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Activation of NF-κB Determines the Sensitivity of Human Colon Cancer Cells to TNFα-Induced Apoptosis

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Tumor necrosis factor alpha (TNFα) generates a potent cytotoxic effect, however many cancer cells are resistant to TNFα-mediated killing and the cause of the differential sensitivity remains to be elucidated. In this study, we demonstrated that TNFα induced cell death in four different human colon cancer cell lines. The degree of cytotoxic effect was different in each cell line, in that HCT-15 was relatively sensitive, while DLD-1, HT-29 and WiDr were relatively resistant. TNFα induced apoptotic changes such as morphological changes, DNA fragmentation and activation of caspase-3 in HCT-15, but to a lesser degree in the others. Transcriptional expression of TNFR1(p55), as well as that of FLICE, Fas, FADD, DR3, FAF, TRADD, and RIP was similar in these cell lines, indicating that the susceptibility to TNFα-induced apoptosis may not be determined by the constitutive expression level of these factors. Interestingly, the cytotoxic effect of TNFα was well correlated with the DNA binding activity of NF-κB in the colon cancer cell lines. Further, the overexpression of a non-phosphorylated mutant form of IκBα enhanced the cytotoxicity of TNFα in the resistant cell line, DLD-1, indicating that NF-κB activity may determine the sensitivity of colon cancer cells to TNFα-induced apoptosis. Thus, our results indicate that modulation of NF-κB activity may provide a useful tool to sensitize colon cancer cells to TNFα treatment.

Key words TNFα; colon cancer; apoptosis; NF-κB

Tumor necrosis factor alpha (TNFα) is a major cytokine that functions immunologically through transcriptional up-regulation of genes encoding inflammatory cytokines.[1] Importantly, TNFα generates potent anti-tumor activity both *in vivo* and *in vitro*, thus, its therapeutic potential against a variety of human cancers has been recognized.[2,3] TNFα initiates its action by binding to two distinct cell surface receptors of TNFR1 (55 kDa) and TNFR2 (75 kDa), which are found on most cell types.[4,5] TNFR1 has been shown to mediate most TNFα-induced effects including cytotoxicity, while signaling through TNFR2 is associated with proliferation. TNFα binds to TNFR1 and the resulting trimerized receptor recruits TRADD (TNFR1-associated death domain protein) *via* interaction between death domains. The death domain of TRADD then recruits FADD (Fas-associated death domain; also called MORT1, mediator of receptor-induced toxicity) in one pathway to activate caspase-8, followed by caspase-3.[6,7] In another pathway, RIP (receptor-interacting protein) binds to TRADD and transduces an apoptotic signal through the death domain.[8] In addition, RIP together with TRAF2 (TNF receptor-associated factor 2) activates NF-κB, which may induce the expression of survival genes.[9—12] Therefore, TNFα appears to protect cells from its own toxic effects by activating NF-κB.

Colorectal cancer is not very susceptible to currently available chemotherapy and immunotherapy, and virtually no enhancement in the survival rate has been achieved from the use of any therapeutic modality in patients with advanced disease.[13,14] It has been noticed that high levels of TNFα in human colon cancer was correlated with longer patient survival,[15] indicating a potential therapeutic application of TNFα for the treatment of human colorectal cancer. However, little has been known about the cytotoxic effects of TNFα on colon cancer cells and the associated molecular and cellular mechanisms.

In this study, we provided evidence that TNFα induced apoptosis that accompanied the morphological changes, DNA fragmentation and activation of caspase-3 in human colon cancer cells. The apoptosis-inducing effect of TNFα on colon cancer cell lines was well correlated with the activation of NF-κB. Further, we found that the overexpression of a non-phosphorylated mutant form of IκBα enhanced the cytotoxic effect of TNFα in the resistant cell line, DLD-1, suggesting a key role of NF-κB that determines cellular resistance to TNFα-induced apoptosis. The results obtained from this study indicate that modulation of NF-κB activity may provide a useful tool to sensitize colon cancer cells to TNFα treatment.

MATERIALS AND METHODS

Cell Lines, Culture and TNFα The human colon cancer cell lines, DLD-1 (ATCC CCL 221), HT-29 (ATCC HTB 38), HCT-15 (ATCC CCL 225), and WiDr (ATCC CCL 218), were obtained from the American type culture collection and maintained in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) in a CO_2 incubator. Recombinant human TNFα was purchased from Biosource International, Inc. (Camarillo, CA). For TNFα treatment, the medium was changed to MEM without FBS supplement after the cells attached to culture dishes.

Cytotoxicity Measurement Cytotoxicity of TNFα was measured by MTT assays using the Cell titer[96] (Beringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Cells were seeded at an initial density of 3000 cells per well in 96-well plates and incubated overnight to allow cells to adhere. Before treatment with TNFα, the culture medium was replaced with the serum-free medium.

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Cytotoxicity was determined after 18 h of TNFα treatment by measuring capacity to convert a tetrazolium salt into a blue formazan product. The percent cytotoxicity represents {100−(OD at 570 nm measured with TNFα treated cells/OD at 570 nm measured with untreated cells)×100}.

Propidium Iodide Staining To assess subdiploid DNA content, total cells were collected and fixed in 70% ethanol in PBS at −20 °C. Cells were then washed and stained with 50 μg/ml propidium iodide (PI) in the presence of 100 μg/ml RNase A for 30 min at 37 °C in darkness. DNA content was analyzed by a FACStar flow cytometer (Becton Dickson, Mountain View, CA). Apoptotic cells with subdiploid DNA staining were found in the 'sub-G_0/G_1' peak and the percentage of such cells was calculated.

Analysis of DNA Fragmentation Agarose gel electrophoresis of oligosomal DNA fragments was carried out as described previously.[16] Briefly, after cells were lysed in a lysis buffer containing 20 mM EDTA, 0.05% (v/v) Triton X-100 and 10 mM Tris–HCl (pH 8.0), the lysates obtained were centrifuged to separate cell debris containing intact chromatin (pellet) from DNA fragments (supernatant). The fragmented DNA in supernatant was precipitated with ethanol and subsequently treated with RNase A and proteinase K. The samples obtained were electrophoresed on a 1.8% agarose gel and the separated DNA was stained with ethidium bromide. DNA fragmentation was also assessed by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay, essentially according to the methods of Liu *et al.*[17] Briefly, cells were fixed with 1% formaldehyde in PBS and incubated in a solution containing 0.2 M sodium cacodylate (pH 7.0), 2.5 mM $CoCl_2$, 25 mM Tris–HCl, 0.25 mg/ml BSA, terminal deoxynucleotidyl transferase (Oncor Inc., Gaithersburg, MD) and 2 nmols of biotin-16-dUTP (Beringer Mannheim) for 30 min at 37 °C. They were then washed with PBS and incubated in a staining solution containing 40 μg/ml fluoresceinated avidine, 4×saline-sodium citrate buffer (1×SSC=0.15 M NaCl, 0.015 M Na-citrate), 0.1% Triton X-100 and 5% non-fat dried milk for 30 min at room temperature in darkness. Following staining, cells were resuspended in a solution of formaldehyde/PBS (1/5, v/v) and fluorescence was measured by a FACStar flow cytometer.

RNase Protection Assay RNase protection assay was performed using a RiboQuant™ multi-probe RNase protection assay kit (Pharmingen, San Diego, CA) with total RNA prepared using a Qiagen RNeasy kit (Qiagen Inc., Chatsworth, CA) following the standard protocols provided by the manufacturer. Briefly, antisense probes for TNFR1(p55) and other genes were synthesized using T7 RNA polymersase with an *in vitro* transcription kit in the presence of 137.5 μM rNTPs, 100 μCi[^{32}P]UTP and hApo-3 template sets. Ten microgram RNA was used to hybridize with ^{32}P-labeled anti-sense probes and then unhybridized RNA was digested by RNase treatment. Protected probes were then resolved on denaturing polyacrylamide gels and analyzed by autoradiography. L32 was analyzed to normalize sampling and technical errors.

Flow Cytometry for Detection of TNFR1(p55) For staining of TNFR1(p55), cells were washed with PBS containing 0.1% BSA, then incubated with 5-μl mouse anti-human TNFR1(p55) antibody (Serotec, Raleigh, NC) for 30 min at 4 °C. After staining, cells were washed twice and incubated with FITC-conjugated goat anti-mouse IgG for 30 min at 4 °C. Irrelevant antibody (normal mouse IgG) was run in parallel as negative controls. Cells were then washed twice and fixed with PBS containing 0.8% paraformaldehyde. Stained cells were analyzed in the FACStar flow cytometer. Data were presented on histograms plotted as fluorescence intensity against cell number and analyzed using a PC-lysis software program (Beckton-Dickson).

Gel-shift Assay Nuclear extract was prepared as previously described,[18] and 10 μg was incubated with ^{32}P-labeled oligonucleotide in a 20-μl reaction mixture containing 10 mM HEPES buffer (pH 7.9), 50 mM KCl, 1 mM DTT, 2.5 mM $MgCl_2$, 10% glycerol, and 1 μg of poly(dI–dC) at 25 °C for 20 min. Following reaction, the mixture was loaded on a 5% nondenaturating polyacrylamide gel containing 0.5×TBE (1×TBE is 0.089 M Tris–borate, 0.089 M boric acid, and 2 mM EDTA). The oligonucleotide used for gel retardation was a consensus NF-κB binding sequence encoding 5′-TCAGGTCACTGTGACCTGA-3′. The oligonucleotide was labeled by Klenow fragment of DNA polymerase and the labeled oligonucleotide was purified by Sephadex G25 column and used as probes for the gel-shift assay.[19]

Immunoblot Analysis of Caspase-3 Cells were lysed in a lysis buffer containing 50 mM Tris (pH 7.5), 137 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 50 mM β-glycerophosphate, 2 mM EDTA, 10 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml pepstatin. Following 30 min incubation on ice, cell lysates were centrifuged at 14000×***g*** for 10 min at 4 °C and the clear supernatants were used for Western blot analysis. Protein concentrations of the lysates were quantified by bicinchoninic acid assay (Pierce, Rockford, IL). Forty microgram protein was subjected to 15% SDS-PAGE, transferred to PVDF membrane (Bio-Rad, Hercules, CA) and probed with antibodies against caspase 3 (Pharmingen) according to the method of Ravi *et al.*[20]

Cell Death Analyses of Transiently Transfected Colon Cancer Cells Cell cultures were cotransfected with a mixture containing pCMV-βgal vector with the pCMV or non-phosphorylated IκBα mutant, pRK-Flag-IκBα^{mt} (SS–AA).[21] The plasmids were co-transfected using LipofectaminPlus® (GibcoBRL, Grand Island, NY) into culture containing 3×10^5 cells per 35 mm dish. To identify the transfected cells, IκBα^{mt} (SS–AA) or control vector was cotransfected with a reporter vector encoding β-galactosidase. After 4 h of transfection, aliquots of each transfected cell culture were seeded to duplicate wells in a 6-well plate (75000 cells/well) and treated with TNFα after the cells had adhered to the bottom of the culture dishes. The cells in one well were treated with vehicle alone and cells in the other well with TNFα in serum-free medium. After 16 h of TNFα treatment, cells were washed with PBS and fixed with a solution containing 0.05% glutaraldehyde, then washed again with PBS. They were then stained to identify the β-galactosidase-expressing cells with a solution containing 0.05% X-gal, 10 mg/ml potassium ferrocyanide, 8 mg/ml potassium ferricyanide and 3 mM $MgCl_2$ in PBS. The blue cells (usually 100—300 cells/well) were counted with a microscope after overnight incubation at 37 °C.

RESULTS

Differential Sensitivity of Human Colon Cancer Cell Lines to TNFα-Induced Cytotoxicity We examined the effects of TNFα on the cell viability of a panel of human colon cancer cell lines, *i.e.*, HCT-15, DLD-1, HT-29 and WiDr, using MTT assays. As shown in Fig. 1, TNFα induced cell death in all human colon cancer cell lines tested. HCT-15 was relatively sensitive in that approximately 50% of cells were killed after 25 ng/ml TNFα treatment for 18 h. In contrast, DLD-1, HT-29 and WiDr were relatively resistant. The percentage of dead cells was approximately 20% when DLD-1 and HT-29 were treated with 100 ng/ml TNFα.

TNFα-Induced Apoptosis in Human Colon Cancer Cells We observed that when HCT-15 cells were treated with TNFα, they became shrunken, curled up, eventually detached and floated into medium, which is a typical phenomenon observed in cells undergoing apoptosis (data not shown). When cellular DNA content of TNFα-treated HCT-15 was analyzed by flow cytometry, a typical profile of cells undergoing apoptosis (Fig. 2) was obtained. A distinct, quantifiable region below G_1 flow cytometry profiles represented a subpopulation of apoptotic cells in which chromatin condensation and DNA degradation reduced the stainability of PI. The percentage of apoptotic cells after treatment with TNFα (25 ng/ml) for 18 h was about 50%. However, the percentage of apoptotic cells in the relatively resistant cells, *i.e.*, DLD-1, WiDr and HT-29, ranged from 4 to 9%. TNFα induced a typical DNA laddering pattern in HCT-15 in both agarose gel electrophoresis and TUNEL assays (Fig. 3). DNA fragmentation was induced by TNFα in a dose- and time-dependent manner as shown in Figs. 3A and 3B. Co-treatment with Actinomycin D enhanced DNA fragmentation in HCT-15 and induced weak DNA fragmentation in DLD-1, HT-29, and WiDr cells (Fig. 3C), suggesting the involvement of *de novo* gene synthesis in the susceptibility of colon cancer cells to TNFα-induced apoptosis.

We tested whether caspase-3, an executor of apoptosis,[22] was activated by TNFα treatment in colon cancer cells. As shown in Fig. 4, the activated form of caspase-3 was detected as early as 4 h after TNFα treatment in HCT-15, while the activation was barely detected at 24 h of TNFα treatment in DLD-1. Taken together, our results showed that TNFα induced apoptosis in human colon cancer cells and indicated that the cytotoxicity induced by TNFα treatment was determined by the susceptibility of the colon cancer cells to undergo apoptosis.

Expression of FLICE, Fas, FADD, DR3, FAP, FAF, TNFR1p55, TRADD, and RIP To gain insight into the mechanism of the differential sensitivity of colon cancer cells

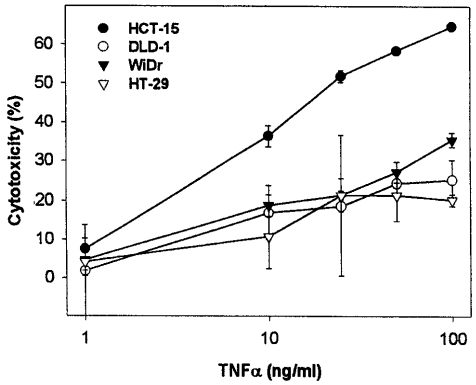

Fig. 1. Cytotoxicity Induced by TNFα in Human Colon Cancer Cell Lines

Human colon cancer cells were seeded in 96-well culture plates and treated with various concentrations of TNFα for 18 h. Cytotoxicity was measured by formation of formazan product using MTT assays as described in Materials and Methods. Each symbol represents the mean±S.E. of triplicate experiments.

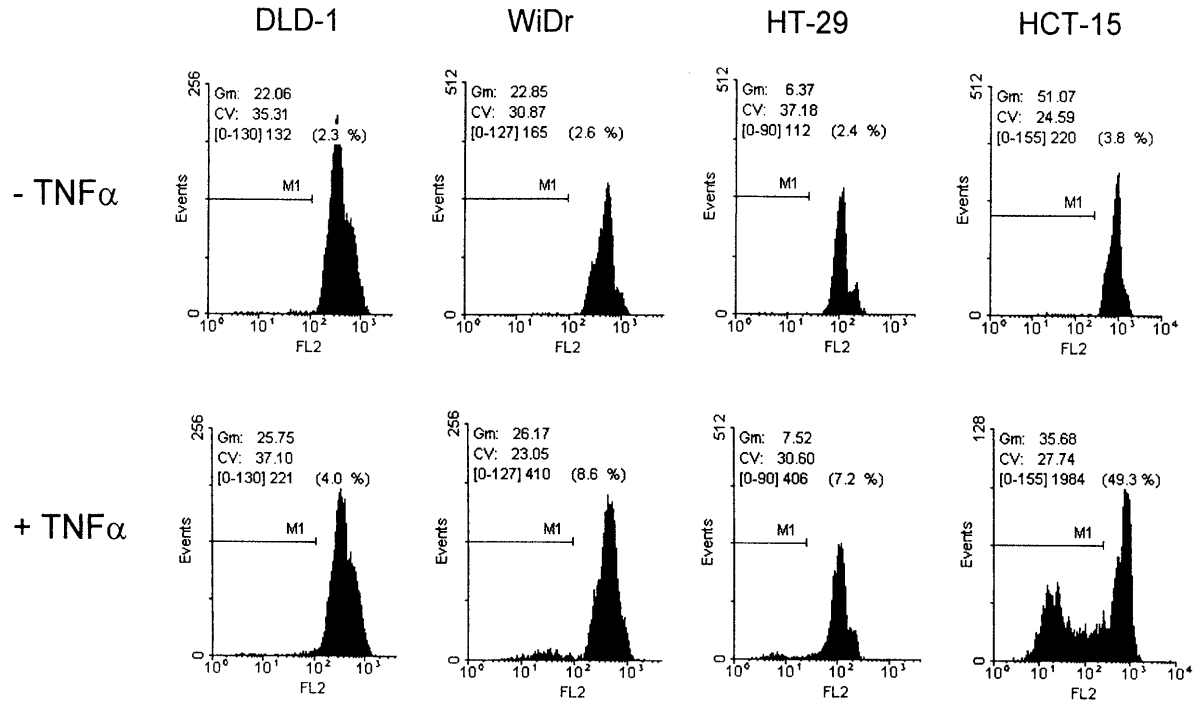

Fig. 2. Propidium Iodide (PI) Staining of TNFα -Treated Colon Cancer Cells

Cells (2.2×10^6 cells) were cultured in 100 mm culture dishes in the presence or absence of 25 ng/ml TNFα for 18 h and stained with PI as described in Materials and Methods. DNA fluorescence histograms of PI-stained cells were obtained by flow cytometry. Apoptotic cells with subdiploid DNA staining are shown (M1) and the percentage of such cells is indicated in parentheses.

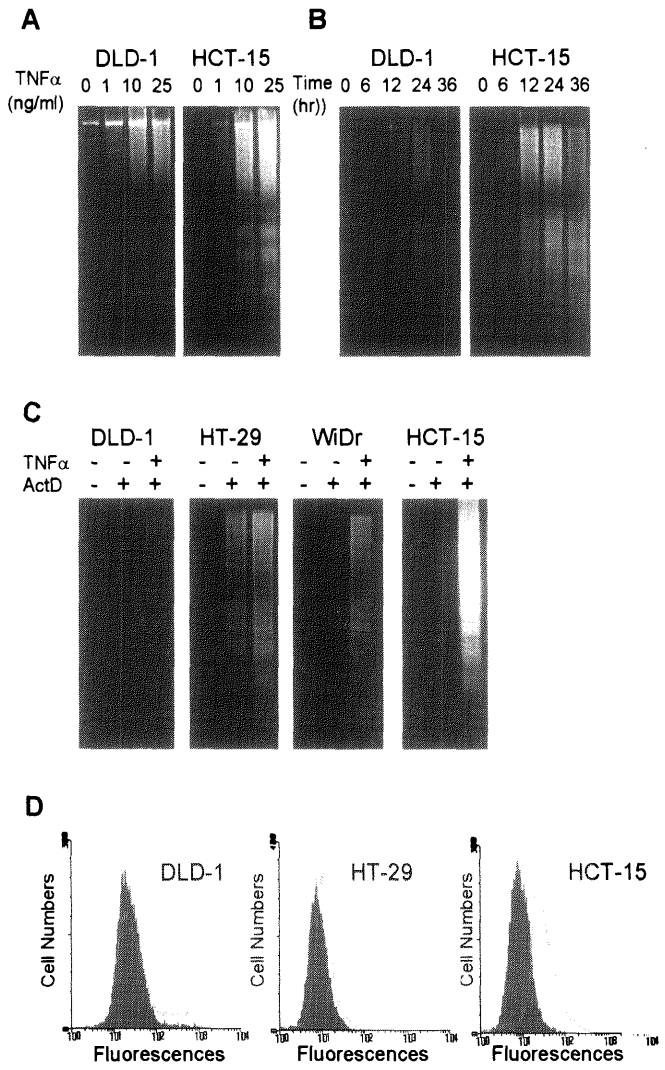

Fig. 3. DNA Fragmentation-Induced by TNFα in Human Colon Cancer Cell Lines

(A) Dose-dependent effects of TNFα: HCT-15 and DLD-1 cells (2×10^6 cells) were treated with or without various concentrations of TNFα for 18 h. (B) Time-dependent effects of TNFα: HCT-15 and DLD-1 cells were treated with or without 25 ng/ml TNFα for different time periods. (C) Enhanced DNA fragmentation induced by Actinomycin D: Colon cancer cells were cultured with or without 50 ng/ml TNFα in the presence of 0.5 μg/ml actinomycin D for 16 h. Fragmented DNA was extracted, subjected to agarose gel electrophoresis, and visualized by ethidium bromide staining as described in Materials and Methods. (D) TUNEL assay: HCT-15, HT-29, and DLD-1 cells were cultured in the presence or absence of 50 ng/ml TNFα for 16 h. Cells were then fixed, labeled with biotin-16-dUTP by the terminal deoxynucleotidyl transferase and stained with avidin-fluorescein isothiocyanate. The stained cells were analyzed with a FACStar flow cytometer as described in Materials and Methods. Filled histogram, without TNFα treatment; open histogram,with TNFα treatment.

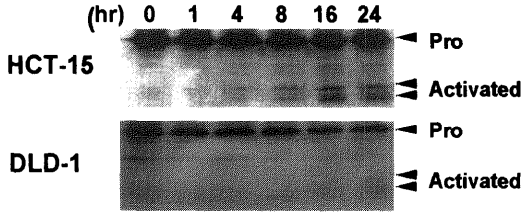

Fig. 4. Immunoblot Analysis of Caspase-3 in TNFα-Treated DLD-1 and HCT-15 Cells

Cells were incubated with 25 ng/ml TNFα for the indicated times and immunoblot analysis was performed with cytosolic fractions obtained as described in Materials and Methods. The positions of the unprocessed (pro) form and processed (activated) forms of caspase-3 are shown.

to TNFα-induced apoptosis, we examined the transcript level of protein factors associated with the TNFα signaling pathway by RNase protection assay. The transcriptional expression of TNFR1(p55), the mediator of most TNF-induced cytotoxic effects, as well as that of caspase-8/FLICE, Fas, FADD, DR3 (death receptor 3), FAF (Fas-associated factor), TRADD, and RIP [4—7] were similar in HCT-15, WiDr and DLD-1 (Fig. 5A). Consistent with the transcript level, the expression of TNFR1 (p55) protein was not significantly different in the colon cancer cell lines (Fig. 5B). The results indicated that the susceptibility of individual cell types to TNFα-induced apoptosis may not be determined by the constitutive expression level of these factors. Interestingly, FAP (Fas-associated phosphatase), an inhibitory phosphatase of the Fas signaling pathway,[23] was expressed in DLD-1 but not in HCT-15 (Fig. 5A). However, FAP may not be a common factor that generated resistance against TNFα treatment since it was not detected in the other resistant cell lines, WiDr (Fig. 5A) and HT-29 (data not shown).

Sensitivity to TNFα-Induced Apoptosis in Colon Cancer Cells Involved Impaired NF-κB Activation The NF-κB transcription factor complex is well known as a critical regulator of immune responses. NF-κB comprises two subunits (p65 and p50), which are held in an inactive complex with IκBα.[24,25] Upon receiving extracellular signals, active NF-κB is released from the complex by inducing the phosphorylation and ubiquitin-mediated proteosomal degradation of IκBα[26,27] Interestingly, NF-κB has been implicated in the counteraction of cytotoxic activity of TNFα in some cells including melanoma, breast cancer and prostate cancer cells.[28—30] However, contradictory results were also obtained in certain types of cancer cells.[31—33] Thus, we questioned whether the activation of NF-κB was correlated with the differential sensitivity of colon cancer cells to TNFα-induced apoptosis. To answer the question, we employed a gel-shift assay using an oligonucleotide probe encoding specific NF-κB binding sequences. The specificity of NF-κB binding to the probe was confirmed by competition with excess unlabeled oligonucleotide and supershift by anti-p50 antibodies (Fig. 6). As shown in Fig. 6, DLD-1, WiDr and HCT-15 exhibited constitutive binding of NF-κB without treatment of TNFα. Treatment with TNFα resulted in the rapid and prolonged induction of NF-κB binding in DLD-1 and WiDr. NF-κB activation was also observed in HCT-15 with TNFα treatment for up to 1 h. However, NF-κB complexes, especially p50 homodimer binding, was significantly diminished after 4 h of TNFα treatment in HCT-15 cells. Recently, Ravi *et al.*[20] reported that ligation of CD95, a potent apoptotic stimulus in lymphocytes, induced proteolytic cleavage of p65 and p50 by caspase-3 and proposed that CD95-induced cleavage of NF-κB subunits sensitized T cells to apoptosis. Since significant activation of caspase-3 was seen only in HCT-15, not in DLD-1 (Fig. 4), the subsequent disappearance of p50 binding activity in EMSA upon TNFα treatment in HCT-15 might be associated with the proteolytic cleavage of p50 by caspase-3.

Overexpression of the IκBα Mutant Enhanced TNFα-Induced Apoptosis To test whether the level of cellular NF-κB activity can affect TNFα sensitivity, IκBα^{mt} (SS-AA), the non-phosphorylated mutant form of IκBα which forms a stable complex with NF-κB,[21] was transiently over-

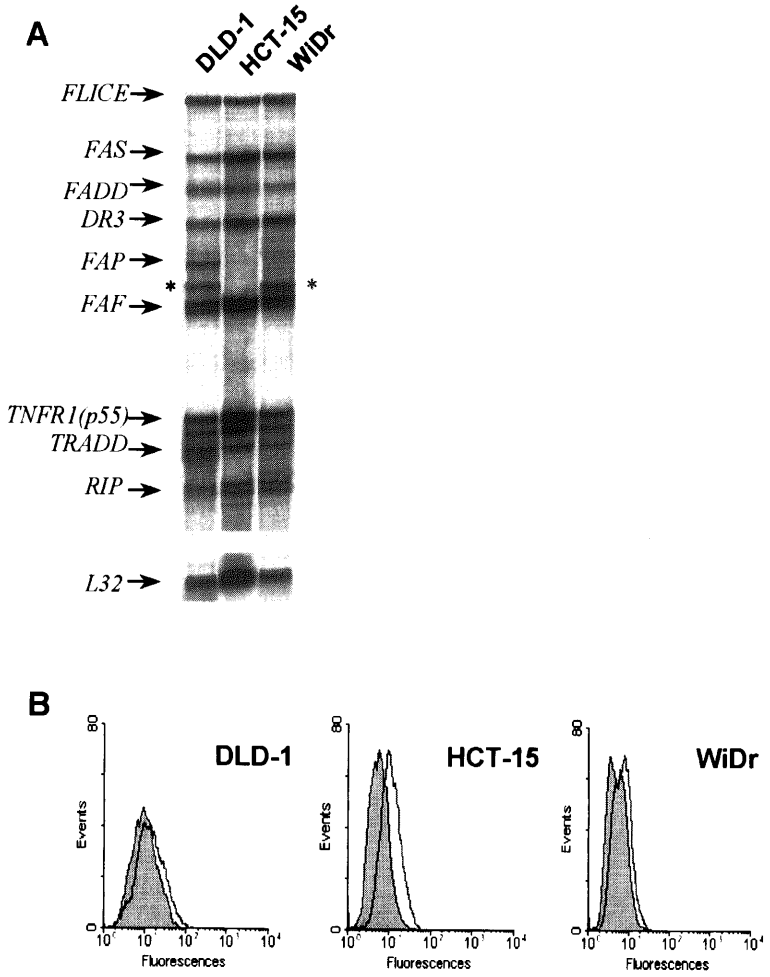

Fig. 5. Expression of TNFR1(p55) and Other Factors Associated with the TNFα Signal Transduction Pathway in Human Colon Cancer Cells

(A) The expression of FLICE, Fas, FADD, DR3, FAP, FAF, TNFR, TRADD, and RIP were determined by RNase protection assay as described in Materials and Methods. Total RNA was extracted from the indicated cell lines and 10 μg RNA was used for each lane. The expression of L32 was monitored as a control. * indicates non-specific band. (B) The expression of TNFR1(p55) protein was determined by flow cytometry using specific antibodies as described in Materials and Methods. Similar results were obtained from at least 3 independent experiments. Filled histogram, stained with normal mouse IgG; open histogram, stained with anti-TNFR1(p55) antibody.

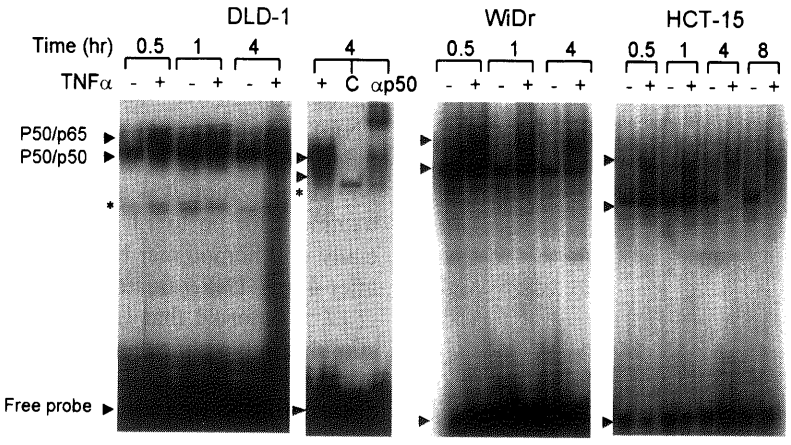

Fig. 6. The NF-κB Activity Determines the Sensitivity of Colon Cancer Cells to TNFα-Induced Apoptosis

DLD-1, WiDr and HCT-15 cells were treated with TNFα (25 ng/ml) for the indicated time periods. Five microgram of nuclear extract was used for each reaction. The reaction mixtures were incubated with P^{32}-labeled oligonucleotide as probe and analyzed by gel-shift assay as described in Materials and Methods. C indicates 100-fold excess amount of unlabeled probe used for competition to show the binding specificity.

expressed in DLD-1. TNFα treatment decreased the viability of DLD-1 cells containing control vector (pCMV), in that the number of blue cells in the wells treated for 16 h with TNFα was about 53% of the number in the untreated wells (Fig. 7). By comparison, about 5% of the cells co-expressing IκBα^{mt} (SS–AA) survived 16 h of treatment. Thus, the results indicated that the inactivation of NF-κB sensitized the DLD-1 cells to TNFα-induced apoptosis.

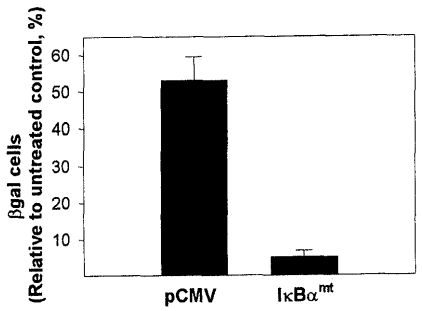

Fig. 7. Effect of the Expression of the IκBα^{mt} on Viability of TNFα-Treated DLD-1 Cells

Cells were cotransfected with 1 μg pCMV-βgal and 10 μg of the indicated expression vector as described in Materials and Methods. Aliquots of the transfected cell cultures were seeded on duplicate wells. After 16 h of incubation with 25 ng/ml TNFα, cells were fixed and stained with X-gal. Each bar represents the ratio of transfected cells in the well treated with TNF to transfected cells in the untreated well. The results represent mean±S.E. values of four separate transfection experiments.

DISCUSSION

Although TNFα induces cytotoxic effects against many different types of human cancer cells, it also causes in the resistance of many cancer cells to TNFα-mediated killing, thus limiting its use in cancer therapy.[34,35] In this study, we showed that TNFα induced apoptosis in colon cancer cells and that the effect of TNFα in individual cell lines was well correlated with the activation of NF-κB following TNFα treatment. Overexpression of the non-phosphorylated mutant form of IκBα enhanced the cytotoxic effect of TNFα on the relatively resistant cell line DLD-1, indicating a key role for NF-κB in cellular resistance in the cells. Similar to our results, activation of NF-κB by a variety of apoptotic stimuli including TNFα, chemotherapeutic drugs, and radiation has been shown to counteract the cytotoxic effects of stimuli in cells such as melanoma, breast cancer and prostate cancer cells *in vitro*.[28—30] Recently, Wang *et al.* showed that inhibition of NF-κB sensitized chemoresistant tumors to TNFα and chemotherapeutic drugs, resulting in tumor regression *in vivo*.[36] However, the role of NF-κB activation in protection of cells from apoptosis has remained controversial. Stable inhibition of NF-κB activation in several cancer cell lines by mutated IκBα did not modify cell death induced by TNFα and chemotherapeutic drugs and NF-κB activation by hyperoxia did not counteract cell death caused by hyperoxia.[32—34] Since such differences might be best explained by cell-type specificities, it may be necessary to characterize the consequences of NF-κB activation in individual cancer cells after TNFα and other chemotherapeutic drug treatment.

Our data suggested that the differential susceptibility of colon cancer cells to TNFα-induced apoptosis was associated with differential NF-κB activity. Although the mechanism by which NF-κB suppresses apoptosis has not been clearly defined, NF-κB may induce cell survival by regulating unidentified, anti-apoptotic genes. In this regard, TRAF1, TRAF2, the inhibitor-of-apoptosis (IAP) proteins, c-IAP1 and c-IAP2, and IEX-1L have recently been identified as target genes of NF-κB transcriptional activity in fibrosarcoma and T-cell leukemia.[37,38] Whether these genes play a role in protecting TNFα-induced apoptosis in resistant colon cancer cells needs to be determined. Our results also suggested that the procedure inhibiting the function of NF-κB may provide the possibility of improved efficacy of TNFα treatment for human colon cancer. From this perspective, our result showing the increased sensitivity of DLD-1 cells to TNFα treatment by transfection of the non-phosphorylated form of IκBα may provide useful information for the development of a novel strategy for colon cancer treatment.

Colonic epithelium is a place where a dynamic balance between cell production at the base and cell death at the surface of the colonic crypts is precisely regulated to maintain the steady-state cell mass.[39,40] Since apoptosis is the final differentiation step for colonic enterocytes, a progressive inhibition of apoptosis may cause a cancerous transformation that further progresses to malignancy in the colon.[41,42] Therefore, understanding how cell death in colonic tissue is regulated could lead to improved treatments and prevention strategies for colon cancer. In some cancers such as Hodgkin's disease, constitutive NF-κB activation has been found to play an important role in generating a transformed phenotype.[43,44] Interestingly, we observed the constitutive DNA binding of NF-κB in a panel of human colon cancer cell lines, which might suggest the involvement of NF-κB in the development and progress of colon cancer. Since TNFα induces apoptosis in the colon cells and NF-κB plays a role in the process, determining the role of NF-κB in the process of colon cancer carcinogenesis is of significant interest.

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REFERENCES

1) Vassalli P., *Annu. Rev. Immunol.*, **10**, 411—452 (1992).
2) Maury C. P., *Acta Med. Scand.*, **220**, 387—394 (1986).
3) Gersa D., *Immunobiology*, **175**, 1—143 (1987).
4) Nagata S., *Cell*, **88**, 355—365 (1997).
5) May M. J., Ghosh S., *Immunol. Today*, **19**, 80—88 (1998).
6) Hsu H., Shu H.-B., Pan M.-G., Goeddel D. V., *Cell*, **84**, 299—308 (1996).
7) Muzio M., Chinnaiyan A. M., Kischkel F. C., O'Rourke K., Shevchenko A., Ni J., Scaffidi C., Bretz J. D., Zhang M., Gentz R., Mann M., Krammer P. H., Peter M. E., Dixit V. M., *Cell*, **85**, 817—827 (1996).
8) Hsu H., Huang J., Shu H.-B., Baichwal V., Goeddel D. V., *Immunity*, **4**, 387—396 (1996).
9) Rothe M., Sarma V., Dixit V. M., Goeddel D. V., *Science*, **269**, 1424—1427 (1995).
10) Wang C.-Y., Mayo M. W., Baldwin A. S., Jr., *Science*, **274**, 784—787 (1996).
11) Beg A. A., Baltimore D., *Science*, **274**, 782—784 (1996).
12) Liu Z.-G., Hsu H., Goeddel D. V., Karin M., *Cell*, **87**, 565—576 (1996).
13) Moetel C. G., Fleming T. R., McDonald J. S., Haller D. G., Laurie J. A., Goodman P. J., Ungerleider J. S., Emerson W. A., Tormey D. D., Glick J. H., Veeder M. H., Mailliard J. A., *New Engl. J. Med.*, **322**, 352—358 (1990).
14) Kato T., Hirai T., *Clin. Surg.*, **45**, 1749—1758 (1990).
15) Ito H., Yagita A., Fujitsuka M., Atomi Y., Tatekawa I., *Jpn. J. Cancer Res.*, **87**, 1160—1164 (1996).
16) Jiang S., Song M. J., Shin E.-C., Lee M.-O., Kim S.-J., Park J. H., *Hepatology*, **29**, 101—110 (1999).
17) Liu Y., Lee M.-O., Wang H.-G., Li Y., Hashimoto Y., Klaus M., Reed J. C., Zhang X.-K., *Mol. Cell. Biol.*, **16**, 1138—1149 (1996).

18) Lee M.-O., Liu Y., Zhang X.-K., *Mol. Cell. Biol.*, **15**, 4194—4207 (1995).
19) Kim C. H., Song Y. H., Park K., Oh Y., Lee T. H., *J. Inflamm.*, **45**, 312—322 (1995).
20) Ravi R., Bedi A., Fuchs E. J., Bedi A., *Cancer Res.*, **58**, 882—886 (1998).
21) Traenckner E. B.-M., Pahl H. L., Henkel T., Schmidt K. N., Wilk S., Baeuerle P. A., *EMBO J.*, **14**, 2876—2883 (1995).
22) Salvesen G. S., Dixit V. M., *Cell*, **91**, 443—446 (1997).
23) Yanagisawa J., Takahashi M., Kanki H., Yano-Yanagisawa H., Tazunoki T., Sawa E., Nishitoba T., Kamishohara M., Kobayashi E., Kataoka S., Sato T., *J. Biol. Chem.*, **272**, 8539—8545 (1997).
24) Brown K., Gerstberger S., Carlson L., Franzoso G., Siebenlist U., *Science*, **267**, 1485—1488 (1995).
25) Ghosh S., Baltimore D., *Nature* (London), **344**, 678—682 (1990).
26) Harhaj E. W., Maggirwar S. B., Good L., Sun S.-C., *Mol. Cell. Biol.*, **16**, 6736—6743 (1996).
27) Lai J.-H., Tan T.-H., *J. Biol. Chem.*, **269**, 30077—30080 (1994).
28) Bakker T. R., Reed D., Renno T., Jongeneel C. V., *Int. J. Cancer*, **80**, 320—323 (1999).
29) Sumitomo M., Tachibana M., Nakashima J., Murai M., Miyajima A., Kimura F., Hayakawa M., Nakamura H., *J. Urology*, **161**, 674—679 (1999).
30) Klefstrom J., Arighi E., Littlewood T., Jaattela M., Saksela E., Evan G. I., Alitalo K., *EMBO J.*, **16**, 7382—7392 (1997).
31) Cai Z., Körner M., Tarantino N., Chouaib S., *J. Biol. Chem.*, **272**, 96—101 (1997).
32) Li Y., Zhang W., Mantell L. L., Kazzaz A. A., Fein A. M., Horowitz S., *J. Biol. Chem.*, **272**, 20646—20649 (1997).
33) Bentires-Alj M., Hellin A.-C., Ameyar M., Chouaib S., Merville M.-P., Bours V., *Cancer Res.*, **59**, 811—815 (1999).
34) Beyaert R., Fiers W., *FEBS Lett.*, **340**, 9—16 (1994).
35) Wallach D., *Trends Biochem. Sci.*, **22**, 107—109 (1997).
36) Wang C.-Y., Cusack J. C., Jr., Liu R., Baldwin A. S., Jr., *Nature Med.*, **5**, 412—417 (1999).
37) Wang C.-Y., Mayo M. W., Korneluk R. G., Goeddel D. V., Baldwin A. S., Jr., *Science*, **281**, 1680—1683 (1998).
38) Wu M. X., Ao Z., Prasad K. V., Wu R., Schlossman S. F., *Science*, **281**, 998—1001 (1998).
39) Gavrieli Y., Sherman Y., Ben-Sasson S. A., *J. Cell Biol.*, **119**, 493—501 (1992).
40) Shaw P., Bovey R., Tardy S., Sahli R., Sordat B., Costa J., *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 4495—4499 (1992).
41) Bedi A., Pasricha P. J., Akhtar A. J., Barber J. P., Bedi G. C., Giardiello F. M., Zehnbauer B. A., Hamilton S. R., Jones R. J., *Cancer Res.*, **55**, 1811—1816 (1995).
42) Kikuchi Y., Dinjens W. N., Bosman F. T., *Virchows Arch.*, **431**, 111—117 (1997).
43) Bargou R. C., Emmerich F., Krappmann D., Bommert K., Mapara M. Y., Arnold W., Royer H. D., Grinstein E., Greiner A., Scheidereit C., Dorken B., *J. Clin. Invest.*, **100**, 2961—2969 (1997).
44) Reuther J. Y., Reuther G. W., Cortez D., Pendergast A. M., Baldwin A. S., Jr., *Genes Dev.*, **12**, 968—981 (1998).