Resistancer of Cancers to Immunologic Cytotoxicity and Adoptive Immunotherapy via X-Linked Inhibitor of Apoptosis Protein Expression and Coexisting Defects in Mitochondrial Death Signaling

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Abstract

The ability of cancers to evade immune surveillance and resist immunotherapy raises a fundamental question of how tumor cells survive in the presence of a competent immune system. Studies to address this question have primarily focused on mechanisms by which tumor cells avoid recognition by or induce tolerance in the immune system. However, little is known about whether cancer cells also acquire an intrinsic ability to resist killing by immune effectors. We find that cancer cells enhance their ability to withstand an attack by cytotoxic immune effector cells via acquisition of specific genetic alterations that interfere with the shared mitochondrial death signaling pathway entrained by granzyme B, IFN-γ, and Apo2 ligand/tumor necrosis factor-related apoptosis inducing ligand (Apo2L/TRAIL), three key mediators of immunologic cell-mediated cytotoxicity. We show that the coexistence of specific mitochondrial signaling defects (either deletion of BAX, overexpression of Bel-xL, or deletion of Smac) with expression of X-linked inhibitor of apoptosis protein decreases the sensitivity of cancer cells to IFN-γ/Apo2L/TRAIL- or granzyme B-induced apoptosis, lymphocyte-mediated cytotoxicity in vitro, and adoptive cellular immunotherapy in vivo. Conversely, negating X-linked inhibitor of apoptosis protein expression or function in tumor cells with defective mitochondrial signaling enables direct activation of caspase-3/-7 by granzyme B or Apo2L/TRAIL, and restores their susceptibility to immunologic cytotoxicity. These findings identify an important mechanism by which cancers evade elimination by immune effector cells and suggest that cancer immunotherapy might be improved by concurrent strategies to alleviate or circumvent the intrinsic mitochondrial death signaling defects that help cancer cells resist immunologic cytotoxicity. (Cancer Res 2006; 66(3): 1730-9)

Introduction

The success of allogeneic hematopoietic cell transplantation in the treatment of certain hematologic malignancies provides evidence of the ability of the immune system to eliminate tumor cells (1). However, the limited efficacy of immunotherapy against most cancers raises a fundamental question of how tumor cells survive in the presence of a competent immune system (2). Studies to address this question have primarily focused on mechanisms by which tumor cells avoid recognition by or induce tolerance in the immune system (3). However, little is known about whether cancer cells also acquire an enhanced ability to resist killing by immune effectors. As strategies to activate antitumor immune responses continue to improve, the intrinsic resistance of tumor cells to immunologic cytotoxicity might pose a significant limitation to the efficacy of cancer immunotherapy.

Natural killer (NK) cells and CTLs employ a diverse range of death signaling mechanisms to eliminate their targets. One mechanism involves calcium-dependent exocytosis of granule proteins, perforin, and granymes (4–6), and another is triggered via engagement of death receptors by Apo2 ligand/tumor necrosis factor-related apoptosis inducing ligand (Apo2L/TRAIL; refs. 7–13). Studies in perforin-deficient mice have confirmed the instrumental role of the granule exocytosis pathway in the elimination of tumor cells by CTLs and NK cells (4–6). IFN-γ-dependent expression of Apo2L/TRAIL also plays a critical role in immune surveillance against tumorigenesis, NK cell–mediated suppression of tumor metastases, and donor T cell–mediated graft-versus-tumor activity (9–13). Both granzyme B and Apo2L/TRAIL transduce death signals via proteolytic activation of effector caspases-3/-7 (14, 15), as well as induction of mitochondrial outer membrane permeabilization via cleavage of the "BH-3 domain only" protein BID to truncated forms that activate BAX and BAK (16–19). The mitochondrial release of cytochrome c results in transactivation of caspase-9 (20–22), and the liberation of Smac/DIABLO (second mitochondria-derived activator of caspase/direct IAP binding protein with low pH) facilitates the activation of effector caspases-3/-7 by alleviating the inhibitory effect of X-linked inhibitor of apoptosis protein (XIAP; refs. 23–31). Although granzyme B and Apo2L/TRAIL are key effectors of immunologic cell-mediated apoptosis, CTLs also express other granzymes (A, K, M, and H) and granule proteins, such as granulysin, which may deliver alternative death signals that operate independently of mitochondrial permeabilization and caspases (32, 33).

We investigated whether the ability of cancer cells to withstand an attack by cytotoxic immune effectors is enhanced by genetic alterations that interfere with the mitochondrial activation of effector caspas. Our studies show that interruption of mitochondrial death signaling via deletion of BAX, overexpression of Bel-xL, or deletion of Smac, decreases the sensitivity of XIAP-expressing
cancer cells to Apo2L/TRAIL- or granzyme B–induced apoptosis, lymphocyte-mediated cytotoxicity in vitro, and adoptive cellular immunotherapy in vivo. Conversely, negating XIAP expression or function in tumor cells with defective mitochondrial signaling restores their susceptibility to immunologic cytotoxicity. These findings indicate that tumor cells with coexisting defects in mitochondrial permeabilization and activation of effector caspsases may evade elimination by immune effector cells. Therefore, cancer immunotherapy may be improved by strategies that alleviate or circumvent intrinsic defects in mitochondrial death signaling in cancers.

Materials and Methods

Cell Lines and Transfection

The HCT116 human colon adenocarcinoma cell line containing wild-type Bax (BAX+/−), Smac, and XIAP (HCT116-WT) was used to generate isogenic BAX-deficient (BAX−/−), Smac-deficient (Smac−/−), or XIAP-deficient (XIAP−/−) derivatives via targeted disruption of BAX, Smac, or XIAP alleles (34–36). HCT116 cells that overexpress c-FLIP (HCT116-FLIP) were generated via the introduction of a retroviral vector encoding human c-FLIP (pMX/IRESCGFpPmiG) into HCT116-WT cells (37). HCT116 cells that overexpress Bcl-xL were generated via the introduction of a retroviral vector encoding human Bcl-xL (pMX/IRESCGFpPmiG) into HCT116-WT cells (HCT116-Bcl-xL; ref. 38) or HCT116-XIAP−/− cells (HCT116-XIAP−/−Bcl-xL). 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).

In vitro Treatments

Exponentially growing cells (2 × 10⁵ per well) were incubated with soluble recombinant human Apo2L/TRAIL (100 ng/mL; Alexis Biochemicals, San Diego, CA), recombinant human IFN-γ (1,000 units/mL; R&D Systems, Minneapolis, MN), and/or the nuclear factor-κB (NF-κB) inhibitor SN-50 (2.5 μmol/L; Calbiochem, La Jolla, CA). Cells (1 × 10⁵/mL) were exposed to recombinant human granzyme B (0.1-1 μg/mL) in the presence of LAK extract (2 μg/mL) in serum-free medium supplemented with 0.1% bovine serum albumin. In some experiments, cells were treated with a synthetic Smac peptide containing eight of the NH2-terminal amino acids of Smac fused to the penetratin sequence of the Drosophila transactivation factor antennapedia (Smac peptide); a corresponding peptide containing an alanine-to-methionine mutation (A1M peptide) was used as a negative control (39).

Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared using NE-PER buffer (Pierce, Rockford, IL). The NF-κB DNA-binding assay was done using [γ-32P]-labeled double-stranded oligonucleotides containing a consensus binding site for NF-κB (5′-GAGGAGCTTTTGGCACTCC-3′; Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (40). DNA-protein complexes were resolved by electrophoresis in 5% nondenaturing polyacrylamide gels and analyzed by autoradiography.

Cell Fractionation and Immunoblot Analysis

The ApoAlert Cell Fractionation kit was used to separate the mitochondrial fraction from the cytosolic fraction, as per the manufacturer’s protocol (Clontech Laboratories, Palo Alto, CA). Whole cell lysates were prepared and resolved by SDS-PAGE (10-50 μg protein/lane), transferred onto Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA), and probed with antibodies against the following proteins: caspase-8 (C-20), FLIP, BAX (N-20), BAK (N-20), Bcl-xL (S-18), caspase-9 (N-17), actin (C-11), and pro-ADP ribose polymerase (PARP; Ab-2; Calbiochem). The cytosolic and mitochondrial fractions were analyzed by immunoblot using antibodies against cytochrome c, cytochrome c oxidase subunit IV (Clontech Laboratories), and Smac (Biomol Research Laboratories, Plymouth Meeting, PA). Immunoreactive protein complexes were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Analysis of Mitochondrial Membrane Potential

Mitochondrial membrane potential depolarization was detected by staining cells with the Mit-E-dye solution containing a fluorescent cationic dye, 1,1′,3,3′-tetraethylenbenzimidazolocarboxyanine iodide (JC-1; Biomol Research Laboratories). Loss of mitochondrial membrane potential (Δψm) was quantified by flow cytometric analysis of the decrease in the 590 nm (red)/527 nm (green) fluorescence intensity emission ratio.

Analysis of Caspase-3/-7 Activity

Cellular caspase-3/-7 activity in cell extracts was assayed colorimetrically in 96-well plates using Ac-DEVAD-pNA substrate (Biomol Research Laboratories). Specific DEVDase activity was calculated and expressed as pmol pNA/min/μg of protein.

Analysis of Cell Death

Apoptosis was quantified by flow cytometric analysis of the average percentage of harvested cells (adherent + floating in the medium) stained with Annexin V (Biovision Inc., Mountain View, CA; mean ± SE, three independent experiments). Induction of cell death was also measured by a 3H-thymidine release assay (29). Percent-specific [3H]-thymidine release was calculated as follows: [(1 − (sample cpm − spontaneous cpm)) / (total cpm − spontaneous cpm)] × 100.

Analysis of tumor cell death by peptide-specific HLA-A2-restricted CD8+ T lymphocytes. The M1 peptide (58-66) derived using 3H-thymidine release assays (29). Percent-specific [3H]-thymidine release by anti-HLA-A2 antibody (BB7.2).

Cell surface expression of HLA-A2 on HCT116 cells was analyzed by flow cytometry. The secretion of IFN-γ by M1 CTLs cocultured with HCT116 cells pulsed with varying M1 peptide concentrations (10⁻³–10⁻⁴ mol/L/E/T ratio, 5:1) was measured by ELISA. The sensitivity of HLA-A2+ HCT116 cells of each genotype to the cytotoxic effects of M1 CTL was examined using 3H-thymidine release assays. HCT116 cells of each genotype were cocultured with M1 cells at varying E/T ratios at 37°C in McCoys’s medium. In some experiments, cells were treated with either Smac peptide or A1M control peptide (5 μg/mL; for 3 hours) or SN-50 (2.5 μmol/L; for 6 hours) and washed prior to pulsing with M1 peptide. M1-loaded 3H-thymidine-labeled cells were cocultured in triplicate in 96-well plates (5 × 10⁵ cells/well) with M1 CTL at varying E/T ratios at 37°C for 4 hours. Cells were harvested onto a filter paper using a cell harvester. Induction of cell death was determined by calculating the percent-specific [3H]-thymidine release as follows: [(1 − (sample cpm − spontaneous cpm)) / (total cpm − spontaneous cpm)] × 100.

Statistical analysis. Linear regression was used to assess the association between the percentage of cell death as a function of E/T ratio for each of the HCT116 cell genotypes and treatments. To adhere to the assumptions of linear regression, a square root transform was applied to the percentage of cell death. To examine influenza matrix protein, was synthesized and purified to >95% purity. A CD8+ M1-peptide-specific, HLA-A2-restricted human T cell line (M1 CTL) was generated and stimulated as previously described (41). Antigen-specific activation of M1 CTLs was confirmed by ELISA (R&D Systems) demonstrating secretion of IFN-γ in supernatants derived from cocultures of M1 effector cells and M1 peptide-pulsed but not unloaded EBV1.24 cells. The peptide-specific and HLA-A2-restricted secretion of target cells by activated M1 CTLs was confirmed by the preferential lysis of M1 peptide-pulsed EBV1.24 cells compared with unloaded cells, and inhibition of target cell lysis by preincubation with anti-HLA-A2 antibody (BB7.2).

Cell surface expression of HLA-A2 on HCT116 cells was analyzed by flow cytometry. The secretion of IFN-γ by M1 CTLs cocultured with HCT116 cells pulsed with varying M1 peptide concentrations (10⁻³–10⁻⁴ mol/L/E/T ratio, 5:1) was measured by ELISA. The sensitivity of HLA-A2+ HCT116 cells of each genotype to the cytotoxic effects of M1 CTL was examined using 3H-thymidine release assays. HCT116 cells of each genotype were cocultured with M1 cells at varying E/T ratios at 37°C in McCoys’s medium. In some experiments, cells were treated with either Smac peptide or A1M control peptide (8 μg/mL; for 3 hours) or SN-50 (2.5 μmol/L; for 6 hours) and washed prior to pulsing with M1 peptide. M1-loaded 3H-thymidine-labeled cells were cocultured in triplicate in 96-well plates (5 × 10⁵ cells/well) with M1 CTL at varying E/T ratios at 37°C for 4 hours. Cells were harvested onto a filter paper using a cell harvester. Induction of cell death was determined by calculating the percent-specific [3H]-thymidine release as follows: [(1 − (sample cpm − spontaneous cpm)) / (total cpm − spontaneous cpm)] × 100.

Statistical analysis. Linear regression was used to assess the association between the percentage of cell death as a function of E/T ratio for each of the HCT116 cell genotypes and treatments. To adhere to the assumptions of linear regression, a square root transform was applied to the percentage of cell death and a log-transform was applied to E/T ratio. Linear models were explored including the main effects of E/T ratio, cell type, treatment, and their two-way interactions. Statistical significance of main effects was defined by an alpha level of 0.05 and for interactions an alpha of 0.10 was used.

Analysis of the Effect of Immune Effectors on S.c. Tumor Xenografts In vivo

Female BALB/c nude mice were injected s.c. with HCT116 cells of defined genotypes (4 × 10⁶ cells per mouse). Forty-eight hours following tumor cell challenge, mice were randomly distributed (14 per group) and

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either left untreated or injected i.v. with 10^6 splenocytes isolated from wild-type BALB/c mice. Tumor size was measured every 5 days.

**Statistical analysis.** Exploratory plots were used to determine the appropriate transformation and linear regression models to consider. Based on these plots, a square-root transform of tumor volume seemed to be most appropriate for linear modeling. Quadratic models achieved appropriate fit of linear regressions. Generalized estimating equations were used to fit linear regression models, accounting for correlation across data points from the same mouse. Separate models were fit for each group (i.e., HCT116 cells of each genotype). Raw data and fitted models (transformed back to the original scale) were used for making qualitative inferences.

**Analysis of the Effect of Immune Effectors on Experimental Hepatic Metastases of Colon Cancer Cells**

Laparotomy was done on anesthetized female BALB/c nude mice, and the spleen was divided into two semi-spleens using titanium clips, leaving the vascular pedicles intact. Following injection of 5 x 10^6 HCT116 cells of each genotype into one of the semi-spleens, the tumor cell–contaminated hemi-spleen was surgically removed, leaving a functional semi-spleen free of tumor cells. The injected tumor cells traffic via the splenic/portal circulation and form tumor deposits in the liver (40). Fifteen days following tumor challenge, mice were randomly distributed (17 per group) and either left untreated or injected i.v. with 10^6 splenocytes isolated from wild-type BALB/c mice. Three mice from each group were euthanized at 45 days after tumor challenge, and the remaining mice (14 from each group) were followed until 90 days after tumor challenge. The livers were sectioned and H&E stained to evaluate gross and microscopic tumors.

**Statistical analysis.** Kaplan-Meier curves were estimated by tumor cell type and treatment group for overall survival and tumor-free survival. Log-rank tests and Cox regression were used to determine if differences between groups were significant (α = 0.05). Hazard ratios and 95% confidence intervals were calculated based on Cox regression results. Incidence of tumor was estimated for each of the tumor cell type and treatment groups. Proportions and 95% confidence intervals were estimated and comparisons were made using Fisher’s exact test.

**Animal Welfare**

All animal protocols were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins Medical Institutions.

**Results**

**Interference with mitochondrial death signaling reduces the susceptibility of XIAP-expressing cancer cells to IFN-γ-mediated activation of Apo2L/TRAIL-induced apoptosis.** IFN-γ-dependent expression of Apo2L/TRAIL mediates NK cell–dependent tumor suppression and donor T cell–mediated graft-versus-tumor activity (9–13). Exposure of colon carcinoma cells (HCT116-WT) to Apo2L/TRAIL resulted in processing of caspase-8, truncation of BID to tBID, loss of mitochondrial membrane potential (ΔΨm), release of mitochondrial cytochrome c and Smac into the cytosol, cleavage/activation of effector caspases (-7/-3), proteolysis of the caspase-3 substrate, PARP, and apoptosis (Fig. 1A-E). Pretreatment of HCT116 cells with IFN-γ increased expression of procaspase-8, BAK, and procaspase-7, and enhanced caspase-3/-7 activation and apoptosis in response to Apo2L/TRAIL (Fig. 1A, D, and E). However, HCT116 derivatives that overexpress caspase-8/FLICE-inhibitory protein (HCT116-FLIP) failed to cleave BID and were resistant to caspase-3/-7 activation or apoptosis in response to IFN-γ and Apo2L/TRAIL (Fig. 1A, D, and E).

Caspase-8–mediated formation of tBID triggers mitochondrial permeabilization via activation of BAX. To assess the requirement of mitochondrial permeabilization in IFN-γ/Apo2L/TRAIL-induced apoptosis of tumor cells, we examined the effect of IFN-γ and Apo2L/TRAIL on HCT116 cells that are either deficient in BAX (34, 38) or overexpress Bcl-xL, a protein that counteracts tBID-mediated activation of BAX (38, 42). Treatment of either Bax-deficient (HCT116-BAX^−/−) or Bcl-xL–overexpressing (HCT116-Bcl-xL) cells with Apo2L/TRAIL-induced formation of tBID, yet failed to induce loss of mitochondrial ΔΨm or mitochondrial release of cytochrome c and Smac (Fig. 1A-C). Compared with parental cells, HCT116-BAX^−/− cells or HCT116-Bcl-xL cells were resistant to caspase-3/-7 activation and apoptosis in response to IFN-γ and Apo2L/TRAIL (Fig. 1A, D, and E).

To determine whether mitochondrial release of Smac is required to counter the inhibition of caspase-3/-7 by endogenous XIAP in tumor cells, we examined the effect of IFN-γ and Apo2L/TRAIL on Smac-deficient (Smac^−/−) or XIAP-deficient (XIAP^−/−) derivatives of HCT116 cells (35, 36). Compared with parental cells, HCT116-Smac^−/− cells were relatively resistant to caspase-3/-7 activation and apoptosis in response to IFN-γ and Apo2L/TRAIL, whereas HCT116-XIAP^−/− cells exhibited relatively greater IFN-γ/Apo2L/TRAIL-induced caspase-3/-7 activation and apoptosis (Fig. 1D and E). To assess whether mitochondrial signaling is required for IFN-γ/Apo2L/TRAIL-induced apoptosis in the absence of XIAP, we examined the effect of IFN-γ and Apo2L/TRAIL on XIAP-deficient HCT116 cells that overexpress Bcl-xL (HCT116-XIAP^−/−/Bcl-xL; Fig. 1F and G). Although forced expression of Bcl-xL inhibited IFN-γ/Apo2L/TRAIL-induced caspase-3/-7 activation and apoptosis in XIAP-proficient HCT116-Bcl-xL cells, it did not confer a similar protection in HCT116-XIAP^−/−/Bcl-xL cells (Fig. 1D-G). Because Bcl-xL and XIAP are induced by NF-κB (43), we examined whether tumor cells with defects in mitochondrial signaling could also be sensitized to IFN-γ/Apo2L/TRAIL-induced apoptosis by SN-50, a specific inhibitor of NF-κB. Treatment with SN-50 inhibited NF-κB DNA-binding activity (Fig. 1H), reduced expression of Bcl-xL and XIAP (Fig. 1C and F), and enhanced apoptosis of HCT116-WT, HCT116-BAX^−/−, HCT116-Bcl-xL, and HCT116-Smac^−/− cells in response to IFN-γ and Apo2L/TRAIL (Fig. 1A, E, and J). These observations show that the BID-BAX-Smac mitochondrial death signaling pathway is essential for IFN-γ/Apo2L/TRAIL-induced apoptosis of tumor cells that express XIAP, but may be circumvented by negating XIAP expression.

**Granzyme B–mediated apoptosis of XIAP-expressing tumor cells is impaired by interference with mitochondrial activation of effector caspases.** We examined the response of HCT116 cells to recombinant granzyme B with LAK extract as a source of perforin. Treatment of parental HCT116 [BAX^+/− (WT)] cells or HCT116-FLIP cells with granzyme B resulted in cleavage of BID (p22) to gtBID (p13), loss of mitochondrial ΔΨm, mitochondrial release of Smac, and equivalent levels of caspase-3/-7 cleavage/activation and DNA fragmentation (Fig. 2A-E). Treatment of either HCT116-BAX^−/− or HCT116-Bcl-xL cells with granzyme B resulted in cleavage of BID to gtBID (Fig. 2B), but did not result in loss of mitochondrial ΔΨm or mitochondrial release of Smac (Fig. 2B and C). Although granzyme B induced direct/primary cleavage of procaspase-3, procaspase-7, and PARP in both HCT116-BAX^−/− and HCT116-Bcl-xL cells (Fig. 2B), it was less effective in inducing caspase-3/-7 activation and DNA fragmentation compared with parental HCT116 cells (Fig. 2D and E). Likewise, granzyme B–mediated caspase-3/-7 activation and DNA fragmentation was impaired in HCT116-Smac^−/− cells compared with parental cells (Fig. 2D and E).

To determine whether the failure to release Smac contributed to the deficiency of caspase-3/-7 activation by granzyme B in HCT116-BAX^−/− or HCT116-Bcl-xL cells, we examined the effect of granzyme B on these cells following treatment with a synthetic peptide...
containing eight of the NH₂-terminal amino acids of Smac (Smac peptide). Because the NH₂-terminal alanine of Smac is critical for binding to XIAP, a corresponding peptide containing an alanine-to-methionine mutation (A1M peptide) was used as a negative control. Treatment with either Smac peptide or A1M peptide alone did not induce caspase-3/-7 activity in HCT116-BAX+/− cells. However, pretreatment with Smac peptide, but not A1M peptide, increased granzyme B–induced caspase-3/-7 activity and DNA fragmentation in both HCT116-BAX+/− and HCT116-Bcl-xL cells (Fig. 2F and G).

To determine whether Smac is required to counteract the caspase-inhibitory effect of XIAP in tumor cells during granzyme...
B–induced apoptosis, we examined the effect of granzyme B on HCT116-XIAP−/− cells, with or without pretreatment with either Smac peptide or A1M peptide. Granzyme B was more effective in inducing caspase-3/7 activation and DNA fragmentation in HCT116-XIAP−/− cells compared with their XIAP-proficient counterparts (Fig. 2D and E). Granzyme B–mediated caspase-3/7 activation or DNA fragmentation in HCT116-XIAP−/− cells was not influenced by either Smac peptide or A1M peptide (Fig. 2F and G). To assess whether mitochondrial signaling is required for granzyme B–induced apoptosis in the absence of XIAP, we examined the effect of granzyme B on XIAP-deficient HCT116 cells that overexpress Bcl-xL (HCT116-XIAP−/−-Bcl-xL; Fig. 1F). Whereas forced expression of Bcl-xL inhibited granzyme B–induced caspase-3/7 activation and DNA fragmentation in XIAP-proficient HCT116-Bcl-xL cells, it did not confer a similar protection in HCT116-XIAP−/−Bcl-xL cells (Fig. 2H and I). These data indicate that BAX-mediated mitochondrial release of Smac is instrumental for granzyme B–induced apoptosis of tumor cells that express XIAP, but may be circumvented by antagonizing XIAP expression or function.

Figure 2. Granzyme B–mediated apoptosis of XIAP-expressing tumor cells is impaired by interference with mitochondrial activation of effector caspases. A, immunoblot analyses of BID, cytosolic Smac, caspase-9, caspase-3, caspase-7, PARP, and actin in lysates of HCT116-BAX+/− (WT) and HCT116-FLIP cells after 10 hours of treatment with granzyme B (1 μg/mL with 2 μg/mL LAK extract). Filled arrowheads, full-length procaspases and PARP; open arrowheads, cleaved proteins. B, immunoblot analyses of BID, cytosolic Smac, caspase-3, caspase-7, PARP, and actin in lysates of HCT116-BAX+/− (WT), HCT116-BAX−/−, and HCT116-Bcl-xL cells after 10 hours of treatment with granzyme B (0.01-1 μg/mL with 2 μg/mL LAK extract). Filled arrowheads, full-length procaspases and PARP; open arrowheads, cleaved proteins. C, effect of granzyme B on mitochondrial membrane potential in HCT116-BAX+/− (WT), HCT116-FLIP, HCT116-BAX−/−, and HCT116-Bcl-xL cells. Cells treated with granzyme B (1 μg/mL with 2 μg/mL LAK extract) for 6 hours were stained with the Mı́t-E-quant solution containing a fluorescent cationic dye (JC-1). The percentage of cells exhibiting loss of mitochondrial membrane potential (ΔΨm) was quantified by flow cytometric analysis of the decrease in the 590 nm (red; log FL2-Y axis)/527 nm (green; log FL1-X axis) fluorescence intensity emission ratio (bottom right quadrant). D, caspase-3/7 (DEVDase) activity (pmol pNA/min/μg of protein) in extracts of HCT116 cells of the indicated genotypes after 8 hours of treatment with granzyme B (1 μg/mL with 2 μg/mL LAK extract). The data represent the percent-specific [3H]thymidine release (columns, mean of three independent experiments; bars, ± SE). E, percentage of death for HCT116 cells of the indicated genotypes after 8 hours of treatment with granzyme B (1 μg/mL with 2 μg/mL LAK extract). F, caspase-3/7 (DEVDase) activity (pmol pNA/min/μg of protein) in extracts of HCT116 cells of the indicated genotypes after 8 hours of treatment with granzyme B (1 μg/mL with 2 μg/mL LAK extract) following pretreatment with either Smac peptide (80 μg/mL) or A1M control peptide (80 μg/mL) for 3 hours. G, percentage of death for HCT116 cells of the indicated genotypes after 8 hours of treatment with granzyme B (1 μg/mL with 2 μg/mL LAK extract) following pretreatment with either Smac peptide (80 μg/mL) or A1M control peptide (80 μg/mL) for 3 hours. H, caspase-3/7 (DEVDase) activity (pmol pNA/min/μg of protein) in extracts of HCT116-XIAP−/−, HCT116-XIAP−/−-Bcl-xL, HCT116-XIAP−/−, and HCT116-XIAP−/−-Bcl-xL cells after 8 hours of treatment with granzyme B (1 μg/mL with 2 μg/mL LAK extract). I, Percentage death of HCT116-XIAP−/−, HCT116-XIAP−/−-Bcl-xL, HCT116-XIAP−/−, and HCT116-XIAP−/−-Bcl-xL cells after 8 hours of treatment with granzyme B (1 μg/mL with 2 μg/mL LAK extract). The data represent the percent-specific [3H]thymidine release (columns, mean of three independent experiments; bars, ± SE).
Interference with mitochondrial death signaling reduces the susceptibility of XIAP-expressing tumor cells to CTL-induced death in vitro. We examined the effect of a CD8+ HLA-A2-restricted human cytotoxic T cell line specific for the M1 peptide (M1 CTL) on M1-pulsed HLA-A2.1+ HCT116 cells or their derivatives (HCT116-FLIP, HCT116-BAX−/−, HCT116-Bcl-xL, HCT116-Smac−/−, HCT116-XIAP−/−, and HCT116-XIAP−/−Bcl-xL). ELISA showed an equivalent secretion of IFN-γ in supernatants of M1 CTL cocultured with M1 peptide-pulsed HCT116 cells of each genotype (Fig. 3A). However, HCT116-FLIP, HCT116-BAX−/−, HCT116-Bcl-xL, and HCT116-Smac−/− cells were all relatively resistant to M1 CTL-induced killing compared with parental HCT116 cells (Fig. 3B). At an effector/target (E/T) ratio of 12.5, the differences in the percentage of cell death between parental HCT116 cells and each HCT116 derivative (HCT116-FLIP, HCT116-BAX−/−, HCT116-Bcl-xL, and HCT116-Smac−/−) were highly significant (P < 0.001 for each pairwise comparison). At higher E/T ratios (≤25:1), the death of HCT116-FLIP cells approached that of parental HCT116 cells, whereas HCT116-BAX−/−, HCT116-Bcl-xL, and HCT116-Smac−/− cells remained significantly resistant to CTL-induced death compared with parental HCT116 cells (P < 0.01 for each pairwise comparison). In contrast, HCT116-XIAP−/− and HCT116-XIAP−/−Bcl-xL cells displayed greater sensitivity to CTL-induced killing compared with their XIAP-proficient counterparts (HCT116 or HCT116-Bcl-xL, respectively; Fig. 3B). We tested whether displacement of XIAP by pretreatment with Smac peptide could increase the sensitivity of HCT116-BAX−/− and HCT116-Bcl-xL cells to M1 CTL-induced killing. Both HCT116-BAX−/− and HCT116-Bcl-xL cells underwent significantly greater CTL-mediated death following pretreatment with Smac peptide versus A1M control peptide (P = 0.008 for HCT116-BAX−/−; P < 0.001 for HCT116-Bcl-xL; Fig. 3C). Inhibition of NF-κB-dependent expression of XIAP by treatment with SN-50 also increased the sensitivity of HCT116-BAX−/− and HCT116-Bcl-xL cells to CTL-mediated death (P < 0.001 for each cell type; Fig. 1H and I, and Fig. 3D). Therefore, interference with mitochondrial release of Smac via loss of BAX or overexpression of Bcl-xL decreases the sensitivity of XIAP-expressing tumor cells to CTL. Conversely, tumor cells with mitochondrial signaling defects are sensitized to immune effector cells by antagonizing XIAP expression or function.

Figure 3. Interference with the mitochondrial death signaling pathway reduces the susceptibility of tumor cells to peptide-specific HLA-A2-restricted CD8+ CTL-induced death in vitro. A, quantification of IFN-γ (pg/mL) by ELISA in supernatants derived from M1 peptide-specific HLA-A2-restricted CD8+ T lymphocytes cocultured for 18 hours with HCT116 cells of each genotype (E/T ratio, 5:1) pulsed with varying concentrations (10−10−10−6 M) of M1 peptide. B, the sensitivity of HLA-A2.1+ HCT116 cells of each indicated genotype to the cytotoxic effects of M1-specific HLA-A2-restricted CD8+ T lymphocytes was examined using [3H]thymidine release assays. [3H]Thymidine-labeled M1 peptide-loaded HCT116 cells of each genotype were cocultured with M1 CTL at the indicated effector/target (E/T) ratios for 4 hours. The data represent the percent-specific killing of HCT116 cells of each genotype determined by calculating the percent-specific [3H]thymidine release (columns, mean of three independent experiments; bars, SE). C, effect of SMac peptide on the sensitivity of HCT116-BAX−/− and HCT116-Bcl-xL cells to the cytotoxic effects of M1-specific CTL. [3H]Thymidine-labeled HCT116-BAX−/− and HCT116-Bcl-xL cells were treated with either Smac peptide (80 μg/mL) or A1M control peptide (80 μg/mL) for 3 hours prior to pulsing with M1 peptide. M1 peptide-loaded tumor cells were cocultured with M1 CTL at the indicated E/T ratios for 4 hours. The data represent the percent-specific killing determined by [3H]thymidine release assays (columns, mean of three independent experiments; bars, SE). D, effect of SN-50 on the sensitivity of HCT116-BAX−/− (WT) to CTL. HCT116-BAX−/− and HCT116-Bcl-xL to the cytotoxic effects of M1-specific CTL. [3H]Thymidine-labeled HCT116 cells of the indicated genotypes were pretreated with SN-50 (2.5 μmol/L) for 6 hours and then washed. SN-50 pretreated or untreated control cells were cocultured with M1 CTL at the indicated effector/target (E/T) ratios for 4 hours. The data represent the percent-specific killing of HCT116 cells of each genotype determined by [3H]thymidine release assays (columns, mean of three independent experiments; bars, SE).
Interference with mitochondrial death signaling curtails the response of XIAP-expressing tumor cells to adoptive cellular immunotherapy in vivo. We examined whether genetic alterations that interfere with mitochondrial death signaling influence the response of tumor cells to immune effectors in vivo. BALB/c nude mice were injected s.c. with either parental HCT116 cells [BAX+/-(WT)] or each of their derivatives (HCT116-FLIP, HCT116-BAX-/-, HCT116-Bcl-xL, HCT116-XIAP-/-, and HCT116-XIAP-/-Bcl-xL) in BALB/c nude mice (4 x 10^6 cells per mouse). Cancers frequently harbor genetic alterations that result in overexpression of Bcl-xL or inactivation of Bax (44, 45), we examined whether these alterations interfere with the elimination of experimental hepatic metastases of colon cancers by immune effector cells (40). Following splenic injection of tumor cells (HCT116, HCT116-BAX-/-, or HCT116-Bcl-xL), BALB/c nude mice exhibited a greater response to adoptive transfer of splenocytes compared with those derived from their XIAP-proficient counterparts (HCT116 or HCT116-Bcl-xL, respectively; Fig. 4B). These data indicate that defects in mitochondrial death signaling could curtail the response of XIAP-expressing tumor cells to adoptive cellular immunotherapy in vivo. Conversely, tumor cells with mitochondrial signaling defects are sensitized to adoptive cellular immunotherapy by negating XIAP expression.

Figure 4. Interference with mitochondrial death signaling curtails the response of XIAP-expressing tumor cells to adoptive cellular immunotherapy in vivo. A, growth of tumors derived from s.c. injection of HCT116 cells of each genotype [HCT116-BAX+/-(WT), HCT116-FLIP, HCT116-BAX-/-, HCT116-Bcl-xL, HCT116-XIAP-/-, and HCT116-XIAP-/-Bcl-xL] in BALB/c nude mice (4 x 10^6 cells per mouse). Columns, mean tumor volumes from 14 mice in each group (bars, ± SE). B, effect of adoptive transfer of splenocytes on the growth of s.c. tumors derived from injection of HCT116 cells of each indicated genotype in BALB/c nude mice. Two days following tumor cell challenge (4 x 10^6 tumor cells per mouse), mice were injected i.v. with 10^6 splenocytes isolated from wild-type BALB/c mice. Columns, mean tumor volumes from 14 mice in each group (bars, ± SE). C, representative images showing the effect of adoptive transfer of 10^6 wild-type splenocytes on the growth of s.c. tumors derived from HCT116 cells of each indicated genotype in BALB/c nude mice.

A comparison; Fig. 4B). At day 30 following adoptive transfer of splenocytes, the mean volume of tumors derived from parental HCT116 cells (<10 mm^3) was also significantly lower than that of tumors derived from either HCT116-FLIP (92 ± 34 mm^3), HCT116-BAX-/- (276 ± 35 mm^3), HCT116-Smac-/- (199 ± 14 mm^3), or HCT116-Bcl-xL (467 ± 48 mm^3) cells (P < 0.001 for each comparison). In contrast, tumors derived from HCT116-XIAP-/- and HCT116-XIAP-/-Bcl-xL cells exhibited a greater response to adoptive transfer of splenocytes compared with those derived from their XIAP-proficient counterparts (HCT116 or HCT116-Bcl-xL, respectively; Fig. 4B and C). These data indicate that defects in mitochondrial death signaling could curtail the response of XIAP-expressing tumor cells to adoptive cellular immunotherapy in vivo. Conversely, tumor cells with mitochondrial signaling defects are sensitized to adoptive cellular immunotherapy by negating XIAP expression.
were either left untreated or injected i.v. after 15 days with $10^6$ splenocytes isolated from wild-type BALB/c mice.

Livers harvested from untreated mice showed gross hepatic tumor nodules at 45 days after splenic implantation of HCT116 cells of all genotypes (three of three mice in each group; Fig. 5A). Livers from mice injected with wild-type splenocytes following implantation of HCT116 cells showed no gross tumors at 45 days (zero of three mice; Fig. 5A). In contrast, gross hepatic tumors were evident in all treated animals at 45 days after tumor challenge with HCT116-BAX<sup>−/−</sup> or HCT116-Bcl-x<sub>L</sub> cells (three of three mice in each group; Fig. 5A).

All untreated controls (14 per group) developed progressive ascites and became moribund within 8 weeks following splenic implantation of HCT116 cells of all genotypes (Fig. 5B). All mice (14 of 14) injected with wild-type splenocytes following implantation of HCT116 cells remained alive and well at 90 days following tumor cell implantation (Fig. 5B), and their livers were free of gross or microscopic tumor (Fig. 5A and C). Of 14 mice injected with wild-type splenocytes following implantation of HCT116-BAX<sup>−/−</sup> cells, 9 mice remained alive at 90 days (Fig. 5B); 3 of the surviving mice showed gross and microscopic evidence of hepatic tumor metastases (Fig. 5A and C, second row), and the other 6 exhibited scar formation without tumors (Fig. 5C, third row). Of 14 mice injected with wild-type splenocytes following implantation of HCT116-Bcl-x<sub>L</sub> cells, 7 mice survived until 90 days (Fig. 5B); 4 of these mice showed gross and microscopic evidence of hepatic metastases (Fig. 5A and C, second row), and the other 3 exhibited scar formation without tumors (Fig. 5C, third row). Although adoptive transfer of splenocytes improved survival of mice challenged with tumor cells of all genotypes, significant differences were found in overall survival at 90 days between mice implanted with HCT116 cells (100%) and those implanted with either HCT116-BAX<sup>−/−</sup> cells (64%; $P = 0.015$) or HCT116-Bcl-x<sub>L</sub> cells (50%; $P = 0.003$), but not between mice implanted with HCT116-BAX<sup>−/−</sup> cells and HCT116-Bcl-x<sub>L</sub> cells ($P = 0.45$). Following adoptive immunotherapy, the tumor-free survival of mice implanted with either HCT116-BAX<sup>−/−</sup> cells (6 of 14; 43%) or HCT116-Bcl-x<sub>L</sub> cells (3 of 14; 21%) was also significantly lower than that of mice implanted with HCT116 cells (14 of 14; 100%; $P = 0.002$ and $P < 0.001$, respectively). The difference in tumor-free survival between mice implanted with HCT116-BAX<sup>−/−</sup> cells and those implanted with HCT116-Bcl-x<sub>L</sub> cells was insignificant ($P = 0.42$).

Figure 5. Genetic impediments to mitochondrial death signaling interfere with elimination of experimental hepatic metastases of colon cancer cells by immune effectors. A, schematic representation of the experimental model of hepatic metastasis of colon cancer (left). Fifteen days following splenic injection of $5 \times 10^6$ HCT116 cells of each indicated genotype, mice (17 per group) were either left untreated or injected i.v. with $10^6$ splenocytes isolated from wild-type BALB/c mice. Three mice from each group were euthanized at 45 days after tumor challenge, and their livers were examined for gross and microscopic tumors. Overall survival was recorded for the remaining animals in each group until 90 days after tumor challenge. All animals were sacrificed at 90 days, and their livers examined for the presence of gross and microscopic tumor. Representative images of livers from mice in each group at 45 days and 90 days after tumor challenge showing the presence/absence of gross tumor metastases (right). B, overall survival and 90-day tumor-free survival of animals injected with HCT116 cells of each indicated genotype and either left untreated or injected i.v. with $10^6$ wild-type splenocytes (14 per group). C, representative images of H&E stained liver sections from surviving mice in each group at 90 days after splenic implantation of tumor cells (magnification, $\times 40$).
Discussion

Immunologists have long understood that normal versus malignant cells of the same tissue type may differ in their capacity to activate the afferent arm of the immune response based on differential expression of genes involved in motility and trafficking, antigen processing and presentation, histocompatibility or T cell costimulatory molecule expression, and cytokine or chemokine production (2, 3, 46). Data presented here show that differential expression of apoptosis-related genes might underlie tumor-specific differences in susceptibility to an effector arm of the immune response, i.e., immunologic cytotoxicity. The resistance of tumor cells to immune cell-mediated apoptosis may contribute to the failure of tumor immunosurveillance, the phenomena of tumor growth despite infiltration by tumor-specific lymphocytes, the failure of tumor vaccines to induce clinical responses despite evidence for priming of effector T cells, or the failure of ex vivo activated CTLs to eradicate tumors following adoptive transfer into cancer patients (2, 47). Moreover, genetic resistance to immunologic cytotoxicity may also underlie the correlation between chemotherapy resistance of a tumor and relapse after allogeneic blood/marrow transplantation (48), or the differential susceptibility of normal versus malignant cells to the cytotoxicity of an allogeneic graft-versus-host reaction (1).

As cancers evolve under adaptive pressure from the host’s immune system, tumor cells may acquire an intrinsic capacity to inhibit the death signaling mechanisms entrained by cytotoxic immune effectors. Cancer cells exhibit significant heterogeneity in their sensitivity to Apo2L/TRAIL (49), and some tumors overexpress endogenous serpins, such as the protease inhibitor 9, which inhibits granzyme B (50). These tumor immune-escape mechanisms subvert either the death receptor- or the granule-exocytosis pathway. Our studies indicate that tumor cells which simultaneously interrupt both granzyme B- and Apo2L/TRAIL-induced death signaling via genetic alterations that interfere with mitochondrial activation of effector caspases have an enhanced ability to withstand an attack by cytotoxic immune effectors (Fig. 6). We show that the coexistence of mitochondrial signaling defects (loss of BAX, overexpression of Bcl-xL, or loss of Smac) in cancer cells which express XIAP decreases their sensitivity to Apo2L/TRAIL- or granzyme B–induced apoptosis, and reduces their susceptibility to lymphocyte-mediated cytotoxicity in vitro and adoptive cellular immunotherapy in vivo. Conversely, negating XIAP expression or function in tumor cells with defective mitochondrial signaling allows the direct activation of caspase-3/-7 by granzyme B or Apo2L/TRAIL and restores their susceptibility to immunologic cytotoxicity. These data are consistent with the ability of Apo2L/TRAIL to induce cytochrome c/caspase-9-independent activation of caspase-3 following Smac-induced inactivation of XIAP (51). Our findings indicate that the susceptibility of tumor cells to immunologic cytotoxicity is determined by the level of expression of XIAP as well as the ability to counteract XIAP-mediated inhibition of effector caspases(-3/-7) via mitochondrial release of Smac. Although BAX plays a dominant role in mitochondrial release of Smac in HCT116 cells that express low basal levels of BAK, IFN-γ-induced expression of BAK may also contribute to Apo2L/TRAIL-induced mitochondrial permeabilization (Fig. 1D,F). In addition to Smac, the mitochondrial release of other proapoptotic proteins, such as Omi/HtrA2, might also play a role in counteracting the inhibition of effector caspases(-3/-7) by XIAP or other IAP family members. As such, cancer cells which express high levels of XIAP and also fail to trigger mitochondrial permeabilization due to coexpression of Bcl-xL do not allow activation of effector caspases(-3/-7) to a threshold required for immune cell–mediated apoptosis. Because expression of Bcl-xL as well as XIAP is induced by NF-κB, tumors with constitutive NF-κB activity harbor coexisting defects in mitochondrial permeabilization and activation of effector caspases. Tumor cells of almost every tissue type frequently acquire the ability to activate NF-κB via a host of genetic alterations, viral proteins, cytokines, and growth factor receptor/nonreceptor tyrosine kinases. Therefore, aberrant activation of NF-κB might be a common denominator of the resistance of cancers to immunologic cytotoxicity.

Although tumor cells with genetic defects in mitochondrial permeabilization and caspase activation might remain susceptible to death signals delivered by other granzymes (A, K, M, and H) or granule proteins (32, 33), our data indicate that they might not be efficiently cleared by immune effector cells. The natural selection of such tumor cells that escape immune surveillance during tumor progression may underlie the limited efficacy of immunotherapy against most common types of human cancer (2). Our findings suggest that cancer immunotherapy, which is currently focused on optimizing the generation or activity of effector cells (46), might be improved via combinatorial strategies to alleviate or circumvent defects in mitochondrial death signaling in cancer cells. Toward this end, targeted inhibition of the specific growth factor receptor–mediated survival signals leading to expression of Bcl-xL and XIAP in tumor cells might enhance the efficacy of immunotherapeutic strategies against cancers.

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Resistance of Cancers to Immunologic Cytotoxicity and Adoptive Immunotherapy via X-Linked Inhibitor of Apoptosis Protein Expression and Coexisting Defects in Mitochondrial Death Signaling

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