Gene therapy that inhibits NF-κB results in apoptosis of human hepatocarcinoma by recombinant adenovirus

Tie-Jun Li, Li-Ping Jia, Xiao-Ling Gao, Ai-Long Huang

INTRODUCTION

Resistance of tumor cells toward induction of apoptosis is one of the main reasons for failure of anticancer treatment[1]. NF-κB is a ubiquitous transcription factor that is activated by a variety of cytokines and mitogens[2] and is thought to be a key regulator of genes involved in inflammation, response to infection, and stress. Classic NF-κB is a heterodimer of p50 (NF-κB-1) and p65 (Rel-A), but proteins that constitute the NF-κB family form a variety of homodimers and heterodimers[3]. NF-κB is retained in an inactive form in the cytoplasm through association with one of the IκB inhibitory proteins, including IκBα, IκBβ and IκBγ[4]. After cellular stimulation, the phosphorylation, ubiquination, and subsequent proteolysis of IκBα in proteosomes enables NF-κB to translocate into the nucleus[5-8], where it regulates the transcription of NF-κB-response genes by interacting with κB binding sites[9,10]. Recently, abundant evidence has implicated cellular NF-κB transcription factors in the control of apoptosis in many systems. It has been suggested to be associated with increased survival in many tumor cells. A number of studies implicated NF-κB in apoptosis resistant tumor cells[11-16]. A superrepressor form of IκBα contains a serine-to-alanine mutation at amino acids 32 and 36, which inhibits signal-induced phosphorylation and subsequent proteosome-mediated degradation of IκBα. This IκB superrepressor has been used to demonstrate that inhibition of NF-κB induces apoptosis through a variety of cancer therapeutic agents and TNF-α[17,18]. Based on this, we have successfully cloned the IκBα gene and constructed the superrepressor IκBαM in Chinese. We have generated recombinant adenovirus AdIκBαM, which will provide a solid basis for the study of IκBαM-mediated antitumor gene therapy. In the present study, we investigated whether the recombinant adenovirus induces the TNF-α-mediated apoptosis in the human hepatocarcinoma cell line (HepG2) and in vivo.

MATERIALS AND METHODS

Cell culture

HepG2 cells, Hela cells and 293 cells were maintained in RPMI 1640 medium with 10% fetal bovine serum, penicil-
lin (100 mg/L), and streptomycin (100 mg/L).

**Construction of recombinant adenovirus AdikBαM**
The full-length cDNA of 1kBα superrepressor (1kBαM), whose serines 32, 36 were mutated into the alanine, was kindly provided by Dr Bing-Rong Liu (from our laboratory). The 1kBαM was injected into the adenoviral shuttle plasmid Track-CMV (a gift from Mr TC He, Molecular Oncology Laboratory, the University of Chicago Medical Center). It contains green fluorescent protein (GFP). We thus constructed the recombinant adenoviral plasmid pAdikBαM. However, pAdikBα did not have replacement of serines 32, 36 with alanines. Recombinant adenoviral plasmids were digested with Pac I. Then, the digested recombinant adenoviral plasmid was transfected into 293 cells with FuGENE™6 transfection reagent (Roche). Viral transfection products (AdikBαM, AdikBα) were monitored by GFP expression. The recombinant adenovirus was selected and purified using standard procedures[9]. To obtain a large quantity of recombinant adenovirus (AdikBαM, AdikBα), the 293 cells were infected and grown for 48 h at 37°C. The infected cells were harvested and centrifuged using a tabletop centrifuge at 1000 r/min for 5 min. The infected cells were resuspended in PBS. The cells were lysed by 4 freeze-thaw cycles to release the virus. The virus was purified through a CsCl gradient. The purified recombinant adenovirus was then titrated by the plaque assay[9], aliquoted, and stored at -70°C until use.

**HepG2 cell analysis in BALB/c nude mice**
All mice whose age was 4 wk, weighing between 15-18 g were provided by SLACAS, China. Number of females was equal to males. Approximately 2 × 10² HepG2 cells in 200 μL of PBS media were injected subcutaneously into the back of 40 BALB/c nude mice. All mice were maintained and handled under specific pathogen-free conditions at the Animal Center in Chongqing University of Medical Sciences. The tumors of 8 mice in each group were directly injected with 2 × 10⁶ plaque-forming units (PFU) of AdikBαM. Eight mice were injected with 2 × 10⁵ pfu of AdikBα. The third group of mice was injected with 2 × 10⁶ pfu of AdikBαM and the fourth group with 2 × 10⁵ pfu of AdikBαM. Groups of control mice were injected with phosphate buffered saline (PBS). All mice were injected 5 times in total, every other day, 100 μL each. After the injection was finished, on the fourth day all mice were killed. The tumor growth curve was drawn. The volume of tumor was calculated according to the formula: Tumor volume = Length × Width² × 0.4.

**Hematoxylin and eosin staining**
HE staining analysis was carried out for evaluation of cell necrosis and apoptosis. The percentage of cells undergoing apoptosis was determined as the number of HE-positive cells in at least 10 randomly selected vision fields of sections obtained from tumors in each group of mice.

**TUNEL analysis of HepG2 cell apoptosis in BALB/c nude mice**
HepG2 cell apoptosis in BALB/c mice was determined using in situ apoptosis staining with the TUNEL staining kit according to the manufacturer's instructions (Roche). Tissues from the mouse tumor were fixed in 4% buffered paraformaldehyde for 4 h and decalcified in 59 mmol/L EDTA, pH 7.8, for 3 wk. The tissue was then dehydrated with different concentrations of ethanol and xylene, and embedded in paraffin. Tissue specimens were cut into 8-μm sections and mounted onto glass slides. Slides were incubated with fresh proteinase K (20 mg/L)-streptavidin-labeled horseradish peroxidase (HRP) at room temperature for 10 min. The slides were covered with a cover glass and incubated at 37°C for 1 h in a humidified chamber. Nonspecific staining was blocked by incubating the slides with blocking buffer at room temperature for 30 min. The slides were incubated with a klenow labeling buffer in the presence of biotin-labeled dNTP for 1.5 h at 37°C. After washing 6 times with PBS, the slides were incubated with streptavidin-conjugated antibody at a 1:50 dilution in Tris buffer, pH 7.4 with PBS and developed by incubation with diaminobenzidine solution for 5 min. Cells undergoing apoptosis were identified by dark brown staining of the nuclei. For quantitative analysis of the percentage of apoptotic cells, a total of 10 random vision fields were evaluated.

**Electron microscopy**
Electron microscopic analysis was carried out for evaluation of cell necrosis and apoptosis.

**Statistical analysis**
Student’s t-test was used for testing the statistical significance of the differences between the groups. A P value of less than 0.05 was considered statistically significant.

**RESULTS**

**Construction and analysis of recombinant adenovirus AdikBαM**
The recombinant adenovirus plasmids were generated by cloning the 1kBαM/1kBα construct into adenoviral shuttle plasmid Track-CMV. The recombinant adenovirus plasmids were digested with Pac I. The digested products were identified by 0.8% agarose gel electrophoresis (Figure 1A). pAdikBαM was digested into 2 fragments: one was about 3 kb, the other probably 30 kb; pAdikBα was digested into fragments of about 4.5 kb and 30 kb. The digested recombinant adenovirus plasmid was transfected into 293 cells. Two days after transfection, the fluorescence was observed (Figure 1B, C). The results showed that we have successfully cloned the 1kBαM/1kBα gene into the plasmid Track-CMV, and the recombinant adenovirus AdikBαM/AdikBα was established. The virus was grown to a titer of 2 × 10¹² pfu/L by purification over a CsCl gradient. AdikBαM could be expressed in HepG2 cells after infection for 48 h (Figure 1D).

**Induction of apoptosis of HepG2 in BALB/c nude mice by AdikBαM in vivo**
HepG2 cells (2 × 10⁵) were injected into the back of 40 BALB/c nude mice subcutaneously. After 2 wk, a tumor
There was a significant inhibition of tumor growth, the tumor growth being stopped. Injection with AdIκBα (2 × 10^12 pfu/L) had a slight effect on tumor growth at first, which then diminished.

**HE staining and TUNEL analysis**

There was a difference in the incidence of cell destruction by HE staining, which was exhibited by almost all mice (Figure 3). BALB/c mice injected with AdIκBα demonstrated a slight effect. In contrast, BALB/c nude mice treated with AdIκBαM (2 × 10^12 pfu/L) exhibited extensive pyknotic nuclei and cell destruction. There were masses of cell necrosis in BALB/c mice injected with PBS, because ischemia in tumor resulted in cell necrosis. The number of apoptotic cells in the AdIκBαM (2 × 10^12) group was 16.8 ± 3.1 (P < 0.01); in the AdIκBαM (2 × 10^11) group 13.1 ± 2.3 (P < 0.01); in the AdIκBαM (2 × 10^10) group 10.1 ± 2.1 (P < 0.01); in the AdIκBα group 5.3 ± 1.8 (P > 0.05); in the PBS control group 3.8 ± 1.8 (P > 0.05). Moreover, to determine if AdIκBαM induced apoptosis in vivo, the tumor was sectioned and analyzed by in situ TUNEL staining (Figure 4). There was significant apoptosis of HepG2 cells infected with AdIκBαM (2 × 10^12 pfu/L) but not of HepG2 treated with control PBS and some apoptosis in HepG2 cells injected with AdIκBα. Furthermore, there was a direct correlation between AdIκBαM dosage and cell apoptosis. The number of apoptotic cells in the AdIκBαM (2 × 10^12) group was 14.7
Resistance to anticancer therapies appears to be mediated by resistance to apoptosis. Therefore, modulation of apoptotic killing in treatment regimens such as TNF, radiation therapy, and certain chemotherapeutic agents implies that NF-κB may play a role in preventing maximal apoptotic killing in many cell types, including tumor cell lines. HepG2 cell line, like other cell lines, does not undergo apoptosis in response to TNF-α. Therefore, we propose that TNF-α acts as a growth factor in the HepG2 cell line, as well as induces production of cytokines and invasive enzymes. Taken together, it implies that NF-κB may play a role in preventing maximal apoptotic killing in treatment regimens such as TNF, radiation therapy, and certain chemotherapeutic agents. Resistance to anticancer therapies appears to be mediated by resistance to apoptosis. Therefore, modulation of NF-κB activity could potentially lead to improved cell killing in the HepG2 cell line and in vivo. Based on this understanding, we have successfully constructed the superrepressor of NF-κB (AdIkBαM), in which 32, 36

Results of electron microscopy
Those cells with dark concentrated nuclei under EM were considered as apoptotic cells. In the AdIkBαM (2 × 10^13) group, there were 1-2 apoptotic cells in a random vision field (× 4000-6000, Figure 5A). Necrotic tissue or cells were observed around the apoptotic cells, but no proliferative phase was found. In the AdIkBαM (2 × 10^13) group, there were 0-2 apoptotic cells in a random vision field (× 4000-6000, Figure 5B). In the AdIkBαM (2 × 10^16) group, apoptotic cells could seldom be found (Figure 5C), whereas in the PBS control and AdIkBdt group, there were few apoptotic cells, and many tumor cells were in the proliferative phase; however, necrotic tumor cells could also be found (Figures 5D, E). Necrotic tumor cells could be found in the AdIkBαM (2 × 10^16) group, probably because AdIkBαM could induce the tumor cells to apoptosis, and then to necrosis. Necrotic tumor cells could be found in the PBS control group probably because the tumor developed too fast and the blood supply was inadequate. Because the results of electron microscopy are the most reliable evidence of apoptosis, we could speculate that recombinant adenovirus could induce apoptosis of human hepatocarcinoma and inhibit the tumor cell proliferation.

DISCUSSION
TNF-α is an important cytokine in the promotion of growth and invasion of cells. It interacts with TNFR I and TNFR II. Signaling through both TNFR I and TNFR II can induce apoptosis[25-22]. Interactions with TNFR I produce a proapoptotic signal by recruitment of TNFR I-associated death domain (TRADD) protein to the death-inducing signaling complex (DISC) of the TNFR I timer[21,24]. TRADD recruits the Fas-associated death domain (FADD), which in turn, recruits caspase 8 and signals apoptosis[21]. Simultaneously, an antiapoptosis pathway involves recruitment of cellular LAP (cLAP), receptor interactive peptide (RIP), and TNFR-associated factor 2 (TNFR2), which leads to activation of NF-κB-inducing kinase (NIK)[20]. This results in phosphorylation of 1κBα and 1κBβ and translocation of NF-κB to the nucleus. This second signal predominates in hepatocarcinoma, and NF-κB translocation to the nucleus plays a role in transcription of several genes, including TNF-α, interleukin-1β, and IL-6, as well as collagenase, stromelysin, and adhesion molecules[26,28]. At the same time, NF-κB translocation inhibits apoptosis in many cell types, including tumor cell lines. HepG2 cell line, like other cell lines, does not undergo apoptosis in response to TNF-α. Therefore, we propose that TNF-α acts as a growth factor in the HepG2 cell line, as well as induces production of cytokines and invasive enzymes. Taken together, it implies that NF-κB may play a role in preventing maximal apoptotic killing in treatment regimens such as TNF, radiation therapy, and certain chemotherapeutic agents. Resistance to anticancer therapies appears to be mediated by resistance to apoptosis. Therefore, modulation of NF-κB activity could potentially lead to improved cell killing in the HepG2 cell line and in vivo. Based on this understanding, we have successfully constructed the superrepressor of NF-κB (AdIkBαM), in which 32, 36

www.wjgnet.com

± 2.4 (P < 0.01); in the AdIkBαM (2 × 10^13) group 12.4 ± 2.2 (P < 0.01); in the AdIkBαM (2 × 10^13) group 8.3 ± 2.0 (P < 0.01); in the AdIkBα group 3.4 ± 1.6 (P > 0.05); in the PBS control group 4.2 ± 1.7 (P > 0.05).

Figure 4 TUNEL was used to evaluate apoptosis of HepG2 cells in BALB/c mice. A: PBS control; B: AdIkBαM (2 × 10^13 pfu/L); C: AdIkBdtM (2 × 10^13 pfu/L); D: AdIkBαM (2 × 10^14 pfu/L); E: AdIkBα (2 × 10^15 pfu/L).

Figure 5 Electron microscopic analysis was used to evaluate apoptosis in HepG2 cells in mice. A: PBS control (× 4000); B: AdIkBαM 2 × 10^10 pfu/L (× 6000); C: AdIkBdtM 2 × 10^10 pfu/L (× 4000); D: AdIkBαM 2 × 10^11 pfu/L (× 6000); E: AdIkBα 2 × 10^12 pfu/L (× 4000).
serines were replaced with alanines and could not be phosphorylated by NIK.

The studies by Duffy et al.20 demonstrated that human head and neck squamous carcinoma cell transfected with IkBaM was significantly restrained. The results of our study with AdIkBaM showed that the HepG2 cell line could be sufficiently infected with this recombinant adenovirus. Furthermore, IkBaM could be expressed stably in HepG2 cells. This was especially prominent with induction by TNF-α. Overexpression of mutated IkBα by an AdIkBaM construct resulted in inhibition of nuclear translocation of NF-κB after TNF-α stimulation of HepG2 cells. Under these conditions, the HepG2 cell underwent extensive apoptosis in response to TNF-α in vitro. There was distinct apoptosis in HepG2 cells treated alone with AdIkBaM and little apoptosis treated with AdIkBα and no apoptosis treated with PBS. This indicated that AdIkBaM could facilitate or induce apoptosis in HepG2 cells in vitro. There are histocytic cells and T cells in BALB/c nude mice, and the BALB/c mice may contain TNF-α in vivo. In light of the results of the experiment in vitro, we did not use TNF-α in vivo. BALB/c mice treated with AdIkBaM (2 × 10^10 pfu/L) exhibited the most extensive inhibition of tumor growth. In contrast, no effect was observed in mice injected with PBS and a slight effect in mice treated with AdIkBα. Mice treated with AdIkBaM (2 × 10^10 pfu/L) underwent extensive apoptosis in vivo. However, there was little apoptosis in mice treated with AdIkBα (2 × 10^12 pfu/L). In this study, we focused particularly on the suppression of tumor growth and the induction of cell apoptosis by the recombinant adenovirus.

Although various degrees of necrosis could be observed by HE staining, more work should be done on whether the recombinant adenovirus could lead to tumor death through inducing the damage of tumor blood vessels. Meanwhile, we should pay more attention to the toxicity of recombinant adenovirus, the first-pass effect of liver, the antigenicity and the targeting of recombinant adenovirus.

In conclusion, the AdIkBaM is expressed in HepG2 cell effectively and stably. It could inhibit the activity of NFκB, and cause increased apoptosis as well as suppression of liver tumor.

REFERENCES

1. Burnett AK, Eden OB. The treatment of acute leukaemia. Lancet 1997; 349: 270-275
2. Baldwin AS Jr, Azizkhan JC, Jensen DE, Beg AA, Coddly LR. Induction of NF-kappaB DNA-binding activity during the G0-to-G1 transition in mouse fibroblasts. Mol Cell Biol 1991; 11: 4943-4951
3. Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S. Rel/NF-kappaB/I kappaB family: intimate tales of association and dissociation. Genes Dev 1995; 9: 2723-2735
4. Chen F, Castranova V, Shi X, Demers LM. New insights into the role of nuclear factor-kappaB, a ubiquitous transcription factor in the initiation of diseases. Clin Chem 1999; 45: 7-17
5. Brown K, Gerstberger S, Carlson L, Franzoso G, Siebenlist U. Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. Science 1995; 267: 1485-1488
6. DiDonato JA, Mercurio F, Karin M. Phosphorylation of I kappa B alpha precedes but is not sufficient for its dissociation from NF-kappa B. Mol Cell Biol 1995; 15: 1302-1311
7. Iimuro Y, Nishiura T, Hellerbrand C, Behrens KE, Schoonhoven R, Grisham JW, Brenner DA. NFkappaB prevents apoptosis and liver dysfunction during liver regeneration. J Clin Invest 1998; 101: 802-811
8. May MJ, Ghosh S. Signal transduction through NF-kappa B. Immunol Today 1998; 19: 80-88
9. Finco TS, Baldwin AS. Mechanistic aspects of NF-kappa B regulation: the emerging role of phosphorylation and proteolyis. Immunity 1995; 3: 263-272
10. Baldwin AS Jr. Series introduction: the transcription factor NF-kappaB and human disease. J Clin Invest 2001; 107: 3-6
11. Wang CY, Mayo MW, Baldwin AS Jr. TNF-α and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. Science 1996; 274: 784-787
12. Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science 1996; 274: 787-789
13. Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. Science 1996; 274: 782-784
14. Diaz-Meco MT, Lallena MJ, Monjas A, Frutos S, Moscat J. Inactivation of the inhibitory kappaB protein kinase/nuclear factor kappaB pathway by Par-4 expression potentiates tumor necrosis factor alpha-induced apoptosis. J Biol Chem 1999; 274: 19606-19612
15. Dudley E, Hornung F, Zheng L, Scherer D, Ballard D, Lennardo M. NF-kappaB regulates Fas/APO-1/CD95- and TCR-mediated apoptosis of T lymphocytes. Eur J Immunol 1999; 29: 878-886
16. Wang CY, Cusack JC Jr, Liu R, Baldwin AS Jr. Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kappaB. Nat Med 1999; 5: 412-417
17. Khoshnan A, Tindell C, Lux A, Bae D, Bennett N, Nel AE. The NF-kappaB B cascade is important in Bc-xL expression and for the anti-apoptotic effects of the CD28 receptor in primary human CD4+ lymphocytes. J Immunol 2000; 165: 1743-1754
18. Hunter RR, Stevenson E, Koncarevic A, Mitchell-Felton H, Esig DA, Kandarian SC. Activation of an alternative NF-kappaB pathway in skeletal muscle during disuse atrophy. FASEB J 2002; 16: 529-538
19. Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH, Ballard DW. Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-kappaB control. Proc Natl Acad Sci USA 1997; 94: 10057-10062
20. Atencio IA, Grace M, Bordens R, Fritz M, Horowitz JA, Hutchins B, Indelicato S, Jacobs S, Kolz K, Maneval D, Musco ML, Shioda J, Venook A, Wen S, Warren R. Biological activities of a recombinant adenovirus p53 (SCH 58500) administered by hepatic arterial infusion in a Phase 1 colorectal cancer trial. Cancer Gene Ther 2006; 13: 169-181
21. Hu B, Zhu H, Qiu S, Su Y, Ling W, Xiao W, QJ. Enhanced TRAIL sensitivity by EIA expression in human cancer and normal cell lines: inhibition by adenovirus E1B19K and E3 proteins. Biochem Biophys Res Commun 2004; 325: 1153-1162
22. Haridas V, Darnay BG, Natarajan K, Heller R, Aggarwal BB. Overexpression of the p80 TNF receptor leads to TNF-dependent apoptosis, nuclear factor-kappa B activation, and c-Jun kinase activation. J Immunol 1998; 160: 3152-3162
23. Yu L, Hamada K, Namba M, Kadomatsu K, Muramatsu T, Matsuura S, Tagaya M. Milkine promoter-driven suicide gene expression and -mediated adenovirus replication produced cytotoxic effects to immortalised and tumour cells. Eur J Cancer 2004; 40: 1787-1794
24. Varfolomeev EE, Boldin MP, Goncharov TM, Wallach D. A potential mechanism of “cross-talk” between the p55 tumor necrosis factor receptor and Fas/APO1: proteins binding to the death domains of the two receptors also bind to each other. J Exp Med 1996; 183: 1271-1275
25. Chinnaiyan AM, Teppar CG, Seldin MF, O’Rourke K, Kischkel FC, Hellbardt S, Kramer PH, Peter ME, Dixit VM. FADD/MORT1 is a common mediator of CD95 (Fas/APO-1)
and tumor necrosis factor receptor-induced apoptosis. *J Biol Chem* 1996; 271: 4961-4965

26 **Hsu H**, Xiong J, Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell* 1995; 81: 495-504

27 **Vincenti MP**, Coon CI, Brinckerhoff CE. Nuclear factor kappaB/p50 activates an element in the distal matrix metalloproteinase 1 promoter in interleukin-1beta-stimulated synovial fibroblasts. *Arthritis Rheum* 1998; 41: 1987-1994

28 **Friedman JM**, Horwitz MS. Inhibition of tumor necrosis factor alpha-induced NF-kappa B activation by the adenovirus E3-10.4/14.5K complex. *J Virol* 2002; 76: 5515-5521

29 **Duffey DC**, Crowl-Bancroft CV, Chen Z, Ondrey FG, Nejad-Sattari M, Dong G, Van Waes C. Inhibition of transcription factor nuclear factor-kappaB by a mutant inhibitor-kappaBalpha attenuates resistance of human head and neck squamous cell carcinoma to TNF-alpha caspase-mediated cell death. *Br J Cancer* 2000; 83: 1367-1374

S- Editor Wang J L- Editor Zhu LH E- Editor Ma WH