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

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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

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.27–33 Adv-IκBdN rendered melanoma cells susceptible to the cytotoxic effects of TNF27 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

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not this approach enhances the pro-apoptotic effect of TNF in glioma cells. We found that Adv-mediated transfer of the IxBδN 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 cell lines such as U-373MG cells were highly resistant to apoptosis by Adv-1xBδN infection in the presence of TNF. To override this resistance mechanism, we transduced other proapoptotic genes with IxBδN gene in the presence of TNF. U-373MG cells showed an increased susceptibility to apoptosis by co-induction of the IxBδN 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 cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum.

Generation of recombinant Adv vectors The Clal/Smal (blunt end) fragment of human IxBδN cDNA, a deletion mutant of IxBx encoding amino acids 37 to 317, from pCMV4-hIxBδD (provided by Dr. Ballard),26 was inserted into the EcoRI (blunt end) site of pCAcc,35 which generated pCA-hIxBδN. The cosmid pAxCAlNL-hBax was generated by inserting the Clal expression cassette from pCA-hIxBδD to the Clal 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 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 pAxCAlNL-hBδN 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. Adv-mediated gene transduction was performed as described previously.35 The AxCALNL-hBax were always co-infected with AxCANCRe37 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 IxBδN (Adv-IxBδN) 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 G0/G1 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% OsO4 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, 106 cells were lysed by incubating them for 15 min on ice in 8× lysis buffer (10 mM Tris/HCl pH 8.0, 0.2% NP40, 1 mM 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 (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. 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 (H2O, pH 7.4), and permeabilized with ace tone/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 (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 IκBdN protein (Fig. 1). The sequence encoding the FLAG epitope was fused in-frame with the N-terminal coding sequence of IκBdN. The apparent molecular size (Mr) of IκBdN 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 IκBdN 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, the MOIs for transduction of 50% of the population (ED50) 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. 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 U251 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).
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-\textit{lacZ} 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-\textit{lacZ} and Adv-\textit{IxBdN} 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). The per-

Fig. 3. A—Microscopic photographs of U251 cells infected with Adv-\textit{lacZ}, Adv-\textit{IxBdN} and/or TNF treatment. The cells were examined 2 days after infection (original magnification \(\times 100\)). The MOI of each Adv and the concentration of TNF are given in parentheses. Upper left panel: U251 cells infected with Adv-\textit{lacZ} (MOI 300), upper right panel: U251 cells infected with Adv-\textit{IxBdN} (MOI 300), lower left panel: U251 cells treated with TNF (30 ng/ml), lower right panel: U251 cells infected with Adv-\textit{IxBdN} (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-\textit{lacZ} (MOI 300) alone, Adv-\textit{IxBdN} (MOI 300) alone, TNF treatment alone (30 ng/ml), Adv-\textit{IxBdN} (MOI 30, 100, or 300) with TNF treatment (30 ng/ml), or TNF treatment (3, 10, or 30 ng/ml) with Adv-\textit{IxBdN} (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-\textit{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, treated with trypan blue exclusion 2 days after infection with Adv-\textit{lacZ} (MOI 300), Adv-\textit{IxBdN} (MOI 300), TNF treatment (30 ng/ml), or Adv-\textit{IxBdN} (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.
percentage 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. 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.

DISCUSSION

Nearly all glioma cells express TNF-R1 receptor, including U251 and T-98G, through which most of the cytotoxic effects of TNF are transduced. However, U251 and T-98G cells do not normally undergo apoptosis when treated with TNF. On the other hand, infection of these
cell lines with Adv-IkBdN 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 IkBdN promoted the pro-apoptotic effect of TNF in these cell lines. In U251 and T-98G cells, IkBdN protein transferred by Adv-IkBdN 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-IkBdN or TNF to the pro-apoptotic 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 association of its p65 subunit with a labile cytoplasmic inhibitor IkB. However, cytoplasmic inhibitor IkB is rapidly degraded after TNF stimulation. The IkBdN gene encodes an IkB protein that lacks the N-terminal 36 amino acids. The deleted portion of the gene encodes serine 32 and 36, which, after phosphorylation by IkB kinase, are targets of degradation via the ubiquitin-proteasome pathway. Therefore, IkBdN escapes proteolytic degradation and binds NF-κB, effectively preventing nuclear importation of NF-κB. Thus, IkBdN 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.
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. 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. 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, generates multiple cytokine responses, and makes glial cells susceptible to natural killer cell-mediated lysis. 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 and invasiveness of gliomas 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. 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 associated with the resistance mechanism, should further improve TNF-mediated therapy.

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