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Constitutive Activation of NF-κB in Human Hepatocellular Carcinoma: Evidence of a Cytoprotective Role

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ABSTRACT

Activation of nuclear factor-κB (NF-κB) can promote or inhibit apoptosis. Oxidative stress is an important mechanism by which certain anticancer drugs kill cancer cells, and is also one of the mechanisms that activate NF-κB. We therefore examined hepatic expression of the NF-κB monomer p65 in human hepatocellular carcinoma (HCC) tissue samples from eight patients and compared it with their respective samples of surrounding liver tissues. We also studied the effect of NF-κB inhibition in human HCC cells exposed to oxidative stress, by infecting HuH7 cells with a recombinant adenovirus carrying mutant IκBα (mIκBα). Cultured HuH7 cells were infected with mIκBα or β-galactosidase (β-Gal) for 24 hr followed by treatment with increasing concentrations of H_2O_2. Cytotoxicity, NF-κB translocation, NF-κB DNA binding, cell proliferation, and apoptosis were determined. The monomer p65 was overexpressed in six of eight human HCC tissues. In HuH7 cells, introduction of mIκBα potently inhibited the translocation, activation, and DNA binding of NF-κB. In control (β-Gal-infected) HuH7 cells, exposure to H_2O_2 produced a dose-dependent increase in apoptosis, regardless of NF-κB status. mIκBα-mediated inhibition of NF-κB activation sensitized HuH7 cells to H_2O_2-induced inhibition of cell growth, and further promoted cell death. Addition of H_2O_2 (200–500 μM) to control or mIκBα-infected HuH7 cells enhanced caspase-3 activity and cleavage. Adenovirus-mediated transfer of mIκBα potently inhibits NF-κB activity in HuH7 cells, and this enhances oxidative stress-induced cell killing.

INTRODUCTION

ONE OF THE MAJOR CHALLENGES in cancer treatment is the resistance of tumor cells to therapy-induced apoptosis, which is considered to be the major mode of cell death with chemotherapeutic agents or radiation therapy. Hepatocellular carcinoma (HCC) is one of the most common hepatic malignancies worldwide, and its incidence in the United States and Australia has risen (El-Serag and Mason, 1999; Law *et al.*, 2000). Because of the poor effects of current treatment modalities, novel approaches of treating HCC are needed.

Oxidative stress is one of the mechanisms by which radiation therapy and certain cancer therapeutic drugs kill cancer cells. The mechanisms include suppression of cell proliferation and triggering of cell death, either by apoptosis or necrosis depending on the cellular context and cell type. Nuclear factor-κB (NF-κB) is a redox-sensitive transcription factor that is universally present in all eukaryotic cells. The 90 or so genes that are governed by NF-κB include cytokines and proinflammatory molecules, cell cycle genes, and genes that either activate and facilitate cell death process, or oppose it and provide cellular protection. To avoid inappropriate unleashing of such a biologically pivotal set of consequences, NF-κB is normally kept inactive in the cytoplasm by binding to inhibitory proteins such as IκB (Karin, 1999). Many agents or stimuli such as cytokines, bacterial products (lipopolysaccharide [LPS]), viruses (e.g.,

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hepatitis C virus [HCV] infection) and their proteins, partial hepatectomy, tumor necrosis factor-α (TNF-α), chemotherapeutic agents, and radiation therapy can activate NF-κB. On stimulation, IκB is phosphorylated at two NH$_2$-terminal serine residues (S^{32} and S^{36}) by an IκB kinase (IKK) complex; this permits ubiquitination and targeting to the 26S proteasome (Henkel *et al.*, 1993; Palombella *et al.*, 1994), where the complex is cleaved to liberate NF-κB dimers. These dimeric complexes of NF-κB proteins are free to enter the nucleus and bind to recognition sites that either stimulate or inhibit transcription of the target genes (Brown *et al.*, 1995).

In many cell types, particularly cancer cells, NF-κB is a potent antiapoptotic factor. Deregulation of apoptosis has been implicated in the pathogenesis of HCC complicating chronic hepatitis B virus (HBV) and HCV infection (Bantel and Schulze-Osthoff, 2003; Staib *et al.*, 2003). However, the underlying mechanisms by which apoptosis is downregulated in the pathogenesis of HCC are incompletely understood. The effects of NF-κB modulation on cell survival depend on both the cell type and the nature of NF-κB inducer (Barkett and Gilmore, 1999). Thus, inactivation of NF-κB sensitizes hepatocytes to TNF-induced apoptosis (Van Antwerp *et al.*, 1996), whereas after partial hepatectomy, TNF-mediated activation of NF-κB is required for hepatocyte survival and progression into the cell cycle (Iimuro *et al.*, 1998; Webber *et al.*, 1998; Plumpe *et al.*, 2000). On the other hand, inhibition of NF-κB by its superrepressor during experimental obstructive cholestasis does not affect the rate of apoptosis (Bird *et al.*, 2003), and actually suppresses hepatic DNA synthesis. In cancer cells, the role of NF-κB inhibition remains even more controversial. Several reports have demonstrated that NF-κB inhibition can sensitize cancer cells, leading to their death by anticancer drugs (Van Antwerp *et al.*, 1996; Wang *et al.*, 1996, 1999; Feig *et al.*, 1999; Patel *et al.*, 2000; Arlt *et al.*, 2001), whereas others found that suppression of NF-κB function failed to change the sensitivity of cancer cells to therapy (Cai *et al.*, 1997; Li *et al.*, 1997; Bentires-Alj *et al.*, 1999). In HCC cells, inhibition of NF-κB suppresses rather than sensitizes the cells to doxorubicin (Adriamycin)-induced apoptosis (Tietze *et al.*, 2000), suggesting that NF-κB may be proapoptotic rather than antiapoptotic in HCC cells. In our work, however, we demonstrated that rat hepatoma ARL-6 cells were relatively more resistant to prooxidant H$_2$O$_2$-induced cell killing compared with normal rat hepatocytes, and inhibition of NF-κB in ARL-6 cells led to sensitization of these cells to H$_2$O$_2$-induced apoptosis (Qiao *et al.*, 2005). NF-κB has become a new target of gene therapy in many malignant conditions. Some NF-κB modulators, especially those with NF-κB-inhibitory capabilities, have been investigated for their efficacy in enhancing the apoptotic response to therapeutic agents, such as BAY 11-7082 (Dai *et al.*, 2004) and bortezomib (PS-341) for the treatment of patients with advanced multiple myeloma (Voorhees *et al.*, 2003; Panwalkar *et al.*, 2004).

In this study, we aimed to examine whether NF-κB inhibition by mutant IκBα (mIκBα) in HuH7 cells (a human HCC cell line) could facilitate the killing effects of the prooxidant H$_2$O$_2$ on these cells. mIκBα has previous been shown to specifically block NF-κB activation in many cell types without affecting other transcription factors (Hellerbrand *et al.*, 1998; Jobin *et al.*, 1998; Feig *et al.*, 1999; Heimberg *et al.*, 2001; Sato *et al.*, 2003).

The results indicate that abrogation of NF-κB activation sensitizes human HCC cells to oxidative stress-induced cell killing and proliferation inhibition.

MATERIALS AND METHODS

Cell culture

HuH7 cells (derived from a human HCC) were obtained from the American Type Culture Collection (Cryosite, Sydney, Australia). They were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Melbourne, Australia) supplemented with 10% fetal calf serum (FCS), streptomycin (100 μg/ml), penicillin (100 μg/ml), and 2 mM L-glutamine in a humidified atmosphere of 5% CO$_2$ at 37°C.

Hepatic tissues from patients with HCC

We collected malignant (HCC) tissues and their matched surrounding nonmalignant liver tissues from eight patients who had liver resection for HCC. These tissues were from four patients with well-differentiated HCC (samples 1–4) and from four patients with poorly differentiated HCC (samples 5–8), all with negative HBV and HCV serology. Written consent was obtained from each patient before tissue collection.

Infection of HuH7 cells with adenoviral vectors

Adenoviral vectors carrying the IκBα superrepressor (mIκBα) or β-galactosidase (β-Gal) gene were used in this study. To obtain high-titer viral vectors, 2×10^7 HEK293 cells (American Type Culture Collection) were infected at a multiplicity of infection (MOI) of 10. The infected cells were maintained under routine culture conditions for several days until a cytopathic effect was evident. Virus-infected HEK293 cells were collected, and viral particles were released by repeated freeze–thaw cycles. The resultant crude virus preparation was purified with a BD Adeno-X virus purification kit (BD Biosciences Clontech, Sydney, Australia), the optical density at 260 nm (OD$_{260}$) was measured, and the virus titer was determined, as per the manufacturer's instructions. HuH7 cells were cultured to 60–70% confluence before being incubated for 4 hr in serum-free medium with adenoviral vectors carrying mIκBα or β-Gal. Cells were then cultured in complete growth medium for up to 24 hr to allow adequate expression of the transgenes before commencement of further experiments.

Infection efficiency in cultured HuH7 cells was determined with β-Gal as the reporter gene, as described elsewhere (Feig *et al.*, 1999). The viral infection rate was determined by calculating the number of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)-stained cells, and by expressing the results as the ratio of X-Gal-positive cells to the total number of cells counted. Infection of cells occurred in the expected dose-dependent manner: with viral concentrations < 200 plaque-forming units (PFU)/cell, HuH7 cells showed virtually no X-Gal staining, whereas abundant X-Gal-positive cells were observed with increasing viral concentrations. On the basis of the X-Gal staining assay, both β-Gal and mIκBα were used at 5000 PFU/cell in most of the subsequent studies unless otherwise specified.

Determination of viability

After addition of adenoviral vectors and/or H_2O_2 for 24 hr, cell viability was determined by 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1; Roche Diagnostics, Sydney, Australia) assay, as per the manufacturer's instructions.

Extraction of cytoplasmic and nuclear proteins

Proteins from cytoplasmic and nuclear fractions of cells were extracted as previously reported (Teoh *et al.*, 2003). Protein concentrations were estimated with a Bradford protein assay kit (Bio-Rad, Hercules, CA).

Assessment of NF-kB activation

Activation of NF-κB was assessed by two complementary methods. Immunofluorescence microscopy was used to examine the translocation of NF-κB p65 subunit, as described previously (Birbach *et al.*, 2002). NF-κB DNA-binding activity was determined by electrophoretic mobility shift assay (EMSA), as reported elsewhere (Teoh *et al.*, 2002). Supershift was not routinely performed with each experiment, as our laboratory has previously confirmed the target identity (Teoh *et al.*, 2002).

TdT-mediated dUTP nick end-labeling assay

The TdT (terminal deoxynucleotidyltransferase)-mediated dUTP nick end-labeling (TUNEL) assay was performed as previously reported (Qiao *et al.*, 2002) with a commercially available assay kit (*In Situ* cell death detection kit; Roche Diagnostics). Briefly, cells were fixed with 3% paraformaldehyde, incubated with TUNEL reagent for 60 min, rinsed with phosphate-buffered saline (PBS), and incubated with Converter-POD; color was developed by incubation with 3,3′-diaminobenzidine (DAB) substrate. After counterstaining with hematoxylin, cells were washed, air dried, mounted, and examined by light microscopy. The number of TUNEL-positive cells was counted and the result expressed as the percentage of positive cells relative to the total number of cells counted.

Determination of protein expression by immunoblotting: Western blot

Sixty micrograms of protein extract from each sample was resolved by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and electrotransferred onto nitrocellulose membranes (Millipore, Bedford, MA), as previously reported (Teoh *et al.*, 2002). The membranes were blocked with 5% nonfat milk and probed overnight with primary antibodies against p65, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), Bax, and Bcl-X_L (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed and incubated with species-specific secondary antibodies conjugated with horseradish peroxidase, which were detected with SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL), and exposed to Hyperfilm (Amersham Biosciences, Castle Hill, Sydney, Australia).

Measurement of caspase-3 activity

Caspase-3 activity was analyzed with an ApoAlert caspase-3 kit (BD Biosciences Clontech), which measures proteolytic cleavage of the chromophore *p*-nitroanilide (pNA). Cells were harvested, washed with PBS, lysed in ice-cold cell lysis buffer, and centrifuged at $14,000 \times g$ for 15 min. Fifty micrograms of supernatant was incubated at 37°C for 3 hr in reaction buffer containing DEVD–pNA. Caspase-3 activity was determined from the count of pNA released, as determined at 405 nm with a Wallac 1420 VICTOR^2 microplate reader (PerkinElmer, Melbourne, Australia).

Assessment of cell proliferation

Cell proliferation was determined by [^{3}H]thymidine incorporation into cellular DNA (Qiao *et al.*, 2001).

Statistical analyses

Results are presented as means $\pm$ SD. Significant differences between groups were determined by Student *t* test.

RESULTS

Expression level of NF-κB in human HCC and nontumorous liver

We compared the expression levels of p65 in eight HCC tissues with their respective levels in surrounding nontumorous liver by Western blot, and revealed that in six patients, p65 was expressed at much higher levels in HCC than in nontumorous tissue (Fig. 1A). In six liver specimens, the constitutive activation of NF-κB p65 was also demonstrated by EMSA (Fig. 1B). Incidentally, we detected a strong binding complex in the cancerous liver of one patient (Fig. 1B). The nature of this complex is not clear. In three cases (patients 3, 4, and 6) in which p65 was richly expressed, p65 was also detected in the nuclear compartments of the HCC tissue (but not in nontumorous liver) (Fig. 1C), indicating constitutive activation. The average expression level of NF-κB was not significantly different between well and poorly differentiated HCCs.

Expression level of NF-κB in HuH7 cells versus nontumorous liver

As HuH7 cells were derived from human HCC, we also compared the basal expression level of NF-κB p65 in this cell type with that in nontumorous tissues obtained from two patients who had liver resection for HCC. By Western blot, p65 was also found to be richly expressed in HuH7 cells compared with two samples of nontumorous tissue (Fig. 1D). EMSA also demonstrated constitutive activation of NF-κB in HuH7 cells compared with noncancerous hepatic tissues (Fig. 1D, arrowhead).

These data suggested that NF-κB is either overexpressed and/or constitutively activated in most HCC tissues.

Infection efficiency of adenoviral vectors in HuH7 cells

On the basis of the above results, we attempted to block NF-κB activity in HCC cells by adenovirus-mediated transfer of
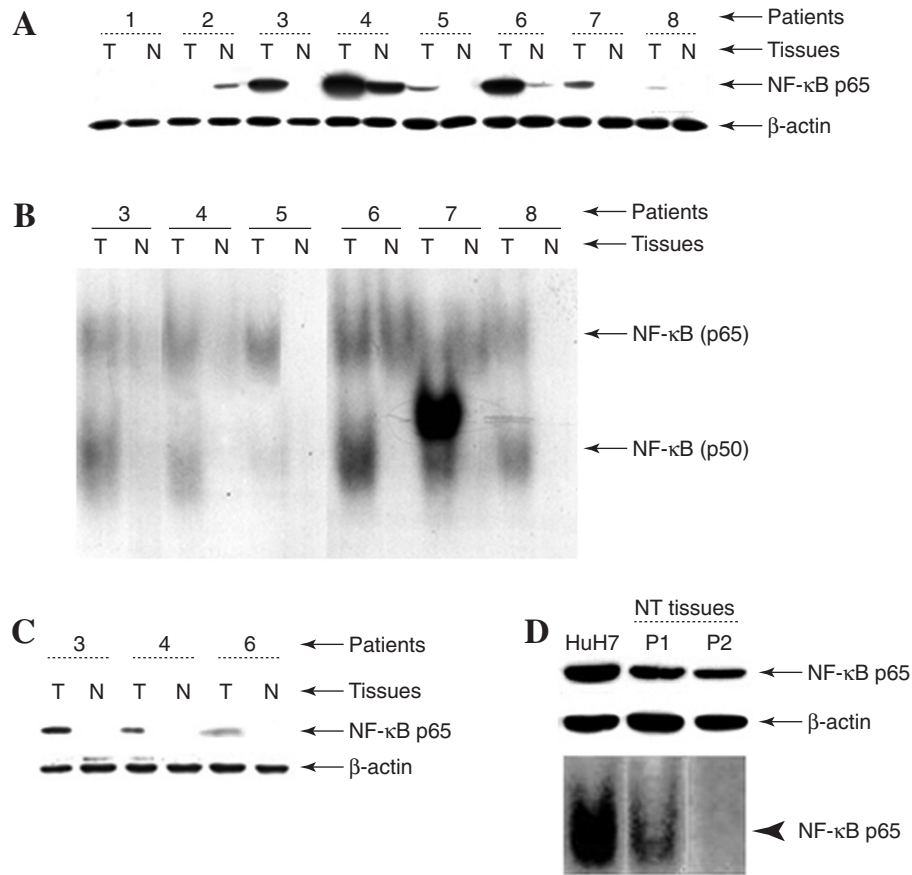
FIG. 1. (**A**) Western blot of basal expression of NF-κB p65 in homogenates of human HCC tissue (T) and of surrounding nontumorous liver tissue (N) from eight patients. (**B**) Constitutive activation of NF-κB p65 in six malignant liver tissues and their respective nonmalignant tissues. (**C**) Detection of NF-κB p65 in the nuclear protein of three human HCC tissues and their nontumorous liver tissues. (**D**) Basal levels of NF-κB p65 in HuH7 cells and in two nontumorous liver tissues. In each Western blot, β-actin was used as a loading control. Results are representative of three repeated experiments.
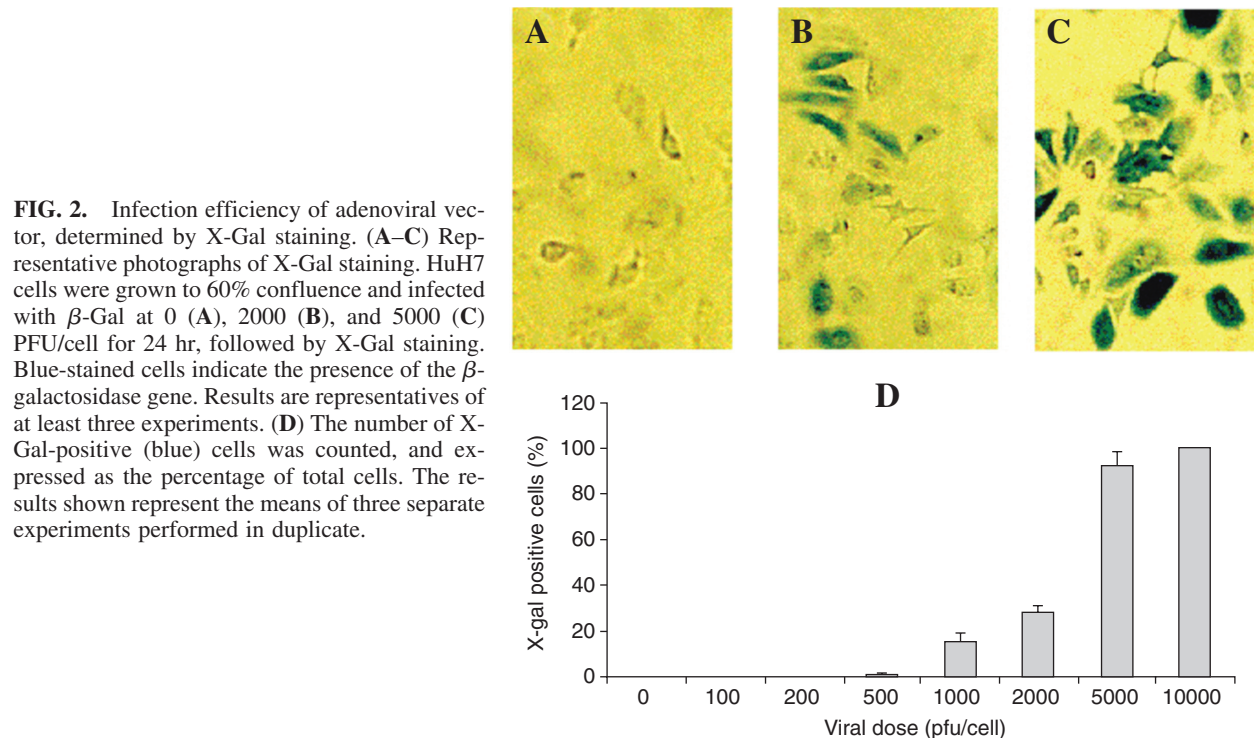

FIG. 2. Infection efficiency of adenoviral vector, determined by X-Gal staining. (**A–C**) Representative photographs of X-Gal staining. HuH7 cells were grown to 60% confluence and infected with β-Gal at 0 (**A**), 2000 (**B**), and 5000 (**C**) PFU/cell for 24 hr, followed by X-Gal staining. Blue-stained cells indicate the presence of the β-galactosidase gene. Results are representatives of at least three experiments. (**D**) The number of X-Gal-positive (blue) cells was counted, and expressed as the percentage of total cells. The results shown represent the means of three separate experiments performed in duplicate.

mIκBα and to determine whether this construct could facilitate growth inhibition and cell death in response to oxidative stress. Initially, we used three different HCC cell lines: HuH7, HepG2, and Hep3B. As the results obtained from these three cell lines were similar, we performed most of the experiments only in HuH7 cells, and the reported data are mostly from this cell line.

To determine the *in vitro* infection efficiency of HCC cells by mIκBα, we used an adenovirus carrying β-galactosidase (β-Gal) as a reporter to determine the number of successfully infected cells. Figure 2 shows the representative results obtained in HuH7 cells. Twenty-four hours after infection with β-Gal adenovirus at various concentrations, cells were viewed by light microscopy, and positively infected cells were determined by X-Gal staining. Cells infected with virus at concentrations below 200 PFU/cell virtually did not show any X-Gal-positive staining. More X-Gal-positive cells were observed with increased viral concentrations above 500 PFU/cell, and the vast majority of cells (>95%) expressed β-Gal after being infected with =5000 PFU/cell (Fig. 2D). Figure 2A–C show representative photographs of X-Gal staining.

Effects of adenoviral infection of HuH7 cells

A single band, of atomic mass 36 kDa, was present in lysates of mIκBα-infected HuH7 cells, whereas in lysates of cells infected with β-Gal no such protein band was observed (Fig. 3A). As the promoter driving IκBα expression is also transcriptionally regulated by NF-κB (Greten and Karin, 2004; Monks *et al.*, 2004), ectopic expression of mIκBα also inhibited endogenous expression of IκBα (Fig. 3A), so that the expression level of mIκBα exceeded endogenous IκBα expression more than 10-fold. As expected, there was no degradation of mIκBα after addition of H_2O_2 (Fig. 3B).
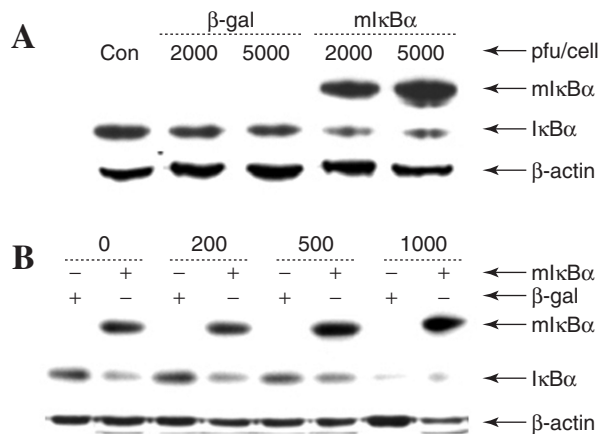
FIG. 3. Expression of mIκBα in HuH7 cells. (**A**) Cells were infected with vehicle (Con) or with adenovirus expressing mIκBα or β-Gal at 2000 and 5000 PFU/cell for 24 hr. (**B**) Cells were infected with adenovirus expressing either mIκBα or β-Gal, each at 5000 PFU/cell for 24 hr, followed by treatment with various concentrations of H_2O_2 for 24 hr. Western blot analysis was performed with polyclonal antibodies against IκBα. β-Actin was used as a loading control. The results are representative of three separate experiments.

Nuclear translocation of p65 is an essential step for NF-κB activation. We therefore examined the effect of mIκBα on p65 translocation. In naive HuH7 cells, p65 was localized virtually entirely in the cytoplasmic compartment (Fig. 4A and C), and mIκBα slightly reduced the basal level of p65 expression, as revealed by Western blot (Fig. 4A). The inhibition of p65 translocation by mIκBα appeared to be dose dependent (Fig. 4B), with mIκBα at 5000 PFU/cell almost completely abrogating p65 translocation. Further studies showed that in cells infected with β-Gal, addition of 500 μM H_2O_2 for 24 hr provoked translocation of p65 to the nucleus (Fig. 4D), and this translocation was profoundly inhibited by mIκBα (Fig. 4E). By EMSA, inhibition of NF-κB translocation led to reduced DNA binding (Fig. 4F). In cells infected with β-Gal followed by H_2O_2 treatment, a specific band corresponding to NF-κB p65 was evident, whereas in cells infected with mIκBα, NF-κB DNA binding was suppressed. Similar to Western blot findings, mIκBα at 5000 PFU/cell almost completely abolished NF-κB DNA-binding activity. These findings for p65 were also observed for p50 (data not shown).

As H_2O_2-induced p65 nuclear translocation occurred in a dose-dependent manner, and cell death was profound after exposure to 1000 μM H_2O_2, we chose 500 μM H_2O_2 for subsequent experiments unless otherwise specified.

Inhibition of NF-κB by mIκBα downregulates NF-κB target genes

To confirm that the introduced mIκBα functionally blocked NF-κB-regulated genes in HuH7 cells, we performed Western blot analysis of intracellular adhesion molecule-1 (ICAM-1). In mIκBα-infected cells compared with β-Gal-infected cells, the basal levels of ICAM-1 was reduced (Fig. 4G), and the H_2O_2-induced increase in ICAM-1 was strongly inhibited (Fig. 4H). Similar results were found with another NF-κB target gene, VCAM-1 (data not shown).

Effects of NF-κB inhibition on proliferation of HuH7 cells

Inhibition of NF-κB by mIκBα did not, itself, significantly suppress cell proliferation (Fig. 5A). Exposure of cells to lower concentrations of H_2O_2 (between 1 and 200 μM) did not significantly affect cellular DNA synthesis (Fig. 5B). However, inhibition of NF-κB accentuated such suppression when cells were exposed to higher doses of H_2O_2 (500 μM) (Fig. 5B).

Effect of NF-κB inhibition on viability of HuH7 cells

Because NF-κB activation favors cell survival, we investigated whether blocking NF-κB sensitized HuH7 cells to oxidative stress-induced cell killing. In cells infected with either β-Gal or mIκBα, incubation of cells with H_2O_2 for 24 hr caused a dose-dependent loss of cell viability. This was substantially greater in cells infected with mIκBα (Fig. 6). Because the earlier studies showed that this concentration of H_2O_2 potently activates NF-κB DNA-binding activity, these results are consistent with the proposal that NF-κB is an important survival factor for maintaining HuH7 cells.

Apart from H_2O_2, we also used bleomycin, a chemotherapeu-
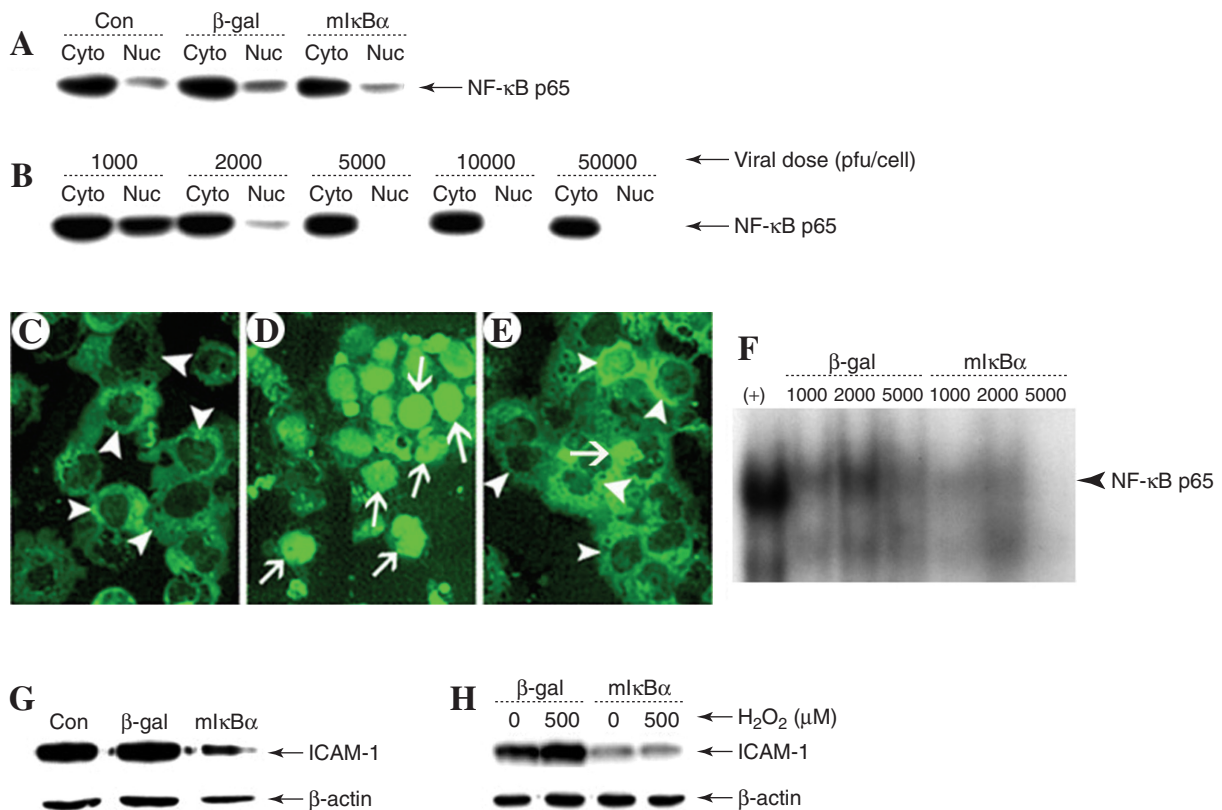
FIG. 4. Effects of *mIκBα* on nuclear translocation of NF-κB p65 subunit in HuH7 cells. (**A**) Basal level of p65 in HuH7 cells, either not infected (Con) or infected at 5000 PFU/cell with adenoviral vector carrying *β*-Gal or *mIκBα*. Cytoplasmic (cyto) and nuclear (nuc) proteins from each sample were prepared and Western blot analysis of p65 was performed, as described in Materials and Methods. (**B**) Cells were infected with the indicated dose of *mIκBα* for 24 hr, followed by treatment with 500 μM H_2O_2 for 24 hr. Analysis of p65 in cytoplasmic and nuclear proteins from each sample was performed as described above. (**C–E**) Immunofluorescence staining for p65 in cells, infected at 5000 PFU/cell with either *β*-Gal (**C** and **D**) or *mIκBα* (**E**) for 24 hr, followed by no further treatment (**C**), or treatment with 500 μM H_2O_2 for 24 hr (**D** and **E**). Arrowheads indicate cytoplasmic distribution of p65, and arrows indicate nuclear staining of p65. (**F**) EMSA of nuclear extracts from HuH7 cells infected with either *β*-Gal or various doses of *mIκBα*, followed by treatment with 500 μM H_2O_2 for 24 hr. The NF-κB subunit p65 band is labeled with an arrowhead. (**G** and **H**) Expression of ICAM-1 in HuH7 cells. Cells were uninfected (Con) or infected at 5000 PFU/cell with *β*-Gal or *mIκBα* for 24 hr (**G**), followed by treatment with or without 500 μM H_2O_2 (**H**). Western blot of ICAM-1 was performed as described in Materials and Methods. *β*-Actin was used as a loading control. The results are representative of three independent experiments performed in duplicate.

tic agent that causes marked oxidative stress to target cells, to test whether inhibition of NF-κB could sensitize the cell-killing effect of this agent. As revealed by WST-1 assay, cells infected with *mIκBα* were more sensitive to bleomycin-induced killing as compared with cells infected with *β*-Gal (data not shown).

Effects of NF-κB inhibition on apoptosis in HuH7 cells

As shown by TUNEL staining, 500 μM H_2O_2 caused massive apoptotic cell death in HuH7 cells (Fig. 7C). Blockade of NF-κB failed to increase the rate of apoptosis (Fig. 7D). On the contrary, apoptotic cell death appeared to be suppressed (Fig. 7E), although the difference between these two groups was not significant. Judging by morphology, cell death in both groups was also via necrosis. More necrotic cells were present in *mIκBα*-infected cells than in *β*-Gal-infected cells.

Mechanisms of cell killing in response to oxidative stress and NF-κB inhibition

Finally, we investigated the possible mechanisms responsible for apoptotic cell death in response to oxidative stress and NF-κB inhibition. We tested whether caspase-3 was activated. As shown in Fig. 8A, caspase-3 was dose dependently activated by 200 and 500 μM H_2O_2, and blockage of NF-κB caused more caspase-3 activation. Western blot confirmed this finding, showing a more marked decrease of procaspase-3 in *mIκBα*-infected cells treated with H_2O_2 (Fig. 8B).

We also measured the expression of two Bcl-2 family proteins: the proapoptotic Bax and anti-apoptotic Bcl-X_L. Bax expression was not altered by viral infection alone or viral infection followed by H_2O_2 (Fig. 8C). In contrast, the expression of Bcl-X_L was suppressed by 500 μM H_2O_2, and *mIκBα* further suppressed its expression (Fig. 8C).

A

B
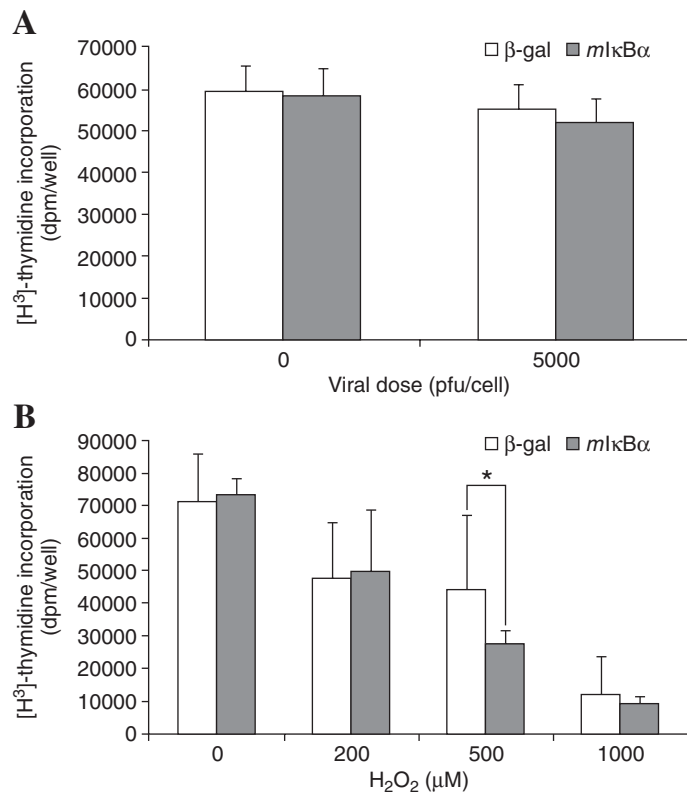
FIG. 5. Effect of NF-κB inhibition on cell proliferation. (**A**) HuH7 cells were infected for 24 hr with β-Gal or mIκBα at 5000 PFU/cell. (**B**) Cells were infected for 24 hr with β-Gal or mIκBα at 5000 PFU/cell followed by exposure to H_2O_2 for 24 hr. DNA synthesis was measured by [^{3}H]thymidine incorporation, as described in detail in Materials and Methods. Each column represents the average β-scintillation count of three separate experiments performed in triplicate. *$p < 0.05$.

DISCUSSION

Activation of the ubiquitous transcription factor NF-κB is protective against cell death in many cell types, particularly (but not exclusively) by apoptosis (Karin and Lin, 2002). It is therefore of interest that many cancers, such as breast cancer, lung cancer, and lymphoma (Rayet and Gelinas, 1999), and HCC (according to other reports), constitutively express high levels of NF-κB (Kaufmann and Earnshaw, 2000; Herr and Debatin, 2001; Chiao et al., 2002). Conversely, several anticancer drugs

FIG. 6. Effect of NF-κB inhibition on viability of HuH7 cells exposed to H_2O_2. At the end of treatment, cell viability was detected by WST-1 assay, as described in Materials and Methods. *$p < 0.05$. Results represent the means of three separate measurements with nine replicates.

potently activate NF-κB, and resistance of certain cancer cells to apoptosis has been attributed to constitutive activation of NF-κB (Chiao et al., 2002; Sato et al., 2003; Panwalkar et al., 2004; Samanta et al., 2004). In the present study, we found that at least six of eight human HCCs (75%) expressed higher levels of the NF-κB Rel protein, p65, than surrounding noncancerous liver, and in at least three of them, this p65 was localized predominantly in the nucleus. The latter finding is consistent with constitutive activation of NF-κB. Thus the present results confirmed and extend earlier studies of high-level NF-κB in HCC liver and show apparently constitutive activation of NF-κB in human HCC. We also found no difference between well and poorly differentiated HCC in terms of NF-κB protein expression. This is in agreement with a previous report (Guo et al., 2005).

Because it is difficult to study the mechanisms and biological implications of NF-κB activation in intact human liver, we addressed these issues in an HCC cell line, HuH7, which also expresses high-level constitutive activation of NF-κB. In this study, we used mIκBα (also termed IκBα superrepressor), transfected via an adenoviral vector, to block NF-κB activation, and examined the effect on cell viability, mechanisms of cell death (especially apoptosis), and proliferation. At the doses that were effective in these experiments, mIκBα could easily be introduced into the HuH7 cells without having any detrimental effects on cell viability or endogenous NF-κB. Expression levels of mIκBα were readily induced up to 10-fold higher than the level of endogenous IκBα. As expected, this protein was not degraded after addition of H_2O_2. Further, the expression of endogenous IκBα, which is itself under NF-κB regulation, was also inhibited by mIκBα.
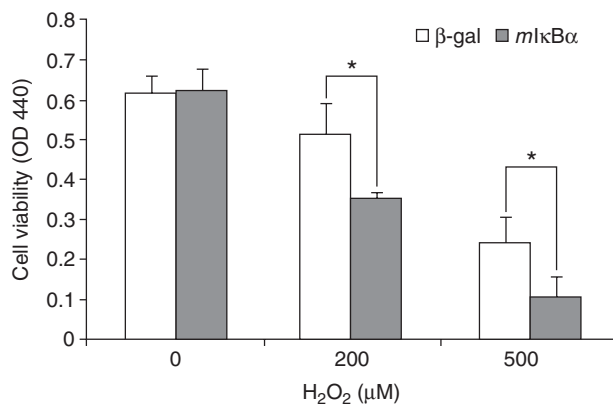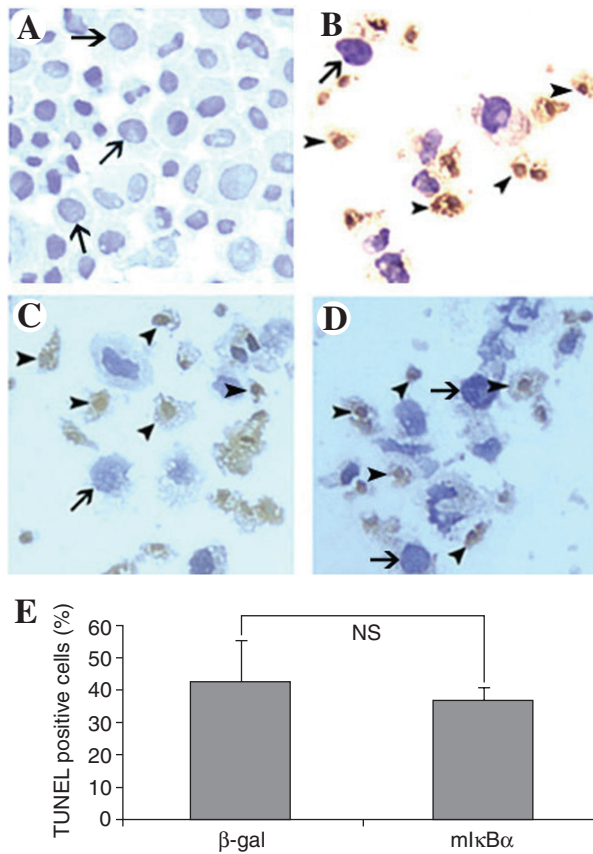
FIG. 7. Detection of apoptosis by TUNEL staining. HuH7 cells were infected at 5000 PFU/cell with β-Gal (**C**) or *m*IκBα (**D**) for 24 hr, followed by treatment with 500 *μM* H₂O₂ for 24 hr. (**A**) Negative control (no TdT was included in the reaction). (**B**) Experimental positive control (cells treated with DNase I for 10 min). Arrowheads represent TUNEL-positive cells, and arrows indicate TUNEL-negative cells. Photographs are representative of three separate experiments. (**E**) Quantitative analysis. NS, no significant difference.

The human liver is constantly exposed to oxidative stress produced as the result of the metabolic production of reactive oxidative species (ROS), such as hydrogen peroxide, superoxide ion, and hydroxyl radicals. ROS can cause oxidative damage to DNA and is therefore a potential mutagenic factor in cancer formation and, in addition, can modulate cell proliferation and cell death in ways that could promote clonal expansion of preneoplastic foci in the liver. On the other hand, some cancer-therapeutic agents kill cells by inducing oxidative stress in mitochondria, a pathway that is opposed by ROS-induced activation of NF-κB. Many apoptotic stimuli, such as calcium ionophore, TNF, interleukin-1, and lipopolysaccharide, can activate NF-κB by distinct intracellular pathways, but a common mechanism involves the synthesis of ROS (Schreck *et al.*, 1991). Thus, NF-κB is a redox-sensitive nuclear transcription factor. Studies have shown that raised intracellular H₂O₂ levels are mechanistically responsible for induction of apoptosis and inhibition of growth in MG132 (an inhibitor of 26S pro-
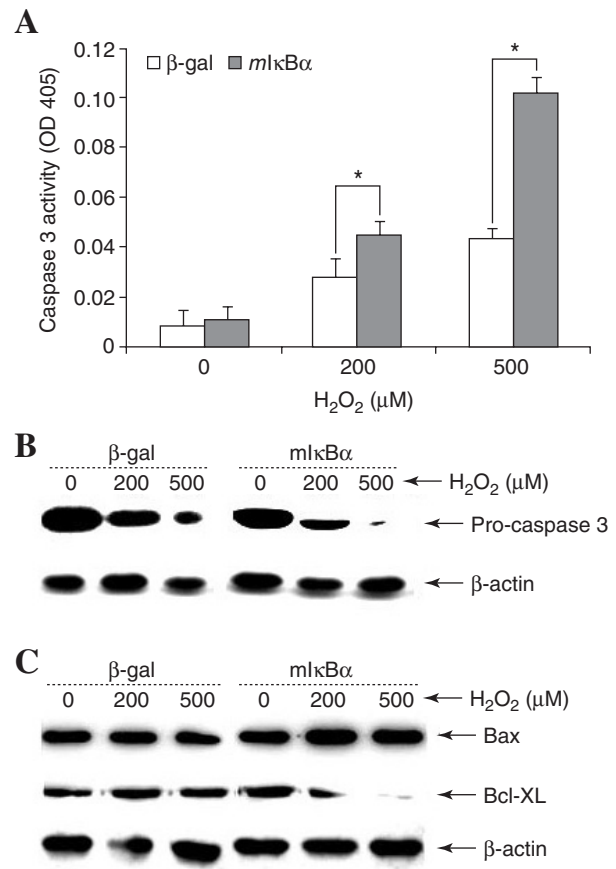
FIG. 8. Effect of NF-κB inhibition on caspase-3, Bax, and Bcl-2 expression. (**A**) Caspase-3 activity was determined in lysates of cells that were infected with or without β-Gal or *m*IκBα at 5000 PFU/cell for 24 hr followed by treatment with 0, 200, and 500 *μM* H₂O₂ for 24 hr. Each column represents the mean of three separate experiments performed in triplicate. *p < 0.05. (**B**) Western blot of caspase-3 in the same samples. (**C**) Protein expression of Bax and Bcl-X_L. β-Actin was used as a loading control. Results shown are representative blots of three separate experiments.

In untreated HuH7 cells, most p65 is located in the cytoplasm. Introduction of *m*IκBα effectively prevented H₂O₂-induced p65 translocation to the nucleus, as judged by Western blot and immunofluorescence staining, and confirmed NF-κB p65/p50 dimeric binding to DNA by EMSA. Such abrogation of NF-κB activation caused the anticipated functional consequences of downregulation of two important downstream target genes, ICAM-1 and VCAM-1.

Previous studies of patients with HCC have shown a marked increase in circulating (Hyodo *et al.*, 1996) and hepatic (Yoong *et al.*, 1998) expression of these cell adhesion molecules, as well as E-selectin. In the present study, we showed that ICAM-1 is highly expressed in HuH7 cells. However, the relationship between the expression of cell adhesion molecules and cancer development or prognosis remains controversial. It is therefore unclear whether modulation of ICAM-1 and VCAM-1 expression by manipulating NF-κB activity would potentially be useful in controlling the local progression of liver cancer, or metastasis from the liver.

teasome)-treated HCC cells (Tuvdendorj *et al*., 2003) as well as in arsenic trioxide-treated acute promyelocytic leukemic cells (Chen *et al*., 2003). The role of NF-κB and ROS in chemically induced hepatocarcinogenesis was supported in one study (Maeda *et al*., 2005).

In the present study, treatment of HuH7 cells with H_2O_2 led to dose-dependent cell killing that was enhanced by NF-κB inactivation. Cell killing was via both apoptosis and necrosis. The fact that blockade of NF-κB activity by *m*IκBα potentiated H_2O_2-induced cell death via apoptotic and nonapoptotic pathways is consistent with growing evidence that NF-κB not only regulates the apoptotic pathways but may also govern pathways that lead to necrosis (Kucharczak *et al*., 2003). In addition, necrosis might be secondary to the apoptosis ("necrogenic apoptosis"), occurring in response to apoptotic stimuli (Xu *et al*., 1998). It seems increasingly likely that at certain stages of their pathways, and particularly in the liver, where the requirement for ATP to execute apoptosis may be high, these two different types of cell death share the same signaling events.

Apoptosis appeared not to be the major mode of cell death when HuH7 cells were exposed to H_2O_2. However, the further accentuation of cell death attributed to NF-κB blockade in the face of H_2O_2 was not apparently by apoptosis. Caspase-3 plays an essential role in apoptosis execution in most cell types, and exposure of HuH7 cells to H_2O_2 caused a dose-dependent increase in caspase-3 activity. Blocking NF-κB further potentiated H_2O_2-induced activation of caspase-3. However, caspase-3 can also play a role in necrosis under some circumstances (El-Hassan *et al*., 2003), especially when ATP is depleted as may be the case in HuH7 cells exposed to H_2O_2. Alternatively, the further accentuation of cell death, which was also not associated with TUNEL-positive cells, could be due to necrosis.

There are many target genes of NF-κB, include such antiapoptotic proteins as cIAP1, cIAP2, TRAF1, TRAF2, Bcl-X_L, and FLIP, and certain proapoptotic proteins such as Bax, Fas, Fas ligand, TRAIL-R1, and TRAIL-R2 (Wang *et al*., 1999; Tietze *et al*., 2000; Heimberg *et al*., 2001; Sato *et al*., 2003; Debatin, 2004). It is therefore possible that activation of NF-κB can inhibit or promote apoptosis. Bax is a strong proapoptotic gene whereas Bcl-X_L possesses strong antiapoptotic properties. In our study, Bax was not affected by NF-κB modulation in HuH7 cells. However, inhibition of NF-κB activity appeared to suppress Bcl-X_L expression. Therefore, we speculated that diminished expression of Bcl-X_L may have contributed to increased apoptosis.

NF-κB is important not only in apoptosis pathways, but also as a transcription factor in the signal transduction of the inflammatory response; this is particularly relevant in cancers in which inflammation plays a pivotal role. The relationship between inflammation and cancer has gained extensive attention (Balkwill and Coussens, 2004; Karin and Greten, 2005; Li *et al*., 2005). Chronic inflammation has been recognized as an important cause of tumor development, accounting for approximately 20% of human cancer. It has been proposed that NF-κB contributes to tumorigenesis through effects on both tumor cells and tumor-associated inflammatory cells (Balkwill and Coussens, 2004). Inhibition of NF-κB in malignant cells would increase susceptibility to apoptosis-inducing agents, through the downregulation of certain antiapoptosis factors such as Bcl-X_L, and in inflammatory cells it would inhibit the expression of growth and sur-

vival factors such as TNF-α and IL-6. Thus, targeting NF-κB could be a useful strategy for cancer therapy (Karin and Greten, 2005).

The effect of NF-κB superrepressor in experimental cancer therapy has been demonstrated in several studies. In one study of hepatocarcinogenesis in multidrug resistance-2 gene (MDR2) knockout mice (Pikarsky *et al*., 2004), it was found that the inflammatory process triggers hepatocyte NF-κB through upregulation of tumor necrosis factor-α (TNF-α) in adjacent endothelial and inflammatory cells. Although inhibition of NF-κB in mice by tetracycline-inducible, liver-specific expression of a superrepressor of NF-κB did not affect the course of hepatitis, or the early phases of hepatocyte transformation, IκB superrepressor-mediated suppression of NF-κB did result in the apoptosis of transformed hepatocytes and suppressed progress to hepatocellular carcinoma, suggesting that NF-κB may not be required for early-phase cancer development (tumor initiation), but is essential for subsequent tumor promotion. Similarly, inhibition of NF-κB by superrepressor reduces the inflammation-induced metastasis of CT26 colon cancer cells to the lungs (Luo *et al*., 2004). Our *in vitro* results support the idea that NF-κB superrepressor may be a potentially useful approach in targeting NF-κB as a therapeutic tool for certain cancer, especially those malignancies that are closely associated with inflammatory or oxidative stress processes.

One of the aims of cancer therapy is to deprive cancer cells of their growth potential. In this study, we showed that inhibition of NF-κB did not, by itself, suppress cell proliferation (as indicated by DNA synthesis) in HuH7 cells. However, blocking NF-κB by *m*IκBα did enhanced the growth-inhibitory effect of H_2O_2, especially when it was used at higher doses. Further studies are needed to clarify what genes are responsible for such growth inhibition.

In conclusion, the current study demonstrated that adenovirus-mediated transfer of *m*IκBα is an efficient way to block the NF-κB pathway *in vitro*. The introduced construct is functional in that it blocks NF-κB translocation, blocks DNA binding, and inhibits the expression of downstream target genes. Inhibition of NF-κB by *m*IκBα has the potential to inhibit HuH7 cell proliferation, and oxidative stress can strongly activate the apoptotic cascade in HuH7 cells. These results warrant further studies into the efficacy and mechanisms of NF-κB inactivation as a new target for HCC gene therapy. Our preliminary *in vivo* results have indicated that HuH7 cells infected with *m*IκBα showed diminished tumorigenic potential in nude mice. We await the completion of this study.

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