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APOPTOTIC SENSITIVITY OF MURINE IAP-DEFICIENT CELLS

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SYNOPSIS

Although numerous studies have implicated the Inhibitor of Apoptosis (IAP) proteins in the control of apoptotic cell death, analyses of murine *Iap*-targeted cells have not revealed significant differences in susceptibility to apoptosis. Here we show that under defined conditions, murine cells lacking XIAP and c-IAP2, but not c-IAP1, exhibit heightened apoptotic sensitivity to both intrinsic and extrinsic apoptotic stimuli.

Keywords

XIAP; c-IAP; apoptosis; tumor necrosis factor; murine fibroblast

INTRODUCTION

The inhibitor of apoptosis (IAP) family of proteins are thought to play a variety of physiological roles in addition to their initially described function as suppressors of programmed cell death. IAPs were first discovered in the genomes of baculoviruses, and cellular orthologs were subsequently identified in an evolutionarily diverse range of organisms [1–4]. IAPs are thought to exert their pro-survival effects primarily through direct binding and inhibition of caspases [5], a family of aspartate-specific cysteine proteases that are the executioners of cell death [6]. While several members of the IAP family have been shown to perturb caspase activity, X-linked Inhibitor of Apoptosis (XIAP) is a more potent inhibitor of caspases than any other family member [7]. Nevertheless, two related IAP proteins, c-IAP1 and c-IAP2, have both been described to exhibit antiapoptotic activity [8,9].

XIAP contains three BIR (baculoviral IAP repeat) domains, which are the defining elements of the IAPs, as well as a carboxy-terminal RING domain, which has been shown to catalyze ubiquitination of target proteins through its role as an E3 ubiquitin ligase. Two domains of XIAP are responsible for direct, high affinity binding to caspases. A region amino-terminal to the second, central BIR binds to effector caspases -3 and -7, while the most carboxy terminal BIR (BIR3) is specific for binding and inhibition of caspase-9. Ectopic expression of XIAP

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has been shown to suppress cell death induced through either the receptor-mediated or the mitochondrial pathways, and experimental evidence strongly supports a critical role for caspase binding in this suppression (reviewed in [10]). XIAP is itself regulated by several inhibitory proteins, including Smac/DIABLO, a nuclear-encoded protein which is localized mitochondrially in healthy cells, but which is released into the cytosol during apoptosis. Smac/DIABLO binds to XIAP in the same domain utilized by XIAP to bind caspases, leading to a displacement of the IAP:caspase interaction and the triggering of caspase-dependent cell death [11-14].

The characterization of genetically modified mice has frequently revealed profound insights into the function of the gene product, and in many cases shed light on the pathogenesis of human diseases in which the orthologous gene is targeted. Interestingly, although XIAP has been well characterized as a potent inhibitor of caspase-3, caspase-7 and caspase-9 *in vitro* and in human cell lines [10] as described above, primary cells derived from mice lacking *Xiap* (also known as *Miha*) have been reported not to show increased sensitivity to apoptotic stimuli [15]. Here we examine this apparently paradoxical finding in more detail, using IAP-deficient cells from matched, littermate controls, using defined apoptotic conditions, and find that cells deficient in XIAP and c-IAP2 exhibit heightened sensitivity to pro-death signals.

RESULTS AND DISCUSSION

To address whether murine XIAP may modulate apoptosis, fibroblasts isolated from lungs of *Xiap*-null mice and control littermates were treated with tumor necrosis factor (TNF), a ligand commonly used to stimulate the extrinsic apoptotic pathway. In cell culture systems, TNF does not kill without the addition of the protein synthesis inhibitor cycloheximide (CHX), in part because TNF is generally thought to induce transcription of genes that suppress apoptosis [16-18]. Interestingly, as shown in Figure 1A, lung fibroblasts lacking XIAP were highly sensitive to treatment with TNF and CHX as compared to their littermate counterparts, suggesting that XIAP does in fact suppress apoptosis in murine cells.

Murine XIAP is closely related to the human protein and contains all the critical components for functioning similarly to its human counterpart [19]. However, previous studies using *Xiap*-null murine embryonic-derived fibroblasts (MEFs) did not reveal sensitivity to TNF [15]. To further examine the responsiveness of *Xiap*-deficient pulmonary fibroblasts, we evaluated the role of murine XIAP in receptor-mediated death using matched littermate MEFs. Intriguingly, the concentration of cycloheximide (CHX) previously used to sensitize cells to TNF-induced death was found to induce killing under these experimental conditions, even in the absence of TNF (data not shown). Using a lower concentration of CHX (0.1 µg/ml), death was potentiated through the TNF receptor without killing the cells with CHX alone. As shown in Figure 1B, *Xiap*-null MEFs were significantly more sensitive to death than their wildtype counterparts. Reintroduction of wildtype murine XIAP into the deficient line (KO+XIAP) demonstrated that this effect was wholly dependent on XIAP, since these cells were protected to approximately the same levels as the wildtypes. Furthermore, reintroduction of a mutated form of XIAP which cannot inhibit caspases (D148A/W310A; KO+mutant) was unable to protect the cells to the same degree as the wildtype protein. These studies were also performed in primary MEFs as well as MEFs generated from distinct embryos with essentially the same results (data not shown). These data further support those from the pulmonary fibroblasts, suggesting that murine XIAP does modulate the apoptotic threshold in a similar, caspase-dependent manner to the human protein.

The lack of XIAP in genetically targeted mice has been suggested to be compensated for by c-IAP1 and c-IAP2 overexpression [15]. Therefore, the levels of these proteins in MEFs from IAP-null mice were examined. As shown in Figure 2A, the level of c-IAP1 was found to be

higher in transformed fibroblasts lacking XIAP, consistent with initial reports [15]. Additionally, XIAP protein levels appeared to be unaffected by the absence of either c-IAP1 or c-IAP2. As previously described [20], c-IAP2 was upregulated in *c-Iap1*-null MEFs.

Since changes in the expression of c-IAPs were observed, the possible contribution of these proteins to protection from receptor-mediated death was also examined. MEFs from mice deficient in *c-Iap1* or *c-Iap2* and corresponding littermates were treated with TNF and CHX to induce apoptosis. Under these experimental conditions, c-IAP1 deficiency did not affect the amount of death observed in response to TNF/CHX treatment (Figure 2B). Interestingly, cells lacking c-IAP2 were significantly more sensitive to TNF and CHX-induced death than their wildtype counterparts, suggesting that unlike c-IAP1, c-IAP2 may play a role in protection from apoptosis under these experimental conditions.

The function of XIAP in inhibiting cell death is to specifically block activation of caspases, leading us to examine caspase activation in IAP-deficient MEFs upon treatment with TNF and CHX. After 8 hours of treatment, a significant amount of cleaved caspase-3 was observed in wildtype MEFs by immunoblot (Figure 2C). This level increased dramatically in TNF/CHX treated *Xiap*-deficient MEFs, consistent with the notion that XIAP is responsible for blocking cleavage and activation of caspase-3. Additionally, reconstitution with a wildtype murine XIAP blocked caspase-3 cleavage even more than the endogenous protein at this timepoint. Supporting the viability studies, no difference in the amount of caspase-3 cleavage was observed between *c-Iap1*-null MEFs and littermate control cells. Caspase-3 activation in the c-IAP2 MEFs also corroborated the findings from the viability studies, showing increased cleavage in treated *c-Iap2*-null MEFs.

Ectopic expression of human XIAP has also been shown to be protective against intrinsic or mitochondrial cell death [13], so the intrinsic apoptotic pathway in IAP deficient MEFs was also examined. As shown in Figure 2D, *Xiap*-null cells were more sensitive to etoposide-induced death than wildtype MEFs, and this phenotype was reversed by reconstitution with wildtype XIAP. Expression of D148A/W310A XIAP was unable to protect against this stimuli in the deficient fibroblasts. These data together indicate not only that murine XIAP modulates the threshold for mitochondrial death, but that similar to human XIAP, the caspase-binding activity is likely to be the primary anti-apoptotic function of the protein. The activity of the c-IAPs in the mitochondrial death pathways was also tested with etoposide. MEFs lacking c-IAP1 were not found to be more sensitive to death, but instead were slightly more resistant than wildtype. However, similar to the effects observed with TNF-induced death, c-IAP2 deficiency resulted in a significant sensitivity to etoposide-induced death, indicating that c-IAP2 can protect from both mitochondrial and receptor-mediated apoptosis.

Previous studies have suggested that murine XIAP, in contrast to the human protein, may be dispensable for protection against apoptosis, since mice deficient in the protein did not display any immediately obvious defects in apoptosis [15]. The studies described here demonstrate that murine XIAP is capable of inhibiting caspase activation to modulate apoptosis. As in human cells, *Xiap*-deficient mouse cells are more sensitive to apoptosis, likely due to an increased activation of the effector caspase-3. This is supported by the data showing that mutation of the caspase-binding residues results in the same phenotype as complete lack of protein. This was observed for apoptosis induced by both the intrinsic (etoposide) and extrinsic (TNF) pathways.

While a lack of murine XIAP was found to result in sensitivity to apoptosis, c-IAP1 deficiency did not affect apoptosis. Interestingly, cell death was increased in the absence of c-IAP2, in parallel with a rise in caspase-3 activation in these cells. This suggested that c-IAP2 may be functioning similarly to XIAP, though it is as yet unclear what the mechanism of the inhibition

of apoptosis might be. One possibility relates to previous studies showing that a lack of c-IAPs can increase production of and sensitivity to TNF [21-23]. This, however, does not account for the sensitivity to etoposide-induced death seen in c-IAP2-deficient MEFs. The similar sensitivity to both intrinsic and extrinsic death signals suggests that c-IAP2 may act at a point where the pathways converge, which could be at the level of caspase-3 activation. However, it has been shown previously that c-IAP2 lacks the residues required to directly inhibit caspase activation [24]. Alternatively, c-IAP2 might affect the function of XIAP in a transcription-independent manner through the IAP inhibitor Smac/DIABLO, for which c-IAP2 is a ubiquitin ligase [25]. Smac/DIABLO is released from the mitochondria upon apoptotic signaling, and binds to XIAP to prevent its association with caspases, allowing apoptosis to proceed [11-14]. It is possible that normally c-IAP2 acts as a homeostatic regulator of spontaneously released Smac/DIABLO, and in the absence of c-IAP2, Smac/DIABLO is better able to neutralize XIAP, abrogating its ability to modulate caspase activation.

In summary, we find that murine XIAP deficiency renders cells more sensitive to apoptosis within a range of concentrations of apoptotic stimuli, both receptor-mediated and mitochondrial, suggesting that it functions similarly to its human homolog. The strength of the apoptotic signal appears to be vital, since modulation of cell death by endogenous XIAP is quickly overwhelmed with greater concentrations of apoptotic stimuli. This may explain the discrepancy with previous studies, and suggests that with further exploration using more sensitive *in vivo* systems, the function of murine XIAP may be further elucidated. The studies presented here suggest that the *Xiap*-null mice will be an important tool with which to study human disorders related to cell death deregulation.

EXPERIMENTAL

Cells

All cells were cultured in DMEM (Mediatech) supplemented with 10% fetal bovine serum (FBS) (Mediatech), 2mM glutamine (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C, 5% CO₂. XIAP (day 12.5), c-IAP1 (day 14.5) and c-IAP2 (day 14) MEFs were isolated from individual embryos from timed matings of XIAP WT male with XIAP heterozygous female or two c-IAP1 heterozygotes according to standard procedures. After two passages, WT and KO embryos were transformed by serial infection with lentiviruses expressing Ras and E1A [26]. Experiments were performed with male XIAP WT and KO cells and female c-IAP1 WT and male c-IAP1 KO cells.

Lungs were removed from male littermate XIAP WT and KO mice, minced, and shaken at 37°C in RPMI1640 with 5% FBS, 1mg/ml collagenase A (Roche) and DNase (Sigma). Suspensions were then expelled through an 18G needle 10 times and suspended in HBSS (Mediatech). Red blood cells were lysed and the remaining cells were placed in culture media and allowed to grow. All experiments were performed between passages 2 and 4.

Mice

XIAP KO mice [15] were backcrossed in the C57BL/6 strain for at least 12 generations. Mice lacking c-IAP1 were generated as described [20]. All mice were housed under specific pathogen-free conditions within the animal care facility at the University of Michigan. The University of Michigan Committee on the Use and Care of Animals approved all experiments.

Reconstitution of XIAP MEFs

Stable reconstitution of *Xiap*-null MEFs was accomplished by infection with a lentivirus expression system (manuscript in preparation) encoding either full length XIAP or a D148A/W310A double mutant generated by site-directed mutagenesis.

Death assays

Cells were treated in triplicate as indicated with media, recombinant murine TNF (Roche) and cycloheximide (Sigma) or etoposide (Bristol-Meyers Squibb) for 24 hours. Floating cells were collected and combined with adherent cells lifted with trypsin-EDTA (Mediatech), and all were resuspended in propidium iodide (PI) buffer (2µg/ml PI [Sigma], 1% bovine serum albumin [Sigma] in 1x PBS) for flow cytometry. Data were collected on a Beckman-Coulter Cytomics FC-500 machine and analyzed using FlowJo (Treestar).

Western blotting

Whole cell lysates were prepared using RIPA lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM DTT, 1mM PMSF in 1x PBS) supplemented with protease inhibitors. Samples were resolved on 4–12% gradient SDS-PAGE gels (Invitrogen), transferred onto nitrocellulose (Invitrogen) and blocked in 5% milk in Tris-buffered saline containing 0.1% Tween (Bio-Rad). Membranes were incubated at room temperature for 1h or overnight at 4°C with the following antibodies: cleaved caspase-3 (Cell Signaling), XIAP (BD Pharmingen), β-actin (Sigma), or rIAP (Robert Korneluk, University of Ottawa, Canada). Secondary horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-rat (GE Healthcare) were used for 1h at room temperature. Enhanced chemiluminescence (GE Healthcare) and Kodak XAR film were used for visualization purposes.

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Abbreviations used

IAP, inhibitor of apoptosis; XIAP, X-linked inhibitor of apoptosis; BIR, baculoviral IAP repeat; TNF, tumor necrosis factor; CHX, cycloheximide; MEF, murine embryonic fibroblast; WT, wild type; KO, knockout; PI, propidium iodide; UT, untreated.

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Figure 1. XIAP modulates apoptosis in murine fibroblasts

A) Lung fibroblasts were treated with mouse TNF (200U/ml) and CHX (1μg/ml) or left untreated (UT) for 24 hours. Floating and adherent cells were harvested together, stained with propidium iodide and analyzed by flow cytometry. Data represent 3 mice per group, and error bars represent standard error. One-way ANOVA was used to calculate significance, and p-values of less than 0.01 are indicated with an asterisk (**).

B) The indicated transformed MEF lines were treated with 200U/ml mTNF and 0.1μg/ml CHX for 24 hours, and cells were harvested and analyzed as in A. All data represent at least 3 independent experiments, with error bars indicating standard error.

Figure 2. Lack of XIAP or c-IAP2 sensitizes cells to apoptosis, while loss of c-IAP1 does not

A) MEF lines were lysed in RIPA lysis buffer. Proteins were separated by SDS-PAGE and immunoblotted with antibodies to XIAP, c-IAP1 and c-IAP2, with β -actin as a loading control.

B) The indicated transformed MEF lines were treated with 200U/ml mTNF and 0.1 μ g/ml CHX for 24 hours. Floating and adherent cells were harvested together, stained with propidium iodide and analyzed by flow cytometry. All data represent at least 3 independent experiments, with error bars indicating standard error.

C) The indicated MEF lines were left untreated or treated with TNF and CHX as in B for 24 hours, then lysed and immunoblotted with antibodies to cleaved caspase-3 and β -actin as a loading control.

D) The indicated transformed MEF lines were treated with 1 µg/ml etoposide for 24 hours, and cells were harvested and analyzed as in B. All data represent at least 3 independent experiments, with error bars indicating standard error.