (*top*) or Ad5LacZ (*bottom*). (*C* and *D*) Immunostaining for the HA-tagged IκBα S32A/S36A was performed in the liver from rats infected with Ad5IκB or Ad5LacZ using anti-HA antibody at 24 h after PH. Approximately 90% of hepatocytes were expressing the HA-tagged IκBα S32A/S36A (*D*). The liver treated with Ad5LacZ had no expression (*C*) (×400). (*E*) NFκB DNA binding activity in the liver after PH was assessed by electrophoretic mobility shift assay using whole liver nuclear extracts prepared from Ad5LacZ-infected or IκB-infected rats at 0 or 0.5 h after PH. Infection with Ad5IκB abolished the activation of NFκB binding activity after PH. For comparison, extracts from untreated or TNF-α–treated NIH3T3 extracts are shown.

2 *D*). As discussed below, cells undergoing both mitosis or apoptosis expressed the HA tag. As a control, no expression of HA was detected in the Ad5LacZ-infected cells (Fig. 2 *C*).

Electrophoretic mobility shift assays demonstrated the early induction of NFκB binding activity after PH in the rats infected with the control Ad5LacZ (Fig. 2 *E*), as previously described during liver regeneration in control rats (6, 8). The constituents of the NFκB binding activity were demonstrated by super-shift of the p50 and p65 subunits (data not shown). On the other hand, infection with Ad5IκB before PH resulted in a decrease in the induction of NFκB binding activity, consistent with effective delivery and expression of the IκB superrepressor.

The IκB superrepressor induces apoptosis during liver generation. Histological examination of uninfected, Ad5LacZ-infected and Ad5IκB-infected rat livers after PH revealed key differences. At time 0, the Ad5LacZ and Ad5IκB livers showed subtle cellular changes with occasional apoptotic cells (Fig. 3, *B* and *C* compared with *A*). However, at 24 h after PH, the Ad5IκB-infected livers demonstrated a marked increase in the number of apoptotic cells (Fig. 3 *F*), as quantified in Fig. 3 *J* (*middle*). Although adenoviral infection alone produced limited apoptosis (Fig. 3 *J*, *left*), the full effect required both Ad5IκB and PH (Fig. 3 *J*, *middle*). The in situ TUNEL assay was used as an independent measure of apoptotic cells (Fig. 3, *G–I*). At 24 h after PH, the percentages of apoptotic cells were ~ 1% in the uninfected livers, 4% in the Ad5LacZ-infected livers, and 24% in the Ad5IκB-infected livers (TUNEL positive cells/hepatocytes) (Fig. 3 *J*, *right*). Thus, blocking of NFκB induction by the IκB superrepressor is associated with a strong stimulus to apoptosis of hepatocytes after PH.

Since TNF-α has been implicated as a potential comitogen

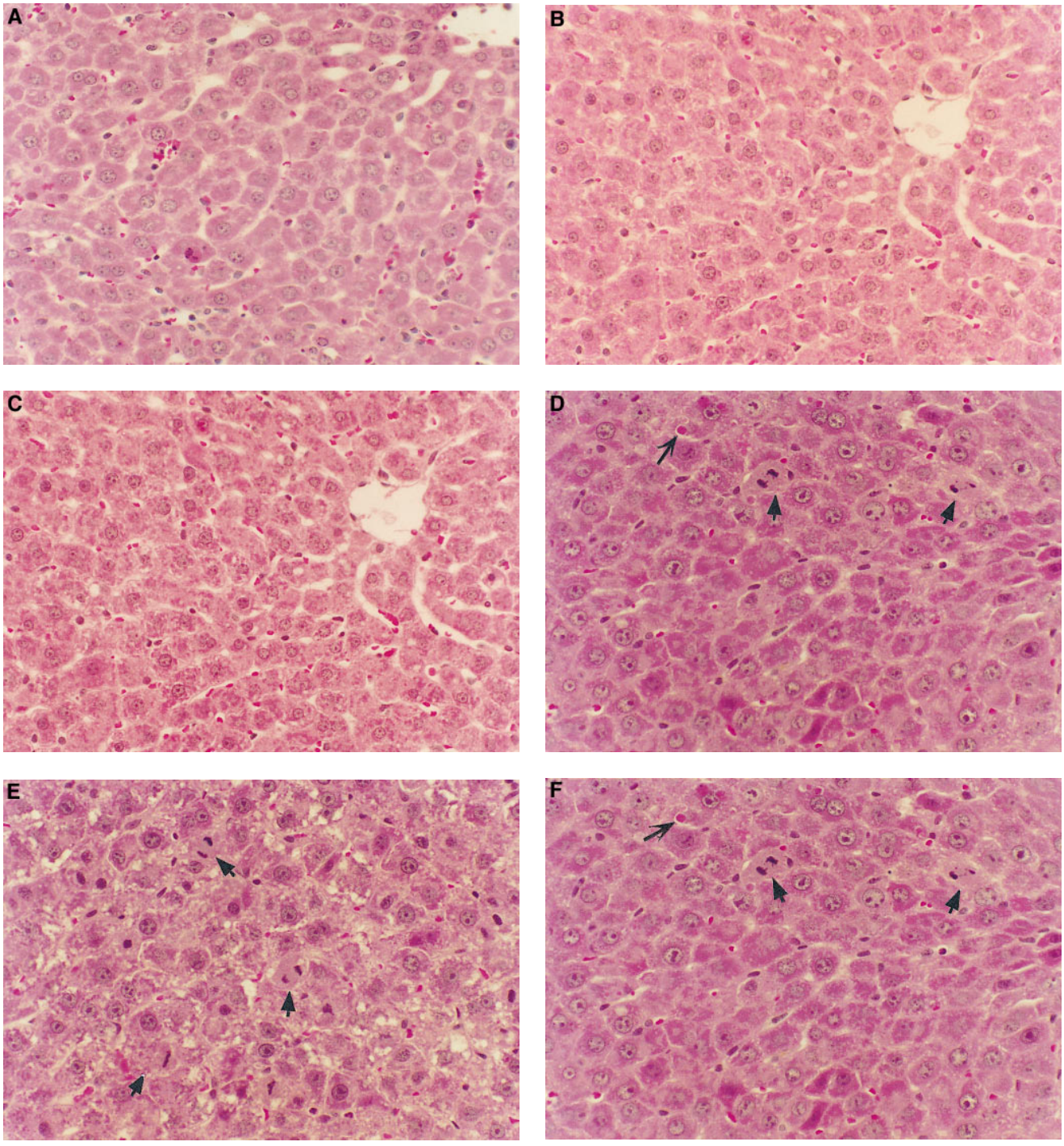

Figure 3. Induction of apoptosis in the liver after PH by IκBα S32A/S36A expression. Uninfected (*A*, *D*, and *G*), Ad5LacZ-infected (*B*, *E*, and *H*), and Ad5IκB-infected (*C*, *F*, and *I*) livers were stained with H&E at 0 (*A–C*) and 24 h (*D–F*) after PH (×400) or underwent in situ TUNEL assay to detect apoptotic cells (*G–I*) (×200). Ad5LacZ-infected (*B*) and Ad5IκB-infected (*C*) liver showed subtle changes with occasional apoptosis. At 24 h after PH, the Ad5IκB caused a dramatic increase in apoptotic cells (*F* and *I*). (*J*) Percentages of apoptotic cells among total hepatocytes were determined by counting apoptotic cells in the H&E staining (*left and middle*) or by TUNEL staining (*right*) as described in Methods. As an additional control, rats that did not undergo PH (24–0 h PH) at the identical time point after intravenous injection. (*K*) TNF-α mRNA expression in the liver after PH was assessed by RT-PCR of whole liver RNA prepared by uninfected, Ad5LacZ-infected, and Ad5IκB-infected rats after PH, as described in Methods. The presence of adenoviral infection either by Ad5LacZ or Ad5IκB led to induced levels of TNF-α mRNA before PH, but did not block TNF-α mRNA.

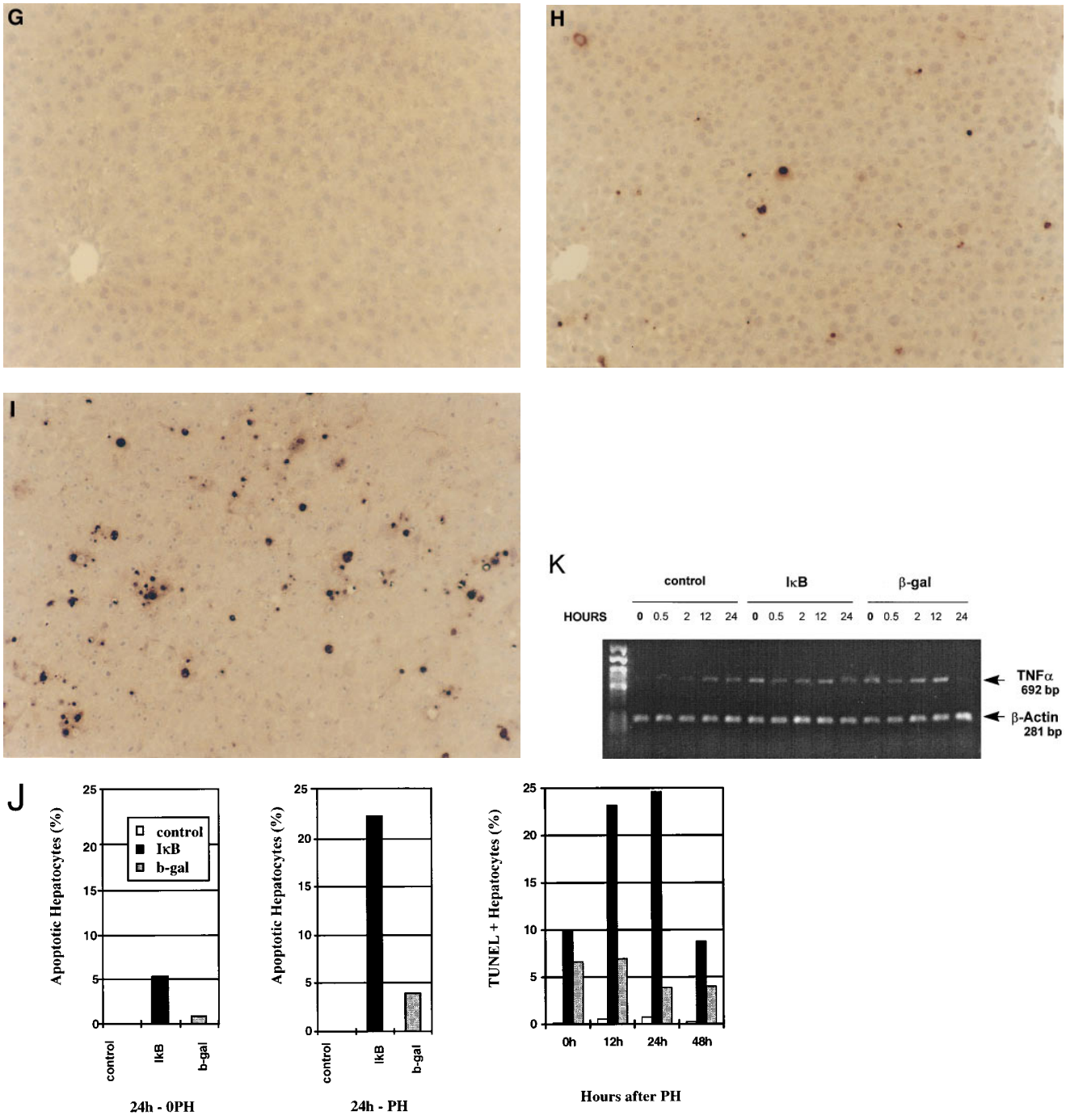

Figure 3 (Continued)

during liver regeneration, we wished to assess its expression in uninfected and infected livers after PH. As previously reported (32), TNF-α mRNA levels are induced by PH in the uninfected control animals (Fig. 3 *K*). Adenoviral infection with either Ad5LacZ or Ad5IκB led to induced levels of TNF-α before PH (Fig. 3 *K*). These TNF-α levels were maintained after PH. Thus, the IκB superrepressor did not inhibit TNF-α expression.

Inhibition of hepatocyte proliferation by IκB superrepressor. DNA synthesis after PH was assessed by BrdU incorporation in uninfected, Ad5LacZ-infected, or Ad5IκB-infected livers (Fig. 4, *A–C*). Both the Ad5LacZ and Ad5IκB infections resulted in an increase in the fraction of hepatocytes in S phase even before PH (Fig. 4 *D*). At 24 and 48 h after PH, DNA synthesis, detected as percentage of BrdU-positive hepatocytes, increased in similar magnitude in the noninfected, Ad5IκB-infected, and Ad5LacZ-infected livers (Fig. 4 *D*). Thus, neither viral infection itself nor expression of the IκB superrepressor inhibited DNA synthesis in hepatocytes during liver regeneration after PH. The effect on hepatocyte mitosis was assessed by quantifying the mitotic index after PH (Fig. 4 *E*, *right*) or without PH as an additional control (Fig. 4 *E*, *left*). Ad5LacZ-

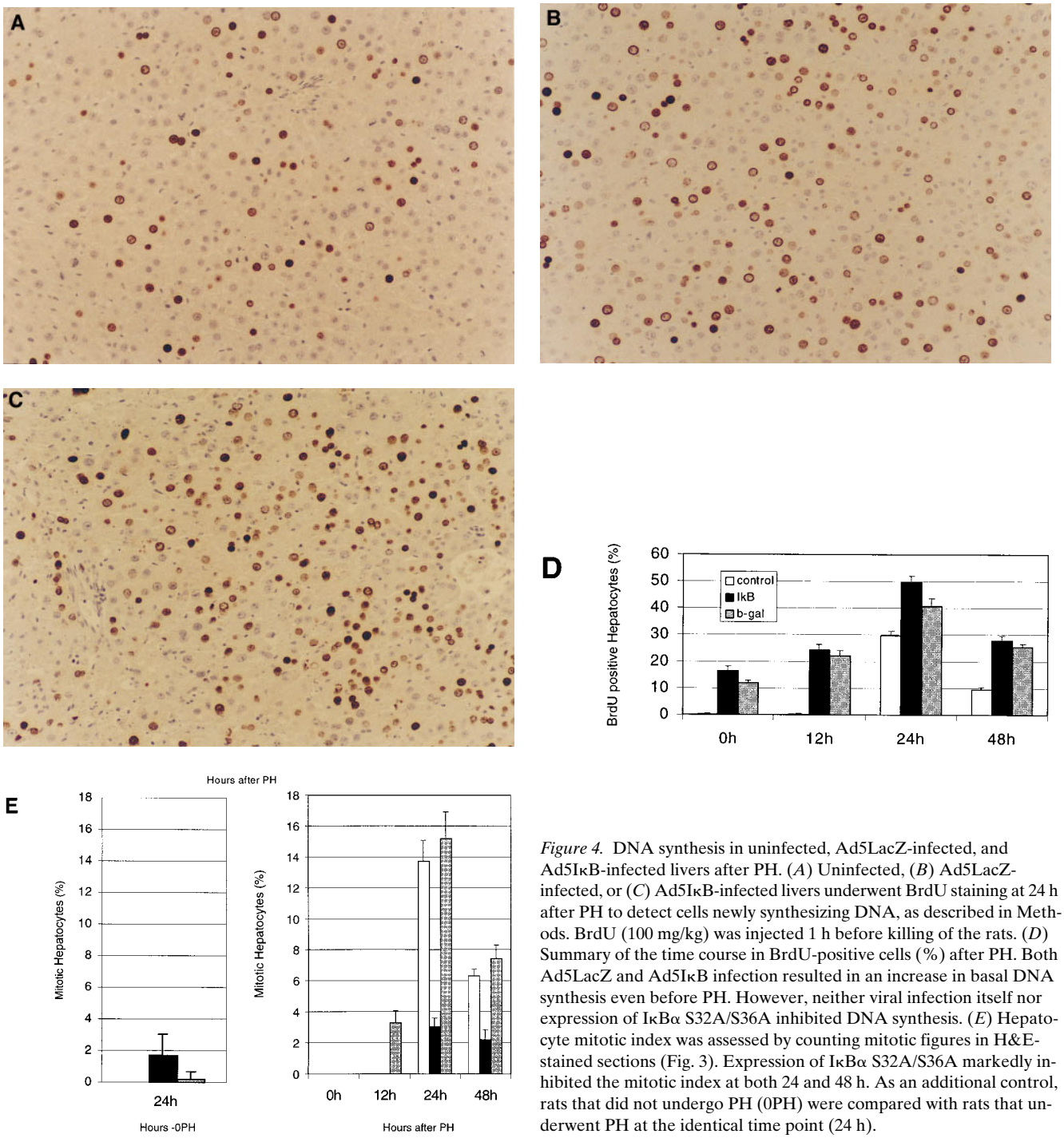

Figure 4. DNA synthesis in uninfected, Ad5LacZ-infected, and Ad5IκB-infected livers after PH. (*A*) Uninfected, (*B*) Ad5LacZ-infected, or (*C*) Ad5IκB-infected livers underwent BrdU staining at 24 h after PH to detect cells newly synthesizing DNA, as described in Methods. BrdU (100 mg/kg) was injected 1 h before killing of the rats. (*D*) Summary of the time course in BrdU-positive cells (%) after PH. Both Ad5LacZ and Ad5IκB infection resulted in an increase in basal DNA synthesis even before PH. However, neither viral infection itself nor expression of IκBα S32A/S36A inhibited DNA synthesis. (*E*) Hepatocyte mitotic index was assessed by counting mitotic figures in H&E-stained sections (Fig. 3). Expression of IκBα S32A/S36A markedly inhibited the mitotic index at both 24 and 48 h. As an additional control, rats that did not undergo PH (0PH) were compared with rats that underwent PH at the identical time point (24 h).

infected livers had an increased mitotic index at 12 h after PH compared with the uninfected livers, and the mitotic index was equally increased at 24 and 48 h after PH, reflecting the elevated fraction of hepatocytes in S phase. In contrast, expression of the IκB was associated with a marked reduction in the mitotic index at both 24 and 48 h. These results are consistent with expression of the IκB superrepressor either inhibiting transition of hepatocytes through G2 during liver regeneration after PH or leading to the massive apoptosis of cells located in late S or G2 phases. Either event would explain the observed reduction of the fraction of hepatocytes in mitosis.

The effect of Ad5IκB on liver function during liver regeneration. The extent of necrosis to hepatocytes was assessed by measuring the liver-specific enzyme ALT in the serum of the rats after PH. As shown in Fig. 5 *A*, there was only a slight increase in serum ALT in the Ad5IκB-infected animals compared with the uninfected and Ad5LacZ-infected rats, suggesting that little leakage of this enzyme had occurred. However, hepatic function was compromised by apoptotic loss of hepatocytes, as indicated by a twofold increase in total bilirubin in the Ad5IκB-treated rats compared with the Ad5LacZ-treated rats (Fig. 5 *B*). Furthermore, incipient liver failure in the

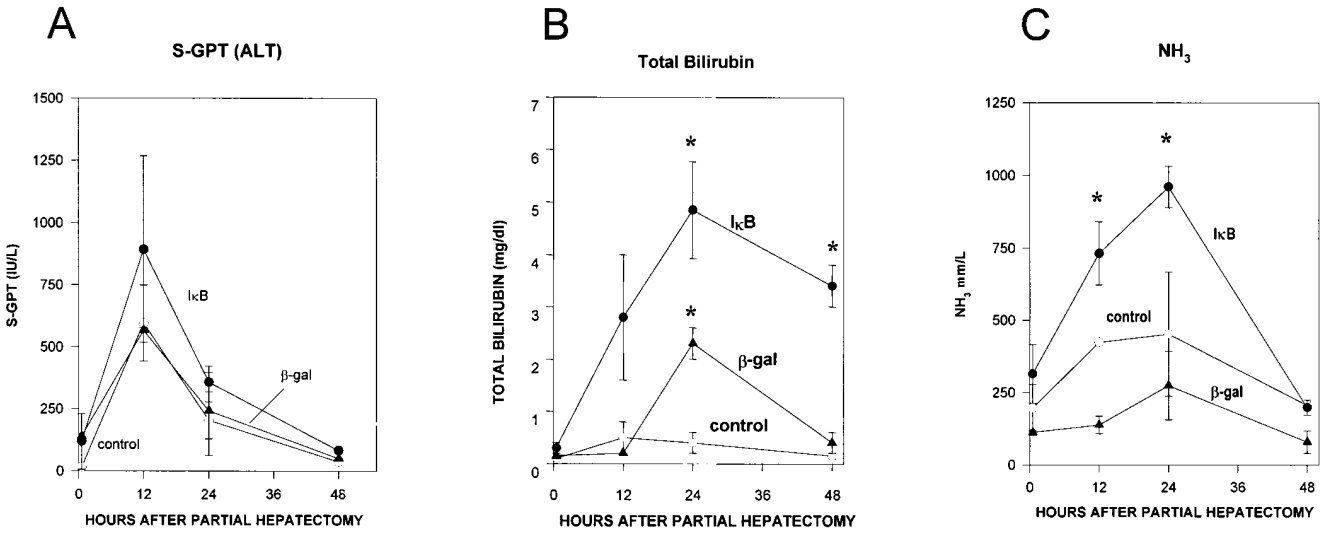

Figure 5. Liver function tests after PH. (*A*) Serum ALT, (*B*) total bilirubin, and (*C*) ammonia were measured in uninfected, Ad5LacZ-infected, and Ad5IκB-infected rats. *Significantly greater than values in uninfected control animals.

Ad5IκB-infected animals was indicated by the increased ammonia level compared with the uninfected and Ad5LacZ-infected animals, which were indistinguishable after PH (Fig. 5 *C*). Thus, the pathophysiological sequelae of expression of the IκB superrepressor resulted in jaundice, as documented by increased total bilirubin, and liver dysfunction as documented by ammonia, both possibly the result of massive cell loss from apoptosis, although more subtle metabolic changes in surviving cells cannot be ruled out. The lack of an increase in ALT reflects the predominant mechanism of cell death, i.e., apoptosis, and not necrosis.

Discussion

After PH, there is a rapid transient induction of DNA binding by NFκB (6–8, 10). The NFκB binding peaks at 1 h after PH and subsequently declines. The NFκB consists of p50/p65 heterodimers, p50 homodimers, and PHF, a complex of p50 and p35 (a truncated p65) (6, 10, 21). The physiological consequences of this induction of NFκB after PH are unknown. It might be a nonspecific response to the physiological stress or PH, it might be required for normal cellular proliferation, or it might fulfill other unknown functions in hepatic regeneration.

We efficiently delivered to the liver a recombinant adenovirus expressing the IκB superrepressor, which has been validated in a variety of cultured cells in transfection experiments and now through viral delivery. After PH, infection with Ad5IκB, but not a control adenovirus, resulted in the massive induction of apoptosis as demonstrated by histological staining and TUNEL analysis. In addition, infection with AD5IκB, but not Ad5LacZ, markedly decreased the mitotic index after PH. These two phenomena, increased apoptosis and failure to progress through the cell cycle, were associated with liver dysfunction, as demonstrated by elevated serum bilirubin and ammonia levels.

The requirement for IL-6 and TNF-α in normal hepatic regeneration after PH has been established by the use of IL-6 and TNF receptor I (TNFRI) knockout mice (1, 9, 33). PH in the IL-6 knockout mice resulted in liver necrosis and liver failure with G1 phase abnormalities including absence of STAT3 activation (9). PH in the TNFR1 knockout mice resulted in decreased DNA synthesis, delayed restoration of liver mass, and increased mortality, with an absence of STAT3 and NFκB activation (33). In both knockout mice, normal hepatic regeneration was restored by the administration of exogenous IL-6 (9, 33), suggesting that TNF-α binds to the TNFR1 of Kupffer cells, inducing secretion of IL-6 (1). The IL-6 then binds to its receptor on hepatocytes, leading to the activation of STAT3.

Similar studies could not be performed to assess the role of NFκB in hepatic regeneration because NFκB p65 knockout mice are embryologically lethal (21). As an alternative approach, we took advantage of the recombinant adenovirus as a gene delivery system to express the IκB superrepressor in the adult rat liver. Adenovirus has been used extensively to efficiently deliver genes specifically to the liver (34–37) in a variety of animals. The adenoviral vector gene delivery leads to short-term expression of genes in hepatocytes (35, 37, 38). In a previous study, an adenoviral vector delivered p53 to the liver, and its expression had no effect on hepatic regeneration or hepatocyte function (35). However, the control experiments in a recent study (39) and our study (Fig. 4) demonstrate that the adenovirus vectors alone induce DNA synthesis in 10% of hepatocytes.

Toxicity of the viral proteins is of concern with these first generation adenoviral vectors, including the induction of apoptosis and immune responses (40, 41). In our study, two different control groups were consistently used: non–viral-infected and control AdLacZ-infected animals. The dose of adenovirus was optimized in pilot experiments at 10^{10} pfu per injection. Using these control groups in short-term experiments, we were able to minimize the adenoviral effect and differentiate it from the specific IκB effect. At 24 h after PH, the number of apoptoic hepatocytes was 1% in uninfected livers, 4% in Ad5LacZ-infected livers, and 24% in Ad5IκB-infected livers (Fig. 3).

Thus, in our study and in previous studies (35, 42, 43), the adenoviral vector is an efficient method to deliver genes of interest to assess their transient effect in the liver in vivo.

Although the factors responsible for induction of NFκB during liver regeneration are still under investigation, the most likely candidate is TNF-α. TNF-α expression is induced during liver regeneration after PH (44). Neutralizing antibodies to TNF-α inhibit hepatocyte DNA synthesis during liver regeneration after PH (45). TNF-α is a potent inducer of NFκB in a variety of cell lines and will induce NFκB in vivo in the liver (8); and NFκB is not activated in TNFRI knockout mice (33). Finally, TNF-α will stimulate DNA synthesis in primary hepatocyte cultures (45). In our study, pretreatment with either Ad5IκB or Ad5LacZ did not block TNF-α mRNA levels as documented by RT-PCR, and pretreatment with Ad5LacZ did not block NFκB.

TNF-α, ionizing radiation, and daunorubicin induce both NFκB and apoptosis. The relationship between NFκB and apoptosis was elucidated recently by studies demonstrating that inhibition of NFκB, either by the IκB superrepressor (19, 20) or in p65 knockout cells (46), results in increased apoptosis by these agents. TNF-α mediates stimulation of NFκB as well as JNK through the TNF receptor–associated protein TRAF2 (TNF receptor–associated factor 2) (47–49). The signal for apoptosis is mediated by the TNF receptor–associated protein TRADD (TNF receptor–associated death domain) which in turn interacts with FADD (Fas-associated death domain protein) leading to activation of proapoptotic proteases (capases) (50, 51). The mechanism by which NFκB blocks apoptosis is unknown. In our study, inhibition of NFκB by the IκB superrepressor revealed an underlying apoptotic stimulus occurring during liver regeneration after PH. As discussed above, a possible explanation is that the enhanced TNF-α expression required for normal liver regeneration activates both apoptotic and NFκB signals in hepatocytes. The antiapoptotic activity of NFκB prevents apoptosis in hepatocytes during normal liver regeneration after PH. Thus, the IκB superrepressor unmasks this apoptotic stimuli, demonstrating a critical role for hepatocyte NFκB in normal liver regeneration.

We have less insight into the mechanism by which inhibition of NFκB results in the decreased mitotic index during liver regeneration at 24 and 48 h after PH. An association of NFκB binding activity with the G0 to G1 transition has been reported (11) but a relationship to the G2 phase of the cell cycle has not been demonstrated. Since DNA synthesis as documented by BrdU labeling is normal despite the inhibition of NFκB, there does not appear to be a block in the G1 phase. Alternatively, inhibition of NFκB may have shifted the mitotic index to a time point that was not measured by our study.

The timing of apoptosis after PH, the entry of the expected fraction of cells into the S phase, and the severity of subsequent inhibition of mitosis suggest that apoptosis is occurring during the S and/or G2 phase in the presence of the IκB superrepressor. On the other hand, if apoptosis occurred predominantly in the G1 phase or the G1/S checkpoint, the apoptosis would occur earlier after PH and the number of S phase cells would be reduced compared with controls. Previous studies have demonstrated that during cell cycle transit, apoptosis may be triggered in cells delayed at the G1/S checkpoint, the G2/M checkpoint, as well as during S phase or M phase when DNA synthesis or spindle formation is perturbed in major ways (44, 52, 53). We propose that inhibition of NFκB results in apoptosis (and not cell-cycle arrest) in the S or G2 phases of cell cycle progression during liver regeneration. A product of an NFκB-responsive gene may be required to block apoptosis and/or enable cell cycle progression.

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