 Requirement of BAX for TRAIL/Apo2L-induced Apoptosis of Colorectal Cancers: Synergy with Sulindac-mediated Inhibition of Bcl-xL

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Abstract

The cornerstone of the systemic treatment of advanced colorectal cancer is 5-fluorouracil. However, 5-fluorouracil-induced apoptosis is dependent on p53, a tumor suppressor gene that is lost or inactivated in at least 85% of human colorectal cancers. Here we show that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/Apo2L triggers caspase-8-mediated truncation of BID, mitochondrial activation of caspase-9, and apoptosis in both p53+/+/ or p53−/− isogenic HCT116 colorectal cancer cells. TRAIL/Apo2L also sensitizes both p53+/+/ or p53−/− colorectal cancer cells to ionizing radiation. In contrast, we find that TRAIL/Apo2L fails to activate caspase-9 or induce apoptosis in isogenic HCT116 colorectal cancer cells that are deficient in BAX, a proapoptotic gene that is mutated in >50% of colorectal cancers of the microsatellite mutator phenotype. Loss of BAX also renders colorectal cancer cells resistant to TRAIL/Apo2L-mediated radiosensitization. We additionally demonstrate that TRAIL/Apo2L-induced death of p53+/+ or p53−/− BAX-proficient but not BAX-deficient colorectal cancer cells is augmented by reducing nuclear factor-κB-dependent expression of Bcl-xL, with either a peptide that disrupts the inhibitor of κB kinase complex or the nonsteroidal anti-inflammatory drug, sulindac sulfide. These results indicate that the combination of TRAIL/Apo2L with either irradiation or sulindac may be highly effective against both p53-proficient and p53-deficient colorectal cancers; however, BAX-deficient tumors may evade elimination by TRAIL/Apo2L-based regimens. Our findings may aid the development and genotype-specific application of TRAIL/Apo2L-based combinatorial regimens for the treatment of colorectal cancers.

Introduction

Colorectal cancer is the second leading cause of cancer deaths in the United States (1). There were an estimated 130,000 new cases and >56,000 deaths attributed to colorectal cancer in the year 2000. Despite revolutionary advances in the identification of genetic alterations involved in the development of colorectal cancer, 5-year survival of patients with metastatic colorectal cancer remains <5% (1, 2). The cornerstone of the current systemic therapy of metastatic colorectal cancers is the antimetabolite 5-FU3 (1). However, the ability of 5-FU to induce apoptosis is dependent on p53, a tumor suppressor gene that is lost or inactivated in at least 85% of human colorectal cancers (3–5). An alternative mechanism of tumor cell death can be triggered by engagement of specific death receptors belonging to the tumor necrosis factor receptor gene superfamily with the “death ligand,” TRAIL/Apo2L (6, 7). The death receptors for TRAIL/Apo2L, TRAIL-R1, and TRAIL-R2, are type I transmembrane proteins containing cytoplasmic sequences, termed “death domains,” that recruit and cross-activate the initiator procaspase-8 (6, 8). Caspase-8 cleaves and activates BID, a “BH-3 domain only” prodeath protein of colorectal cancer cells. To BAX-proficient but not BAX-deficient colorectal cancer cells is augmented by reducing nuclear factor-κB-dependent expression of Bcl-xL, with either a peptide that disrupts the IκB kinase complex or the nonsteroidal anti-inflammatory drug, sulindac sulfide.

Materials and Methods

Cell Lines and Cell Culture. The HCT116 human colon adenocarcinoma cell line containing wild-type p53 and one intact BAX allele (p53+/+BAX+/− genotype) and isogenic p53-deficient (p53−/−) or BAX-deficient (BAX−/−) derivatives of HCT116 cells generated by disruption of either p53 or BAX alleles by gene targeting, have been described previously (4, 16). HCT116 cells of each genotype (p53+/+, BAX+/−; p53−/−, and BAX−/−) were provided by Dr. Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD). Cells were cultured at 37°C and 5% CO2 in McCoy’s 5A medium supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Irradiation. IR (500 cGy) was delivered with a 137Cs dual source γ-cell irradiator.

Immunoblot Assays. Cell lysates were prepared as described (13), and 50–100 μg of protein were resolved by SDS-PAGE, transferred onto Immobilon-P membrane, and probed with antibodies against Bax, Bcl-xL, Bax, β-actin, or tubulin. The blots were visualized by enhanced chemiluminescence. A representative immunoblot is shown in Supplementary Figure 4.
bilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA), and probed with antibodies against BAX (N-20), BID (C-20), caspase-8 (C-20), caspase-3, caspase-9 (H-170), FLIP (G-11), Bcl-x\textsubscript{\textminus} (S-18), IKK\beta (H-470), and actin (C-11; Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive protein complexes were visualized with enhanced chemiluminescence (Amer sham, Arlington Heights, IL).

IKK Kinase Assays. IKK complexes were immunoprecipitated from whole cell extracts (500 μg) using an antibody against IKK\beta (M-280; Santa Cruz Biotechnology). One-half of the immunoprecipitate was subjected to a kinase assay at 30°C for 30 min in kinase buffer [20 mM HEPES (pH 7.6), 3 mM MgCl\textsubscript{2}, 10 μM ATP, 3 μCi [γ-\textsuperscript{32}P]ATP, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM p-nitrophenyl phosphate, 300 μM Na\textsubscript{2}VO\textsubscript{4}, 1 mM benzamidine, 2 μM phenylmethylsulfonyl fluoride, 10 μg/ml apro tin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM DTT] containing 500 ng of GST-I\textsubscript{IKK} fusion protein (GST-I\textsubscript{IKK}; 1:317; Santa Cruz Biotechnology) as substrate. The kinase reaction was terminated by addition of 2 × SDS-PAGE sample buffer, and subjected to SDS-PAGE and autoradiography (13). The remaining half of the immunoprecipitate was subjected to immunoblot analysis, as described above.

Inhibition of the IKK Complex. Inhibition of the IKK\β-NEMO interaction and NF-κB activation was achieved by incubation of HCT116 cells with 200 μM of a cell-permeable peptide spanning the IKK\β NBD (17). The sequence of the WT NBD indicating the Antennapedia homeodomain (lowercase) and the IKK\β (uppercase) segments and the mutant peptide (MU NBD) with the positions of the W\textsubscript{\textgamma}A mutations (underlined) are indicated below:

Wild type: drqikwiffqnrrmkwkkTALDASALQTE

Mutant: drqikwiffqnrrmkwkkTALDWSWLQTE

[Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and W, Trp]. Both peptides were supplied as a 20-mM solution in DMSO (Genemed Synthesis Inc., South San Francisco, CA). Results for DMSO controls were not different from controls.

EMSAs. Nuclear extracts were prepared as described (13). Double-stranded oligonucleotides containing either a consensus binding site for NF-κB (5’-GGGGACATTCC-3’; Santa Cruz Biotechnology) were \textsuperscript{\textgamma} labeled using polyadenylate kinase and [γ-\textsuperscript{32}P]ATP. Nuclear extracts (2.5–5 μg) were incubated with ~1 μl of labeled oligonucleotide (20,000 cpm) in a 20-μl incubation buffer [10 mM Tris, 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 2% glycerol, and 1–2 μg of poly(dI-dC)] for 20 min at 25°C. The specificity of NF-κB DNA-binding activity was confirmed by competition with excess cold wild-type or mutant oligonucleotide or supershift with an antibody against p65/RelA(Geneca, Montreal, Canada), as described (13).

DNA-protein complexes were resolved by electrophoresis in 5% nondenaturing polyacrylamide gels and analyzed by autoradiography and densitometry (Molecular Dynamics).

Analysis of Cell Death. Cells were assessed for morphological features of apoptosis (condensed chromatin and micronucleation) by microscopic visualization. Cell viability was assessed at the indicated intervals by trypan blue dye exclusion and harvested cells adherent + floating in the medium. Cell survival was measured by scoring ≥500 cells in each group, and the average percentage of viability (mean ± SE) was calculated from three different experiments.

Results

TRAIL/Apo2L-mediated Apoptosis of Colorectal Cancer Cells Is p53-independent but Requires BAX. HCT116 colorectal cancer cells have wild-type p53 (p53\textsuperscript{+/+}) and undergo apoptosis in response to 5-FU (4). Isogenic derivatives of HCT116 cells in which both p53 alleles were disrupted by gene targeting (p53\textsuperscript{–/–}) are nearly completely resistant to 5-FU-induced apoptosis compared to wild-type cells with p53\textsuperscript{+/+} genotype (4). To investigate whether TRAIL/Apo2L can trigger death of p53-deficient colorectal cancers, we compared the sensitivity of both p53\textsuperscript{+/+} and p53\textsuperscript{–/–} HCT116 cells to recombinant human TRAIL/Apo2L. In contrast, the p53-dependent induction of apoptosis by 5-FU, isogenic p53\textsuperscript{–/–} and p53\textsuperscript{+/+} HCT116 cells exhibited equivalent sensitivity to TRAIL/Apo2L-induced apoptosis (Fig. 1, a–c). Moreover, exposure to TRAIL/Apo2L augmented the sensitivity of both p53\textsuperscript{+/-} and p53\textsuperscript{+/-} HCT116 cells to IR (Fig. 1, b and c). These data indicate that TRAIL/Apo2L-induced apoptosis and radiosensitization of colorectal cancer cells is independent of p53.

To determine whether BAX is required for TRAIL/Apo2L-induced death of colorectal cancers, we studied isogenic derivatives of HCT116 cells that differ only in the presence or absence of the BAX gene (16). Of HCT116 cells, 94% have an intact BAX allele (BAX\textsuperscript{+/+}) and express functional BAX protein. BAX-deficient HCT116 cells (BAX\textsuperscript{–/–}) were generated by targeted inactivation of the wild-type BAX allele in a BAX heterozygote (16). The loss of BAX expression in BAX\textsuperscript{–/–} cells was confirmed by Western blot analysis (Fig. 1a). Exposure of HCT116 cells of each genotype (p53\textsuperscript{+/-}/BAX\textsuperscript{+/-}; p53\textsuperscript{+/-}/BAX\textsuperscript{–/–}; and BAX\textsuperscript{–/–}) to TRAIL/Apo2L resulted in activation of caspase-8, and caspase-8-mediated proteolysis of caspase-3 and Bid (Fig. 1d). The formation of tBID in BAX-proficient HCT116 cells (p53\textsuperscript{+/-}/BAX\textsuperscript{+/-}; and p53\textsuperscript{+/-}) triggered the release of cytochrome c from mitochondria, resulting in the transactivation cleavage and activation of caspase-9 (Fig. 1d). In contrast, BAX\textsuperscript{–/–} HCT116 cells failed to activate caspase-9 and were completely resistant to TRAIL/Apo2L-induced apoptosis or TRAIL/Apo2L-mediated radiosensitization (Fig. 1, b–d).

TRAIL/Apo2L-induced Death of p53\textsuperscript{+/-} or p53\textsuperscript{–/–} BAX-proficient Colorectal Cancer Cells Is Augmented by Reducing NF-κB-dependent Expression of Bcl-x\textsubscript{\textgamma}. The release of mitochondrial cofactors and cell survival is determined by the relative concentrations of pro- and antiapoptotic members of the Bcl-2 family. BAX is counteracted by Bcl-x\textsubscript{\textgamma}, a prosurvival member of the Bcl-2 family (15). In addition to Bcl-x\textsubscript{\textgamma}, death receptor-induced activation of BAX is inhibited by the caspase-8/FLICE inhibitory protein (FLIP; Ref. 18). The expression of both Bcl-x\textsuperscript{\textgamma} and FLIP is induced by NF-κB (14, 19), a family of dimeric transcription factors that is frequently activated by specific genetic alterations in human colorectal cancers (e.g., activating mutations of ras genes; Ref. 11). HCT116 cells have a K-ras mutation, and exhibited NF-κB DNA-binding activity in EMSA (Fig. 2a). Immunoblot analyses demonstrated that HCT116 cells expressed both Bcl-x\textsuperscript{\textgamma} and FLIP (Fig. 2a). Signal-dependent activation of NF-κB requires phosphorylation-dependent degradation of IκB proteins by an IKK complex comprising the regulatory protein NEMO (NF-κB essential modifier; IκK-γ) in association with two kinases, IKKα and IKKβ (17). An amino-terminal α-helical region of NEMO interacts with six α\textsubscript{\textgamma}-region residues in the COOH-terminal of IKKβ and IKKα, termed the NEMO binding domain (NBD; Ref. 17). A cell-permeable peptide spanning the IKKβ NBD (consisting of the region T735 to E745) fused with a sequence derived from the Antennapedia homeodomain for membrane translocation (WT NBD) blocks the interaction of NEMO with the IKK complex and inhibits activation of NF-κB; a corresponding mutant NBD peptide (W739 and W741 mutated to alanines; MU NBD) does not disrupt signal-induced formation of the IKK-NEMO complex (17). Immune complex kinase assays using IKKβ immunoprecipitates showed that WT NBD but not MU NBD reduced IKK activity in both BAX\textsuperscript{+/-} or BAX\textsuperscript{–/–} HCT116 cells (Fig. 2a). Accordingly, treatment of BAX\textsuperscript{+/-} or BAX\textsuperscript{–/–} HCT116 cells with the WT NBD inhibited NF-κB DNA binding activity in EMSA and reduced NF-κB-dependent expression of both Bcl-x\textsubscript{\textgamma} and FLIP (Fig. 2a). To determine whether inhibition of NF-κB-dependent expression of Bcl-x\textsubscript{\textgamma} can augment TRAIL/Apo2L-induced apoptosis of colorectal cancers, we treated BAX\textsuperscript{–/–} or BAX\textsuperscript{+/-} HCT116 cells with TRAIL/Apo2L in the presence of either WT NBD or MU NBD. Treatment with WT NBD but not MU NBD augmented the death of BAX\textsuperscript{–/–} HCT116 cells in response to TRAIL/Apo2L (Fig. 2b). In contrast, WT NBD failed to

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sensitize BAX<sup>−/−</sup> HCT116 cells to TRAIL/Apo2L-induced death (Fig. 2b).

**Augmentation of TRAIL/Apo2L-induced Death of p53<sup>−/−</sup> or p53<sup>−/−</sup> BAX-proficient Colorectal Cancer Cells by Sulindac Sulfide.** The IKK complex can be directly inhibited by sulindac sulfide, a nonsteroidal anti-inflammatory drug that is used as a chemopreventive agent for patients with predisposition to colorectal cancer (20, 21). Exposure of HCT116 cells of each genotype (p53<sup>−/−</sup>BAX<sup>−/−</sup>; p53<sup>−/−</sup>; and BAX<sup>−/−</sup>) to sulindac resulted in inhibition of IKK<sub>α</sub> kinase activity, repression of NF-κB DNA-binding activity, and a corresponding reduction in expression Bcl-x L and FLIP (Fig. 2a). To determine whether sulindac-mediated reduction of Bcl-x<sub>L</sub> expression can augment TRAIL/Apo2L-induced apoptosis of colorectal cancers, we treated BAX<sup>−/−</sup> or BAX<sup>−/−</sup> HCT116 cells with TRAIL/Apo2L in the presence of sulindac sulfide. Exposure to sulindac increased TRAIL/Apo2L-induced death of BAX-proficient HCT116 cells (p53<sup>−/−</sup>BAX<sup>−/−</sup>; and p53<sup>−/−</sup>BAX<sup>−/−</sup>) in a dose-dependent manner (Fig. 2, c and d). In contrast, BAX-deficient HCT116 cells (BAX<sup>−/−</sup>) remained resistant to TRAIL/Apo2L-induced death even in the presence of sulindac (120 μM; Fig. 2, c and d).

**Discussion**

The development of colorectal cancer involves the acquisition of specific genetic aberrations that inhibit apoptosis (22). Such aberrations not only facilitate survival in hypoxic tumor microenvironments and tumor progression but also confer resistance to anticancer agents (23, 4). Unraveling mechanisms to unleash the death program in tumor cells that resist chemotherapeutic agents or radiation could aid the design of effective therapeutic interventions against colorectal cancers. Our studies indicate that colorectal cancer cells that resist 5-FU by virtue of loss of p53 function remain susceptible to death signaling pathways activated by TRAIL/Apo2L. Moreover, TRAIL/Apo2L also radiosensitizes HCT116 colorectal cancer cells in a p53-independent fashion. However, we find that TRAIL/Apo2L-induced apoptosis and radiosensitization of colorectal cancer cells requires BAX for tBID-mediated mitochondrial activation of caspase-9. Although BAX-deficient colorectal cancer cells are able to activate caspase-3 and BID in response to TRAIL/Apo2L, their inability to form the caspase-9/Apaf-1/cytochrome c complex renders them resistant to TRAIL/Apo2L-induced death. Previous observations have indicated that tBID activates both BAX and BAK, resulting in mitochondrial dysfunction and cell death; MEFs and hepatocytes lacking both BAX and BAK but not the corresponding cells lacking only one of these genes are completely resistant to tBID-induced apoptosis (9). Whereas these observations indicate that other multidomain proapoptotic Bcl-2 family members such as BAK can also initiate mitochondrial dysfunction in normal murine cells (MEFs and hepatocytes) in response to diverse death signals (9), our studies suggest that BAK (expressed in HCT116 cells) does not substitute for BAX in TRAIL/Apo2L-induced mitochondrial dysfunction and death of colorectal cell lines.

**Fig. 1.** TRAIL/Apo2L-mediated apoptosis of HCT116 cells is p53-independent but requires BAX. a, Western-blot analysis of p53 and BAX expression in isogenic HCT116 cells of the indicated genotypes. b, phase contrast photomicrographs of HCT116 cells of the indicated genotypes (p53<sup>−/−</sup>BAX<sup>−/−</sup>; p53<sup>−/−</sup>; and BAX<sup>−/−</sup>) after treatment with either TRAIL/Apo2L, IR, or both TRAIL/Apo2L and IR. c, survival of HCT116 cells of the indicated genotypes after 48 h of the indicated treatments (mean of three independent experiments; bars, ± SE). d, Western-blot analyses of procaspase-8, BID (p22), BAX, procaspase-3, and caspase-9 [the inactive zymogen (procaspase-9) and the active subunit resulting from its cleavage (caspase-9)] in whole-cell lysates of HCT116 cells of the indicated genotypes after 24 h of the indicated treatments.

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cancer cells. The BAX gene contains an unstable tract of eight consecutive deoxyguanosines in the third coding exon (spanning codons 38–41) that is mutated in >50% of colorectal cancers of the microsatellite mutator phenotype (10). Our results suggest that such mismatch repair-deficient cancers may develop resistance to TRAIL/Apo2L through selection of BAX-deficient tumor cells.

In addition to frameshift mutations in BAX, TRAIL/Apo2L-induced death of colorectal cancers can also be inhibited by Bcl-xL, a NF-κB-inducible gene that sequesters tBID and prevents BAX-mediated mitochondrial apoptosis (11). This mechanism of resistance may operate in colorectal cancers that have constitutive activation of NF-κB via mutations in K-ras (≈50% of all colorectal cancers) or overexpression of the EGFR (≈72% of all colorectal cancers; Refs. 11, 12). Our results indicate that TRAIL/Apo2L-induced death of p53+/− or p53−/− BAX-proficient colorectal cells is augmented by reducing NF-κB-dependent expression of Bcl-xL with either a peptide that disrupts the IkB kinase complex or the nonsteroidal anti-inflammatory drug, sulindac sulfide. Sulindac has been primarily used in the management of colonic and rectal adenomas in patients with familial adenomatous polyposis (21, 24). Administration of sulindac to patients with familial adenomatous polyposis results in steady-state plasma levels of 10–15 μM (25). Sulindac sulfide is concentrated in the colonic epithelium to levels that are at least 20-fold higher than those achieved in the serum (25). The concentrations of sulindac sulfide used in our in vitro studies to potentiate TRAIL/Apo2L-induced apoptosis (60–120 μM) reflect the levels seen in colonic epithelium but exceed pharmacological plasma levels. Whereas our results provide a biological rationale for combining TRAIL/Apo2L and sulindac for treatment of p53-proficient or p53-deficient colorectal cancers, additional studies are required to evaluate and optimize the therapeutic ratio of such regimens.

Our results indicate that the combination of TRAIL/Apo2L with either irradiation or sulindac may be highly effective against both p53-proficient and p53-deficient colorectal cancers; however, BAX-deficient tumors may evade elimination by TRAIL/Apo2L-based regimens. In addition to defining key molecular determinants of TRAIL/Apo2L-induced apoptosis (Fig. 3), our findings may aid the development and genotype-specific application of TRAIL/Apo2L-based combinatorial regimens for the treatment of colorectal cancers.

Fig. 2. TRAIL/Apo2L-induced death of p53+/+ or p53−/− BAX-proficient, but not BAX-deficient, HCT116 colorectal cancer cells is augmented by reducing NF-κB-dependent expression of Bcl-xL. α, effect of cell-permeable peptides spanning the IKK β NBD [wild-type (WT) or mutant (MU)] or sulindac sulfide on IKK β kinase activity (immune-complex kinase assay), NF-κB DNA binding activity (EMSA), and expression of Bcl-xL and FLIP (Western blot analysis) in HCT116 cells of the indicated genotypes. β, survival of BAX−/− and BAX−/− HCT116 cells after 48 h of treatment with TRAIL/Apo2L in the presence or absence of either WT NBD or MU NBD (mean of three independent experiments; bars, ± SE).

β, survival of BAX−/− and BAX−/− HCT116 cells after 48 h of treatment with TRAIL/Apo2L in the presence or absence of either WT NBD or MU NBD (mean of three independent experiments; bars, ± SE).

β, survival of HCT116 cells of the indicated genotypes after 48 h of treatment with the indicated concentrations of TRAIL/Apo2L and sulindac (mean of three independent experiments; bars, ± SE).

β, survival of HCT116 cells of the indicated genotypes after 48 h of treatment with sulindac (60 μM) or the combination of TRAIL/Apo2L and sulindac.

β, survival of HCT116 cells of the indicated genotypes after 48 h of treatment with the indicated concentrations of TRAIL/Apo2L and sulindac (mean of three independent experiments; bars, ± SE).

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Fig. 3. Schematic representation of the molecular determinants of TRAIL/Apo2L-induced apoptosis of colorectal cancers. Engagement of death receptors with TRAIL/Apo2L cross-activates caspase-8 and triggers caspase-8-mediated proteolytic activation of caspase-3 and BID. Our studies indicate that TRAIL/Apo2L-induced apoptosis of colorectal cancer cells requires BAX for tBID-mediated release of cytochrome c and mitochondrial activation of caspase-9. Activation of NF-κB by genetic aberrations (such as mutations of ras or overexpression of EGFR) may protect colorectal cancers from TRAIL/Apo2L by inducing expression of survival genes that inhibit caspase-8 (FLIP) or sequester tBID (Bcl-xL). Conversely, TRAIL/Apo2L-induced death of colorectal cancers may be augmented by agents that reduce Bcl-xL expression via inhibition of NF-κB (peptidomimetic compounds that disrupt the IKK complex or sulindac sulfide).

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References


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