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MOLECULAR TARGETS FOR THERAPY (MTT)

Regulation and targeting of antiapoptotic XIAP in acute myeloid leukemia

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XIAP is a member of the inhibitors-of-apoptosis family of proteins, which inhibit caspases and block cell death, with prognostic importance in AML. Here we demonstrate that cytokines regulate the expression of XIAP in leukemic cell lines and primary AML blasts. Inhibition of phosphatidylinositol-3 kinase (PI3K) with LY294002 and of the mitogen-activated protein kinase (MAPK) cascade by PD98059 resulted in decreased XIAP levels (34±8.7 and 23±5.7%, respectively). We then generated OCI-AML3 cells with constitutively phosphorylated Akt (p473-Akt) by retroviral gene transfer. Neither these nor Akt inhibitor-treated OCI-AML3 cells showed changes in XIAP levels, suggesting that XIAP expression is regulated by PI3K downstream effectors other than Akt. The induction of XIAP expression by cytokines through PI3K/MAPK pathways is consistent with its role in cell survival. Exposure of leukemic cells to chemotherapeutic agents decreased XIAP protein levels by caspase-dependent XIAP cleavage. Targeting XIAP by XIAP antisense oligonucleotide resulted in downregulation of XIAP, activation of caspases and cell death, and sensitized HL-60 cells to Ara-C. Our results suggest that XIAP is regulated by cytokines through PI3K, and to a lesser degree through MAPK pathways. Selective downregulation of XIAP expression might be of therapeutic benefit to leukemic patients.
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Introduction

Inhibitors-of-apoptosis proteins (IAP) are a family of related proteins that suppress cell death by inhibiting both upstream and terminal caspases.[1–8] These proteins suppress apoptosis induced by a variety of stimuli, including tumor necrosis factor, Fas, menadione, staurosporine, etoposide, Taxol, and growth factor withdrawal.[1,4,5,9] XIAP is the most widely expressed IAP. Its mRNA has been found in all adult and fetal tissues examined, except peripheral blood leukocytes.[5] It is also the most potent inhibitor of caspases, with an inhibition constant (K_i) in the low nanomolar range.[2,3,10] The caspase-inhibiting activity of XIAP is negatively regulated by at least three proteins: XIAP-associated factor I (XAF1),[11,12] Smac/Diablo,[13,14] and HtrA2 (Omi).[15–19] The expression of XIAP is translationally regulated by a cap-independent mechanism mediated by an internal ribosome entry site, a unique sequence located in the 5′ untranslated region (UTR) of XIAP,[20] and is transcriptionally regulated by the NF-κB-signaling pathway.[21] In turn, XIAP may regulate NF-κB expression.[22,23] The level of XIAP is also post-translationally regulated by the proteasome degradation system, and XIAP itself exhibits ubiquitin ligase (E3) activity.[24,25] Recent studies suggested that XIAP is also involved in other biological processes such as modulation of receptor-mediated signal transduction.[26] Taken together, XIAP plays crucial roles in multiple cellular functions.[27]

In addition to being a caspase inhibitor, XIAP can also undergo caspase-mediated cleavage. Endogenous XIAP can be cleaved during apoptosis induced by various stimuli.[28–30] XIAP is cleaved into two fragments, BIR1–BIR2 and BIR3–RING, by caspases activated during Fas-induced apoptosis,[28] and in T-lymphocyte apoptosis.[29] The BIR1–BIR2 fragment actually inhibits Fas-induced apoptosis, but it is less efficient and less stable than full-length XIAP. The BIR3–RING fragment is a specific inhibitor of caspase-9.[28]

Given the antiapoptotic properties of XIAP and the frequent resistance of leukemic cells to programmed cell death, understanding XIAP regulation and modulating XIAP gene expression could help to more efficiently induce apoptosis and overcome resistance of leukemic cells to apoptosis. Cytokines generated by stromal cells in the bone marrow microenvironment are required for growth and survival of hematopoietic cells.[31,32] Cytokines have been reported to regulate IAPs in endothelial cells.[33] Recently, we reported that the expression of survivin, another member of the IAP family, is regulated by cytokines in myeloid leukemias through both the mitogen-activated protein kinase kinase/extracellular-signal regulated kinase (MAPK/ERK) and the phosphatidylinositol-3 kinase (PI3K) pathways.[34] We have reported that XIAP expression has prognostic importance in AML.[35] However, XIAP expression and regulation have not been analyzed in detail in hematopoietic malignancies. In this study, we investigated the effects of growth-stimulatory cytokines and various anticancer agents on XIAP expression in leukemia. Here, we report that XIAP expression is induced by cytokines through the PI3K, and to a lesser degree through the MAPK pathways. Numerous anticancer agents decreased XIAP protein levels by inducing caspase-mediated XIAP cleavage in leukemic cells. Noting the antiapoptotic function of XIAP, selective downregulation of XIAP expression could be of therapeutic benefit, and here we demonstrate that targeting XIAP by XIAP antisense oligonucleotides induced caspase activation and sensitized Ara-C-induced cell death in HL-60 cells. Taken together, these data propose an important role for XIAP in myeloid leukemias, and suggest different approaches to its downregulation.

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Materials and methods

Cell cultures

Human leukemic cell lines were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS), 1 mM L-glutamine, and 50 μg/ml penicillin/streptomycin. For Mo7e and TF-1 cell cultures, the medium was supplemented with GM-CSF at a concentration of 100 and 12 U/ml, respectively (Immunex, Seattle, WA, USA). Bone marrow specimens from AML patients with high (>70%) blast counts were obtained after signed informed content, according to institutional guidelines. Mononuclear cells were purified by Ficoll-Hypaque (Sigma Chemical Co., St Louis, MO, USA) density-gradient centrifugation, and cultured in AIM-V medium (Gibco-BRL) containing 10% FCS. XIAP antisense oligonucleotide (TAGGACTTGTCCACCTTTTC), located 14–33 bases upstream of the translation start site (ISIS-102369), and its control oligonucleotide (TAGGACTACCTGTCCTTTTC) (ISIS-119192) were provided by ISIS Pharmaceuticals (Carlsbad, CA, USA). All the experiments were carried out at least three times, and the results are expressed as means $\pm$ s.e.

Treatment of cells

For cytokine stimulation, OCI-AML3 cells at a density of 0.2×10^6/ml in serum-free RPMI 1640 medium, or primary AML blasts at a density of 1×10^6/ml in serum-free AIM-V medium, were treated with 100 U/ml GM-CSF, 50 ng/ml G-CSF (Amgen, Thousand Oaks, CA, USA), 100 ng/ml stem cell factor (SCF; Amgen), or a combination of these factors for 48 h. To block the MAPK- and PI3K-signaling pathways, the MEK-specific inhibitor PD98059[36,37] (CalBiochem, La Jolla, CA, USA), the PI3K-specific inhibitor LY294002[38] (Sigma), and Akt-specific inhibitor (CalBiochem)[39] were included in cell cultures, respectively. To study the effect of chemotherapeutic drugs on XIAP protein levels, cells at a density of 1×10^6/ml were treated with various concentrations of anticancer drugs, including Ara-C (1–5 μM), doxorubicin (100–500 ng/ml), vincristine (2–10 ng/ml), and Taxol (10–50 ng/ml) for up to 48 h. The drug concentrations used to efficiently kill cells were based on their IC_{50}, and adjusted experimentally. IDN-1529 and IDN-1965[40] are two irreversible, cell-permeable, broad-spectrum caspase inhibitors, developed by Idun Pharmaceuticals (La Jolla, CA, USA). For caspase inhibition, 20 μM IDN-1529 or IDN-1965 was added to the cells 1 h prior to drug administration. To target XIAP expression, HL-60 cells (3×10^6) were suspended in Nucleofector kit solution T (100 μl) (Amaxa biosystems, Germany), mixed with 3–9 μg of either XIAP antisense oligonucleotide or the control oligonucleotide, and nucleo-transfected with a Nucleofector (program K17, Amaxa biosystem), following the manufacturer's instruction. For combination treatment of XIAP antisense oligonucleotide and Ara-C, various concentrations of Ara-C were added to cells 4 h after nucleo-transfection of HL-60 cells with oligonucleotides.

Akt constructs and retroviral infection of OCI-AML3 cells

A *neo*^{r} retrovirus encoding a constitutively activated Akt gene ΔAkt(Myr$^+$)[41] was generously provided by Dr Richard Roth (Stanford University, Stanford, CA, USA). The Akt gene contains the myristylation domain (Myr$^+$) of v-Src, which localizes Akt to the lipid-rich membrane. Moreover, this Akt gene lacks the PH domain. Although the PH domain of Akt serves to localize Akt to the cell membrane, it also turns off Akt activity. Therefore, it was removed from the Akt cDNA and replaced with the v-Src myristylation domain.[41] The LNL6[42] empty retroviral vector was used as a control, and was generously provided by Dr A Dusty Miller (Fred Hutchinson Cancer Center, Seattle, WA, USA). Plasmid DNAs containing recombinant retroviruses were transfected into the retroviral packaging cell lines ψ2 or PA317 with lipofectin (Life Technologies, Gaithersburg, MD, USA), and retroviruses were passed sequentially from one cell line to the other to amplify their titers as described.[43] OCI-AML3 cells were infected with viral stocks prepared from PA317 cells as described.[43] Briefly, 1×10^6 OCI-AML3 cells were infected with the different viral supernatants at titers of approximately 10^5 focus forming units/ml in 2 ml of RPMI-1640 medium (Life Technologies) with 5% FBS (Atlanta Biologicals, Atlanta, GA, USA) and 1 μg/ml polybrene (Sigma, St Louis, MO, USA) for 4 h, and then the cells were diluted five-fold for an additional 48 h of incubation. The cells were collected by centrifugation, and plated at 5×10^4 cells/well in 96-well flat-bottom plates (Corning, Corning, NY, USA) in RPMI 1640 medium containing 10% FBS and 2 mg/ml G418 (Life Technologies) for 3 weeks. Fresh medium (containing G418) was added every 3 days. In contrast, no cells grew from mock-infected cells plated in the same conditions. The G418^r cells were isolated as pools, which initially contained approximately 10^4 different virally infected cells. Single colonies were selected by limiting dilution. Two clones (clone 1 and clone 8) of OCI-AML3 cells with the highest p-473 Akt level and OCI-AML3 with LNL6 vector control[42] were used to compare survivin and XIAP protein levels by immunoblotting.

Cell death assays

Cell viability was determined by trypan blue exclusion. Early apoptotic events were detected by the flow-cytometric measurement of externalized phosphatidyl serine[44] with the Annexin-V-FLUOS Staining Kit (Roche Diagnostics Corp., Indianapolis, IN, USA). The activation of caspases was measured by flow cytometry, using a fluorescein-conjugated cell-permeable peptide (FAM-VAD-FMK) that irreversibly binds to activated caspases (caspase-1 to -9) (CaspaTag, Intergen, Purchase, NY, USA).

Reverse transcription and TaqMan polymerase chain reaction

OCI-AML3 cells were treated with various chemotherapeutic agents, as described above. RNA was isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed with random heximers (Roche Applied Science, Indianapolis, IN, USA) by AMV reverse transcriptase (Boehringer Mannheim) at 42°C for 1 h. Polymerase chain reaction (PCR) amplification mixtures (25 μl) contained cDNA, XIAP forward primer (5′CCCAAATTGCAGATTTATCAACG3′), XIAP reverse primer (5′TGCATGTGTCTCAGATGGCC3′), XIAP probe (5′ATCTGGGAAGCAGAGATCATTTTGCCTTAGAC3′), and TaqMan Universal PCR master mix (PE Applied Biosystems, Foster City, CA, USA). Thermal cycle conditions included holding the reactions at 50°C for 2 min and at 95°C for 10 min, and cycling for 40 cycles between 95°C for 15 s and 60°C for 1 min. RNA levels of β-2M were used as internal controls. Results were collected and analyzed by an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems).

Immunoblotting

Cells were washed twice with PBS buffer, and lyzed at a density of 2×10^4 cells/μl in 2 × protein lysis buffer (0.25 M Tris-HCl, 2% SDS, 4% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue). For detection of p-Akt, cells were lyzed in the buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM PMSF, and 10 ng/ml aprotinin. An equal amount of cell lysate was loaded onto a 12% SDS-PAGE gel (Bio-Rad, Hercules, CA, USA). After electrophoresis, the proteins were transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were blotted overnight at 4°C, with PBS buffer containing 7% nonfat dry milk (Bio-Rad) and 0.3% Tween-20, followed by incubation with the monoclonal XIAP antibody (1:500; Transduction Laboratories and PharMingen, San Diego, CA, USA), polyclonal caspase-3 antibody (CPP32, 1:1000; PharMingen), caspase-7, -8, and -9 antibodies (1:1000; Cell Signaling Technology, Inc., Beverly, MA, USA), survivin antibody (1:1000; R&D Systems, Minneapolis, MN, USA), Akt antibody and p473-Akt-specific antibody (1:1000; Cell Signaling Technology, Inc.) or anti-β-actin (Sigma Chemical Co., St Louis, MO, USA) for 2 h at room temperature. The membranes were washed three times with PBS buffer containing 0.3% Tween-20, and probed with a second antibody. After washing, the membranes were reacted with ECL buffer (Amersham Pharmacia Biotech), and the signals were detected by phosphoimager Storm 860 (Molecular Dynamics Version 4.0; Molecular Dynamics, Sunnyvale, CA, USA) and quantitated by Scion Image software (Scion Corp., Frederick, MD, USA). β-Actin was used as loading control.

Results

Expression of XIAP protein in leukemic cell lines

To study the regulation of XIAP expression in hematopoietic malignant cells, we first compared XIAP protein levels in various leukemic cell lines by immunoblot analysis. Our results demonstrate that all leukemic cell lines examined expressed XIAP including HL-60, HL-60 Dox, NB-4, KG-1, KG-1a, OCI-AML3, Mo7e, TF-1, THP-1, and U937. Among them, OCI-AML3, Mo7e, TF-1, and THP-1 expressed relatively higher levels of XIAP, while HL-60, KG-1, and K562 expressed relatively low levels (not shown). Previously, we have demonstrated that all primary AML samples also express XIAP at different levels.[35]

Effects of cytokines on XIAP expression in leukemic cells

A combination of cytokines is required for survival and growth of hematopoietic cells. To examine whether cytokines can promote hematopoietic cell survival by modulating antiapoptotic proteins, we tested the effects of cytokines on XIAP expression in established leukemic cell lines and primary leukemic cells, culturing cells with various hematopoietic cytokines (GM-CSF, G-CSF, SCF) alone or in combination. All of these factors induced XIAP expression in HL-60 cells (Figure 1a), with combinations being more effective than single cytokines. Similar results, although less pronounced, were observed in OCI-AML3 cells (not shown), reflecting the relatively high levels of endogenous XIAP protein in OCI-AML3 cells. Among the primary AML patient samples used for studying cytokine effects, all expressed XIAP protein at baseline, and the three cytokines as well as their combinations further increased XIAP expression ($n = 3$). Results from a representative patient sample are shown in Figure 1b.

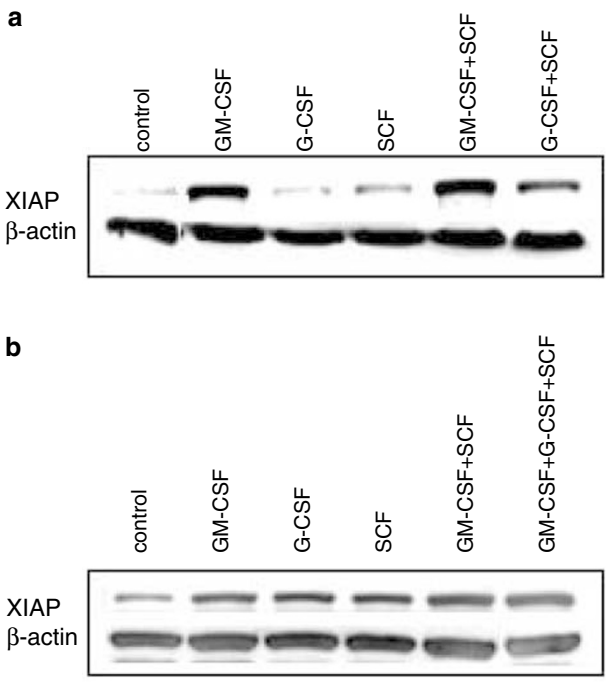

Figure 1 Induction of XIAP protein levels by cytokines. HL-60 cells (a) and primary AML blast cells (b) were cultured in serum-free medium with GM-CSF (100 U/ml), G-CSF (50 ng/ml), SCF (100 ng/ml) or combinations of these factors for 48 h. Cells were lyzed and XIAP levels were analyzed by immunoblotting, using lysates equivalent to 0.2×10^6 cells.

In summary, XIAP expression in both cell lines and primary samples of myeloid leukemias is increased by cytokines.

Regulation of XIAP expression by PI3K and MAPK signal transduction pathways

Hematopoietic cytokines can signal through an array of cross-talking kinase pathways. We therefore examined the role of two major signaling pathways, the MAPK and PI3K pathways, in the regulation of XIAP. Using the specific MEK inhibitor PD98059 (20 μM), we observed a moderate decrease in XIAP levels ($23 \pm 5.7\%$) in OCI-AML3 cells (Figure 2a). Treatment of OCI-AML3 cells with the PI3K specific inhibitor LY294002 (10 μM) resulted in a greater decrease of XIAP protein ($34 \pm 8.7\%$) (Figure 2a). XIAP expression was inhibited by the MAPK and PI3K inhibitors in the presence or absence of GM-CSF or serum. Similar results were obtained with HL-60 cells (data not shown).

Since PI3K exhibits both lipid kinase and protein kinase activity, and activates a number of signaling proteins such as Akt (PKB), PKC, and kinases of the MAPK pathway,[45] we wanted to know whether the regulation of XIAP by the PI3K pathway is mediated through Akt. We generated OCI-AML3 cells constitutively expressing p473-Akt by retroviral transfer and selection of stable clones with highest p473-Akt levels. We compared XIAP levels in two of these clones with the vector control cells. As shown in Figure 2b, our results demonstrated that although PI3K inhibition negatively regulates XIAP levels, XIAP expression was

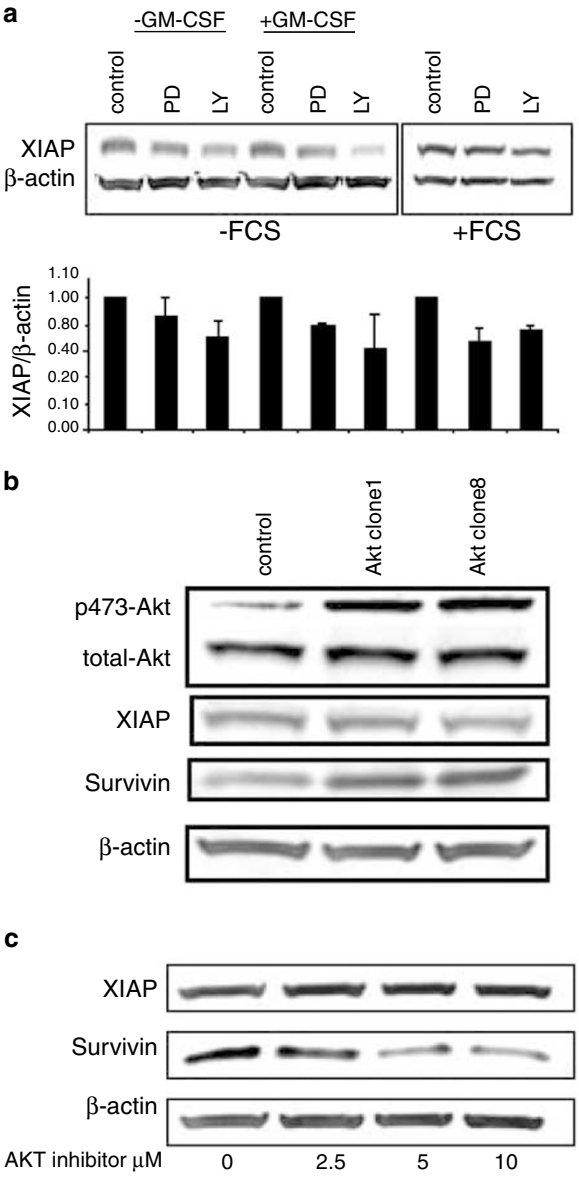

Figure 2 Regulation of XIAP expression through the MAPK and PI3K pathways. (a) Inhibition of XIAP expression by the MEK inhibitor PD98059 and the PI3K inhibitor LY294002. OCI-AML3 cells were cultured in serum-free RPMI-1640 medium with or without GM-CSF (100 U/ml), or with 10% FCS. Either PD98059 (20 μM) or LY294002 (10 μM) was included in cultures. Cells were harvested at 48 h, and XIAP levels were determined by immunoblotting. (b) Expression of XIAP and survivin in Akt-overexpressing OCI-AML3 cells was analyzed by immunoblotting. (c) OCI-AML3 cells were treated with various concentrations of Akt inhibitor. Cell lysates were prepared at 48 h, and expressions of XIAP and survivin were analyzed by immunoblotting.

unchanged in p473-Akt-overexpressing OCI-AML3 cells. Interestingly, the expression of survivin, another member of IAPs, was induced in p473-Akt-overexpressing OCI-AML3 cells (Figure 2b). To further confirm our observation, we treated OCI-AML3 cells with an Akt inhibitor from CalBiochem. This inhibitor, at low concentrations (IC50 = 5 ± 1.7 μM), specifically inhibits Akt. As shown in Figure 2c, exposure to up to 10 μM of Akt inhibitor did not change XIAP levels, while the expression of survivin was decreased.

XIAP expression in leukemic cells was decreased by MEK inhibition, and more significantly by PI3K inhibition, suggesting

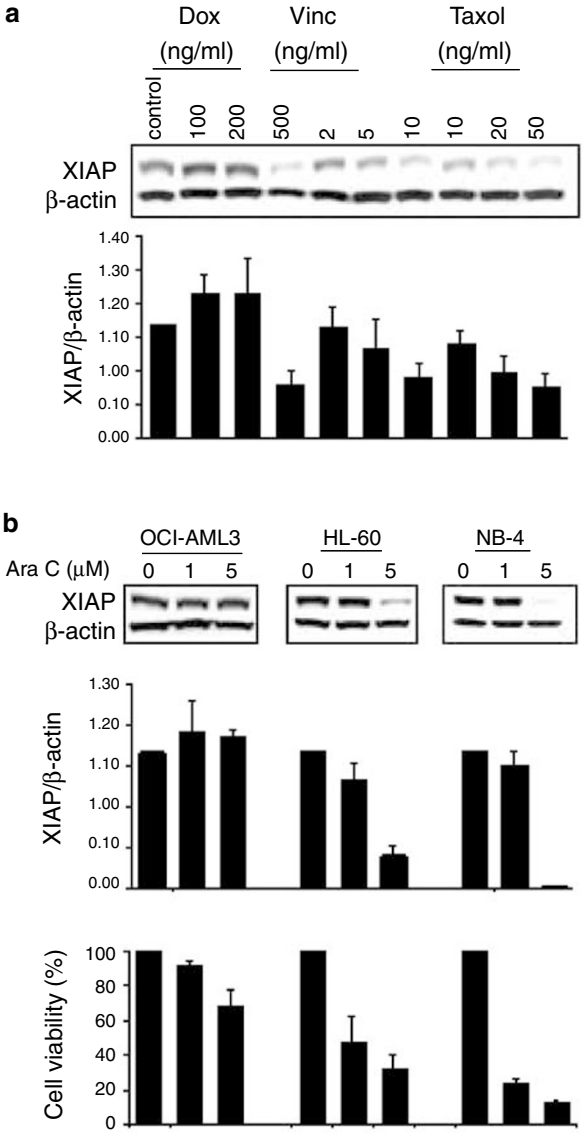

Figure 3 Effects of anticancer drugs on XIAP protein expression. XIAP protein levels were determined by immunoblotting analysis. (a) OCI-AML3 cells were cultured with Dox, vincristine (Vinc) or Taxol for 48 h. (b) OCI-AML3, HL-60, and NB-4 cells were cultured with Ara-C for 48 h. XIAP levels and corresponding cell viabilities are presented.

that XIAP expression is regulated mainly through the PI3K pathway, and to a lesser degree through the MAPK pathway. The regulation of XIAP by the PI3K pathway appears to be affected by downstream mediators other than Akt.

Induction of caspase-dependent XIAP cleavage by anticancer agents

To examine the effects of commonly used anticancer agents on XIAP protein expression in leukemic cells, we treated OCI-AML3 cells with various concentrations of Ara-C (1–5 μM), doxorubicin (Dox) (100–500 ng/ml), vincristine (2–10 ng/ml), and Taxol (10–50 ng/ml) for 48 h, and XIAP expression was analyzed by immunoblotting. We found significantly decreased XIAP levels in OCI-AML3 cells treated with Dox, vincristine, and Taxol, but not with Ara-C (Figure 3). As shown in Figure 3a,

XIAP protein levels were decreased by more than 50% in response to Dox (500 ng/ml), vincristine (10 ng/ml), and Taxol (50 ng/ml). However, Ara-C in concentrations up to 5 μM slightly increased rather than decreased XIAP protein in OCI-AML3 cells after 48 h (Figure 3b). In contrast, under the same conditions, the XIAP protein levels in HL-60 cells were 24%, and in NB-4 cells 2% of that of untreated cells (Figure 3b). Interestingly, OCI-AML3 cells were more resistant to Ara-C-induced cell death than HL-60 and NB-4 cells (Figure 3b).

To understand the mechanism of drug-induced changes in XIAP protein levels, we performed quantitative TaqMan PCR on OCI-AML3 cells treated with various anticancer drugs. We did not detect significant alterations in XIAP RNA levels in treated cells comparing with untreated cells, suggesting that the change is not transcriptional. We also noticed that the decrease in the XIAP protein levels produced by Dox was accompanied by the appearance of a band at about 30 kDa (Figure 4).[30] This 30-kDa protein (*I* in Figure 4) can be detected by immunoblotting analysis using a monoclonal antibody against XIAP (Transduction Laboratories) in a time-dependent manner, suggesting that it represents a fragment of XIAP, generated possibly through caspase-mediated cleavage previously described.[28–30] Longer treatment resulted in further degradation (II in Figure 4) of the XIAP protein. Treatment with other drugs, including Ara-C, vincristine, and Taxol, also induced XIAP cleavage (Figure 5a), and cell viabilities were decreased more than 50%, as determined by trypan blue exclusion. The caspase inhibitor IDN-1529 (as well as IDN-1965, results not shown) at 20 μM completely inhibited the cleavage of XIAP induced by the drugs studied (Figure 5a). Immunoblotting with caspase-3 antibody demonstrated that caspase-3, an effector caspase, was activated by the aforementioned drugs, and that activation was blocked by IDN-1529 and IDN-1965 (Figure 5b), demonstrating that XIAP cleavage is caspase-dependent. In addition to caspase-3, caspase-8 and caspase-7 were activated by these drugs, and the activation of these caspases was blocked by caspase inhibitors (Figure 5c). Cleavage of caspase-9, an initiator caspase, was not inhibited by IDN-1529 or IDN-1965 (not shown).

Downregulation of XIAP expression induced caspase activation and cell death in HL-60

To investigate the role of XIAP and search for ways to selectively eliminate leukemic cells, we targeted XIAP expression in HL-60 cells with a XIAP antisense oligonucleotide obtained from ISIS. Like other suspension cells, HL-60 cells are very difficult to transfect. After many failed attempts, we successfully transfected HL-60 cells using a nucleotransfection method developed recently by Amaxa Biosystems (Germany). With the optimized combination of solution and program, cell killing due to nucleotransfection was minimal ($\leqslant$10%, as determined by Annexin-V positivity, see Figure 6). XIAP antisense oligonucleotide and its control oligonucleotide were introduced to HL-60 cells suspended in solution T, using a Nucleofector (Amaxa Biosystems). The K17 program was used for electroporation. As shown in Figure 6a, XIAP expression was downregulated at 48 h by XIAP antisense oligonucleotide (3–9 μg) in a dose-dependent manner. XIAP antisense (9 μg) or control oligonucleotides was chosen to transfect 3×10^6 HL-60 cells in the subsequent experiments. Accompanying XIAP downregulation, caspases were activated, as determined by CaspaTag (Figure 6b), and cell viability was decreased, as measured by cell count (Figure 6c)

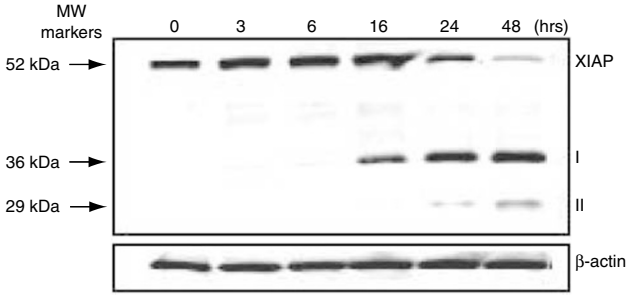

Figure 4 Decrease of XIAP protein levels in OCI-AML3 cells in response to Dox is time-dependent, and accompanied by XIAP cleavage. OCI-AML3 cells were harvested at different times after exposure to Dox (500 ng/ml) and lyzed. Immunoblotting analysis was performed to measure XIAP protein levels. Degradation products of XIAP are marked I and II.

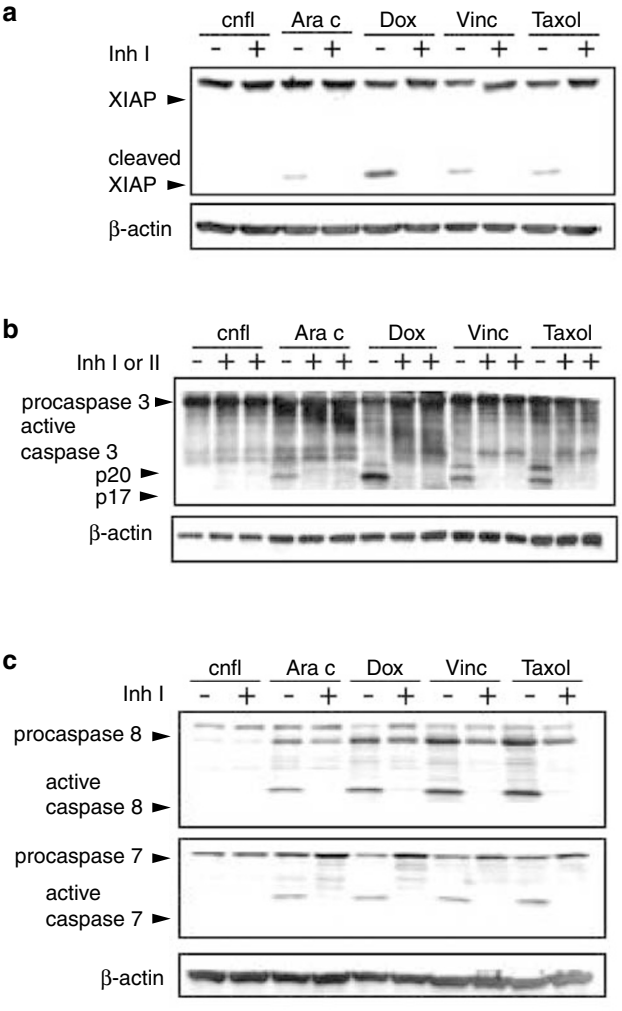

Figure 5 Effects of various drugs on XIAP protein levels, and caspase activation with or without caspase inhibitors. OCI-AML3 cells were treated with 2 μM Ara-C, 500 ng/ml Dox, 10 ng/ml vincristine (Vinc) or 50 ng/ml Taxol for up to 48 h, with or without the caspase inhibitor IDN-1529 (Inh I) or IDN-1965 (Inh II) (20 μM). (a) Immunoblotting analysis of XIAP protein cleavage at 24 h. (b) Caspase-3 processing at 48 h measured by immunoblotting. (c) Immunoblotting analysis of caspase-8 and -7 processing at 48 h.

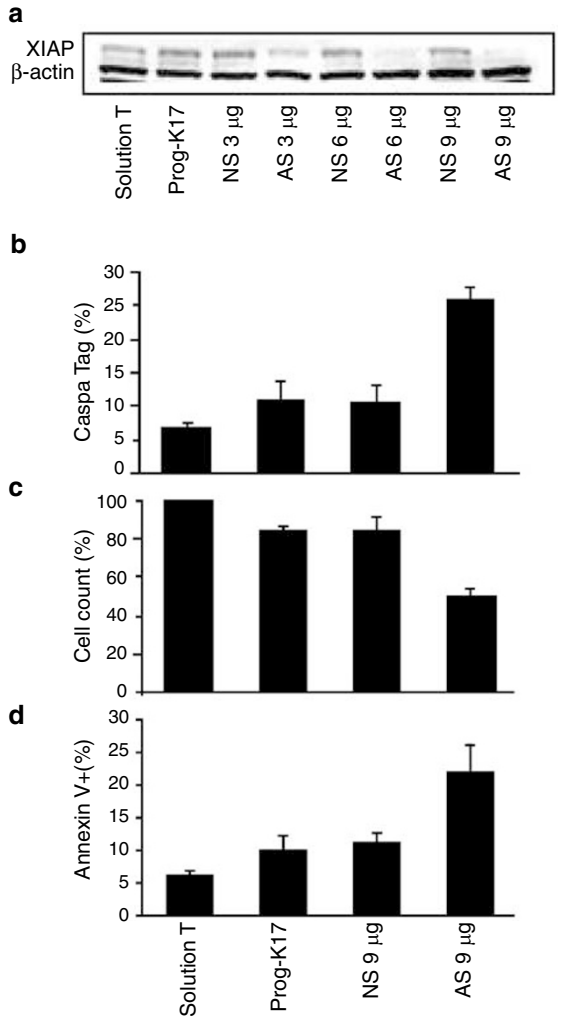

Figure 6 Downregulation of XIAP expression by antisense oligonucleotide, resulting in caspase activation and apoptosis. HL-60 cells were treated for 48 h with XIAP antisense oligonucleotide, and its control oligonucleotide by nucleotransfection. (a) Downregulation of XIAP protein levels by XIAP antisense oligonucleotide analyzed by immunoblotting. (b) Downregulation of XIAP expression-induced caspase activation analyzed by CaspaTag. (c,d) Downregulation of XIAP expression-induced cell death determined by cell count and Annexin-V+ cells, respectively. NS, control oligonucleotide; AS, XIAP antisense oligonucleotide.

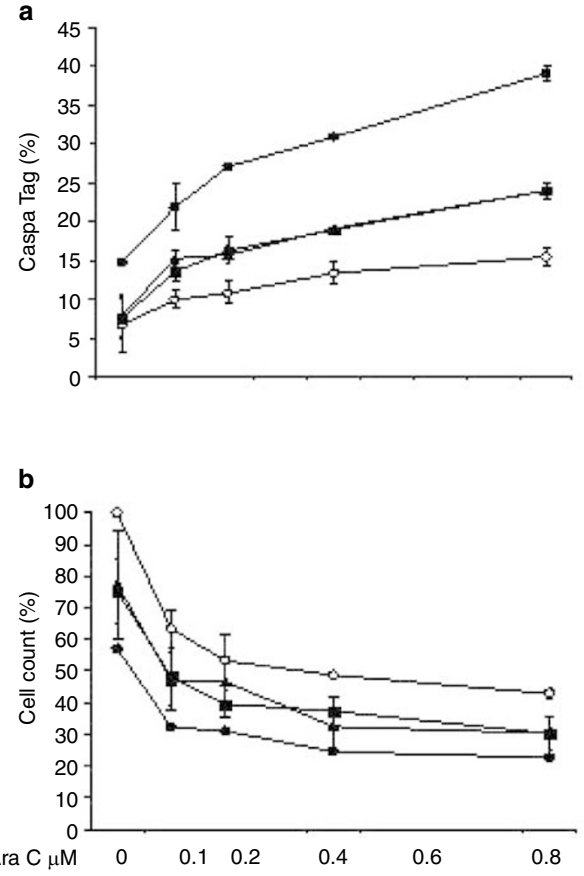

Figure 7 Downregulation of XIAP expression by antisense oligonucleotide sensitizes Ara-C-induced cell death in HL-60 cells. (a) Activation of caspases by XIAP antisense oligonucleotide and Ara-C analyzed by CaspaTag. (b) Decrease of viable cells by XIAP antisense oligonucleotide and Ara-C determined by cell count. ○, solution T; ■, program 17; ▲, nonsense; and ●, antisense oligonucleotide.

and Annexin-V positivity (Figure 6d) at 48 h. The control oligonucleotide had no effect on XIAP expression, caspase activation, and cell viability (Figure 6).

Ara-C is the most commonly used chemotherapeutic agent in treating AML. Like any other chemotherapeutic drugs, its use is associated with the development of toxicity and resistance. Since XIAP is a caspase inhibitor, downregulation of XIAP expression would be expected to sensitize leukemic cells to chemo-induced cell death. To test this hypothesis, we treated HL-60 cells with XIAP antisense oligonucleotide in combination with various concentrations of Ara-C. Downregulation of XIAP sensitized to Ara-C induced cell death. As shown in Figure 7, in the presence of XIAP antisense oligonucleotide, Ara-C at 0.1 μM induced more than 20% activation of caspase, while 0.6–0.8 μM of Ara-C was required to achieve the same effect with nonsense control (Figure 7a). Consequently, cell viability was reduced to 30% by 0.1 μM Ara-C with XIAP antisense oligonucleotide, an effect that required 0.8 μM Ara-C with nonsense controls (Figure 7b).

Discussion

Better understanding of the regulation of IAPs and targeting XIAP expression in leukemia will help us to overcome IAP-mediated chemoresistance and induce leukemic cell death. Our previous study demonstrated that survivin is expressed in myeloid leukemias and survivin levels are cytokine-regulated in myeloid leukemias through both the MAPK and PI3K pathways.[34,46] In this study, we examined the regulation of XIAP in myeloid leukemias, extending our previous studies that documented prognostic importance of XIAP protein levels in AML. Hematopoietic cytokines induced XIAP expression in both leukemic cell lines and primary AML samples, and XIAP expression was regulated through the PI3K and, to a lesser degree, the MAPK/ERK pathways. In addition, several commonly used anticancer agents such as Ara-C, Dox, vincristine, and Taxol decreased XIAP protein levels, apparently through caspase-dependent cleavage of XIAP. Downregulation of XIAP induced caspase activation and sensitized cell death in leukemic cells.

Cytokines are potent regulators of hematopoietic cell survival and proliferation. For example, recent studies from our laboratory demonstrated that hematopoietic cytokines strongly increase survivin expression levels in myeloid leukemia cell lines and in primary AML samples.[34] Our present study demonstrates that XIAP expression is regulated by various cytokines in hematopoietic malignancies. Others have observed that survivin and XIAP expression are increased in endothelial cells in response to mitogenic growth factors.[33,47] Therefore, growth factors in general may exert their antiapoptotic effects, at least in part, by increasing IAP expression, supporting the role of IAP in inhibiting cell death and promoting cell survival.

XIAP expression is regulated by various mechanisms at both the transcriptional and translational levels.[20,21] Our previous studies demonstrated that survivin expression is regulated through the MAPK/ERK and PI3K signaling pathways. Here we demonstrate that XIAP is also regulated through these two signaling pathways, but in contrast to survivin, expression of XIAP seems slightly more sensitive to PI3K signaling. Interestingly, the signal transduction pathways involved in the regulation of survivin and XIAP (in response to hematopoietic cytokines such as GM-CSF) appear to diverge downstream of PI3K: while GM-CSF induces LY294002-sensitive increases in both survivin and XIAP levels, modulating Akt activity in the same cellular context appears to selectively affect survivin expression, suggesting that XIAP regulation involves a different, as yet unidentified, PI3K-dependent signaling pathway. These results are consistent with recent evidence from neurons indicating that PI3K activation gives rise to Akt-dependent and independent signals, sometimes with opposing functional consequences.[48] We need to keep in mind that the Akt overexpressed in OCI-AML3 cells lacks the PH domain. Even though it is unlikely, one cannot rule out the possibility that this domain is important in the regulation of XIAP expression. Studies from another group have suggested that XIAP regulates phospho-Akt expression.[49,50] Therefore, the mechanism of XIAP regulation through the PI3K pathway requires further investigation.

The mechanism by which Ara-C and Dox increase XIAP expression in certain cultured AML cells is unclear. Upregulating antiapoptotic IAP expression may be one means used by cells attempting to survive stress. This notion is supported by a recent study from Holcik *et al*,[20] showing that, in cell lines, XIAP protein levels were translationally increased and regulated through an internal-ribosome-entry-site motif in response to irradiation. We have previously reported that Ara-C increases Bcl-2, another antiapoptotic protein, in AML cells *in vivo*.[51]

IDN-1529 and IDN-1965 are two broad-spectrum caspase inhibitors. They were reported to inhibit various caspases, including caspase-9.[40] However, in our study, they did not inhibit the activation of caspase-9. Nevertheless, IND-1529 or IDN-1965 inhibited the activation of caspase-8, -3, and -7, which was sufficient for the inhibition of XIAP cleavage, indicating that XIAP cleavage is mediated by caspases other than caspase-9.

Apoptosis is a genetically controlled process regulated by multiple anti- and proapoptotic portents. XIAP is a potent caspase inhibitor, and suppresses apoptosis induced by various stimuli.[2,3,52] Decrease in antiapoptotic XIAP level would shift this balance toward apoptosis, promote spontaneous cell death of otherwise healthy cells, and sensitize cell death induced by various stimuli. Downregulation of XIAP has been shown to induce apoptosis in chemoresistant human ovarian cancer cells[53] and sensitize non-small-cell lung carcinoma to low-dose γ-irradiation.[54] Strategies to negatively regulate XIAP by its inhibitor Smac/Diablo are being developed. Recent studies have shown that synthetic Smac/Diablo peptides and Smac agonists enhance chemotherapeutic agents or Trail-induced cell death.[55,56] Our present study demonstrates that downregulation of XIAP by the antisense oligonucleotide in leukemic cells activates caspases and induces cell death. Chemotherapy is still the primary therapy for treating AML patients, and the biggest challenge is chemoresistance. Downregulation of XIAP expression will decrease the threshold of cell death, and increase the efficiency of chemotherapy-induced cell death. Our study showed that XIAP downregulation in combination with Ara-C is more effective in killing HL-60 cells.

In our hands, nucleofection appears to be the most effective method to successfully introduce oligonucleotide cells in suspension. A drawback of this technique is that the combination of solutions and 'programs' has to be optimized for every cell line to achieve high transfection efficiency and low toxicity. Even the same cells from different sources or different batches may respond differently to the same treatment. This is reflected in Figures 6 and 7. The batch of HL-60 cells in Figure 6 was more sensitive to XIAP downregulation-induced cell death than those in Figure 7. However, the general trend is the same, and the differences in killing efficiency do not change our overall conclusion.

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