Adenovirus-mediated Gene Transduction of IκB or IκB Plus *Bax* Gene Drastically Enhances Tumor Necrosis Factor (TNF)-induced Apoptosis in Human Gliomas

Nobusada Shinoura,[1, 2] Naoki Yamamoto,[1] Yoko Yoshida,[1] Takashi Fujita,[3] Nobuto Saito,[2] Akio Asai,[2] Takaaki Kirino[2, 4] and Hirofumi Hamada[1, 5, 6]

[1]Department of Molecular Biotherapy Research, Cancer Chemotherapy Center, Cancer Institute, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170-8455, [2]Department of Neurosurgery, Tokyo University, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, [3]Department of Tumor Cell Biology, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-0021, [4]CREST (Core Research for Evolutional Science and Technology), Tokyo and [5]Department of Molecular Medicine, Sapporo Medical University, Sapporo, 060-8556

Tumor necrosis factor-α (TNF), which was initially supposed to be a promising cancer therapeutic reagent, does not kill most types of cancer cells partly due to the activation of an anti-apoptotic gene, *NF-κB*. NF-κB forms an inactive complex with the inhibitor kappa B alpha (IκBα), which is rapidly phosphorylated and degraded in response to various extracellular signals. To disrupt this protective mechanism, we introduced an *inhibitor kappa B alpha (IκBdN)* gene, a deletion mutant gene lacking the nucleotides for the N-terminal 36 amino acids of IκBα, into human glioma cells (U251, T-98G, and U-373MG) via an adenoviral (Adv) vector in addition to treatment of the glioma cells with recombinant TNF. Immunohistochemical analysis revealed that NF-κB was translocated to nuclei by TNF treatment in U251 and T-98G cells, but not in U-373MG cells. Neither transduction of IκBdN nor treatment with TNF protein alone induced apoptosis in U251 and T-98G cells, whereas both cell lines underwent drastic TNF-induced apoptosis after transduction of IκBdN. On the other hand, U-373MG cells were refractory to TNF-induced apoptosis even when they were transduced with the *IκBdN* gene. U-373MG cells underwent drastically increased apoptosis when co-transduced with the *IκBdN* and *Bax* gene in the presence of TNF. Adv-mediated transfer of IκBdN or IκBdN plus Bax may be a promising therapeutic approach to treat gliomas through TNF-mediated apoptosis.

Key words: Apoptosis — IκB — TNF — Bax — Gene therapy

Tumor necrosis factor-α (TNF) has been reported to induce tumor regression and to enhance the antitumoral effect of conventional therapies by various mechanisms, such as promotion of leukocyte infiltration in tumors[1, 2] and generation of multiple cytokine responses.[3] However, TNF does not exhibit a direct cytotoxic effect on most glioma cell lines.[4–6] Instead, TNF promotes the growth of U-373 glioma cells.[7] Therefore, conventional therapies such as radiation,[8–10] chemotherapy[11] and immunotherapy[12, 13] have been used in combination with TNF treatment to augment the therapeutic effect in patients with malignant gliomas.

TNF induces the activation of NF-κB,[14] which plays a key role in the anti-apoptotic mechanism after TNF induction.[15–17] The prototypical form of NF-κB is a heterodimeric complex containing two DNA-binding subunits, p50 and p65.[18–21] The NF-κB p65 subunit serves as an intracellular receptor for the inhibitor kappa B alpha (IκBα), which prevents the nuclear import of NF-κB.[22–24]

Various extracellular signals, such as TNF, rapidly uncouple the IκBα-dependent cytoplasmic retention of NF-κB.[25] The sensitivity to TNF-induced apoptosis was enhanced by stable expression of the N- and C-terminal phosphorylation mutant of IκBα in primary mouse and human fibroblast, human Jurkat lymphoma and T24 bladder carcinoma lines.[17] The IκBα lacking the N-terminal 36 amino acids (IκBdN) also escaped proteolytic breakdown and served as a superantagonist of NF-κB by inhibiting its nuclear translocation.[26] Several therapeutic approaches have been reported to utilize adenovirus (Adv)-IκBdN for the inactivation of NF-κB, thereby inducing apoptosis.[27–31] Adv-IκBdN rendered melanoma cells susceptible to the cytotoxic effects of TNF,[27] and induced apoptosis in bladder cancers with activated NF-κB.[30]

In our previous reports, Adv-mediated transduction of Fas ligand, a member of the TNF superfamily, effectively induced apoptosis.[32] We have also reported that Adv-mediated transduction of Fas receptor,[33] a member of the TNF receptor superfamily, or Bax,[34] a member of Bcl-2 family, induced apoptosis in gliomas. In this study, we transduced the *IκBdN* gene via Adv and exposed glioma cells to recombinant human TNF to evaluate whether or

[6] To whom correspondence should be addressed at the Cancer Chemotherapy Center, Cancer Institute.
  E-mail: hhamada@jfcr.or.jp

not this approach enhances the pro-apoptotic effect of TNF in glioma cells. We found that Adv-mediated transfer of the *IκBdN* gene in combination with TNF induced drastic apoptosis in U251 and T-98G glioma cell lines, which have been shown to be resistant to TNF-mediated apoptosis. However, some cells such as U-373MG cells were highly resistant to apoptosis by Adv-IκBdN infection in the presence of TNF. To override this resistance mechanism, we transduced other proapoptotic genes with *IκBdN* gene in the presence of TNF. U-373MG cells showed an increased susceptibility to apoptosis by co-induction of the *IκBdN* plus *Bax* gene with TNF treatment. This therapeutic modality would be effective in treating gliomas which are refractory to TNF-mediated apoptosis.

MATERIALS AND METHODS

Cell lines The U251 glioma cell line was obtained from the Tumor Repository at the Division of Cancer Treatment, National Cancer Institute (Frederick, MD). The T-98G and U-373MG glioma cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). The U251, T-98G and U-373MG cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS).

Generation of recombinant Adv vectors The *Cla*I/*Sma*I (blunt end) fragment of human IκBαdN cDNA, a deletion mutant of IκBα encoding amino acids 37 to 317, from pCMV4-hIκBαdN (provided by Dr. Ballard),[26] was inserted into the *Eco*RI (blunt end) site of pCAcc,[35] which generated pCA-hIκBdN. The cosmid pAxCA-hIκBdN was generated by inserting the *Cla*I expression cassette from pCA-hIκBdN to the *Cla*I site of the cosmid pAxcw.[36] pAxCALNL-hBax, a cosmid encoding *Bax* gene, was generated as described previously.[34] The on/off switching unit CALNL-hBax consisted of the CAG promoter, the *neo* gene and poly(A) sequence flanked by a pair of loxP sites, *Bax* gene and another poly(A) signal.[37] The CALNL-hBax without NCre expresses the *neo* gene, but does not express the *Bax* gene. In the presence of NCre, which contains the Cre recombinase coding region,[37] the *neo* gene between the loxP sites is excised, and the CA promoter and *Bax* gene are joined together, resulting in the Adv expressing *Bax* gene under the control of the CA promoter.[37]

The cosmids pAxCA-hIκBdN and pAxCALNL-hBax were transfected with the genomic DNA-terminal protein complex of adenovirus type 5 (Ad5dlX), and the recombinant Advs were generated according to the method described by Miyake *et al.*[36] Multiplicities of infection (MOIs) were determined by viral titration using the 293 plaque forming assay as described previously.[37] Adv-mediated gene transduction was performed as described previously.[35] The AxCALNL-hBax were always co-infected with AxCANCre[37] at an MOI ratio of 2:1.[34] The total MOI of Adv used to infect each cell was kept the same in all experiments, by supplementing with the control Adv-lacZ.[32]

Assessment of cell death The degree of cell death was assessed by determining the percentage of cells which had died, the percentage of hypodiploid cells, and the degree of DNA fragmentation. These assays were performed using the cell lines two days after infection with Adv for IκBdN (Adv-IκBdN) and/or treatment with recombinant human TNF (Pepro Tech, #300-01A, London, UK).

After adherent and detached cells were combined together, the cells were stained with 0.2% trypan blue, and the percentage of dead cells was determined using a hemocytometer.

The percentage of hypodiploid cells was determined by the method described previously.[32] Briefly, ethanol-permeabilized cells were stained with propidium iodide, and then analyzed with CELLQuest software on a FACScan (Becton Dickinson, San Jose, CA). The DNA fluorescence gate was programmed to exclude cell aggregates and debris. The percentage of cells that had undergone apoptosis was assessed to be the ratio of the fluorescent area smaller than the G_0/G_1 peak to the total area of fluorescence. Two samples of cells for each experimental condition were analyzed.

DNA fragments in apoptotic cells were detected using the "APO-BRDU" kit (Pharmingen, San Diego, CA), according to the manufacturer's instructions. Briefly, the 3′-hydroxyl ends of the DNA in apoptotic cells were labeled with brominated deoxyuridine triphosphate nucleotides (Br-dUTP) by using terminal deoxynucleotidyl transferase. Br-dUTP was stained with fluorescein isothiocyanate (FITC)-labeled anti-BrdU monoclonal antibody. The samples were stained with propidium iodide and analyzed by FACScan.

Electron microscopy For transmission electron microscopy, the cells were first fixed in 0.1 *M* sodium phosphate buffer containing 2.5% glutaraldehyde at a pH of 7.5. They were then fixed in 0.1 *M* sodium phosphate buffer containing 1% OsO_4 at a pH of 7.2. The cells were embedded in Epon 812 (TAAB, Berkshire, UK), and sliced into 60 nm sections. The ultrathin sections were contrasted with uranyl acetate and lead citrate, and then examined with a Hitachi H7000 transmission electron microscope (Tokyo).

Immunoblot analysis Immunoblot analysis was performed using an ECL kit (Amersham, Buckinghamshire, England), as described previously.[32] Briefly, 10^6 cells were lysed by incubating them for 15 min on ice in 8× lysis buffer (10 m*M* Tris/HCl pH 8.0, 0.2% NP40, 1 m*M* EDTA), and the protein content of the supernatant was quantified using the DC protein assay kit (Bio-Rad, Hercules, CA), according to the manufacturer's instructions. An equal volume of 2× Laemmli buffer was added to the

supernatant, and this was boiled for 5 min. Equal amounts of protein from each extract (30 μg per lane) were separated using 10% polyacrylamide gels and transferred onto nitrocellulose membranes. After having been blocked with 5% dried milk in TBS (10 mM Tris-HCl pH 7.5, 150 mM sodium chloride), the membranes were incubated with the primary antibody for 1 h at 25°C. The primary antibody used was murine anti-FLAG M2 monoclonal antibody (Eastman Kodak, #IB13026, New Haven, CT). After washing, the membranes were incubated for 1 h at 25°C with 12 μl (per 6 ml) of horseradish peroxidase-conjugated rabbit anti-mouse IgG+A+M (H+L) (Zymed Laboratories, #61-6420, San Francisco, CA) for FLAG. Staining was carried out using the ECL kit, according to the manufacturer's instructions.

Immunohistochemistry Immunostaining was performed as described previously.[38] Briefly, cells were plated on plastic chamber slides (SonicSeal Slide Wells; Nunc, Naperville, IL), at 3 days after Adv-IκBdN infection and 3 h after TNF treatment, fixed with 4% paraformaldehyde for 10 min at 25°C, and permeabilized with acetone/methanol (1:1, v/v) for 2 min at room temperature. After having been blocked with phosphate-buffered saline (PBS) containing 5 mg/ml bovine serum albumin (BSA) and 50 mM glycine for 1 h at room temperature, cells were treated with mouse anti-NF-κB p50 monoclonal antibody[39] (1:1000 dilution) in PBS containing 0.04% Tween 20 (PBST) at 4°C overnight. They were washed with PBST, and treated with goat anti-mouse FITC-labeled IgG (1:500 dilution, ICN Pharmaceuticals, #55514, Aurora, OH) for 2 h at 25°C. Then, the cells were washed with PBST, and subjected to confocal microscopic observation using a MicroRadiance (Bio-Rad).

RESULTS

Expression of IκBdN in U251 cells infected with Adv-IκBdN Transduction of Adv-IκBdN into U251 cells induced the expression of IκBαdN protein (Fig. 1). The sequence encoding the FLAG epitope was fused in-frame with the N-terminal coding sequence of IκBαdN.[26] The apparent molecular size (Mr) of IκBαdN protein, which consists of amino acids 37 to 317 of IκBα combined with FLAG protein, was 39 kD, i.e., larger than the theoretical molecular size (Fig. 1). As shown in Fig. 1, the expression of FLAG tag proteins increased in a MOI-dependent manner, indicating that infection of Adv-IκBdN induced expression of IκBαdN protein in U251 cells.

Translocation of NF-κB to nuclei in U251, T-98G and U-373MG cells To evaluate the translocation of NF-κB and the effect of IκBdN protein on intracellular NF-κB, immunohistochemical analysis using FITC-conjugated anti-NF-κB p50 antibody was performed (Fig. 2). As described previously,[40] the MOIs for transduction of 50%

of the population (ED$_{50}$) in U251, T-98G and U-373MG were 27, 49, 17, respectively. At MOI 300, 100% of the cells in the respective cell lines were transduced with Adv vector.[40] In U251 cells infected with control Adv-lacZ, both cytoplasm and nuclei were stained with this antibody, indicating that NF-κB protein was partly translocated to nuclei (Fig. 2A, upper left panel). In U251 cells treated with TNF alone, only nuclei were stained, indicating that the TNF treatment led to the translocation of NF-κB into nuclei (Fig. 2A, lower left panel). In T-98G cells infected with control Adv-lacZ, only cytoplasm was stained with this antibody (Fig. 2B, upper left panel), whereas both cytoplasm and nuclei were stained in T-98G cells treated with TNF alone (Fig. 2B, lower left panel), indicating that the TNF treatment also led to the translocation of NF-κB into nuclei. In contrast, NF-κB was not translocated to nuclei in U-373MG cells by TNF treatment (Fig. 2C, lower left panel). On the other hand, in U251, T-98G or U-373MG cells infected with Adv-IκBdN, only cytoplasm was stained, irrespective of TNF treatment (Fig. 2, A–C, upper and lower right panels), suggesting that IκBdN protein inhibited the import of NF-κB into nuclei, leading to inactivation of NF-κB.

TNF-mediated apoptosis in U251 and T-98G glioma cells after infection with Adv-IκBdN We evaluated the effect of infecting U251 and T-98G cells with Adv-IκBdN on apoptosis when these cells were exposed to TNF. These two cell lines were infected with Adv-IκBdN at a MOI of 300 and/or treated with TNF at 30 ng/ml. The percentage of cell death and degree of apoptosis, in particular the percentage of hypodiploid cells and DNA fragmentation, were analyzed. Infection with Adv-IκBdN effectively killed U251 cells only in combination with TNF (Fig. 3A).

The percentage of cell death in U251 was determined using different concentrations of TNF or different MOIs of Adv-IκBdN to evaluate whether the proapoptotic effect of this approach was due to NF-κB inactivation (Fig. 3B).
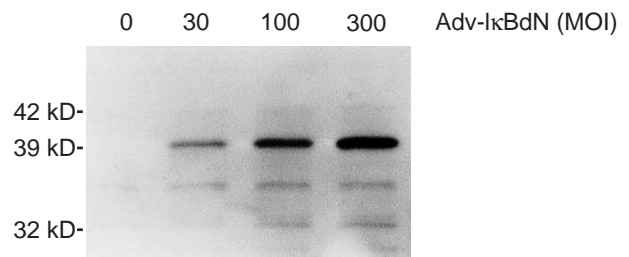

Fig. 1. Immunoblot analysis of the IκBdN protein extracted from U251 glioma cells 2 days after infection with Adv-IκBdN at various MOIs. The MOI of Adv-IκBdN used to infect U251 cells is given in parentheses. Lane 1, U251; lane 2, U251 infected with Adv-IκBdN (30); lane 3, U251 infected with Adv-IκBdN (100); lane 4, U251 infected with Adv-IκBdN (300).

A

Adv-lacZ   Adv-IκBdN

TNF(−)

TNF(+)

B

Adv-lacZ   Adv-IκBdN

TNF(−)

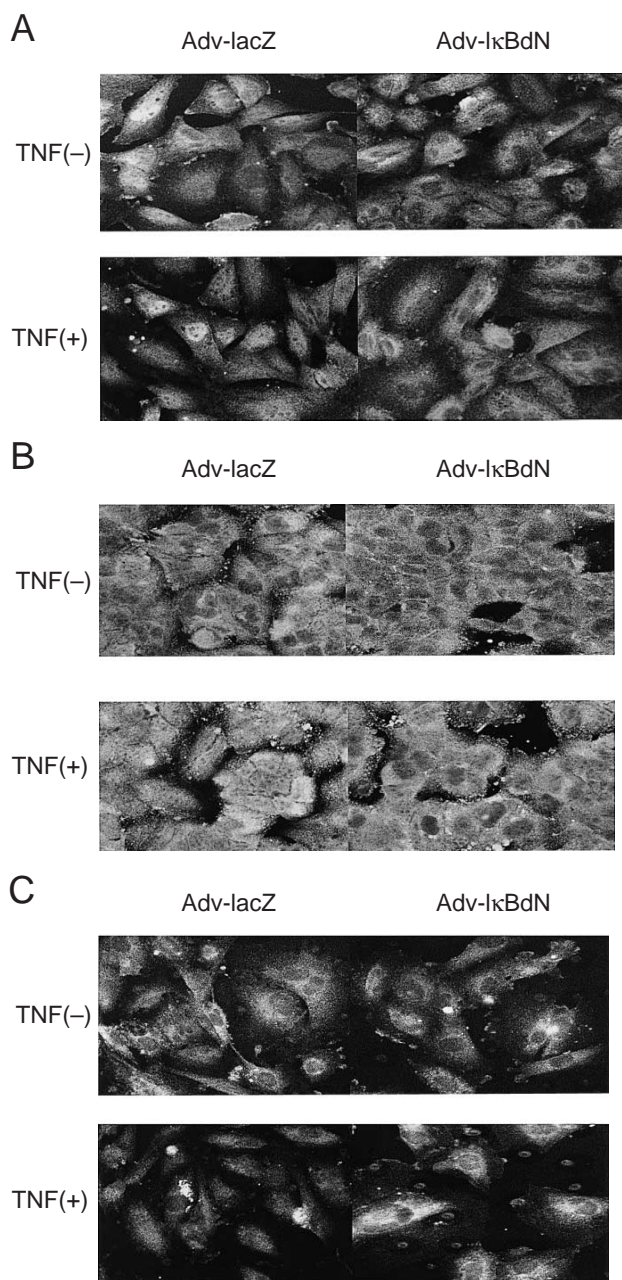TNF(+)

C

Adv-lacZ   Adv-IκBdN

TNF(−)

TNF(+)

Fig. 2. Confocal microscopic photographs of U251 (A), T-98G (B), and U-373MG (C) cells infected with Adv-IκBdN, Adv-lacZ and/or TNF treatment, and stained with FITC-labeled NF-κB p50 antibody. The cells were stained 3 days after infection, and 7 h after TNF treatment (original magnification ×600). The MOI of each Adv and the concentration of TNF are given in parentheses. Upper left panel: U251 (A), T-98G (B), or U-373MG (C) cells infected with Adv-lacZ (MOI 300), upper right panel: U251 (A), T-98G (B), or U-373MG (C) cells infected with Adv-IκBdN (MOI 300), lower left panel: U251 (A), T-98G (B), or U-373MG (C) cells treated with TNF (30 ng/ml), lower right panel: U251 (A), T-98G (B), and U-373MG (C) cells infected with Adv-IκBdN (MOI 300) in the presence of TNF (30 ng/ml).

The percentage of dead cells in U251 cells infected with Adv-IκBdN (MOI 300) and exposed to TNF (30 ng/ml) (77±2.3%) was significantly greater than the percentage of dead cells in U251 cells infected with either Adv-IκBdN (300) (14±2.7%) ($P<0.001$, Student's t test) or treated with TNF alone (30) (17±1.6%) ($P<0.001$, Student's t test) (Fig. 3B). The percentage of dead cells increased in U251 cells as the MOI of Adv-IκBdN or the concentration of TNF increased (Fig. 3B), suggesting that NF-κB activation was closely involved in the proapoptotic effect of this approach. Similarly, the percentage of dead T-98G cells infected with Adv-IκBdN in combination with TNF treatment (75±6.2%) was significantly greater than that infected with Adv-IκBdN without TNF treatment (8.2±1.1%) ($P<0.001$, Student's t test) or that with TNF treatment alone (12±1.2%) ($P<0.001$, Student's t test) (Fig. 3C).

The percentage of hypodiploid cells reflects the degree of apoptosis. The percentage of hypodiploid cells in the two cell lines infected with Adv-IκBdN and treated with TNF was significantly greater (76±3.9% and 57±0.2% for U251 and T-98G cells, respectively) than that in the cell lines infected with Adv-IκBdN alone (0.9±0.0% and 2.8±0.1% for U251 and T-98G cells, respectively) ($P<0.05$ and $P<0.01$, respectively, Student's t test) or exposed to TNF alone (3.2±0.4% or 3.8±1.4%, for U251 and T-98G cells, respectively) ($P<0.05$ and $P<0.01$, respectively, Student's t test) (Fig. 4). Similarly, the Br-dUTP uptake assay for detecting cells with fragmented DNA revealed that a remarkably high percentage of U251 cells infected with Adv-IκBdN and exposed to TNF (60%) contained fragmented DNA, whereas a low percentage of U251 cells infected with either Adv-IκBdN alone (0.3%) or treated with TNF alone (0.3%) contained fragmented DNA (Fig. 5A). U251 cells underwent apoptosis more markedly than T-98G cells, partly because the Adv transduction in U251 cells was more efficient compared with that in T-98G cells (data not shown). Although the degree of apoptosis in U251 cells was greater than that in T-98G cells, the results were relatively similar (Fig. 5B). The percentage of fragmented DNA in T-98G cells infected with Adv-IκBdN and exposed to TNF was 11%, whereas the percentage of fragmented DNA in T-98G cells infected with Adv-IκBdN alone or treated with TNF alone was 0.5% or 0.6%, respectively (Fig. 5B). All the assays demonstrate that infection with Adv-IκBdN in the presence of TNF induces significant apoptosis in glioma cells.

Electron microscopic analysis of U251 and T-98G cells infected with Adv-IκBdN in combination with TNF treatment revealed condensed chromatin in the nuclei (Fig. 6: U251, upper right panel; T-98G, lower right panel), which is a feature of apoptotic cell death.

In summary, infection with Adv-IκBdN in the presence of TNF significantly induced apoptotic cell death relative

to either infection with Adv-IκBdN or treatment with TNF alone in U251 and T-98G cells.

Bax induced an enhanced apoptosis in U-373MG cells which was resistant to this approach Infection with

Adv-IκBdN in combination with TNF treatment did not effectively kill U-373MG cells, even though the Adv-mediated transduction efficiency of U-373MG cells was similar to that of U251 cells (Fig. 7, A and B).[33] The per-
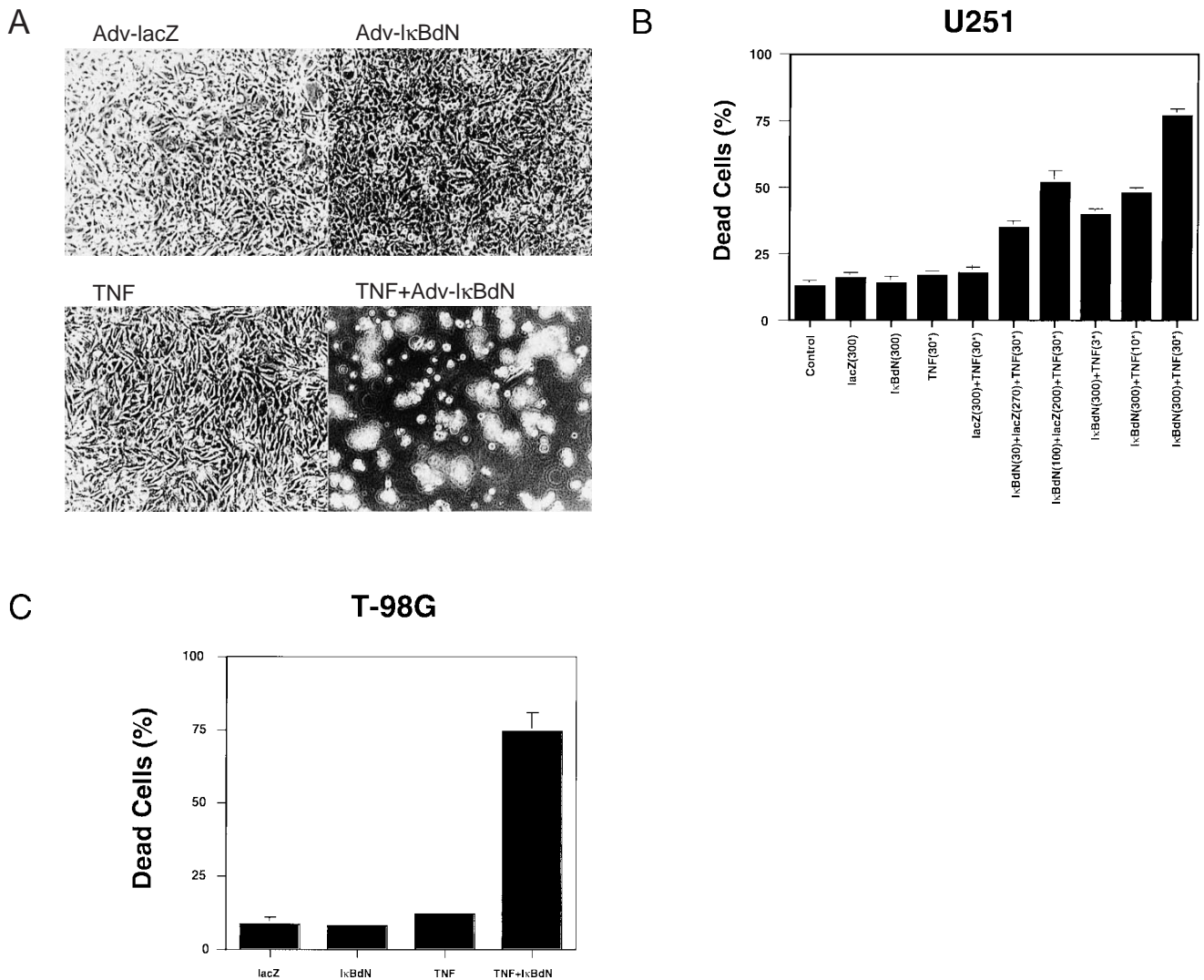

Fig. 3. A—Microscopic photographs of U251 cells infected with Adv-IκBdN, Adv-lacZ and/or TNF treatment. The cells were examined 2 days after infection (original magnification ×100). The MOI of each Adv and the concentration of TNF are given in parentheses. Upper left panel: U251 cells infected with Adv-lacZ (MOI 300), upper right panel: U251 cells infected with Adv-IκBdN (MOI 300), lower left panel: U251 cells treated with TNF (30 ng/ml), lower right panel: U251 cells infected with Adv-IκBdN (300) in the presence of TNF (30 ng/ml). B—Percentage of dead cells in U251 cells, measured by trypan blue exclusion 2 days after infection with Adv-lacZ (MOI 300) alone, Adv-IκBdN (MOI 300) alone, TNF treatment alone (30 ng/ml), Adv-IκBdN (MOI 30, 100, or 300) with TNF treatment (30 ng/ml), or TNF treatment (3, 10, or 30 ng/ml) with Adv-IκBdN (MOI 300) infection. Percentage of dead cells indicates the percentage of trypan blue-permeable cells. The total MOI was kept constant (300) by supplementing with Adv-lacZ. A number with an asterisk (∗) indicates the concentration of TNF (ng/ml). The mean±standard deviation of the percentage of dead cells in six preparations for each experimental condition is shown. C—Percentage of dead cells in T-98G cells, measured by trypan blue exclusion 2 days after infection with Adv-lacZ (MOI 300), Adv-IκBdN (MOI 300), TNF treatment (30 ng/ml), or Adv-IκBdN (MOI 300) plus TNF treatment (30 ng/ml). Percentage of dead cells indicates the percentage of trypan blue-permeable cells. The mean±standard deviation of the percentage of dead cells in six preparations for each experimental condition is shown.
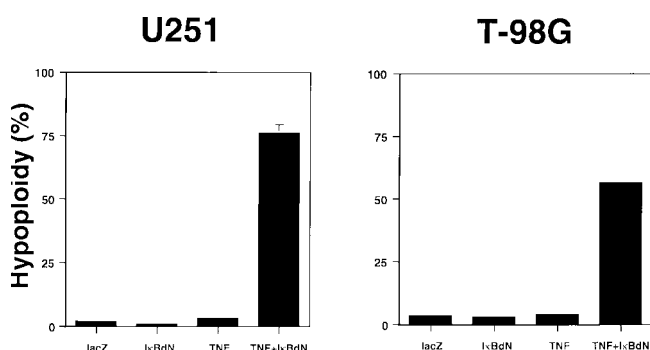
U251 T-98G

Fig. 4. FACS analysis of the percentage of hypodiploid cells, 2 days after infection of U251 and T-98G cells with Adv-lacZ (MOI 300), Adv-IκBdN (MOI 300), TNF treatment (30 ng/ml), or Adv-IκBdN (MOI 300) plus TNF treatment (30 ng/ml). The mean±standard deviation of the percentage of hypodiploid cells in two preparations for each experimental condition is shown.

centage of dead cells in Adv-IκBdN-infected U-373MG cells exposed to TNF (16±1.5%) showed a similar level to that of cells infected with control Adv-lacZ (15±2.3%) or Adv-IκBdN alone (14±2.8%), or treated with TNF alone (13±3.0%) (Fig. 7B). Therefore, we attempted to render U-373MG cells susceptible to apoptosis by combined induction of the *Bax* gene. We previously showed that Bax protein could be abundantly induced by Adv-mediated genetic transduction.[34] However, infection of Adv-Bax, or Adv-Bax and Adv-IκBdN, or Adv-Bax with TNF treatment showed similar, only moderate enhancement of cell death (28±6.9%, 27±2.1%, or 28±6.4%, respectively) (Fig. 7, A and B). In contrast, combined infection of Adv-Bax and Adv-IκBdN in the presence of TNF induced a drastically increased cell death (68±4.9%) compared with those of the former groups (Fig. 7, A and B). Similar results were obtained using the assay for apoptosis. The percentage of hypodiploid cells in U-373MG cells co-infected with Adv-Bax and Adv-IκBdN, and treated with TNF was much greater (37%) than that in cells infected with Adv-Bax (13%), Adv-Bax and Adv-IκBdN (7.9%) or Adv-Bax with exposure to TNF (16%) (Fig. 7C). Similarly, the Br-dUTP uptake assay for detecting cells with fragmented DNA revealed that a remarkably high percentage of U-373MG cells co-infected with Adv-Bax and Adv-IκBdN, and exposed to TNF (79%) contained fragmented DNA, whereas a lower percentage of U-373MG cells infected with either Adv-Bax (6.8%), Adv-Bax and Adv-IκBdN (4.7%) or Adv-Bax in the presence of TNF (9.8%) contained fragmented DNA (Fig. 7D). The results indicate that co-infection of Adv-Bax and Adv-IκBdN in the presence of TNF induces drastic apoptosis in U-373MG cells, which were refractory to apoptosis induction by infection with Adv-IκBdN in the presence of TNF.
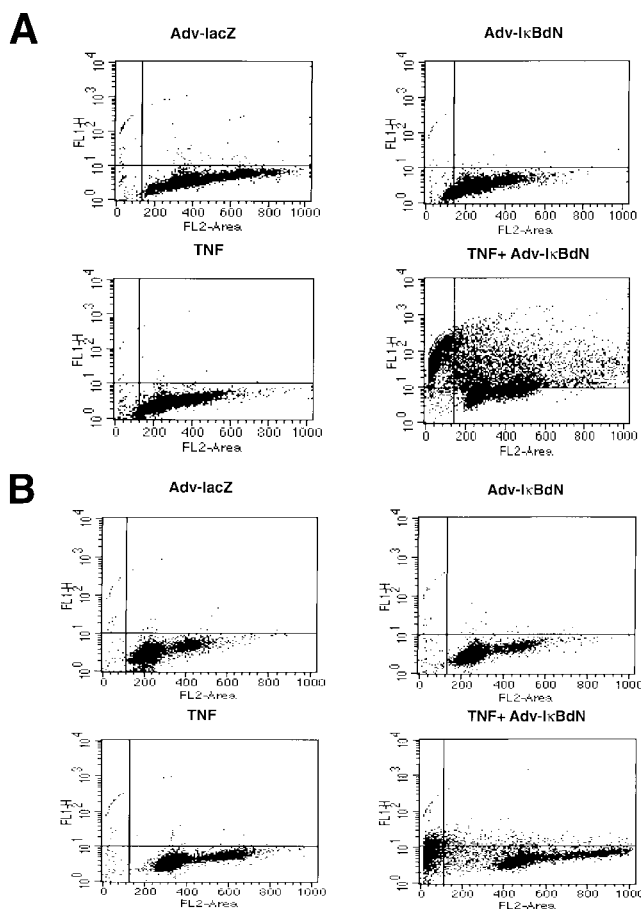

Fig. 5. DNA fragmentation of U251 (A) and T-98G (B) infected with Adv-IκBdN, Adv-lacZ and/or treated with TNF. The assay was performed as described in "Materials and Methods," 2 days after infection. The *X*-axis represents the propidium iodide-related fluorescence and the *Y*-axis represents the Br-dUTP-related fluorescence. Cells in the upper left and upper right areas represent apoptotic cells with fragmented DNA. The MOI of Adv-IκBdN or Adv-lacZ, and the concentration of TNF are given in parentheses. Upper left panel: U251 (A) or T-98G (B) cells infected with Adv-lacZ (MOI 300), upper right panel: U251 (A) or T-98G (B) cells infected with Adv-IκBdN (MOI 300), lower left panel: U251 (A) or T-98G (B) cells treated with TNF (30 ng/ml), lower right panel: U251 (A) or T-98G (B) cells infected with Adv-IκBdN (MOI 300) in the presence of TNF (30 ng/ml).

DISCUSSION

Nearly all glioma cells express TNF-R1 receptor, including U251[41] and T-98G,[42] through which most of the cytotoxic effects of TNF are transduced.[43] However, U251 and T-98G cells do not normally undergo apoptosis when treated with TNF. On the other hand, infection of these
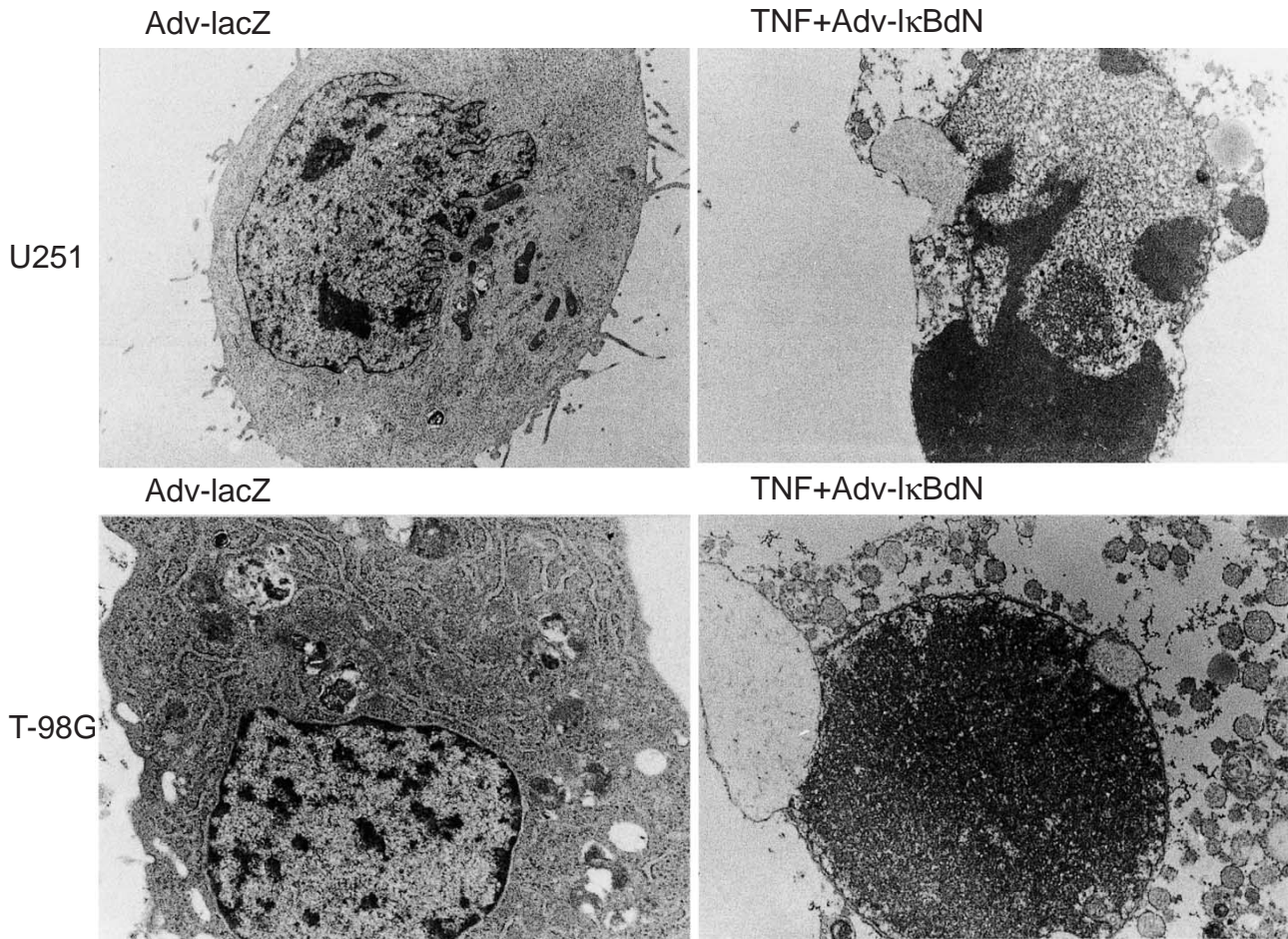
Adv-lacZ

TNF+Adv-IκBdN

U251

Adv-lacZ

TNF+Adv-IκBdN

T-98G

Fig. 6. Ultrastructural analysis of U251 and T-98G cells after infection with Adv-IκBdN (MOI 300) concomitantly with TNF treatment (30 ng/ml) or Adv-lacZ (MOI 300). Upper left panel: U251 cells 2 days after infection with Adv-lacZ (×4000), upper right panel: U251 cells 2 days after infection with Adv-IκBdN (MOI 300) in the presence of TNF (30 ng/ml) (×10000), lower left panel: T-98G cells 2 days after infection with Adv-lacZ (×10000), lower right panel: T-98G cells 2 days after infection with Adv-IκBdN (MOI 300) in the presence of TNF (30 ng/ml) (×10000). Condensation of chromatin appeared in U251 (upper right panel) and T-98G (lower right panel) cells 2 days after infection with Adv-IκBdN in combination with TNF exposure.

cell lines with Adv-IκBdN induced significant TNF-mediated apoptosis. The anti-apoptotic mechanism of TNF-mediated apoptosis is most likely due to activation of NF-κB, because suppression of NF-κB activity by IκBdN promoted the pro-apoptotic effect of TNF in these cell lines. In U251 and T-98G cells, IκBdN protein transferred by Adv-IκBdN inhibited the import of NF-κB into the nuclei, which was induced by TNF treatment alone, consequently leading to the inactivation of NF-κB (Fig. 2, A and B). The dose-effect relationship of Adv-IκBdN or TNF to the proapoptotic effect in U251 cells (Fig. 3A) also suggests that NF-κB inactivation contributed to the promotion of the cytopathic effect of this approach. Nuclear importation of NF-κB is hindered because of the high-affinity associa-

tion of its p65 subunit with a labile cytoplasmic inhibitor IκB.[22–24] However, cytoplasmic inhibitor IκB is rapidly degraded after TNF stimulation.[25] The *IκBdN* gene encodes an IκB protein that lacks the N-terminal 36 amino acids. The deleted portion of the gene encodes serine 32 and 36, which, after phosphorylation by IκB kinase, are targets of degradation via the ubiquitin-proteasome pathway.[44, 45] Therefore, IκBdN escapes proteolytic degradation and binds NF-κB, effectively preventing nuclear importation of NF-κB. Thus, IκBdN is a constitutively active "superantagonist" of NF-κB. Inhibition of NF-κB function results in suppression of its anti-apoptotic activity, potentiating the proapoptotic effect in cells treated with TNF.[15–17]
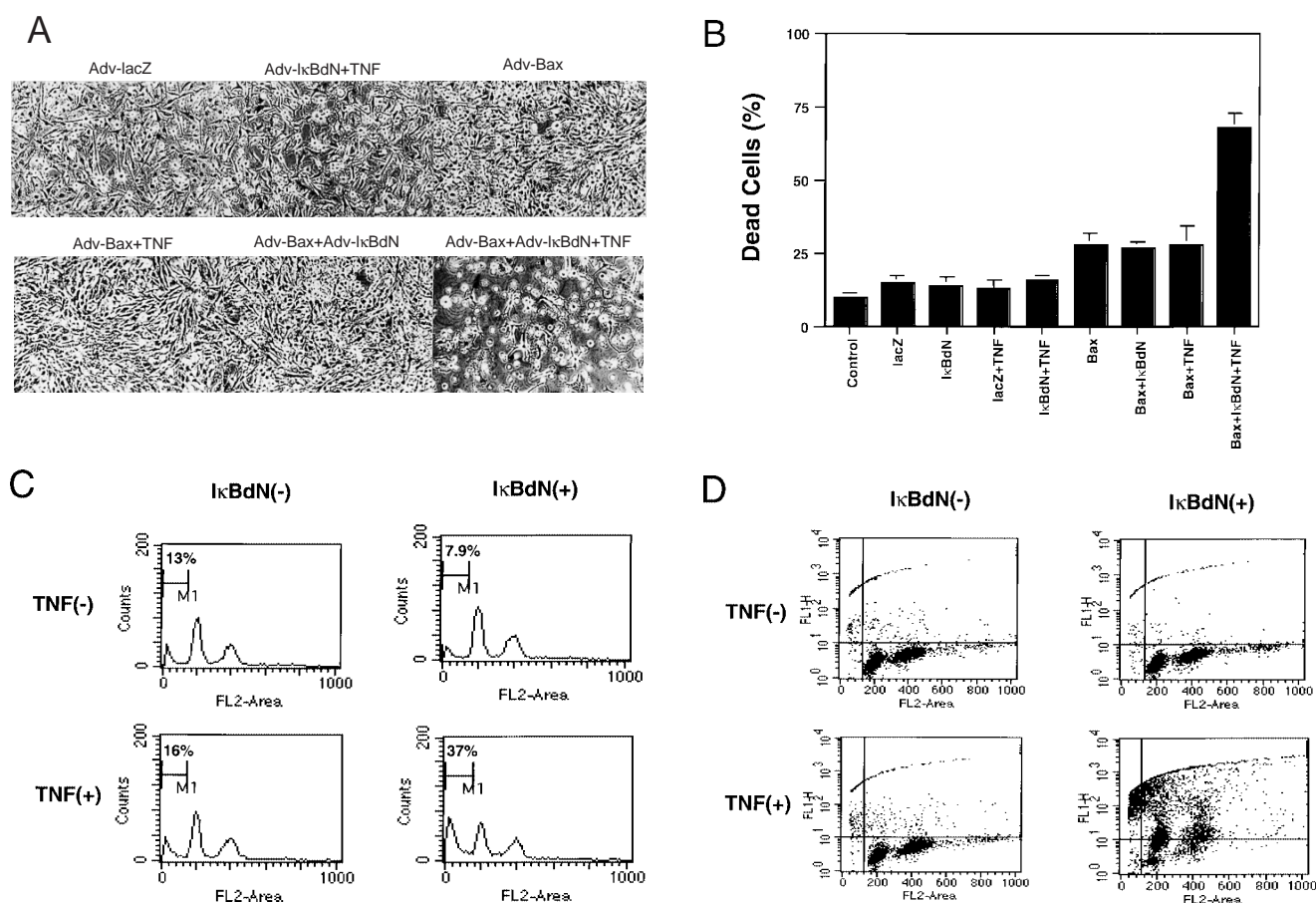
Fig. 7. A—Microscopic photographs of U-373MG cells infected with Adv-Bax, Adv-IκBdN, Adv-lacZ and/or TNF treatment. The cells were examined 3 days after infection (original magnification ×100). The MOI of each Adv and the concentration of TNF are given in parentheses. Adv-Bax was always co-infected with Adv for NCre (Adv-Cre), as described in "Materials and Methods." The total MOI was kept constant (345) by supplementing with Adv-lacZ. Upper left panel: U-373MG cells infected with Adv-lacZ (MOI 345), upper middle panel: U-373MG cells infected with Adv-IκBdN (MOI 300), Adv-lacZ (MOI 45) in the presence of TNF (30 ng/ml), upper right panel: U-373MG cells infected with Adv-Bax (MOI 30), Adv-Cre (MOI 15), and Adv-lacZ (MOI 300), lower left panel: U-373MG cells infected with Adv-Bax (MOI 30), Adv-Cre (MOI 15), and Adv-lacZ (MOI 300) in the presence of TNF (30 ng/ml), lower middle panel: U-373MG cells infected with Adv-Bax (MOI 30), Adv-Cre (MOI 15), and Adv-IκBdN (MOI 300), lower right panel: U-373MG cells infected with Adv-Bax (MOI 30), Adv-Cre (MOI 15), and Adv-IκBdN (MOI 300) in the presence of TNF (30 ng/ml). B—Percentage of dead cells in U-373MG cells, measured by trypan blue exclusion 3 days after infection with various combinations of Adv-lacZ, Adv-IκBdN, or Adv-Bax (plus Adv-Cre) with or without TNF treatment (30 ng/ml). Percentage of dead cells indicates the percentage of trypan blue-permeable cells. The total MOI was kept constant (345) by supplementing with Adv-lacZ. The mean±standard deviation of the percentage of dead cells in six preparations for each experimental condition is shown. C—FACS analysis of the percentage of hypodiploid cells, 3 days after infection of U-373MG cells with Adv-Bax (plus Adv-Cre) and Adv-lacZ or Adv-IκBdN, with or without TNF treatment (30 ng/ml). The MOI of each Adv and the concentration of TNF are given in parentheses. The total MOI was kept constant (345) by supplementing with Adv-lacZ. Upper left panel: U-373MG cells infected with Adv-Bax (MOI 30), Adv-Cre (MOI 15), and Adv-lacZ (MOI 300), upper right panel: U-373MG cells infected with Adv-Bax (MOI 30), Adv-Cre (MOI 15), and Adv-IκBdN (MOI 300), lower left panel: U-373MG cells infected with Adv-Bax (MOI 30), Adv-Cre (MOI 15), and Adv-lacZ (MOI 300) in the presence of TNF (30 ng/ml), lower right panel: U-373MG cells infected with Adv-Bax (MOI 30), Adv-Cre (MOI 15), and Adv-IκBdN (MOI 300) in the presence of TNF (30 ng/ml). D—DNA fragmentation of U-373MG cells 3 days after infection with Adv-Bax (plus Adv-Cre) and Adv-lacZ or Adv-IκBdN, with or without TNF treatment (30 ng/ml). The panels in Fig. 7D correspond to those in Fig. 7C.

There are several advantages to combining the infection of gliomas with Adv-IκBdN with TNF treatment. This approach markedly potentiates TNF-mediated apoptotic cell death in two glioma cell lines, U251 and T-98G, which are refractory to TNF-induced apoptosis. In addition, this approach would not be expected to damage

normal brain because normal brain lacks TNF-binding receptors, whereas most gliomas have TNF-binding sites.[46] Indeed, when TNF was administered intra-arterially to patients with brain tumors, the side effects were mild and controllable, and the neurological symptoms improved in half of the patients without tumor regression.[47] Therefore, this approach may be highly effective in killing glioma cells, while sparing normal brain tissue. In addition to the direct cytotoxic effect, TNF administration exerts immunological effects which would greatly augment the *in vivo* antitumoral effect. TNF treatment induces immune cell migration,[1, 2] generates multiple cytokine responses,[3, 5] and makes glioma cells susceptible to natural killer cell-mediated lysis.[48] These immunological responses would induce an *in vivo* bystander effect of Adv-IκBdN infection in the presence of TNF. Further investigations are needed to evaluate the *in vivo* efficacy of this system, including the bystander effect. Finally, the suppression of angiogenesis[49] and invasiveness of gliomas[50] by TNF treatment may also enhance the *in vivo* therapeutic effect of this approach in patients with malignant gliomas.

On the other hand, not all cell lines use NF-κB to protect against TNF-induced apoptosis. The inhibition of NF-κB does not alter TNF-induced apoptosis in MCF-7 cells, indicating that TNF-mediated apoptosis in these cells is independent of NF-κB.[51] Also, U-373MG cells did not exhibit TNF-mediated apoptosis even after infection with Adv-IκBdN (Fig. 7, A and B). In contrast to U251 and T-98G cells, NF-κB was not translocated to nuclei of U-373MG (Fig. 2C). Thus, the signal from TNF receptor to NF-κB (antiapoptotic) was not so strong as that of U251 or T-98G. It is likely that the proapoptotic signal from TNF receptor to FADD was also weak in U-373MG cells, and therefore inactivation of NF-κB did not remarkably increase the TNF-mediated proapoptotic signal in U-

373MG cells (Fig. 7). On the other hand, Bax induction was highly beneficial to overcome the resistance to the IκBdN plus TNF treatment in U-373MG cells (Fig. 7). Bax is reported to play a critical role in chemotherapy-induced apoptosis. Expression levels of Bax in cancers determine the cytotoxic effect of chemotherapy.[52-54] Chemotherapeutic agents also induce cancer cell death through upregulation of Bax.[55] Therefore, our result might be consistent with the report by Wang *et al.*,[56] who had shown that Adv-IκBdN sensitizes chemoresistant tumors to a chemotherapeutic compound, CPT-11. In their report, the inactivation of NF-κB by Adv-IκBdN and the activation of Bax by chemotherapy might have synergistically promoted tumor regression. It remains to be determined precisely why the IκBdN plus TNF treatment did not elicit apoptosis in U-373MG cells, and why the co-treatment with Bax overrode this resistance mechanism.

Since some cancer cells are resistant to the IκBdN-mediated therapies, for efficient cancer therapy utilizing TNF, we need to choose an adequate combination with the therapeutic genes, i.e., *IκBdN* alone or *IκBdN* plus *Bax*. Our approach, which utilized the *Bax* gene to override the resistance mechanism, should further improve TNF-mediated therapy.

ACKNOWLEDGMENTS

REFERENCES

1) Fukumura, D., Salehi, H. A., Witwer, B., Tuma, R. F., Melder, R. J. and Jain, R. K. Tumor necrosis factor alpha-induced leukocyte adhesion in normal and tumor vessels: effect of tumor type, transplantation site, and host strain. *Cancer Res.*, **55**, 4824–4829 (1995).

2) Wright, J. L. and Merchant, R. E. Effects of an intratumoral injection of human recombinant tumor necrosis factor-alpha on cerebrovascular permeability and leukocytic infiltration in a rat glioma model. *Acta Neuropathol. (Berl.)*, **93**, 78–86 (1997).

3) Tada, M., Sawamura, Y., Sakuma, S., Suzuki, K., Ohta, H., Aida, T. and Abe, H. Cellular and cytokine responses of the human central nervous system to intracranial administration of tumor necrosis factor alpha for the treatment of malignant gliomas. *Cancer Immunol. Immunother.*, **36**, 251–259 (1993).

4) Del Maestro, R. F., Lopez-Torres, M., Mcdonald, W. B., Stroude, E. C. and Vaithilingam, I. S. The effect of tumor necrosis factor-alpha on human malignant glial cells. *J. Neurosurg.*, **76**, 652–659 (1992).

5) Sakuma, S., Sawamura, Y., Tada, M., Aida, T., Abe, H., Suzuki, K. and Taniguchi, N. Responses of human glioblastoma cells to human natural tumor necrosis factor-alpha: susceptibility, mechanism of resistance and cytokine production studies. *J. Neurooncol.*, **15**, 197–208 (1993).

6) Zuber, P., Accolla, R. S., Carrel, S., Diserens, A. C. and De Tribolet, N. Effects of recombinant human tumor necrosis factor-alpha on the surface phenotype and the growth of human malignant glioma cell lines. *Int. J. Cancer*, **42**, 780–786 (1988).

7) Lachman, L. B., Brown, D. C. and Dinarello, C. A. Growth-promoting effect of recombinant interleukin 1 and

tumor necrosis factor for a human astrocytoma cell line. *J. Immunol.*, **138**, 2913–2916 (1987).

8) Staba, M. J., Mauceri, H. J., Kufe, D. W., Hallahan, D. E. and Weichselbaum, R. R. Adenoviral TNF-alpha gene therapy and radiation damage tumor vasculature in a human malignant glioma xenograft. *Gene Ther.*, **5**, 293–300 (1998).

9) Gridley, D. S., Archambeau, J. O., Andres, M. A., Mao, X. W., Wright, K. and Slater, J. M. Tumor necrosis factor-alpha enhances antitumor effects of radiation against glioma xenografts. *Oncol. Res.*, **9**, 217–227 (1997).

10) Huang, P., Allam, A., Perez, L. A., Taghian, A., Freeman, J. and Suit, H. D. The effect of combining recombinant human tumor necrosis factor-alpha with local radiation on tumor control probability of a human glioblastoma multiforme xenograft in nude mice. *Int. J. Radiat. Oncol. Biol. Phys.*, **32**, 93–98 (1995).

11) Walther, W., Stein, U. and Pfeil, D. Gene transfer of human TNF alpha into glioblastoma cells permits modulation of mdr1 expression and potentiation of chemosensitivity. *Int. J. Cancer*, **61**, 832–839 (1995).

12) Takaoka, T., Yoshida, J., Mizuno, M. and Sugita, K. Transfection-induced tumor necrosis factor-α increases the susceptibility of human glioma cells to lysis by lymphokine-activated killer cells: continuous expression of intercellular adhesion molecule-1 on the glioma cells. *Jpn. J. Cancer Res.*, **85**, 750–755 (1994).

13) Harada, K., Yoshida, J., Mizuno, M., Sugita, K., Kurisu, K. and Uozumi, T. Growth inhibition of subcutaneously transplanted human glioma by transfection-induced tumor necrosis factor-alpha and augmentation of the effect by gamma-interferon. *J. Neurooncol.*, **22**, 221–225 (1994).

14) Baeuerle, P. A. and Henkel, T. Function and activation of NF-kappa B in the immune system. *Annu. Rev. Immunol.*, **12**, 141–179 (1994).

15) Beg, A. A. and Baltimore, D. An essential role for NF-κB in preventing TNF-α-induced cell death. *Science*, **274**, 782–784 (1996).

16) Wang, C.-Y., Mayo, M. W. and Baldwin, A. S., Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. *Science*, **274**, 784–787 (1996).

17) Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R. and Verma, I. M. Suppression of TNF-α-induced apoptosis by NF-κB. *Science*, **274**, 787–789 (1996).

18) Bours, V., Villalobos, J., Burd, P. R., Kelly, K. and Siebenlist, U. Cloning of a mitogen-inducible gene encoding a kappa B DNA-binding protein with homology to the rel oncogene and to cell-cycle motifs. *Nature*, **348**, 76–80 (1990).

19) Ghosh, S., Gifford, A. M., Riviere, L. R., Tempst, P., Nolan, G. P. and Baltimore, D. Cloning of the p50 DNA binding subunit of NF-kappa B: homology to rel and dorsal. *Cell*, **62**, 1019–1029 (1990).

20) Nolan, G. P., Ghosh, S., Liou, H. C., Tempst, P. and Baltimore, D. DNA binding and I kappa B inhibition of the cloned p65 subunit of NF-kappa B, a rel-related polypeptide. *Cell*, **64**, 961–969 (1991).

21) Ruben, S. M., Dillon, P. J., Schreck, R., Henkel, T., Chen, C. H., Maher, M., Baeuerle, P. A. and Rosen, C. A. Isolation of a rel-related human cDNA that potentially encodes the 65-kD subunit of NF-kappa B. *Science*, **251**, 1490–1493 (1991).

22) Baeuerle, P. A. and Baltimore, D. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science*, **242**, 540–546 (1988).

23) Beg, A. A., Ruben, S. M., Scheinman, R. I., Haskill, S., Rosen, C. A. and Baldwin, A. S., Jr. I kappa B interacts with the nuclear localization sequences of the subunits of NF-kappa B: a mechanism for cytoplasmic retention. *Genes Dev.*, **6**, 1899–1913 (1992).

24) Ganchi, P. A., Sun, S. C., Greene, W. C. and Ballard, D. W. I kappa B/MAD-3 masks the nuclear localization signal of NF-kappa B p65 and requires the transactivation domain to inhibit NF-kappa B p65 DNA binding. *Mol. Biol. Cell*, **3**, 1339–1352 (1992).

25) Henkel, T., Machleidt, T., Alkalay, I., Kronke, M., Ben-Neriah, Y. and Baeuerle, P. A. Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. *Nature*, **365**, 182–185 (1993).

26) Brockman, J. A., Scherer, D. C., Mckinsey, T. A., Hall, S. M., Qi, X., Lee, W. Y. and Ballard, D. W. Coupling of a signal response domain in IκBα to multiple pathways for NF-κB activation. *Mol. Cell. Biol.*, **15**, 2809–2818 (1995).

27) Bakker, T. R., Reed, D., Renno, T. and Jongeneel, C. V. Efficient adenoviral transfer of NF-kappa B inhibitor sensitizes melanoma to tumor necrosis factor-mediated apoptosis. *Int. J. Cancer*, **80**, 320–323 (1999).

28) Goodman, D. J., Von Albertini, M. A., Mcshea, A., Wrighton, C. J. and Bach, F. H. Adenoviral-mediated overexpression of I(kappa)B(alpha) in endothelial cells inhibits natural killer cell-mediated endothelial cell activation. *Transplantation*, **62**, 967–972 (1996).

29) Iimuro, Y., Nishiura, T., Hellerbrand, C., Behrns, K. E., Schoonhoven, R., Grisham, J. W. and Brenner, D. A. NFkappaB prevents apoptosis and liver dysfunction during liver regeneration. *J. Clin. Invest.*, **101**, 802–811 (1998).

30) Sumitomo, M., Tachibana, M., Ozu, C., Asakura, H., Murai, M., Hayakawa, M., Nakamura, H., Takayanagi, A. and Shimizu, N. Induction of apoptosis of cytokine-producing bladder cancer cells by adenovirus-mediated IkappaBalpha overexpression. *Hum. Gene Ther.*, **10**, 37–47 (1999).

31) Thomas, L. H., Friedland, J. S., Sharland, M. and Becker, S. Respiratory syncytial virus-induced RANTES production from human bronchial epithelial cells is dependent on nuclear factor-kappa B nuclear binding and is inhibited by adenovirus-mediated expression of inhibitor of kappa B alpha. *J. Immunol.*, **161**, 1007–1016 (1998).

32) Shinoura, N., Yoshida, Y., Sadata, A., Hanada, K., Yamamoto, S., Kirino, T., Asai, A. and Hamada, H. Apoptosis by retrovirus- and adenovirus-mediated gene transfer of Fas ligand to glioma cells: implications for gene therapy. *Hum. Gene Ther.*, **9**, 1983–1993 (1998).

33) Shinoura, N., Ohashi, M., Yoshida, Y., Kirino, T., Asai, A., Hashimoto, M. and Hamada, H. Adenovirus-mediated overexpression of Fas induces apoptosis of gliomas. *Cancer Gene Ther.* (1999), in press.

34) Shinoura, N., Yoshida, Y., Asai, A., Kirino, T. and Hamada, H. Relative level of expression of Bax and Bcl-X$_L$ determines the cellular fate of apoptosis/necrosis induced by the overexpression of Bax. *Oncogene* (1999), in press.

35) Yoshida, Y. and Hamada, H. Adenovirus-mediated inducible gene expression through tetracycline-controllable trans-activator with nuclear localization signal. *Biochem. Biophys. Res. Commun.*, **230**, 426–430 (1997).

36) Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., Takamori, K., Tokuda, C. and Saito, I. Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. *Proc. Natl. Acad. Sci. USA*, **93**, 1320–1324 (1996).

37) Kanegae, Y., Lee, G., Sato, Y., Tanaka, M., Nakai, M., Sakaki, T., Sugano, S. and Saito, I. Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. *Nucleic Acids Res.*, **23**, 3816–3821 (1995).

38) Watanabe, N., Iwamura, T., Shinoda, T. and Fujita, T. Regulation of NFκB1 proteins by the candidate oncoprotein BCL-3: generation of NF-κB homodimers from the cytoplasmic pool of p50-p105 and nuclear translocation. *EMBO J.*, **16**, 3609–3620 (1997).

39) Yoneyama, M., Suhara, W., Fukuhara, Y., Fukuda, M., Nishida, E. and Fujita, T. Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J.*, **17**, 1087–1095 (1998).

40) Yoshida, Y., Sadata, A., Zhang, W., Saito, K., Shinoura, N. and Hamada, H. Generation of fiber-mutant recombinant adenoviruses for gene therapy of malignant glioma. *Hum. Gene Ther.*, **9**, 2503–2515 (1998).

41) Kato, T., Sawamura, Y., Tada, M., Sakuma, S., Sudo, M. and Abe, H. p55 and p75 tumor necrosis factor receptor expression on human glioblastoma cells. *Neurol. Med. Chir. (Tokyo)*, **35**, 567–574 (1995).

42) Morgavi, P., Cimoli, G., Ottoboni, C., Michelotti, A., Conte, P., Parodi, S. and Russo, P. Sensitization of human glioblastoma T98G cells to VP16 and VM26 by human necrosis factor. *Anticancer Res.*, **15**, 1423–1428 (1995).

43) Tartaglia, L. A., Rothe, M., Hu, Y. T. and Goeddel, D. V. Tumor necrosis factor's cytotoxic activity is signaled by the p55 TNF receptor. *Cell*, **73**, 213–216 (1993).

44) Regnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z. and Rothe, M. Identification and characterization of an IkappaB kinase. *Cell*, **90**, 373–383 (1997).

45) Didonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E. and Karin, M. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature*, **388**, 548–554 (1997).

46) Maruno, M., Yoshimine, T., Isaka, T., Ghulam Muhammad, A., Nishioka, K. and Hayakawa, T. Cellular targets of exogenous tumour necrosis factor-alpha (TNF alpha) in human gliomas. *Acta Neurochir. (Wien)*, **138**, 1437–1441 (1996).

47) Yoshida, J., Wakabayashi, T., Mizuno, M., Sugita, K., Yoshida, T., Hori, S., Mori, T., Sato, T., Karashima, A., Kurisu, K., Kiya, K. and Uozumi, T. Clinical effect of intra-arterial tumor necrosis factor-alpha for malignant glioma. *J. Neurosurg.*, **77**, 78–83 (1992).

48) Kondo, S., Yin, D., Takeuchi, J., Morimura, T., Miyatake, S. I., Nakatsu, S., Oda, Y. and Kikuchi, H. Tumor necrosis factor-α induces an increase in susceptibility of human glioblastoma U87-MG cells to natural killer cell-mediated lysis. *Br. J. Cancer*, **69**, 627–632 (1994).

49) Niida, H., Takeuchi, S., Tanaka, R. and Minakawa, T. Angiogenesis in microvascular endothelial cells induced by glioma cells and inhibited by tumor necrosis factor *in vitro*. *Neurol. Med. Chir. (Tokyo)*, **35**, 209–214 (1995).

50) Iwasaki, K., Rogers, L. R., Barnett, G. H., Estes, M. L. and Barna, B. P. Effect of recombinant tumor necrosis factor-alpha on three dimensional growth, morphology, and invasiveness of human glioblastoma cells *in vitro*. *J. Neurosurg.*, **78**, 952–958 (1993).

51) Cai, Z., Korner, M., Tarantino, N. and Chouaib, S. IκBα overexpression in human breast carcinoma MCF7 cells inhibits nuclear factor-κB activation but not tumor necrosis factor-α-induced apoptosis. *J. Biol. Chem.*, **272**, 96–101 (1997).

52) Chresta, C. M., Masters, J. R. W. and Hickman, J. A. Hypersensitivity of human testicular tumors to etoposide-induced apoptosis is associated with functional p53 and a high bax:bcl-2 ratio. *Cancer Res.*, **56**, 1834–1841 (1996).

53) Strobel, T., Swanson, L., Korsmeyer, S. and Cannistra, S. A. Bax enhances paclitaxel-induced apoptosis through a p53-independent pathway. *Proc. Natl. Acad. Sci. USA*, **93**, 14094–14099 (1996).

54) Wagener, C., Bargou, R. C., Daniel, P. T., Bommert, K., Mapara, M. Y., Royer, H. D. and Dorken, B. Induction of the death-promoting gene bax-a sensitizes cultured breast-cancer cells to drug-induced apoptosis. *Int. J. Cancer*, **67**, 138–141 (1996).

55) Boersma, A. W. M., Nooter, K., Burger, H., Kortland, C. J. and Stoter, G. Bax upregulation is an early event in cisplatin-induced apoptosis in human testicular germ-cell tumor cell line NT2, as quantitated by flow cytometry. *Cytometry*, **27**, 275–282 (1997).

56) Wang, C.-Y., Cusack, J. C., Jr., Liu, R. and Baldwin, A. S., Jr. Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-κB. *Nat. Med.*, **5**, 412–417 (1999).