Impact of XIAP protein levels on the survival of myeloma cells

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Funding: this work has been supported by La Ligue Nationale Contre le Cancer (équipe labélisée 2005) and La Région des Pays de la Loire. Grégoire Desplanques is supported by the French-Italian University (UFI, Grenoble, France).

Manuscript received June 11, 2008. Revised version arrived September 17, 2008. Manuscript accepted September 18, 2008.

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ABSTRACT

Background

XIAP is the best characterized and the most potent direct endogenous caspase inhibitor and is considered a key actor in the control of apoptotic threshold in cancer cells. In this report, we specifically addressed XIAP regulation and function in myeloma cells.

Design and Methods

XIAP and its endogenous inhibitor XAF-1 protein levels and their regulation were assessed by immunoblot analysis in myeloma cell lines or primary myeloma cells. XIAP knockdown by RNA interference was used to evaluate XIAP impact on *in vitro* drug sensitivity and *in vivo* tumor growth.

Results

Our results indicate that myeloma cells expressed high levels of XIAP protein that were tightly regulated during growth factor stimulation or stress condition. Of note, an increased XIAP level was evidenced during the blockade of the canonical cap-dependent translation by the mTOR inhibitor rapamycin, supporting the hypothesis of a functional IRES sequence in XIAP mRNA. In addition, caspase-mediated XIAP cleavage correlated to an apoptotic process occurring upon cell treatment with the proteasome inhibitor bortezomib. Importantly, XIAP knockdown using RNA interference enhanced drug sensitivity and decreased tumor formation in NOD/SCID mice. Finally, myeloma cells also expressed the XIAP inhibitor XAF-1 that interacted with XIAP in viable myeloma cells.

Conclusions

Altogether, our data argue for a delicate control of XIAP function in myeloma cells and stimulate interest in targeting XIAP in myeloma treatment.

Key words: XIAP, myeloma, apoptosis.

Citation: Desplanques G, Giuliani N, Delsignore R, Rizzoli V, Bataille R, and Barillé-Nion S. Impact of XIAP protein levels on the survival of myeloma cells. Haematologica 2009; 94:87-93. doi: 10.3324/haematol.13483

Introduction

Cancer occurs or progresses because the malignant cells fail to die in response to chemotherapy, irradiation or immune response. X-linked inhibitor of apoptosis protein (XIAP) is the best characterized and the most potent direct endogenous inhibitor of caspases and is thus considered to be a key physiological regulator of cell death.[1,2] Indeed XIAP inhibits the upstream caspase-9 that binds to its BIR3 domain, and the downstream caspase-3 and -7 that bind to its BIR2 domain. XIAP expression appears elevated in many cancer cells often conferring resistance to chemotherapy-induced cell death. Therefore, defining mechanisms involved in the regulation of XIAP in cancer cells is of particular interest. Regulation of XIAP expression occurs at multiple levels. Various signaling pathways, including NF-κB, PI3K and MAPK, mediate *Xiap* gene transcription. Moreover, XIAP mRNA harbors a putative internal ribosome entry site (IRES) sequence within its 5' untranslated region that may allow an alternative translation process during cell stresses compromising canonical cap-dependent translation leading to apoptosis.[3] Finally, XIAP protein that can be processed by proteosomal degradation is highly regulated by its interaction with at least 3 specific endogenous inhibitors: (i) SMAC/Diablo and Omi/HtrA2, both localized in the mitochondria in viable cells and released in the cytoplasm during apoptosis where they neutralize XIAP anti-caspase activity,[4,5] and (ii) the tumor suppressor gene XAF-1 that antagonizes XIAP activity in non-apoptotic cells.[6]

Despite recent progress, multiple myeloma (MM) is still an incurable cancer and the search for new agents and effective strategies against this disease remains a high priority.[7] Two classes of XIAP inhibitors are currently under development in cancer therapy:[8] (i) BIR3 inhibitors or Smac-mimetics that block the activity of the caspase-9, and (ii) BIR-2 inhibitors that bind to caspase-3 and -7 that were reported to induce apoptosis as single agents in a broad range of cancer cell lines in contrast to BIR3 inhibitors. Previous data have reported that XIAP levels were modulated in myeloma cell lines during *in vitro* drug or cytokine treatment.[9,10] However, to our knowledge, the impact of XIAP on MM biology has not yet been specifically addressed in this cancer. Therefore, we concentrated our attention on this attractive anti-cancer target in MM. Our results indicate that myeloma cells display high levels of XIAP protein whose level is tightly controlled by myeloma growth factors, an IRES sequence and drug treatment in correlation with the apoptotic process. Moreover, XIAP extinction by RNA interference led to *in vitro* increased drug sensitivity and *in vivo* decreased tumor formation. Finally, our data revealed that XAF-1 interferes with XIAP in viable myeloma cells modulating its activity in myeloma cells. Thus, XIAP emerges as a relevant target in MM and our results argue for the use of XIAP inhibitors in MM treatment.

Design and Methods

Cell lines and culture conditions

The XG1, XG6, NAN1, NAN2, NAN3, NAN4, NAN6 human myeloma cell lines (HMCLs) had been previously established in our laboratory.[11] U266, OPM2, KMS12PE, KMS18 and NCI-H929 HMCLs were available commercially. MM1-S was a gift from Dr ST Rosen (Chicago, IL, USA). These cell lines were maintained in RPMI-1640 medium supplemented with 5% FCS, 2 mM glutamine and 5.10^{-5}M 2β mercapto ethanol supplemented with recombinant IL6 or not.[11]

Primary cells

Primary myeloma cells were purified from bone marrow, blood or pleural effusion samples of patients with MM using CD138 immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described.[12] Informed consent was provided according to the Helsinki Declaration of the World Medical Association, and the University Hospital of Nantes review board approved these studies.

Reagents and antibodies

IL6, IGF-1 and TRAIL were from R&D Systems (Minneapolis, USA), and melphalan and dexamethasone from Sigma-Aldrich (St Louis, Missouri, USA). The mTOR inhibitor was purchased by Alexis Biochemicals. The proteasome inhibitor bortezomib was kindly provided by OrthoBiotech and the NFκB inhibitor AS602868 by Merck Serono International SA. Anti-XIAP antibody was purchased from BD Biosciences and from Abcam (for immunoprecipitation), anti-XAF1 from Abcam, anti-survivin and anti-HtrA2/Omi from R&D Systems, anti-p27 KIP-1 from BD Pharmingen, anti-PARP-1 (Ab2) from Calbiochem, anti-cox IV (3E11) from Cell Signaling, anti-tubulin from Amersham, anti-phospho-4EBP1 (Thr 37/46) from Ozyme, anti-Smac/DIABLO from Alexis and anti-actin from Chemicon International.

RNA interference by siRNA or shRNA

For transient RNA interference experiments, 10 μM of siRNA XIAP or control siRNAs from Ambion were mixed with 5.10^{6} U266 cells in buffer R and electrofected using the protocol T-01 of the Amaxa Nucleofector™ apparatus (Amaxa, Cologne, Germany). For stable RNA interference experiments using lentiviral delivery, short hairpin (sh)RNA corresponding to the targeted sequence of XIAP mRNA[13] 5' AAG TGT CCC ATG TGC TAC ACA 3' or to the bacterial LacZ were cloned and lentiviral particules were prepared as previously described.[14]

Immunoblot analysis

Samples for western blot analysis were prepared as described.[14] Fifty micrograms of proteins were loaded for each lane and separated by 10% or 12.5% SDS-PAGE, then electrotransfered to PVDF membranes. Western blot analysis was performed by standard techniques with ECL detection (Pierce) for XIAP, p27,

XAF1, HtrA2/Omi, Smac/DIABLO, P-4EBP1, PARP-1, tubulin, COX IV and actin. Protein loading was checked with anti-actin antibody.

Subcellular fractionation

For the mitochondrial and cytoplasm fractions, cells were washed once with ice-cold PBS, then incubated in hypotonic buffer (5 mM HEPES pH 7.4 , 25 mM sucrose, 2 mM EDTA, 2 mM aprotinin, 5 mM leupeptin, 0.4 mM Pefabloc) for 30 min on ice. Cells were disrupted with a dounce homogenizer for 15 min on ice. Immediately after homogenization, the lysate was centrifuged at 500 g for 10 min to eliminate the nuclear pellet and unbroken cells. The collected supernatant was centrifuged at 10,000 g for 15 min to obtain the mitochondrial pellet and the cytoplasmic supernatant. The mitochondrial pellet was further lysed on lysis buffer. The nuclear fraction was prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents based on the manufacturer's instructions (Pierce, Rockford, IL, USA).

Immunoprecipitation

Cells (25×10^6) were lysed in 1% CHAPS-containing lysis buffer. Whole-cell lysates were obtained, precleared with Protein A-Sepharose, and incubated overnight with 7 μg of XIAP antibody. The immunocomplexes were captured with protein A. Beads were pelleted, washed three times, and boiled in SDS sample buffer. The presence of immunocomplexes was determined by immunoblotting analysis.

Cell viability and proliferation analysis

Cell viability and proliferation were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After 48 h of incubation with AS602868 (10 μM), dexamethasone (1 μM), melphalan (1 μM) or TRAIL (10 ng.mL-1), cells were incubated with 50 μL of 2.5 mg/mL MTT (Sigma-Aldrich) for 3 h 30 min. Absorption at 570 nm wavelength was measured after total solubilization of formazan crystals by 100 μL of lysis solution. The inhibition of cell growth induced by drugs was expressed as a percentage compared to untreated corresponding cells.

Subcutaneous xenograft model in NOD/SCID mice

Four to eight week old non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice were obtained from Charles River Laboratories (L'Arbresle, France) and were housed and monitored at the core facility of the INSERM UMR892. This facility is approved by the French Association for Accreditation of Laboratory Animal Care and is maintained in accordance with the regulations and standards of the INSERM institute and the French Department of Agriculture. Mice (n=18) were inoculated subcutaneously (s.c.) into the right flank with 2×10^7 cells in 100 μL of NCI-H929. Caliper measurements of the longest perpendicular tumor diameters were taken 4 times (days 0, 9, 14 and 20) to estimate the tumor volume, using the following formula:

$$4\pi/3x(width/2)^2x(length/2)$$

representing the three dimensional volume of an ellipse. Animals were sacrificed when their tumors reached 2 cm or when the mice became moribund. Tumors were then frozen and kept for immunoblot analysis.

Statistics analysis

The two-way ANOVA test was used for statistical analysis.

Results

Study of XIAP protein expression in human myeloma cells and its regulation

A panel of HMCLs was first screened for XIAP expression using immunoblot analysis. As shown in Figure 1A for representative cell lines, all HMCLs expressed similar high levels of XIAP protein (Figure 1A, top panel). Secondly, purified primary myeloma cells obtained from patients with MM also displayed detectable XIAP but at variable levels from barely detectable (P2-3) to levels close to those detected in HMCLs (P5) (Figure 1A, bottom panel). However, no correlation between XIAP level and disease status could be evidenced in our series of samples. Interestingly, a significant induction of XIAP expression could be detected in myeloma cells treated by both main MM survival factors, i.e. IL6 and IGF-1, as shown in Figure 1B for the HMCL XG6. Moreover, in an attempt to evaluate whether the IRES sequence previously reported in 5'UTR XIAP mRNA was indeed functional in myeloma cells, cells were treated by the mTOR inhibitor rapamycin that inhibits the cap-dependent protein translation requiring the mTOR substrate 4EBP1 phosphorylation, then XIAP levels were assessed by immunoblot in rapamycin treated cells in comparison with untreated cells. Figure 1C indicates that efficient inhibition of cap-dependent protein translation evidenced by dramatic inhibition of 4EBP1 phosphorylation, potently induced XIAP expression in myeloma cells compared to control cells. This effect was also observed for the expression of the CDK inhibitor p27, whose mRNA also contains an IRES sequence. This cap-independent maintenance of XIAP level during rapamycin treatment suggests a functional IRES in XIAP mRNA in myeloma cells. Furthermore, XIAP regulation was determined during the apoptotic process induced by the proteasome inhibitor bortezomib. After as little as eight hours of drug-treatment, detection of a 29 kDa XIAP product was observed by immunoblot analysis (Figure 1D). The 29 kDa XIAP product resulted from a caspase-dependent XIAP cleavage during apoptosis since it was completely inhibited by the incubation of cells with the pancaspase inhibitor z-VADfmk prior to bortezomib treatment (Figure 1E). XIAP cleavage further increased at 16 h after drug treatment leading to the disappearance of the 55 kDa full length XIAP protein at 24 h. Importantly, the kinetic decrease of full length XIAP associated with the kinetic increase in apoptotic cell number (Figure 1D). These results indicate a close correlation between intact XIAP protein disappearance and apoptosis in myeloma cells.

XIAP knockdown enhanced drug resistance in myeloma cells

To further delineate the role of XIAP in myeloma cell apoptosis control, XIAP knockdown was performed by RNA interference strategy. As U266 was the most efficiently tranfected cell line among HMCLs, XIAP was first knocked down in these cells using siRNA. XIAP expression in U266 cells was decreased as soon as day 1 post-transfection and up to day 3, as detected by immunoblot analysis (Figure 2A, top panel) with no impact on other IAP (*data not shown*). Of interest, specific XIAP knockdown, that had no impact on the proliferation of untreated cells, significantly enhanced U266 sensitivity to both conventional drugs used in MM treatment, i.e. dexamethasone and melphalan and new targets previously defined in MM, such as TRAIL[15] (an inductor of apoptosis through the extrinsic pathway) or the NF-κB inhibitor AS602868[16] (Figure 2A, bottom panel). In order to extend these results to other HMCLs, RNA interference experiments using short hairpin RNA (shRNA) delivered by lentiviral particules were also performed on both hard-to-transfect HMCLs NCI-H929 and MM1-S. Decreased XIAP expression in shXIAP-transduced cells (shXIAP) compared to control-transduced cells (shCt) was determined in both HMCLs by immunoblot analysis depicted for NCI-H929 at day 7 in Figure 2B (top panel). Efficient XIAP knockdown in NCI-H929 cells also led to enhanced drug sensitivity (Figure 2B, bottom panel). The same results were obtained in MM1-S cell line (*data not shown*).

XIAP knockdown decreased myeloma cell tumorigenicity

In addition, prolonged XIAP knockdown obtained in shXIAP-transduced NCI-H929 cells (up to three weeks) allowed us to test the impact of XIAP extinction on tumor formation in *in vivo* experiments. Of major importance, XIAP knockdown potently restrained NCI-H929 cell capacity to form tumor in the subcutaneous xenograft model using NOD/SCID mice compared control cells (Figure 3A). Indeed, mice engrafted with shCt-transduced NCI-H929 cells developed measurable tumors at day 14 that exponentially grew until their sacrifice. In contrast, shXIAP-transduced cells led to slightly detectable tumors that did not grow any further during the experiments. Difference in tumor size between shXIAP- versus shCt-transduced cells was highly significant at day 20. Immunoblot analysis (Figure 3B) indicated that XIAP expression was still knocked down at the end of the experiment.

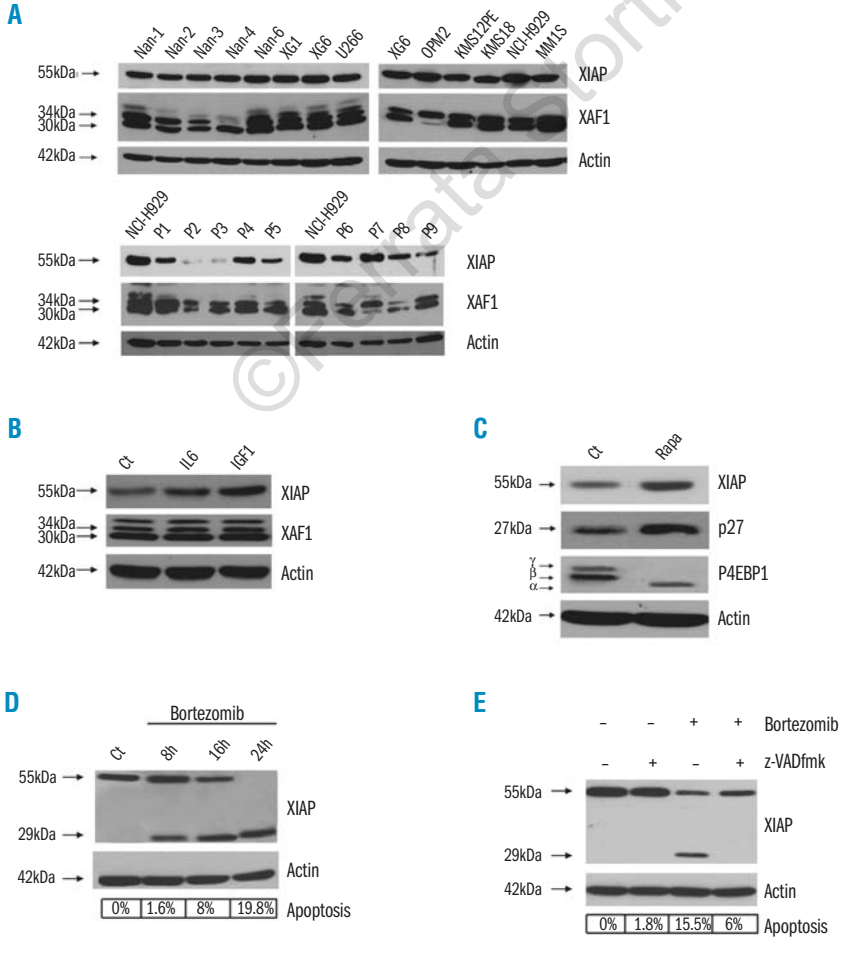

Figure 1. XIAP and XAF-1 expression in myeloma cells. (A) XIAP expression was assessed by immunoblot analysis in HMCLs (top panel) and in a series of primary myeloma cells (P1-P9) (bottom panel). XAF-1 expression evaluated as indicated. Protein loading was controlled with anti-actin mAb. (B) XIAP and XAF-1 protein expression in the XG6 HMCL after culture in presence of IL6 (10 ng/mL corresponding to 385 nmol/mL) or IGF-1 (100 ng/mL corresponding to 13.1 μmol/mL) for 48h. (C) XIAP, p27 and p4EBP1 protein expression in untreated XG6 cells (Ct) or after rapamycin (Rapa) treatment (100 nM) for 24h. 3 isoforms of P4EBP41 were detected (a: hypophosphorylated isoform, b and g: Thr 37, 46- and 37, 46, 65, 70-phosphorylated isoforms respectively). (D) XIAP protein expression during bortezomib 10 nM treatment for 8, 16 and 24h. Specific apoptosis was evaluated in each condition by Apo2.7 immunostaining. (E) XIAP protein expression during bortezomib treatment in presence of the pancaspase inhibitor z-VADfmk (50 μM added one hour prior to bortezomib treatment) or not. Specific apoptosis was evaluated in each condition by Apo2.7 immunostaining.

XIAP and its inhibitor XAF-1 interacted in the cytoplasm in myeloma cells

As previously described, viable myeloma cells express SMAC and Omi/HtrA2 and as expected, these inhibitors were detected in the mitochondria fraction in viable myeloma cells in contrast to XIAP that was mainly detected in the cytoplasmic fraction (Figure 4A). Interestingly, we pointed out for the first time that HMCLs as well as primary myeloma cells also expressed XAF-1 (Figure 1A). Levels of XAF-1 varied among our panel of HMCLs or primary myeloma cells. Interestingly, neither IL6 nor IGF-1 could induce XAF-1 expression in myeloma cells in contrast to XIAP (Figure 1B). Furthermore, we demonstrated that XAF-1 could be detected in cytoplasmic as well as in nuclear enriched fractions in myeloma cells (Figure 4A). Therefore, XIAP and XAF-1 colocalized in cytoplasm in myeloma cells and we further demonstrated that they physically interacted as proved by co-immunoprecipitation experiments (Figure 4B).

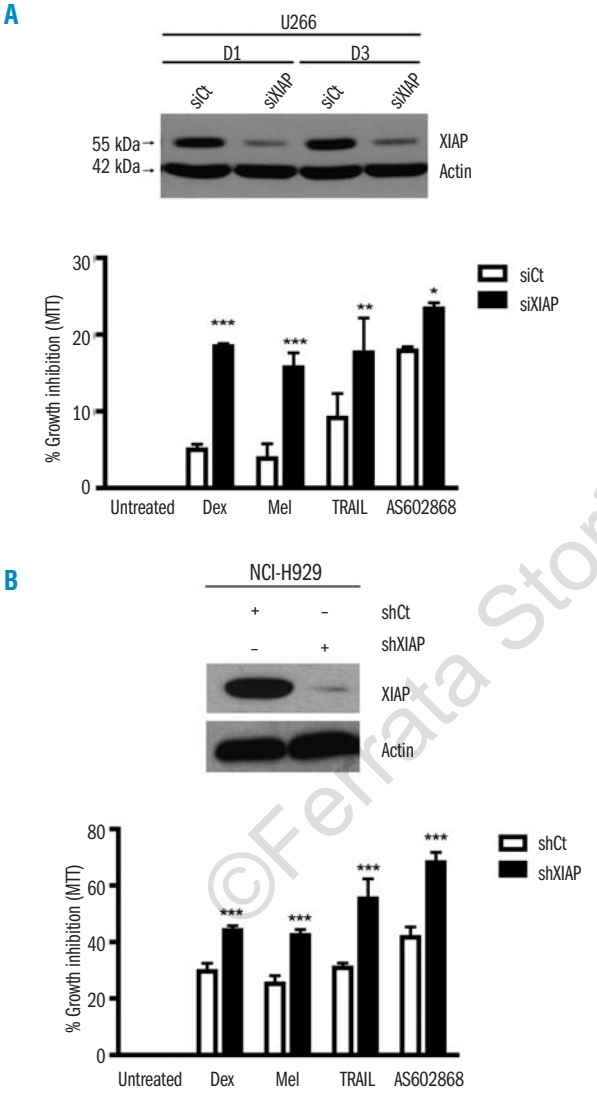

Figure 2. XIAP knockdown increased drug sensitivity. XIAP knockdown was performed by RNA interference in both HMCLs U266 transduced by siRNA (A) and NCI-H929 transduced by shRNA (B) and evaluated by immunoblot analysis at day 1 (D1) and D3 in U266 (siXIAP) and at D7 in NCI-H929 (shXIAP) compared to control transduced cells (siCt or shCt). Myeloma cell growth was quantified by MTT test after treatment with dexamethasone (Dex) 10^{-6}M, melphalan (Mel) 1 μM, TRAIL 10 ng/mL or AS602868 10 μM (columns, mean; bars, SD; n=3 and n=6 respectively for siRNA and shRNA experiments) and related to cell growth in corresponding untreated cells. Results were then expressed in % of cell growth inhibition compared to untreated cells. The two-way ANOVA test was used for statistical analysis (*p<0.05; **p<0.01; ***p<0.001).

Discussion

In this report, we demonstrate that myeloma cells express high levels of XIAP regulated by (i) IL6 and IGF-1, (ii) the mTOR and cap-dependent translation inhibitor rapamycin, and (iii) a caspase-mediated cleavage during drug treatment. Moreover, specific XIAP knockdown sensitized these cells to apoptosis and strongly decreased tumor formation. Finally, myeloma cells also express the novel XIAP inhibitor XAF-1 that may interfere with the caspase inhibitor function of XIAP.

Even though HMCLs display significant genetic heterogeneity (including IgH translocations, chromosome deletions or ploidy abnormalities),[17] XIAP levels were found to be high and stable among HMCLs, as observed in many cancer cell lines. This suggests that strong XIAP

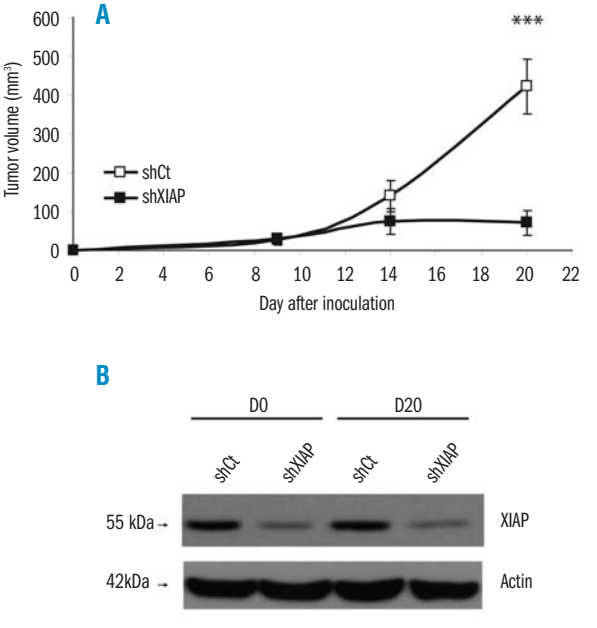

Figure 3. XIAP knockdown decreased NCI-H929 tumorigenicity. NCI-H929 transduced with shRNA control (shCt) or XIAP shRNA (shXIAP) were inoculated subcutaneously in NOD/SCID mice. (A) Tumor formation and measurement were assessed for 20 days (columns, mean; bars, SD; n=18). The two-way ANOVA test was used for statistical analysis (*** = p<0.001). (B) XIAP expression in tumors at the beginning (D0) and the end point (D20) of the experiment using immunoblot analysis.

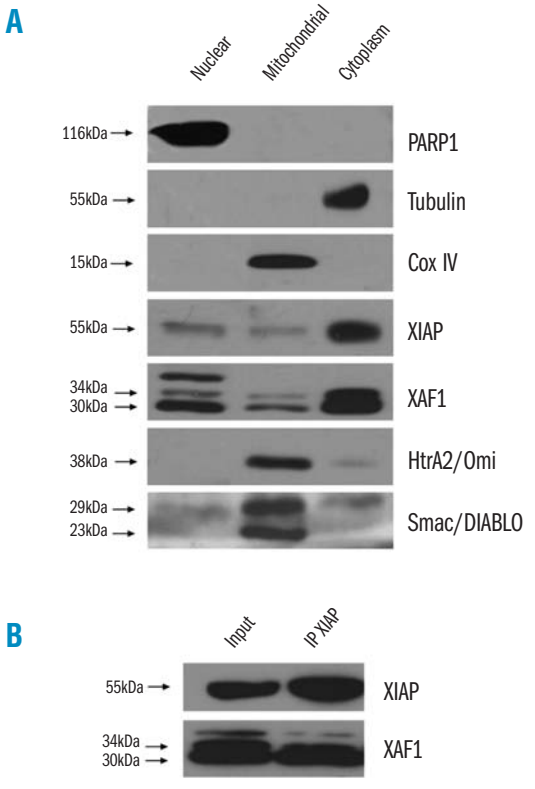

Figure 4. XIAP and XAF-1 colocalized in the cytosolic fraction and were co-immunoprecipitated. (A) XIAP and XAF-1 localizations were evaluated by immunoblot analysis after subcellular fractionation of NCI-H929 cells. Enrichment of fractions was verified by expression of PARP1, COX IV and tubulin for nuclear, mitochondrial and cytoplasmic fraction respectively. (B) XIAP and XAF-1 co-immunoprecipitation in NCI-H929 cells. First lane (Input) corresponds to the cell lysate used for immunoprecipitation (IP) and second lane (IP XIAP) corresponds to the lysate obtained after IP.

expression may be related to a high degree of malignancy. In contrast, XIAP protein level varied among patients but a larger series of samples should be evaluated to evidence a bioclinical impact of XIAP protein level in patients. XIAP expression was increased by both myeloma growth factors IL6 and IGF-1 that activate Jak/STAT, MAPK, PI3K or NFκB signaling pathways by either direct or indirect mechanisms in myeloma cells.[18] The IGF-1-induced XIAP expression, observed in leukemic cells,[19] has also been reported in the MM1-S HMCL in which PI3K, NFkB and MAPK pathways have been implicated.[9] Moreover, XIAP expression decreased during treatment by the proteasome inhibitor bortezomib, as previously observed,[10] and underwent a rapid caspase-mediated cleavage, leading to both fragments corresponding to BIR1-BIR2 and BIR3-RING domains that are less efficient caspase-inhibitors.[20] Importantly, this decrease in intact XIAP correlated with apoptosis induction. Finally, we show that inhibition of the canonical cap-dependent translation by the mTOR inhibitor rapamycin, was associated with increased levels of XIAP as well as CDK inhibitor $p27^{Cip/Kip}$ that both contain an IRES sequence in their 5'UTR mRNA.[3,21] Thus, even though we cannot exclude other molecular mechanisms, our results suggest that XIAP may participate through its IRES sequence in myeloma cell protection under conditions of compromised cap-dependent translation such as rapamycin treatment. As rapamycin appeared to be an agent of potential therapeutic interest in MM, inducing G1 cell cycle arrest but no cell death,[22] our data raise the possibility that the increase in XIAP level observed upon rapamycin treatment could limit its own antimyeloma effect and even counteract the action of antimyeloma treatments. Recent publications also reported negative feedback loops in cancer cells treated by mTOR inhibitors involving the activation of AKT or MAPK signaling pathways that may be detrimental to rapamycin in cancer therapy.[23,24] We further demonstrate in this report that specific XIAP knockdown in 3 HMCLs significantly increased cell sensitivity to various antimyeloma drugs suggesting that XIAP indeed participates in the drug resistance observed in MM. In line with this hypothesis, Nakagawa *et al.* reported that XIAP might play a role in worsening the prognosis of MM patients because of drug resistance.[25] Of particular interest, prolonged XIAP knockdown in myeloma cells also led to a dramatic decrease in tumor development in the subcutaneous xenograft NOD-SCID model. These data indicate that high basal XIAP level was necessary to allow *in vivo* tumor growth. Altogether our results corroborate those from Chauhan *et al.* who have recently reported that targeting SMAC with the SMAC mimetic LBW242, considered as potent XIAP inhibitor, increased cell death in MM.[26] Finally, we show for the first time that myeloma cells express the XIAP inhibitor XAF-1. Indeed, we demonstrate that XIAP and XAF-1 colocalized in the cytoplasmic fraction of myeloma cells and formed complexes that may neutralize caspase-inhibitory activity of XIAP. Of note, *XAF-1* gene mapped on 17p13 as *p*53 gene and 11% of patients with MM harbor the genetic aberration del(17p) that was powerful and independent predictor of survival.[27] Even though several studies have focused on the *p*53 gene, formal demonstrations of its deregulation are currently lacking, and involvement of other genes like *XAF-1* cannot be excluded and should be investigated.

Altogether, our data demonstrate that a decrease in XIAP level in myeloma cells shifts the balance toward apoptosis and strongly stimulate interest in targeting XIAP by already available XIAP inhibitors in MM treatment.

Authorship and Disclosures

GD: carried out experiments; NG: designed the experiments and participated in writing the manuscript; RdS, VR: discussed data; RB: provided primary myeloma cells and analyzed data; SB-N: designed the experiments, analyzed data and wrote the manuscript.The authors reported no potential conflicts of interest.

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