Advances in Brief

X-Linked Inhibitor of Apoptosis Protein (XIAP) Is a Nonredundant Modulator of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)-Mediated Apoptosis in Human Cancer Cells

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Abstract

Although the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to play an important role in the immunosurveillance of neoplasia, apoptotic factors that modulate the sensitivity of cancer cells to TRAIL are poorly understood. The inhibitor of apoptosis proteins (IAPs) have generated considerable interest as potential targets for cancer therapy, but the lack of a phenotype in X-linked IAP (XIAP) knockout mice has generated speculation that IAP function may be redundant. Using gene targeting technology, we show that disruption of the gene encoding XIAP in human cancer cells did not interfere with basal proliferation, but caused a remarkable sensitivity to TRAIL. These results demonstrate that XIAP is a nonredundant modulator of TRAIL-mediated apoptosis and provide a rationale for XIAP as a therapeutic target.

Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; also known as Apo 2 ligand) is a member of the tumor necrosis factor family of ligands that can initiate apoptosis by a pathway triggered by its interaction with death receptors, sometimes referred to as the extrinsic pathway (1). Recent studies using TRAIL-null knockout mice have shown that the TRAIL effector pathway plays an important role in the selective killing of spontaneous or transplanted human tumor cells by TRAIL-expressing T cells and natural killer cells (2). The initiation of apoptosis in cancer cells by soluble TRAIL is, therefore, a promising strategy for cancer therapy (2), particularly for cell types that have mutations in p53 and the associated apoptotic defects in the intrinsic pathway (1).

During both naturally occurring neoplasia and subsequent chemotherapy, cancer cells develop defects that make them less likely to undergo apoptosis and thereby provide a selective growth advantage. One mechanism of resistance to apoptotic stimuli involves the overexpression of the inhibitor of apoptosis (IAP) family of proteins, which include closely related X-linked IAP (XIAP), cIAP-1, and cIAP-2 (3, 4). These proteins have been shown to prevent apoptosis by binding to initiator and effector caspases and thereby protecting them from cleavage activation (3, 5). The importance of XIAP relative to the other IAP family members remains conjectural (3). XIAP-null mice are completely normal and have no detectable defect in apoptosis. In such mice, there is a compensatory up-regulation of cIAP-1 and -2, implying that XIAP may be redundant with other IAP family members (6). Transgenic animals that globally overexpress XIAP have shown no increase in cancer incidence (7). In contrast, the *Drosophila* homologue of XIAP seems to play an important role in cell proliferation, with loss of function resulting in embryonic cell death (8). Although contributing to our understanding of how XIAP may function, these diverse studies fail to provide a clear prediction as to how XIAP inhibition might affect human cancer cells (9).

An antiapoptotic effect of XIAP expression on the TRAIL effector pathway has been inferred from experiments that have relied on the forced expression of either XIAP or of endogenous inhibitors of IAPs (5), Smac/DIABLO and XIAP-associated factor 1 (XAF1). Efforts to enhance TRAIL-mediated apoptosis with Smac peptides (10, 11) and small molecules designed to inhibit IAPs (12) have yielded promising results, but the precise targets of these molecules are not fully defined (9). In this report, we describe a genetic approach to examine the consequences of XIAP loss of function in human cancer cells.

Materials and Methods

Cell Culture and TRAIL Treatment. The human colon cancer cell lines HCT116, DLD-1, and their derivatives were cultured in McCoy's 5A medium supplemented with 10% FCS and penicillin/streptomycin. Cells were treated by adding medium containing TRAIL (Alexis Biochemicals, Montreal, Canada) at the indicated final concentrations. According to the manufacturer's recommendations, TRAIL enhancer was added at a final concentration of 10 times the respective TRAIL concentration to facilitate TRAIL receptor cross-linking.

Targeted Deletion of the Human *XIAP* Locus. The strategy used for creating knockouts with AAV vectors was as described in Hirata *et al.* (13). The targeting construct pAAV-Neo-XIAP was made by PCR, using bacterial artificial chromosome clone RP1-315G1 (Invitrogen, Carlsbad, CA) as the template for the homology arms. Details of the vector design and sequences of all PCR primers are available from the authors on request. Stable G418-resistant clones were initially selected in the presence of 0.4 mg/ml Geneticin (Invitrogen, Carlsbad, CA) and then were routinely propagated in the absence of selective agents.

Microscopic Analysis. Cells were collected by incubation in trypsin/EDTA followed by centrifugation and were fixed in a solution containing 3.7% formaldehyde, 0.5% NP40, and 10 μg/ml Hoechst 33258 in PBS. Apoptotic indices were determined by visual scoring of at least 300 nuclei.

Clonogenic Survival. For each drug concentration, 10^3 and 10^4 cells were plated into duplicate 100-mm dishes and allowed to attach. Cells were then treated with the indicated final concentrations of TRAIL for 24 h. After the treatment period, cells were washed and incubated in medium without TRAIL for an additional 14 days. Colonies with more than 50 cells were counted after staining with crystal violet.

Immunoblotting. Protein extracts were resolved via SDS-PAGE and transferred to polyvinylidene difluoride membranes. Filters were blocked in Tris-buffered saline containing 0.1% Tween 20 and 5% BSA, and then were incubated at room temperature with primary antibody in blocking buffer for 16 h. All of the antibodies used in this study were purchased from Cell Signaling Technology (Beverly, MA).

Fig. 1. Strategy for disruption of the human *XIAP* gene. *A,* the endogenous locus is shown along with the targeting construct. A targeted insertion was made in Exon 2, 188 bp after translation start (*ATG*). *HA,* homology arm; *IRES,* internal ribosome entry site; *Neo,* geneticin-resistance gene; *R-ITR,* right inverted terminal repeat; *L-ITR,* left inverted terminal repeat; *triangles,* loxP sites. Primers (*P1, P2*) used for PCR analysis are indicated above the endogenous and targeted loci. *AAV-Neo-XIAP,* the targeting construct; *pA,* polyadenylation signal. *B,* PCR analysis in wild-type (*WT*) sibling (*SIB*) clones that have randomly integrated the knockout construct and in XIAP knockout (*KO*) clones that have homologously integrated the knockout construct. Primers P1 and P2 were used for amplification across the insertion site. In the *WT (SIB) Lanes,* two products corresponding to both the endogenous locus and the nonhomologous, or random, integration of the KO construct (primers P1 and P2 are within the homology arms). In the *KO Lanes,* one product corresponding to the homologous integration of the KO construct. *C,* Western blot with an XIAP-specific antibody showing the presence of XIAP protein in WT cells, and its absence in KO cells.
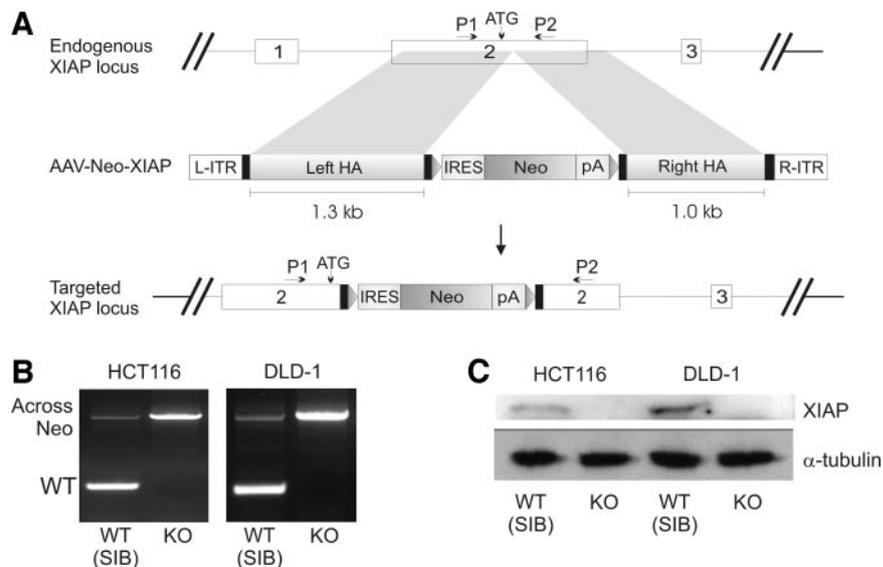
Results and Discussion

Generation of Viable *XIAP*-Null Cells. To directly address whether XIAP affects the sensitivity of human cancer cells to the TRAIL pathway, we used gene targeting technology to knock out *XIAP* in two different colorectal carcinoma cell lines (Fig. 1*A*). Both of these cell lines are diploid with a single X-chromosome and were chosen because of their extensively characterized responses to therapeutic agents (14). One cell line, HCT116, harbors wild-type *p53* alleles. In DLD-1 cells the *p53* gene is biallelically mutated. Knockout clones from both parental cell lines were identified by PCR (Fig. 1*B*) and the absence of XIAP protein was confirmed by Western blot (Fig. 1*C*).

Apoptosis in *XIAP*-Null Cells. Levels of apoptosis in wild-type (XIAP-WT) and knockout (XIAP-KO) cells were measured by microscopic analysis of stained nuclei. Untreated cultures of XIAP-KO and XIAP-WT cells, derived from both HCT116 and DLD-1, contained very few (0–1%) apoptotic cells (Fig. 2*A*). Treatment with TRAIL resulted in an increase in apoptosis, but this increase was markedly greater in the XIAP-KO cell population (Fig. 2*A*).

To assess how loss of XIAP might affect long-term growth and proliferative potential, we examined colony formation in both untreated cells and cells treated with TRAIL. The cell lines tested had similar plating efficiencies irrespective of *XIAP* genotype. However, significant differences between TRAIL-treated XIAP-WT and XIAP-KO cells were observed (Fig. 2*B*). Over a broad range of TRAIL doses, XIAP-KO cells exhibited markedly reduced clonogenic survival and proliferation.

XIAP-Dependent Alteration of Caspase Processing. Next, we explored the biochemical changes in caspase processing that followed treatment with TRAIL. XIAP has been shown to bind *in vitro* to effector caspases-3 and -7 as well as to caspase-9, a protease that plays an important role in the processing of both caspases-3 and -7 (3). Lysates from TRAIL-treated XIAP-WT and XIAP-KO cells were immunoblotted with anti-caspase antibodies. Quantitative and qualitative differences in the cleavage products of caspase-3 were observed (Fig. 3). Overall, TRAIL-treated XIAP-WT cells, which underwent relatively little apoptosis, exhibited low amounts of caspase-3-derived cleavage products, whereas TRAIL-treated XIAP-KO cells had markedly higher levels of cleaved caspase-3. The time-dependent appearance of low-molecular weight caspase-3 protein also differed between XIAP-WT and XIAP-KO cells (Fig. 3), suggesting that XIAP pro-

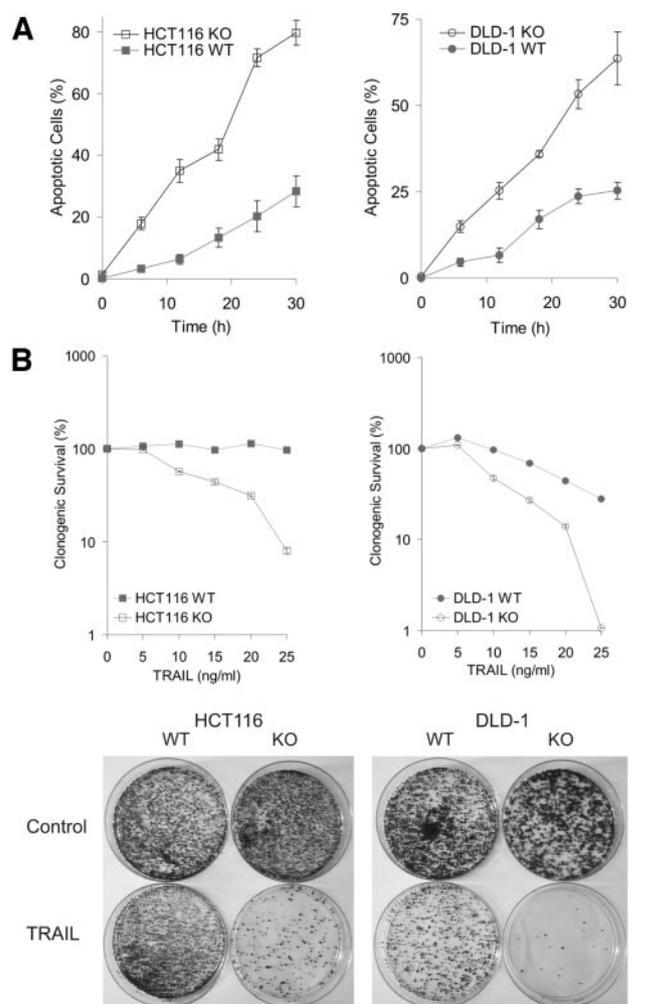Fig. 2. *XIAP*-null human cancer cells are sensitive to *TRAIL. A,* HCT116-derived XIAP-*WT* and XIAP-*KO* cells were treated with 75 ng/ml TRAIL; DLD-1-derived XIAP-WT and XIAP-KO cells were treated with 35 ng/ml TRAIL. Cells were harvested at the indicated time points after treatment and were stained with Hoechst 33258. Fixed nuclei were examined under fluorescence for evidence of standard apoptotic changes. *B, top,* to assess survival after TRAIL treatment, we treated cells with various concentrations of TRAIL, as indicated, for 24 h. Equal proportions of treated cells were then plated into fresh medium and were incubated without drug for an additional 10 days. Colonies of more than 50 cells were scored. *Bottom,* representative plates untreated (*Control*) or treated with 25 ng/ml TRAIL (*TRAIL*) after staining with crystal violet.
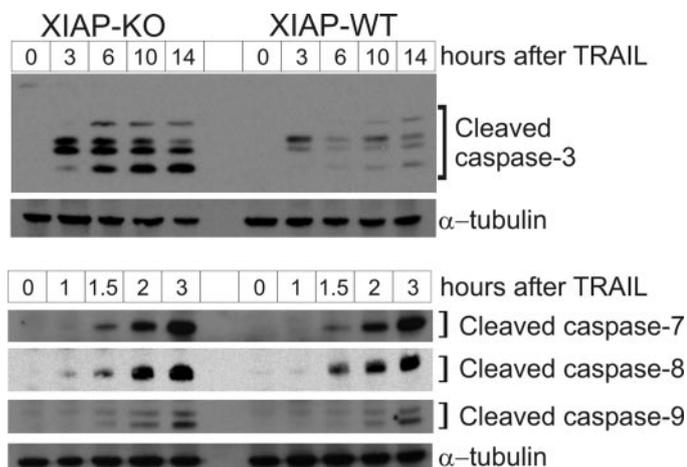
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Fig. 3. X-linked inhibitor of apoptosis protein (*XIAP*) affects caspase-3 processing. HCT116-derived cells were treated with 75 ng/ml tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*), collected at the indicated time points, and were lysed in sample buffer. Cell lysates were immunoblotted with antibodies against caspase-3, caspase-7, caspase-8, and caspase-9. Membranes were stripped and reprobed with an antibody directed against α-tubulin to assess protein loading. *XIAP-KO,* XIAP-knockout cells; *XIAP-WT,* XIAP-wild-type cells.

tected the cleaved intermediate from further processing. This phenotype is consistent with biochemical studies that have demonstrated that the binding of XIAP to caspase-3 requires initial procaspase-3 cleavage and that XIAP can target activated caspase-3 for ubiquitin-mediated degradation (3). In contrast, despite previous reports that XIAP can associate with caspase-7 and caspase-9, we found that the proteolytic activation of these caspases was similar in XIAP-WT and XIAP-KO cells (Fig. 3). Likewise, no appreciable differences in the processing of caspase-8 were observed.

These data conclusively show that (*a*) XIAP was not essential for cancer cell viability; (*b*) elimination of XIAP caused a marked increase in the sensitivity to TRAIL-mediated apoptosis and a corresponding decrease in clonogenic survival; and (*c*) the regulation of caspase-3 by XIAP is a likely mechanism for TRAIL resistance. That the phenotypes observed were consistent in two different cell lines, with *p53* wild-type and *p53* mutant backgrounds, suggests that XIAP is likely to be important for the modulation of TRAIL sensitivity in many cancer cell types. The cell lines generated in this study should prove useful for additional studies of XIAP, its interaction with caspases and other proapoptotic proteins, and its potential roles in the modulation of other apoptotic stimuli.

TRAIL has been shown to selectively affect neoplastic cells (1). This selectivity has generated considerable enthusiasm for TRAIL as a potential therapy with a favorable therapeutic index (1, 2). Our results demonstrate that the sensitivity of cancer cells to TRAIL can be increased by inhibiting the function of XIAP. Additional studies are clearly warranted to determine whether such a strategy would be effective *in vivo*.

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