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Nuclear Factor-κB-inducible Death Effector Domain-containing Protein Suppresses Tumor Necrosis Factor-mediated Apoptosis by Inhibiting Caspase-8 Activity*

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Activation of the transcription factor nuclear factor-κB (NF-κB) has been found to play an essential role in the inhibition of tumor necrosis factor (TNF)-mediated apoptosis. NF-κB regulates several antiapoptotic molecules including inhibitors of apoptosis, Bcl-2 family proteins (A1 and Bcl-X_L), and IEX-IL. Here we report that the expression of a small death effector domain (DED)-containing protein, NDED (NF-κB-inducible DED-containing protein), depends on the activation of NF-κB. The inhibition of NF-κB by IκBα, a natural inhibitor of NF-κB, suppressed NDED mRNA expression induced by TNF. The restoration of NDED in NF-κB null cells inhibited TNF-induced apoptosis. Intriguingly, unlike the caspase-8 inhibitor cellular FADD-like interleukin-1β converting enzyme-inhibitory protein (c-FLIP), NDED suppressed TNF-mediated apoptosis by inhibiting TNF-induced caspase-8 enzymatic activity but not the processing of caspase-8. Furthermore, NDED could not inhibit etoposide-mediated apoptosis that is independent of caspase-8 activation. Our results provide the first demonstration that NF-κB transcriptionally induces the DED-containing protein to suppress TNF-mediated apoptosis by inhibiting caspase-8 activity, which offers new insight into the antiapoptotic mechanism of NF-κB.

Activation of the TNF[1] receptor I (TNFRI) by TNF leads to the recruitment of the death domain-containing protein TNFRI-associated death domain protein and receptor-interacting protein to the receptor complex (1, 2). Along with the receptor-interacting protein and other signaling molecules, TNFRI-associated death domain protein can activate the NF-κB signaling pathway or trigger the caspase cascade by interacting with FADD (Fas-associated death domain protein) in the absence of NF-κB activation (1–3). FADD recruits and activates caspase-8. Both caspase-8 and FADD contain death effector domains (DED) that were found to play a critical role for the protein-protein interaction and caspase activation (4, 5). Active caspase-8 promotes cell death by cleaving the cytosolic Bid protein, a proapoptotic family member of Bcl-2 (6, 7). Truncated Bid translocates to mitochondria, resulting in the release of cytochrome *c* from mitochondria to the cytosol (6–8). Cytochrome *c*, along with ATP and Apaf-1, a mammalian homologue of Ced-4, recruits and activates procaspase-9. The active caspase-9 amplifies apoptosis by the cleavage of downstream effector caspases (9–11). Although biochemical and genetic studies have indicated that Bid plays an important role in promoting death receptor-mediated apoptosis (6–8), it is not known whether the cleavage of Bid is modulated by antiapoptotic molecules.

NF-κB is a stress-responsive transcription factor that plays important roles in inflammation and infection (2, 12, 13). Classical NF-κB is a heterodimer composed of p50 and p65/RelA and is sequestered in the cytoplasm by the IκB group of inhibitory proteins. Proinflammatory cytokines such as TNF activate IκB kinase to phosphorylate the conserved N-terminal region of IκB proteins. The phosphorylated IκB is ubiquitinated and subsequently degraded by the 26S proteasome, resulting in the nuclear translocation of NF-κB where it activates transcription (2, 12, 13). Recently, we and others (14–19) demonstrated that NF-κB plays an essential role in inhibiting TNF-mediated apoptosis. Several important antiapoptotic molecules have been identified that are transcriptionally regulated by NF-κB. These molecules include inhibitors of apoptosis family proteins, TNFR-associated factor family proteins, Bcl-2 family members A1 and Bcl-X_L, and IEX-1L (20–25). However, overexpression of these molecules cannot completely inhibit TNF-induced apoptosis, indicating that NF-κB may regulate other genes to suppress apoptosis.

During the process of searching for other NF-κB-inducible genes, we found that one of the TNF-induced genes, previously named *GG2–1* (27) or *SCC-S2* (28), was transcriptionally regulated by NF-κB. Interestingly, this gene has one DED domain and partially inhibits TNF killing by unknown mechanisms (28). Because we found that *GG2–1* or *SCC-S2* was regulated by NF-κB as presented here, for convenience we renamed it NDED (NF-κB-inducible death effector domain-containing protein). Given that the DED-containing protein plays a role in the death signal transduction (1–3), this study was undertaken to determine whether NDED is an authentic NF-κB-regulated

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[1] The abbreviations used are: TNF, tumor necrosis factor; TNFRI, TNF receptor I; NF-κB, nuclear factor-κB; FADD, Fas-associated death domain protein; DED, death effector domain; NDED, NF-κB-inducible DED-containing protein; SSC, squamous cell carcinoma cells; IκBα, inhibitor of kappa Bα; SR-IκBα, super-repressor form of IκBα; c-FLIP, cellular FADD-like interleukin-1β converting enzyme-inhibitory protein; DFF, DNA fragmentation factor; DISC, death-inducing signaling complex.

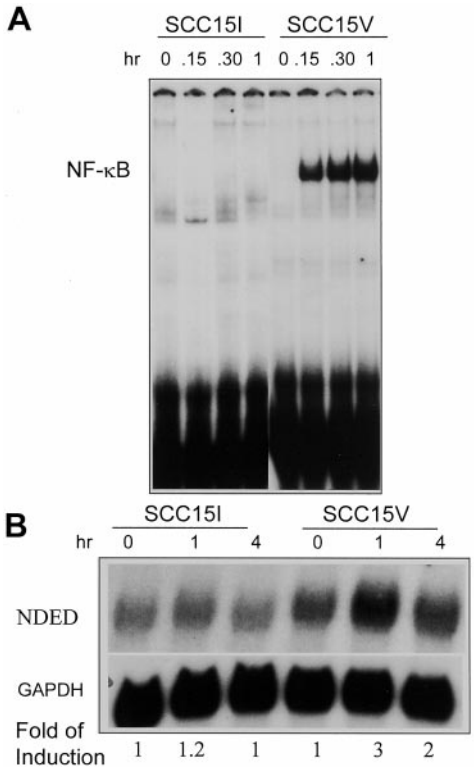

FIG. 1. **NDED expression is induced by TNF through the activation of NF-κB in squamous cell carcinoma.** *A*, TNF-induced nuclear translocation of NF-κB is inhibited by adenovirus-mediated delivery of SR-IκBα. The cells were infected with adenovirus-expressing SR-IκBα or control virus at a multiplicity of infection of 1:500. Twenty-four h after infection, the cells were treated with TNF for the indicated time, and the nuclear extracts were prepared. Five μg of proteins were used for the electrophoretic mobility shift assay as described under "Experimental Procedures." *B*, the induction of NDED expression by TNF depends on the activation of NF-κB. Adenovirus infection was performed as described for *A*. Twenty-four h after infection, the cells were treated with TNF for the indicated time. For the prevention of apoptosis, cells were pretreated with caspase inhibitor Z-VAD at a concentration of 20 μM for 30 min before TNF stimulation. Total RNA was extracted with Trizol reagent and 20 μg of RNA were used for Northern blot analysis. The membrane was probed with a ^{32}P-labeled human NDED cDNA probe. For loading control, the blot was stripped and reprobed with ^{32}P-labeled GAPDH cDNA probe. TNF stimulation of NDED mRNA expression was quantified using a PhosphorImager and normalized with GAPDH. The fold inductions were expressed as ratios of radioactivities in treated and untreated cells.

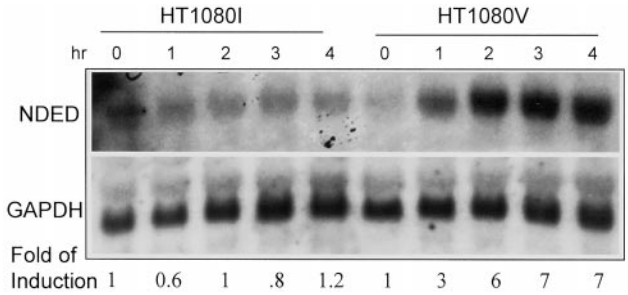

FIG. 2. **NDED expression depends on the activation of NF-κB in HT1080 fibrosarcoma cells.** HT1080V and HT1080I cells were treated with TNF for the indicated times. Northern blots were performed as described in the Fig. 1*B* legend. TNF stimulation of NDED mRNA expression was quantified using a PhosphorImager and normalized with GAPDH. The -fold inductions were expressed as ratios of radioactivities in treated and untreated cells.

antiapoptotic molecule and how NDED suppresses apoptosis. Our results demonstrated that the overexpression of NDED suppressed TNF-mediated apoptosis in NF-κB null cells. Intriguingly, NDED inhibited TNF-induced caspase-8 activity

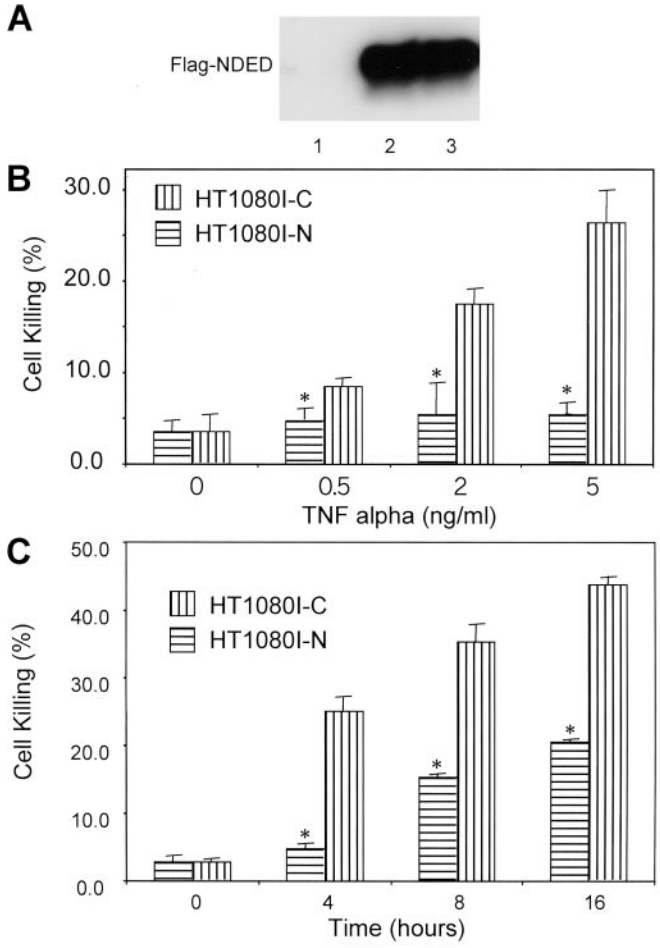

FIG. 3. **NDED expression in NF-κB null cells partially inhibits TNF-mediated apoptosis.** *A*, the establishment of HT1080I cells stably expressing NDED. HT1080I cells were co-transfected with pCMV2-FLAG-NDED vector or control empty vector pcDNA3.1 (−) using LipofectAMINE. Twenty-four h after transfection, the cells were selected with G418 (600 μg/ml) for 2 weeks. About 50 clones were screened by monoclonal antibody against the FLAG epitope. Six positive clones that expressed similar levels of NDED protein were pooled (HT1080I-N). *Lane 1* represents control cells (HT1080I-C) expressing the G418-resistant marker; *lanes 2* and *3* represent HT1080I cells stably expressing FLAG-NDED. *B* and *C*, NDED inhibited cell killing by TNF. Cells were treated with the indicated concentrations of TNF for 8 h (*B*) and with TNF (5 ng/ml) for the indicated time periods (*C*). The detached and attached cells were collected, and the dead cells were determined by the trypan blue exclusion assay. The assays were performed in triplicate, and the result represents the average values from three independent experiments. Statistical differences between each group were determined by the Student's *t* test. *, $p < 0.01$.

but not the processing of caspase-8, subsequently resulting in inhibition of Bid cleavage and caspase-3 activation.

EXPERIMENTAL PROCEDURES

Cell Culture—Human head and neck squamous cell carcinoma cells SCC15 (ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Human fibrosarcoma cells HT1080V and HT1080I cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal calf serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 200 μg/ml hygromycin (15).

Reverse Transcription Polymerase Chain Reaction and the Establishment of Stable Cell Lines—To acquire the full-length encoding region for NDED, total RNA was prepared from TNF-treated HT1080V cells. One μg of total RNA was reverse transcribed with reverse transcriptase and amplified using an reverse transcription-polymerase chain reaction kit according to manufacturer protocol (Roche Molecular Biochemicals). The 5′ and 3′ primers used for amplification of the NDED coding region were 5′-CCGATGGCCACAGATGTCTT-3′ and 5′-1GCTCATATGT-

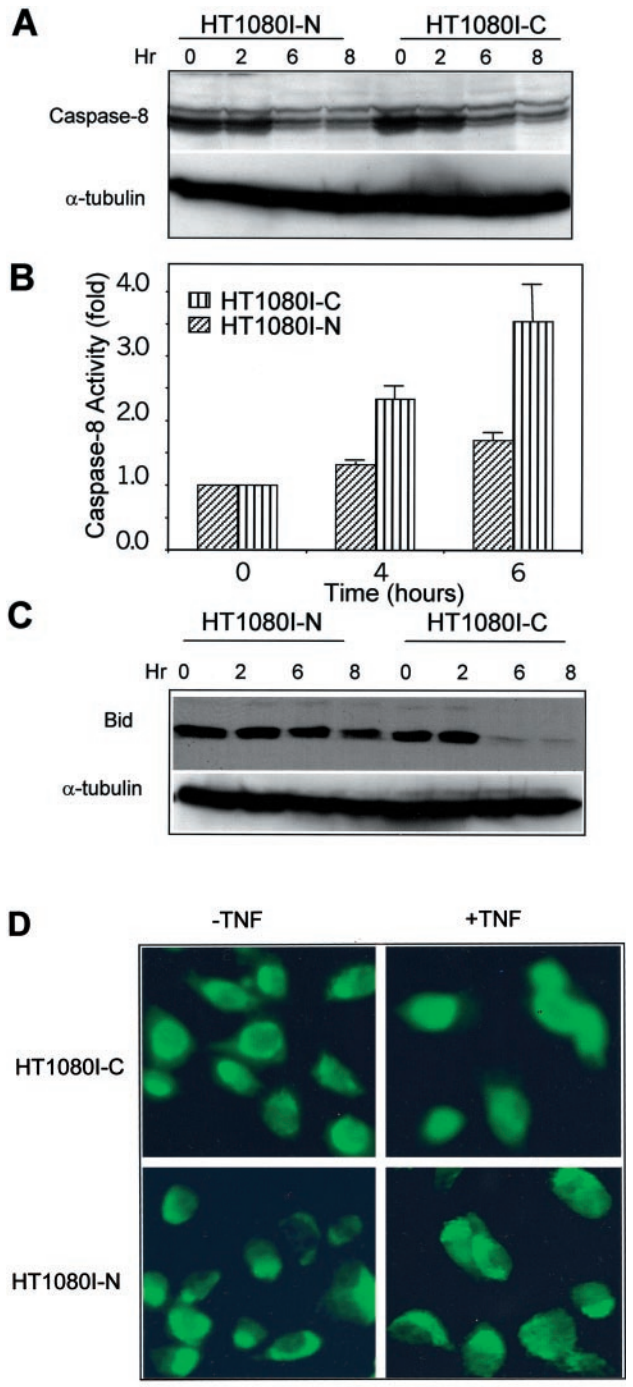

FIG. 4. **NDED inhibits caspase-8-mediated cleavage of Bid but not the processing of caspase-8.** *A*, NDED did not inhibit the processing of caspase-8 induced by TNF. HT1080I-N and HT1080I-C cells were treated with TNF for the indicated times. The detached and attached cells were collected, and whole-cell protein extracts were prepared. Fifty μg of proteins from each group were resolved by 15% SDS-polyacrylamide gel electrophoresis. The blot was probed with a monoclonal antibody against caspase-8 (1:1,000). As an internal control, the blot was stripped and reprobed with an antibody against α-tubulin (1:2,000). *B*, NDED inhibited TNF-induced caspase-8 activity. HT1080I-N and HT1080I-C cells were treated with TNF for 4 or 6 h. The detached and attached cells were harvested and lysed in lysis buffer. Two hundred μg of proteins were incubated with caspase-8 substrate *p*-nitroanaline-Ile-Glu-Thr-Asp for 2 h at 37 °C. The reaction was measured with a plate reader at 405 nm. *C*, the processing of Bid was inhibited by NDED. Cell treatment and the Western blot assays were performed as described for *A*. The blot was probed with goat polyclonal antibody against Bid. As a loading control, the blot was stripped and reprobed with antibody against α-tubulin. *D*, the cytosolic release of cytochrome *c* was inhibited in HF1080I-N cells but not in HT-1080I-C cells. Both HT1080I-N and HT1080I-C cells were treated with TNF for 3–4 h. After TNF treatment, the cells were washed gently and fixed with 4% paraformaldehyde. Cells were stained with a primary monoclonal antibody against cytochrome *c* (1:200) and with fluorescein isothiocyanate-conjugated secondary antibody. The results were examined and photographed under a fluorescence microscope using a filter set for fluorescein isothiocyanate.

TCTCTTCATC-3′. The full-length NDED cDNA fragment was purified and subcloned into a pCR3.1 vector using a TA cloning kit (Invitrogen). To add a FLAG epitope to the N terminus of NDED, the full-length NDED cDNA fragment was then subcloned into the pCMV2-FLAG vector. Automated sequence analysis of both strands confirmed that the cDNA encoding the NDED fragment was in the expression frame. HT1080I cells stably expressing NDED were made by co-transfecting in HT1080I cells with pCMV2-FLAG-NDED vector or empty control vector and pcDNA3.1 (−) vector containing the G418 selectable marker using LipofectAMINE according to manufacturer instructions (Life Technologies, Inc.). Two days after transfection, cells were selected for resistance to 600 μg/ml G418 (Life Technologies, Inc.). Individual clones expressing pCMV-FLAG-NDED were screened after 2 weeks of selection with a monoclonal antibody against the FLAG epitope (Sigma). Positive clones expressing NDED were pooled and designated as HT1080I-N cells, and cells expressing empty vector as HT1080I-C cells.

Northern Blot Analysis—After treatment with TNF (5 ng/ml) for different time periods, cells were lysed with Trizol reagent, and total RNA was isolated according to manufacturer protocol (Life Technologies, Inc.). Fifteen-μg aliquots of total RNA were separated on a 1.4% agarose-formaldehyde gel, transferred onto a nylon membrane, and cross-linked with a UV cross-linker. Blots were hybridized overnight with a random-primed ^{32}P-labeled probe at 42 °C in 50% formamide, 5× SSC, 1× 50 mM Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.25% polyvinylpyrrolidene, 0.25% Ficoll, and 5 mM EDTA, and 150 μg of denatured salmon sperm DNA. The probes were generated with a random-primed labeling kit (Amersham Pharmacia Biotech) in the presence of [α-^{32}P]dCTP (ISN) and purified with a micro G-50 Sephadex column (Amersham Pharmacia Biotech). After hybridization, the blots were washed twice in 2× SSC/0.1% SDS for 10 min at 42 °C and twice in 0.1× SSC/0.1% SDS for 20 min at 42 °C. Blots were scanned by a PhosphorImager, and the signals were normalized with internal control glyceraldehyde-3-phosphate dehydrogenase.

Electrophoretic Mobility Shift Assays—Cells were infected with adenovirus-expressing super-repressor IκBα or a control virus as described previously (16). Twenty-four h after infection, the cells were treated with TNF for the indicated times. Nuclear extracts were prepared for electrophoretic mobility shift sssays as described previously (15, 22). Five-μg aliquots of nuclear extracts were preincubated with 1 μg of poly(dI-dc) in binding buffer (10 mM Tris, pH 7.7, 50 mM NaCl, 20% glycerol, 1 mM dithiothreitol, and 0.5 mM EDTA) for 10 min at room temperature. Approximately 20,000 cpm of ^{32}P-labeled DNA probe containing the class I major histocompatibility complex NF-κB site (5′-CAGGGCTGGGGATTCCCCATCTCCACAGTTTCACTTC-3′) was then added, and binding proceeded for 15 min. The complexes were separated on a 5% polyacrylamide gel and exposed for autoradiography (15, 22).

Western Blot Analysis—Whole-cell extracts were prepared as described previously (22). The extracts were subjected to sodium dodecyl sulfate, 10 or 15% polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane by a semi-dry transfer apparatus (Bio-Rad). Proteins were probed with primary antibody and visualized by using an ECL kit (Amersham Pharmacia Biotech) according to manufacturer instructions. For internal control, the blots were stripped with 62.5 mM Tris buffer, pH 6.8, containing 100 mM 2-mercaptoethanol and 2% SDS at 60 °C for 1 h and reprobed for α-tubulin (22). Primary antibodies were from the following sources: Monoclonal antibodies against human caspase-8 (1:1,000), PharMingen; monoclonal antibody against α-tubulin (1:2,000), Sigma; and goat polyclonal antibody against Bid (1:1,000), R&D Systems.

Trypan Blue Exclusion and Cell Death Enzyme-linked Immunosorbent Assay—Cells were treated with a variety of concentrations of TNF for the indicated times, and cell viability was determined with a trypan blue exclusion assay. For the cell death enzyme-linked immunosorbent assay, 5×10^5 cells were plated onto 12-well plates the day before stimulation. The cells were treated with TNF (5 ng/ml) for 6 h. Twenty-μl aliquots of supernatant were used to assess DNA fragmentation and histone release from the nucleus. These measurements were performed according to manufacturer protocol (Roche Molecular Biochemicals).

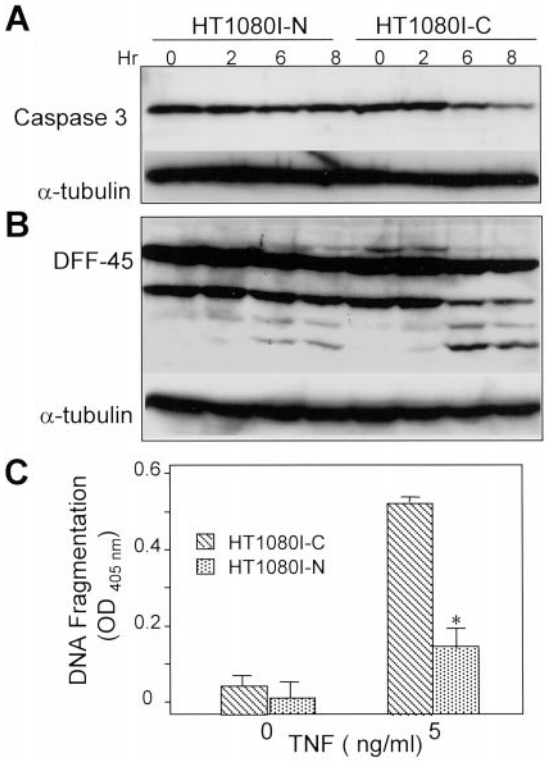

FIG. 5. **Caspase-3 activation was inhibited in HT1080I-N cells but not in HT1080I-C cells.** *A*, the processing of caspase-3 was inhibited in HT1080I-N cells but not in HT1080I-C cells. Both HT1080I-N and HT1080-C cells were treated with TNF as described in the Fig. 3*A* legend. The whole extracts were prepared and probed with a polyclonal antibody against caspase-3. As an internal control, the blot was stripped and probed with a monoclonal antibody against α-tubulin. *B*, the cleavage of caspase-3 substrate DFF was inhibited in HT1080I-N cells but not in HT1080I-C cells. Whole-cell extracts were probed with polyclonal antibody against DNA fragmentation factor-45. As an internal control, the blot was stripped and reprobed with an anti-α-tubulin antibody. *C*, DFF was inhibited in HT1080I-N cells but not in HT1080I-C cells. The supernatants were collected from TNF-treated HT1080I-N and HT1080I-C cells and frozen at −70 °C. Twenty μl of supernatants were measured by cell death enzyme-linked immunosorbent assay as recommended by the manufacturer. The reactions were measured with a plate reader by measuring the optical density at the wavelength of 405 nm. The assay was performed in triplicate, and the result represents one of three independent experiments. Statistical significance was determined by the Student's *t* test. *, $p < 0.01$.

Immunofluorescence Staining—5×10^4 cells were plated on microscope coverslips in 24-well plates the day before stimulation. Two-three h after TNF treatment, the cells were gently washed with PBS and fixed with 4% paraformaldehyde. After blocking with normal goat serum for 30 min, the cells were incubated with primary monoclonal antibody against cytochrome *c*, and immunocomplexes were detected with fluorescein isothiocyanate-conjugated secondary antibody against mouse IgG. The coverslips were inverted on a drop of Vectashield mounting media (Fisher Scientific) on slides, examined, and photographed by a fluorescence microscope using a filter set for fluorescein isothiocyanate.

RESULTS

The Expression of NDED Induced by TNF Depends on the Activation of NF-κB—NDED was originally found to be induced by ionizing irradiation and TNF in several cell lines including squamous cell carcinoma cells (SCC) (26, 27). Because TNF is a potent activator of NF-κB (1–3), we were interested in determining whether the induction of NDED expression depends on the activation of NF-κB. To inhibit the activation of NF-κB induced by TNF, we utilized the adenoviral delivery of a modified form of IκBα, the super-repressor of IκBα (SR-IκBα) (14). SR-IκBα, which contains two mutations at serines 32 and 36, cannot be phosphorylated by IκB kinase and subsequently degraded by proteasome, thereby preventing NF-κB translocation to the nucleus (16, 21). As shown in Fig. 1*A*, TNF-induced nuclear translocation was totally blocked in human head and neck cancer SCC15 cells infected with adenovirus-expressing SR-IκBα (SCC15I) but not in the cells infected with control virus (SCC15V). Northern blot analysis demonstrated that NDED mRNA was rapidly induced in SCC15V cells (3-fold) but weakly in SCC15I cells (1.2-fold) 1 h after TNF stimulation, as quantified by a PhosphorImager, indicating that NDED was an early NF-κB-responsive gene (Fig. 1*B*). Control blotting with glyceraldehyde-3 phosphate dehydrogenase (GAPDH) indicated equivalent loading for the different experimental conditions (Fig. 1*B*).

Previously, we established an HT1080 human fibrosarcoma cell line (HT1080I) that stably expresses SR-IκBα. This cell line, but not control cell line (HT1080V), potently suppresses NF-κB activation and is sensitive to TNF-induced apoptosis (15). Thus, we also utilized this cell model system to determine whether the expression of NDED was regulated by NF-κB. As shown in Fig. 2, TNF stimulation in HT1080V cells also resulted in a substantial increase in the steady-state levels of NDED mRNA (3-fold within 1 h). In contrast, NDED mRNA expression was suppressed in HT1080I cells, in which NF-κB activation was inhibited by SR-IκBα. Together, these results demonstrated that TNF-induced NDED mRNA expression depended on the activation of NF-κB.

NDED Inhibits TNF-mediated Apoptosis in NF-κB Null Cells—Because NDED is one of the NF-κB-induced gene products, we next examined whether NDED played a role in the inhibition of TNF-mediated apoptosis. To test this, it was very important to determine whether the overexpression of NDED inhibited apoptosis in NF-κB null cells, because growing evidence suggests that some molecules can utilize the NF-κB signaling pathway to inhibit apoptosis (21, 28, 29). Because the apoptotic signaling pathways in the HT1080I cell model system have been well characterized (15, 16, 20, 22), this model was utilized to determine whether the restoration of NDED inhibited TNF-mediated apoptosis under conditions of inactivation of NF-κB. HT1080I cells were stably transfected with the expression vector for NDED or an empty vector as control. Ten positive clones expressing NDED were identified and pooled as HT1080I-N cells based on their level of protein expression. The antibiotic-resistant clones, which expressed empty vector, were pooled and designated as control HT1080I-C cells (Fig. 3*A*). As shown in Fig. 3, *B* and *C*, HT1080I-C cells were killed after TNF stimulation in a time- and dose-dependent manner. In contrast, cell death in HT1080I-N cells induced by TNF was potently inhibited by the expression of NDED. Both DNA fragmentation assay and annexin V staining confirmed that TNF-mediated killing depended on the mechanism of apoptosis in this cell model system as described previously (data not shown). These results demonstrated that under an NF-κB null condition, the overexpression of NDED was capable of inhibiting TNF-induced apoptosis, suggesting that NDED is an authentic NF-κB-induced antiapoptotic molecule.

NDED Suppresses Caspase-8 Activity but Not the Processing of Procaspase-8—The caspase-8 inhibitor, c-FLIP, also called I-FLICE, FLAME-1, casper, Clarp, or CASH, has been found to inhibit TNF- and Fas-mediated apoptosis (30–36). c-FLIP contains two DED domains and a caspase-like domain similar to that in caspase-8 but lacks key features of proteolytic enzymes. c-FLIP binds to the DED domains of both FADD and caspase-8 and inhibits the processing and activation of caspase-8 (30, 37). Because NDED contains a DED domain, we first determined whether NDED, similar to c-FLIP, could block the processing of caspase-8 induced by TNF. As shown in Fig. 4*A*, the kinetics

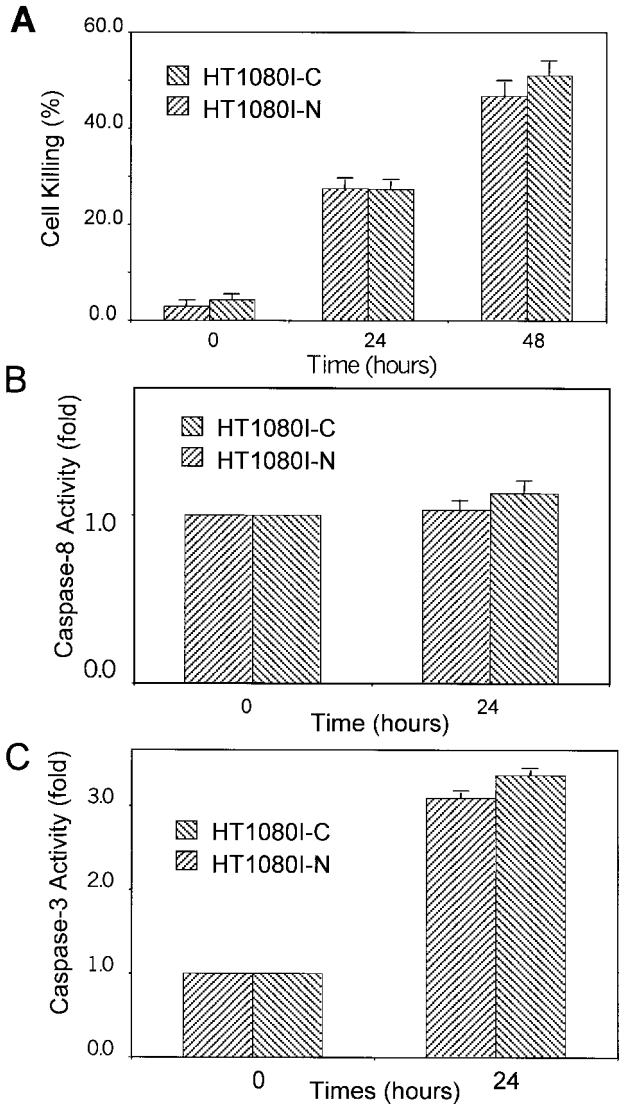

FIG. 6. **NDED does not inhibit the etoposide-induced caspase cascade.** *A*, NDED did not inhibit cell killing by etoposide. Cells were treated with etoposide for 24 or 48 h. Cell viability was determined by the trypan blue exclusion assay. The assays were performed in triplicate, and the results represent two independent experiments. *B*, etoposide did not induce caspase-8 activity. Cells were treated with etoposide for 24 h, and the caspase-8 assay was performed as described in the Fig. 4*B* legend. *C*, NDED did not inhibit caspase-3 activity induced by etoposide. Cell treatment was performed as described for *B*. The whole-cell protein extracts were incubated with caspase-3 substrate *p*-nitroanaline-Asp-Glu-Val-Asp. The reactions were measured with a plate reader at 405 nm. The results represent the average value from two independent experiments. Statistical significance was determined by the Student's *t* test. *, $p > 0.05$.

of the processing of procaspase-8 was identical in both HT1080I-N and HT1080I-C cells after TNF stimulation, as detected by the Western blot analysis. The result suggested that, unlike c-FLIP, NDED functioned downstream to caspase-8 processing. Because we could not detect cleavage of caspase-8 in our cell model system by using the Western blot assay (20, 22), the caspase-8 activity assay was assessed directly to examine whether NDED modulated TNF-induced active caspase-8 enzymatic activity using specific caspase-8 substrate. Surprisingly, despite the fact that caspase-8 was processed with similar kinetics in both HT1080I-C and HT1080I-N cells, the activity of caspase-8 was potently suppressed in HT1080I-N cells but not in HT1080I-C cells after TNF treatment (Fig. 4*B*). A proapoptotic family member of Bcl-2, Bid, has been found to be a specific substrate of caspase-8 during TNF- and Fas-mediated apoptosis (6–7). The truncated Bid promotes and/or amplifies apoptosis by inducing the release of cytochrome *c* from mitochondria to the cytosol (6–7). To confirm that caspase-8 activity was inhibited by NDED, we compared the cleavage of Bid in HT1080I-N with HT1080I-C cells. As shown in Fig. 4*C*, the cleavage of Bid was significantly blocked in HT1080I-N cells compared with HT1080I-C cells, indicating that caspase-8 activity was suppressed. (Fig. 4*B*). We also examined the distribution of cytochrome *c* by immunofluorescence staining. As shown in Fig. 4*D*, untreated cells exhibited a punctate cytoplasmic staining of cytochrome *c*, indicating mitochondrial localization. In contrast, TNF-treated HT1080I-C cells but not HT1080I-N cells exhibited very diffuse staining patterns in the entire cells, indicating that the release of cytochrome *c* from mitochondria to the cytosol was inhibited in HT1080I-N cells.

In addition to the cleavage of Bid, caspase-8 can also directly process executing caspases such as caspase-3 to induce apoptosis (3, 9). Thus, we also examined whether the activation of caspase-3 was affected by the inhibition of caspase-8. As shown in Fig. 5, the processing of caspase-3 was partially inhibited by NDED after TNF treatment in HT1080I-N cells but not in HT1080I-C cells. Consistent with this, the cleavage of DNA fragmentation factor (DFF) 45, a caspase-3 substrate, was significantly inhibited by the overexpression of NDED in HT1080I-N cells but not in HT1080-C cells after TNF treatment. The cleavage of DFF-45 leads to the release and activation of DFF-40 to induce DNA fragmentation and chromatin condensation (38). At the late stage of apoptosis, the cell membrane is broken, and the fragmented DNA induced by DFF-40 is released to the cell culture medium, which can be detected by the cell death enzyme-linked immunosorbent assay (22). As shown in Fig. 5*C*, after TNF stimulation there was significantly less DNA fragmentation and histone release in HT1080I-N cells compared with that from HT1080I-C cells.

NDED Does Not Inhibit Caspase-8-independent Apoptosis—The results described above strongly suggested that NDED inhibited apoptosis by inhibiting caspase-8 activity. However, it was possible that NDED might also inhibit other caspases and/or apoptotic signaling. According to our current model, there are two primary pathways to induce apoptosis (3, 9). One is mediated by the death receptors such as TNFRI, which is caspase-8-dependent. Another is activated by stress inducers such as the chemotherapeutic drug etoposide or ionizing radiation. These inducers stimulate the release of cytochrome *c* and sequentially activate caspase-9 and caspase-3 to induce apoptosis, which is independent of caspase-8 activation (3, 9). Using the HT1080 cell model system, we previously confirmed that etoposide-mediated apoptosis was independent of caspase-8 (22). Thus, by examining whether NDED inhibited etoposide-mediated apoptosis, we could determine whether NDED also inhibited other caspase activity and/or apoptotic signaling pathway. Both HT1080I-N and HT1080I-C cells were treated with etoposide for 24–48 h, and cell viability was assessed. As shown in Fig. 6*A*, the same degree of cell death was induced in both HT1080I-C and HT1080I-N cells after etoposide stimulation, indicating that the overexpression of NDED could not suppress etoposide-mediated apoptosis. Consistent with our previous observation, caspase-8 activity was not induced in either HT1080I-N or HT1080I-C cells (Fig. 6*B*). Most importantly, caspase-3 activity was equivalently induced in both HT1080I-N and HT1080I-C cells by etoposide, indicating that NDED did not suppress caspase-3 activity (Fig. 6*C*). Taken together, the results suggest that NDED specifically

inhibited TNF-mediated but not etoposide-mediated apoptosis by suppressing caspase-8 activity.

DISCUSSION

Our results presented here demonstrate that NDED is a novel NF-κB-inducible antiapoptotic molecule. Because NDED inhibited TNF-mediated apoptosis by inhibiting caspase-8 activity but did not suppress etoposide-mediated apoptosis, it is likely that NDED specifically inhibited TNF-induced caspase-8 activation but not other caspase cascades. Previous studies indicated that NF-κB-regulated antiapoptotic genes inhibited TNF-mediated apoptosis at two steps in the caspase cascades (20, 22). By the induction of TNFR-associated factors 1 and 2 and cellular inhibitors of apoptosis 1 and 2, NF-κB suppresses the TNF-induced processing of caspase-8 (20, 21). By induction of the Bcl-2 family protein A1, NF-κB suppresses TNF-mediated mitochondrial damage and cytochrome *c* release (22, 24). Interestingly, overexpression of these genes could not fully suppress TNF-mediated apoptosis. The effect of NDED on the inhibition of the active caspase-8 activity would seem to complement NF-κB-regulated antiapoptotic molecules during NF-κB-mediated suppression of TNF-mediated apoptosis (20–24).

Both caspase-8 and FADD have been found to play an essential role in TNF- and Fas-mediated apoptosis. In the early stage of Fas-mediated apoptosis, the death-inducing signaling complex (DISC) is formed, resulting in the cleavage and activation of caspase-8 (3, 4, 5). c-FLIP has been found to bind with FADD and caspase-8 and to interfere with DISC formation and the processing of caspase-8 (30, 37). However, under physiological conditions, DISC formation could not be detected in TNF-mediated apoptosis. This may have been caused by transient interaction between caspase-8 and FADD (3, 20, 33). For this reason, we were unable to determine whether NDED inhibited DISC formation and activation in TNF-mediated apoptosis. Because NDED could not block the processing of caspase-8, it suggests that NDED inhibited caspase-8 activation downstream of DISC formation. Currently, we do not know how NDED inhibits caspase-8 activity. From the recruitment of caspase-8 to the receptor, to the processing of caspase-8, and to activation of caspase-8 *in vivo*, many signaling molecules are involved. Recently, for example, the Ced-4-homologous protein, FLASH, has been found to play a critical role in caspase-8 activation (39). Despite considerable efforts, our co-immunoprecipitation studies could not detect NDED interacting with caspase-8 and FADD. This result may be because of the transient interaction between the active caspase-8 and NDED, or because of the fact that active caspase-8-NDED complexes may be very unstable and could not be pulled down under the conditions for co-immunoprecipitation that we utilized. In fact, although the FADD-caspase-8 interaction induced by Fas was readily detected by immunoprecipitation, thus far we and others (20, 33) have been unable to detect the FADD and caspase-8 interaction after TNF stimulation. Interestingly, we found that NDED immunoprecipitated a 35-kDa protein, the molecular weight of which was larger than that of FADD, but reacted with a monoclonal antibody against the FADD death domain.[2] NDED might collaborate with this molecule to inhibit caspase-8 activity. However, it also is possible that this unidentified molecule may be involved in caspase-8 activation and is a potential inhibitory target for NDED. Finally, given the fact that several DED-containing proteins have been found to play a role in signal transduction (2, 3, 40), NDED may inhibit TNF-mediated apoptosis by regulating the signaling pathway and/or phosphorylation of components of the cell death machinery.

[2] Z. You and C.-Y. Wang, unpublished observation.

Elevated expression of c-FLIP has been linked recently to the escape of tumors from immune surveillance (41–44). c-FLIP was detected in metastatic cutaneous melanoma lesions but not in surrounding normal melanocytes from human patients, indicating that c-FLIP/I-FLICE up-regulation may be associated with tumorigenesis (41, 42). The ectopic expression of c-FLIP in a Moloney murine leukemia virus-induced lymphoma was sufficient to allow tumor cells to escape from T cell-dependent immunity *in vivo* and *in vitro*. Murine B lymphoma cells transduced with c-FLIP rapidly develop into aggressive tumors, showing a high rate of survival and growth (43). The tumor-progressive activity of c-FLIP is mediated by the prevention of the death receptor-induced apoptosis triggered by conventional T cells. These observations suggest that death receptor signaling pathways may play an important role in controlling tumorigenesis and tumor progression (44). There is growing evidence that NF-κB is associated with oncogenic transformation by providing an antiapoptotic function (28, 29, 45). Constitutive activation of NF-κB has been found in several human tumors including Hodgkin's lymphoma, prostate cancer, and breast cancer (46–48). Because NDED is regulated by NF-κB, it will be very interesting to examine the expression level of NDED in NF-κB-related tumors. Elevated NDED, like c-FLIP, may promote tumor progression and plays a critical role in the escape of tumors from immune surveillance by inhibiting death receptor-mediated apoptosis.

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