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Expression of αB-crystallin overrides the anti-apoptotic activity of XIAP

Jee Suk Lee, Hye Young Kim, Na Young Jeong, Sang Yeob Lee, Young Geol Yoon†, Yung Hyun Choi, Chunlan Yan, In-Sun Chu, Hyungjong Koh, Hwan Tae Park, and Young Hyun Yoo

Department of Anatomy and Cell Biology and Mitochondria Hub Regulation Center, College of Medicine, Dong-A University, Busan, Republic of Korea (J.S.L., H.Y.K., N.Y.J, Y.G.Y., Y.H.Y.); Department of Rheumatology, College of Medicine, Dong-A University, Busan, Republic of Korea (S.Y.L.); Department of Biochemistry and Research Institute of Oriental Medicine, Dongeui University College of Oriental Medicine, Busan, Republic of Korea (Y.H.C.); Department of Toxicology, Zhejiang University School of Public Health, Hangzhou, Zhejiang, China (C.Y.); Korean Bioinformation Center, KRIBB, Daejeon, Republic of Korea (I.C.); Department of Pharmacology and Mitochondria Hub Regulation Center, College of Medicine, Dong-A University, Busan, Republic of Korea (H.K.); Department of Physiology, Medical Science Research Institute, College of Medicine, Dong-A University, Busan, South Korea (H.T.P.)

†Present Address: Department of Biomedical Science, Jungwon University, 85 Munmu-ro, Goesan-eup, Goesan-gun, Chungbuk 367-805, South Korea (Y. G. Y.)

Although crystallins are major structural proteins in the lens, α-crystallins perform non-lens functions, and αB-crystallin has been shown to act as an anti-apoptotic mediator in various cells. The present study was undertaken to examine whether αB-crystallin expressed in human malignant glioma cells exerts anti-apoptotic acitivity. In addition, we sought to elucidate the mechanism underlying any observed anti-apoptotic function of αB-crystallin in these cells. Three glioma cell lines, U373MG, U118MG, and T98G, were used. We observed that only the U373MG cell line expresses αB-crystallin, whereas the other 2 glioma cell lines, U118MG and T98G, demonstrated no endogenous expression of αB-crystallin. We next observed that the silencing of αB-crystallin sensitized U373MG cells to suberoylanilide hydroxamic acid (SAHA)–induced apoptosis and that αB-crystallin associates with caspase-3 and XIAP. Because XIAP is the most potent suppressor of mammalian apoptosis through the direct binding with caspases, we assessed whether XIAP also plays an anti-apoptotic role in SAHA-induced apoptosis in αB-crystallin-expressing U373MG cells. Of note, the silencing of XIAP did not alter the amount of cell death induced by SAHA, indicating that XIAP does not exert an anti-apoptotic activity in U373MG cells. We then determined whether the ectopic expression of αB-crystallin in glioma cells caused a loss of the anti-apoptotic activity of XIAP. Accordingly, we established 2 αB-crystallin over-expressing glioma cell lines, U118MG and T98G, and found that the silencing of XIAP did not sensitize these cells to SAHA-induced apoptosis. These findings suggest that αB-crystallin expressed in glioma cells overrides the anti-apoptotic activity exerted by XIAP.

Keywords: αB-crystallin, apoptosis, glioma, SAHA, XIAP.

α-Crystallin is a major protein in the eye lens of vertebrates and is found as large aggregates of 2 closely related subunits, αA and αB.[1] Both αA-crystallin and αB-crystallin are also distributed outside the vertebral eye lens.[2,3] Thus, entirely different non-lens roles for α-crystallins have been suggested.

Previous studies have shown that αB-crystallin is a member of the conserved small heat shock family of proteins and acts as a morecular chaperone induced by various stress stimuli, coferring cytoprotection by suppressing aggregation of denatured protein.[4] αB-crystallin is also responsible for the control of the cell cycle and neoangiogenesis in the process of metastases

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Corresponding Author: Young Hyun Yoo, MD, PhD, Department of Anatomy and Cell Biology and Mitochondria Hub Regulation Center, College of Medicine, Dong-A University, 3-1 Dongdaesin-dong, Seo-gu, Busan 602-714, Republic of Korea (yhyoo@dau.ac.kr).

and invasion.[5] Accumulating evidence indicates that αB-crystallin negatively regulates apoptosis.

Apoptosis is a cellular process that plays crucial roles in multiple physiological and pathological events and is regulated by positive and negative regulators in series.[6] Apoptosis is distinguished by nuclear condensation and DNA fragmentation[7] and is executed by an activity of a family of cysteine proteases known as caspases.[8] Caspases, which are divided into initiators (caspases-2, -8, -9, and -10) and effectors (caspase-3, -6, and -7) based on their relative position in the signaling cascade,[9] are expressed in essentially all metazoan cells as catalytically inactive zymogens known as procaspases.[10] Of importance, caspase-3 plays a role in executing apoptosis.[11] To date, many proteins have been identified as substrates of caspase-3.[12]

To date, several cancer cells, such as gliomas, breast carcinomas, and renal cell carcinomas, have been demonstrated to express αB-crystallin,[13–17] and the expression of αB-crystallin correlates with poor clinical outcomes in various cancers.[15,18] The expression of αB-crystallin protects cancer cells from apoptosis and contributes to the aggressive behavior of diverse carcinomas,[15,19] and chemotherapy has been shown to enhance the expression of αB-crystallin in cancer cells.

Irrespective of accumulating evidence supporting that αB-crystallin provides anti-apoptotic protection against various apoptotic effectors, little progress has been made in understanding the molecular mechanisms underlying their action. αB-crystallin inhibits autocatalytic maturation of caspase-3 preventing.[20] αB-crystallin prevents apoptosis induced by staurosporine by interacting with Bax and Bcl-2 and inhibiting their translocation from the cytosol to the mitochondria.[21] More recently, Sreekumar et al. have shown that αB-crystallin protects stressed retinal pigment epithelial cells from apoptosis by inhibiting caspase-3 and PARP (poly [ADP-ribose] polymerase) activation.[22] αB-crystallin was observed to prevent apoptosis induced by hydrogen peroxide treatment by interacting with p53 in the cytoplasm and preventing its translocation to mitochondria.[23] In addition, alphaB-crystallin was known as a novel p53-target gene and required for p53-dependent apoptosis.[24] αB-crystallin overexpression decreases cell apoptosis by preventing RAS activation and inhibiting the RAF/MEK/ERK pathway.[25]

Glioblastoma multiforme (GBM) is the most common type of brain tumor and is known as an aggressive, highly invasive, and destructive tumor among human cancers.[26,27] Moreover, GBM responds poorly to conventional therapy.[28] Previous studies showed that the level of αB-crystallin expression is elevated in glioblastoma.[29] αB-crystallin is elevated in highly migratory glioblasma cells, where it plays a functional role in apoptosis resistance.[30] αB-crystallin is known to enable Bcl2-Like 12 to block the activation of capase-3 and -7, thereby contributing to GBM pathogenesis and its hallmark biological properties.[31]

A histone deacetylase inhibitor (HDACi), suberoylanilide hydroxamic acid (SAHA), was demonstrated to be effective against various glioma cell lines at a range of 1–10 μM.[32] Histone acetylation is maintained by histone acetyl transferases (HATs) and histone deacetylases (HDACs), and changes in the histone acetylation patterns are correlated with gene transcription and cause many diseases.[33] HDACs selectively induce apoptosis in many tumor cell types.[34] However, nontumorigenic cells clearly showed resistance to the cell death induced by HDAC inhibitors.[33]

The present study was undertaken to examine whether αB-crystallin expressed in human malignant glioma cells exerts anti-apoptotic acitivity. We observed that αB-crystallin endogenously and ectopically expressed in U373MG human glioma cells inhibits the cytotoxicity by SAHA and various anticancer agents. Furthermore, we observed that αB-crystallin expressed in human glioma cells overrides the anti-apoptotic activity exerted by XIAP.

Materials and Methods

Materials

The following reagents were obtained commercially: rabbit polyclonal anti-human caspase-3, caspase-9, caspase-12, HSP60, survivin, GAPDH and mouse monoclonal anti-human caspase-7, HSP70, HSP90 antibodies, and temozolomide (TMZ) from Santa Cruz Biotechnology; mouse monoclonal anti-human poly(ADP-ribose) polymerase (PARP) antibody from Oncogene; rabbit polyclonal anti-human caspase-6 and caspase-8 antibodies from Cell Signaling; mouse monoclonal anti-cytochrome *c* antibodies from BD Transduction Laboratories; FITC-conjugated goat anti-rabbit, horse anti-mouse IgG antibodies from Vector; HRP-conjugated donkey anti-rabbit and sheep anti-mouse IgGs from Amersham Pharmacia Biotech; mouse monoclonal anti-human β-actin antibodies, Hoechst 33342, dimethyl sulfoxide (DMSO), RNase A, proteinase K, propidium iodide (PI), valproic acid (VPA), resveratrol, etoposide, doxorubicin, blasticidin and cisplatin (CisPt) from Sigma-Aldrich; 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) and mitotrackers from Molecular Probes; SAHA and MS-275 from Alexis Biochemicals; and SuperSignal West Pico enhanced chemiluminescence Western blotting detection reagent from Pierce.

Cell Culture

T98G, U118MG, U373MG, and ARPE19 cells were obtained from the American Type Culture Collection (ATCC). The culture medium used throughout these experiments was DMEM/F-12 (ARPE19) and RPMI-1640 media (GibcoBRL) with 10% fetal bovine serum (FBS; GibcoBRL), 20 mM HEPES and 100 μg/mL penicillin in 5% CO_2 at 37°C.

αB-Crystallin Plasmid Construct and Establishment of 3x Flag-αB-Crystallin Over-Expressing Glioma Cell Lines

Human full-length αB-crystallin cDNA (*CRYAB*; accession number: NM_001885) was purchased from 21 Century Human Gene Bank and subcloned into pcDNA6-3xFlag. pcDNA6-3xFlag was constructed by inserting the 3xFlag tag sequence between *Nhe*I and *Eco*RI sites of pcDNA6 (Invitrogen). The primers used were as follows: CRYABFwd (5′-AAGAATTCATGGACATCGCCATCCACCACCCC-3′) and CRYABRev (5′-TTTCT AGACTATTTCTTGGGGGCTGCGGTGACAGC -3′). The *Eco*RI and *Xba*I sites are underlined respectively. The PCR-amplified αB-crystallin gene was cloned between the *Eco*RI and *Xba*I sites, which generated in-frame fusion with 3xFlag. All constructs were verified by sequencing. U118MG and T98G cells were transfected using lipofectamine 2000 (Invitrogen) reagent according to the manufacturer's instructions. The transfected cells were incubated for 2 days, and stable cell lines were then selected with changes of fresh medium containing blasticidin (10 μg/mL) for 4 weeks. Single-cell clones were isolated by limiting dilutions and subsequently analyzed for an increase of αB-crystallin protein expression relative to identically cloned empty vector controls.

Transient Transfection of XIAP

Human full-length XIAP cDNA (BIRC4; accession number: BC032729) was purchased from OpenBioSystem (OpenBioSystem). Human XIAP cDNA was inserted between NheI and EcoRI sites of MCS A in the pIRES plasmid (Clontech; catalog # 631605) containing 2 multiple cloning sites (MCS) A and B that allows expression of 2 genes of interest. Transient transfection of XIAP was performed using the lipofectamine 2000 (Invitrozen).

Cell Viability Assay

Cell viability was determined by the Vi-Cell cell counter (Beckman Coulter), which performs an automated trypan blue exclusion assay.

Nuclear Morphology Analysis of Apoptosis

Cell suspensions were cytospun onto clean fat-free glass slides using a cytocentrifuge. Centrifuged samples were fixed for 10 min in 4% paraformaldehyde and stained in 4 μg/mL Hoechst 33 342 for 30 min at 4°C. Cells were observed and photographed under an epifluorescence microscope by an observer who was blinded to the experimental group. To quantify cells showing nuclear fragmentation, the total cell number (100 cells from each experiment) was counted using differential interference contrast (DIC) optics.

Quantification of DNA Hypoploidy and Cell Cycle Phase Analysis by Flow Cytometry

Ice-cold 95% ethanol with 0.5% Tween 20 was added to the cell suspension to a final concentration of 70% ethanol. Fixed cells were pelleted and washed in 1% BSA-PBS solution. Cells were re-suspended in 1 mL PBS containing 11 Kunitz U/mL RNase, incubated at 4°C for 30 min, washed once with BSA-PBS, and re-suspended in PI solution (50 μg/mL). After cells had been incubated at 4°C for 30 min in the dark and washed with PBS, DNA content was measured on an Epics XL (Beckman Coulter), and data were analyzed using Multicycle software, which allowed a simultaneous estimation of cell cycle parameters and apoptosis.

Western Blot Analysis

Equal amounts of proteins were run on 7.5%–15% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech) and reacted with each antibody. Immunostaining with antibodies was performed using SuperSignal WestPico enhanced chemiluminescence substrate and detected with LAS-3000 Plus (Fuji Photo Film).

Immunocytochemistry and Confocal Microscopy

Cells grown in 2-well chamber slides were treated as indicated, fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100 for 15 min, and then blocked with 2% bovine serum albumin for 1 h at room temperature (RT). Cells were probed with mouse monoclonal anti-cytochrome *c* (1:75) overnight at 4°C, followed by staining with goat anti-mouse Alexa Fluor 488 antibody (1:100, Molecular Probes for 1 h at RT). After washing, cells were mounted with SlowFade Light antifade reagent (Molecular Probes) and analyzed by confocal microscopy. To visualize the mitochondria in living cells, 20 nM mitotracker CMXRos was added and incubated for 20 min. Fluorescent images were observed and analyzed under Zeiss LSM 700 laser-scanning confocal microscope.

siRNA

For XIAP depletion, pre-designed siRNA constructs were purchased from Dharmacon (ON-TARGET plus siRNA J-004098-14-0050). αB-crystallin target sequence (5′- AAU UGA CCA GUU CUU CGG AGA -3′) was synthesized by Dharmacon Research through the ready-to-use option using 21-nucleotide RNA with 3′-dTdT overhangs. As a negative control, the same nucleotides were scrambled to form a non-targeting combination. siRNAs were transfected using siPORT Amine (Ambion) in Opti-MEM (GibcoBRL). Cells grown to a confluency of 40%–50% in 6-well plates were transfected with 200 nM final siRNA concentration per well.

Twenty-four hours after transfection, apoptotic stimuli were applicated.

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the cultured cells using the Qiagen RNeasy Mini Kit (Quiagen). Total RNA (2 μg) was reverse transcribed using oligo-dT primers and M-MLV reverse transcriptase. RT-PCR products containing αB-crystallin and β-actin were amplified using gene a specific primer (αB-crystallin: F- TCC CCA GAG GAA CTC AAA GTT AAG, R- GGC GCT CTT CAT GTT TCC A, β-actin: F-TGA CGG GGT CAC CCA CAC TGT GCC CAT, R- CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG). PCR (40 μL) was performed in reaction mixes containing 3 μL cDNA, 10 μM primers, 2.5 μg/mL Go Tag Flexi DNA polymerase (Promega), 50 mM KCl, 10 mM Tris-HCl (pH, 9.0), 3.0 mM $MgCl_2$, and 0.2 mM dNTPs. PCR was performed in a 9700 Thermocycler (Perkin-Elmer), and the general thermocycling conditions were as follows: 1 cycle of 2 min of initial denaturation at 95°C, followed by 30 cycles of 30 s at 95°C, annealing for 40 s at 55°C and reaction for 100 s at 72°C, followed by a final 5 min extension at 72°C. PCR products were separated by gel electrophoresis using 2% SeaKem LE agarose in 1X TAE buffer.

Assay of Mitochondrial Membrane Potential (MMP)

3,3′ Dihexyloxa carbocyanine iodide ($DIOC_6$) was added directly to the cell culture medium (1 μM final concentration). After 30 min incubation, the cells were pelleted and resuspended with PBS. The cells were submitted to flow cytometry on the Epics WL (Beckman Coulter) to measure the MMP. Data were acquired and analyzed using EXPO32 ADCXL 4 color software. The analyzer threshold was adjusted on the forward scatter channel to exclude noise and most of the subcellular debris.

Co-immunoprecipitation (Co-IP)

Cell extracts that were incubated with antibodies were precipitated with protein A-Sepharose beads. Immunoprecipitated proteins were separated on SDS-PAGE, and Western blot analysis was performed as described. Each Co-IP experiment was confirmed via reciprocal IP.

Statistical Analysis

Four independent experiments were conducted in vitro. The results are expressed as the means ± standard deviations from 4 experiments, each performed in triplicate. Statistical significance of differences was determined using the paired Kruskal-Wallis nonparametric test. A P value $<.05$ was considered to be statistically significant.

Results

αB-Crystallin Is Expressed in Human U373MG Glioma Cells

We first screened the expression of αB-crystallin in 3 glioblastoma cell lines: U373MG, U118MG, and T98G. An RT–PCR assay showed the expression of the αB-crystallin gene in U373MG cells but not in U118MG and T98G cells (Fig. 1A). A Western blot assay further confirmed the expression of the αB-crystallin protein in U373MG cells but not in U118MG and T98G cells (Fig. 1A). To examine the prevalence of the expression of αB-crystallin, we evaluated gene expression profile data from The Cancer Genome Atlas Network.[35] Our test on *CRYAB* expression between normal tissues and subtypes (proneural, neural, classical, and mesenchymal) from the cohort of GBM showed that the expression of *CRYAB* was significantly up-modulated in the neural subtype ($P < .001$).

Human Glioma U373MG Cells Undergo Apoptosis After Treatment with SAHA.

To explore the anti-apoptotic role of αB-crystallin in glioma, αB-crystallin-expressing U373MG cells were treated with a histone deacetylase inhibitor, SAHA. SAHA treatment significantly decreased the viability of U373MG cells in a dose-dependent manner (Fig. 1B). Because the viability of the U373MG cells treated with 10 μM for 48 h was approximately 50%, this concentration was used solely in our further analyses. At 10 μM, SAHA significantly reduced the viability of U373MG cells in a time-dependent manner (Fig. 1C). Further assays were then undertaken to determine whether U373MG cells treated with SAHA undergo apoptosis. Western blot assays demonstrated the production of caspase-3, -6, -7, -8, -9, and 12 cleavage products in cells treated with SAHA. Western blotting assay also showed the degradation of PARP and the production of its cleavage product (Fig. 1D). Confocal microscopy demonstrated that SAHA induced the release of cytochrome *c* from the mitochondria in U373MG cells (Fig. 1E), and Hoechst staining showed that the cells treated with SAHA had fragmented atypical nuclei (Fig. 1F). By flow cytometry, we found that SAHA increased the subdiploid apoptotic population in the U373MG cells (Fig. 1F). These data suggested that the cell death induced by SAHA in U373MG cells is apoptotic. Although SAHA treatment decreased the level of the αB-crystallin protein, the levels of other HSPs (60, 70, and 90) were sustained. Anti-apoptotic factors, such as survivin and XIAP, were substantially downregulated by the SAHA treatment (Fig. 1G).

siRNA Against αB-crystallin Sensitizes U373MG Cells to SAHA-Induced Cell Death

To examine whether the silencing of αB-crystallin sensitized the U373MG cells to SAHA-induced apoptosis, the

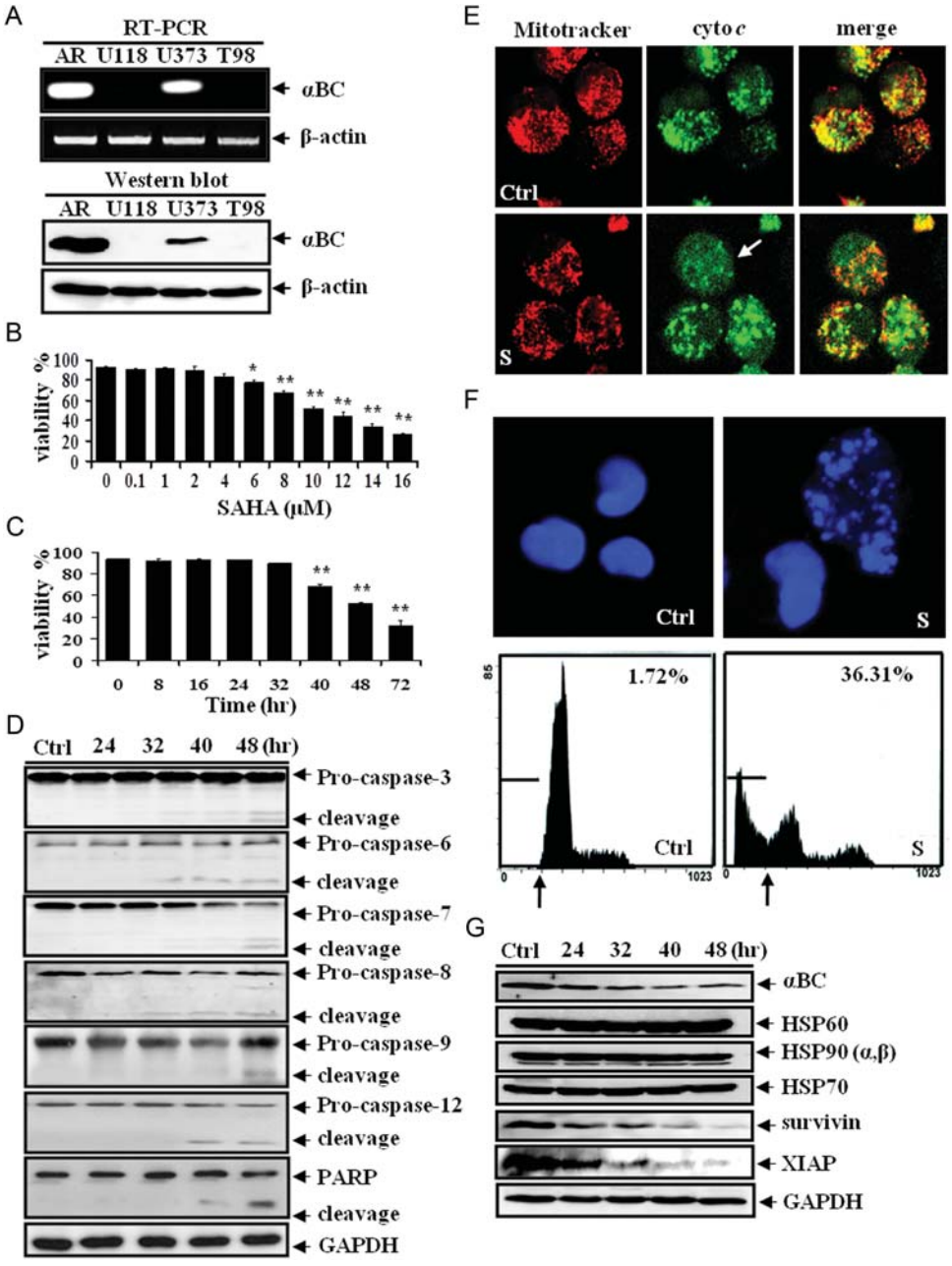

Fig. 1. SAHA-induced apoptosis was mediated by αB-crystallin in U373MG cells. ARPE-19 cells that endogenously expressed αB-crystallin were used as a positive control. (A) Expression of αB-crystallin in untreated cells. Total RNA was extracted from cultured ARPE-19, U118MG, U373MG and T98G cells (upper panel). Western blot analysis showing the expression level of αB-crystallin protein (lower panel). β-actin, a loading control. (B and C) Viability as determined using the trypan blue exclusion assay. Cells were treated with 10 μM SAHA for 48 h. $*P < .05$, $**P < .01$. The asterisks indicate a significant difference compared with the untreated control. (D) Western blots of caspases and PARP. The degradation of PARP and the production of cleavage products of PARP and caspase subtypes were demonstrated. (E) Confocal microscopic images showing the localization of cytochrome *c* (cyto *c*). Mitochondria were labeled by incubating the cells with 20 nM Mitotracker Red for 20 min at 37°C before fixation. (F) Hoechst staining conducted on the cells harvested 48 h after SAHA treatment (upper panel). Representative histograms showing cell cycle progression and induction of apoptosis (lower panel). (G) Western blots of αB-crystallin, Hsp60, Hsp90, Hsp70, survivin and XIAP. AR, ARPE-19 cells. U118, U118MG cells. U373, U373MG cells. T98, T98G cells. Ctrl, control. S, SAHA. αBC, αB-crystallin.

cells were treated with SAHA at 2 μM, a dose that alone did not reduce the viability of the U373MG cells. Of note, the silencing of αB-crystallin by siRNA significantly sensitized the U373MG cells to SAHA and led to cell death (Fig. 2A). Flow cytometry showed that the silencing of αB-crystallin augmented the accumulation of subdiploid apoptotic cells (Fig. 2B), and confocal microscopy showed that the αB-crystallin siRNA

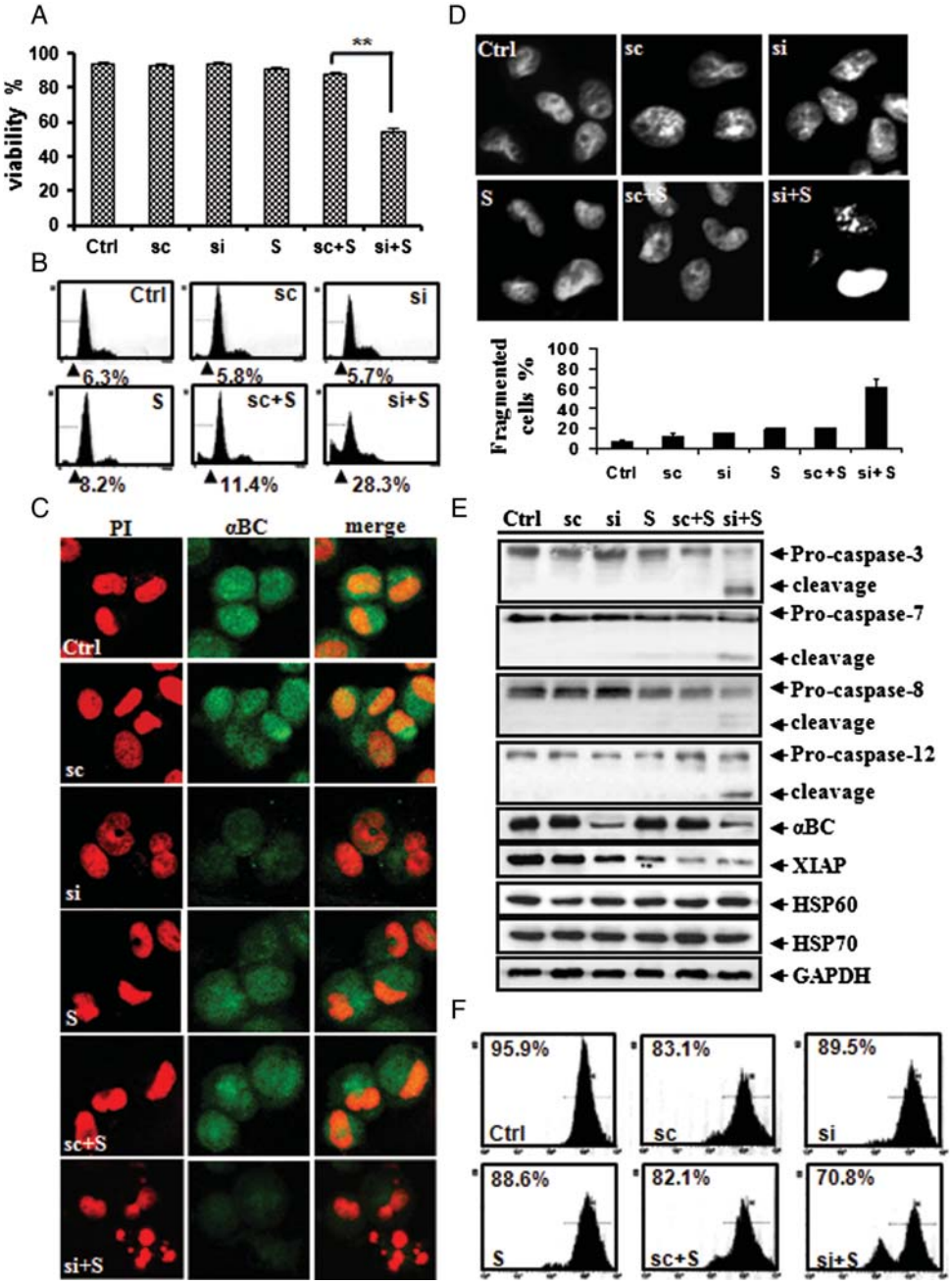

Fig. 2. Silencing of αB-crystallin by siRNA significantly sensitized U373MG cells to SAHA-induced cell death. Assays were performed on U373MG cells at 48 hs after treatment with a sublethal dose, 2 μM SAHA, with or without αB-crystallin siRNA or scrambled RNA. (A) Viability as determined by the trypan blue exclusion assay. αB-crystallin siRNA significantly sensitizes U373MG cells to SAHA-induced cell death. $^{**}P < .01$. The asterisks indicate a significant difference compared to the experimental control treated with scrambled RNA and SAHA. (B) Representative histograms the showing cell cycle progression and induction of apoptosis. (C) Confocal microscopy showing that a specific siRNA knockdown markedly depleted αB-crystallin at 48 h after treatment. PI, propidium iodide labeling of nuclei (Red). (D) Nuclear morphology (upper panel) after Hoechst staining. Percentage of cells with condensed or fragmented nuclei is depicted in the diagram (lower panel). (E) Western blot assay on apoptosis-related proteins. GAPDH, a loading control. (F) Flow cytometry showing mitochondrial membrane potential. See Fig. 1 for other definitions. Si, small interfering RNA directed against αB-crystallin; sc, scramble control.

markedly depleted αB-crystallin in the U373MG cells treated with SAHA, which showed nuclear condensation or fragmentation (Fig. 2C). Hoechst staining showed that the silencing of αB-crystallin enhanced the nuclear fragmentation and condensation (Fig. 2D). A Western blotting assay showed that the silencing of

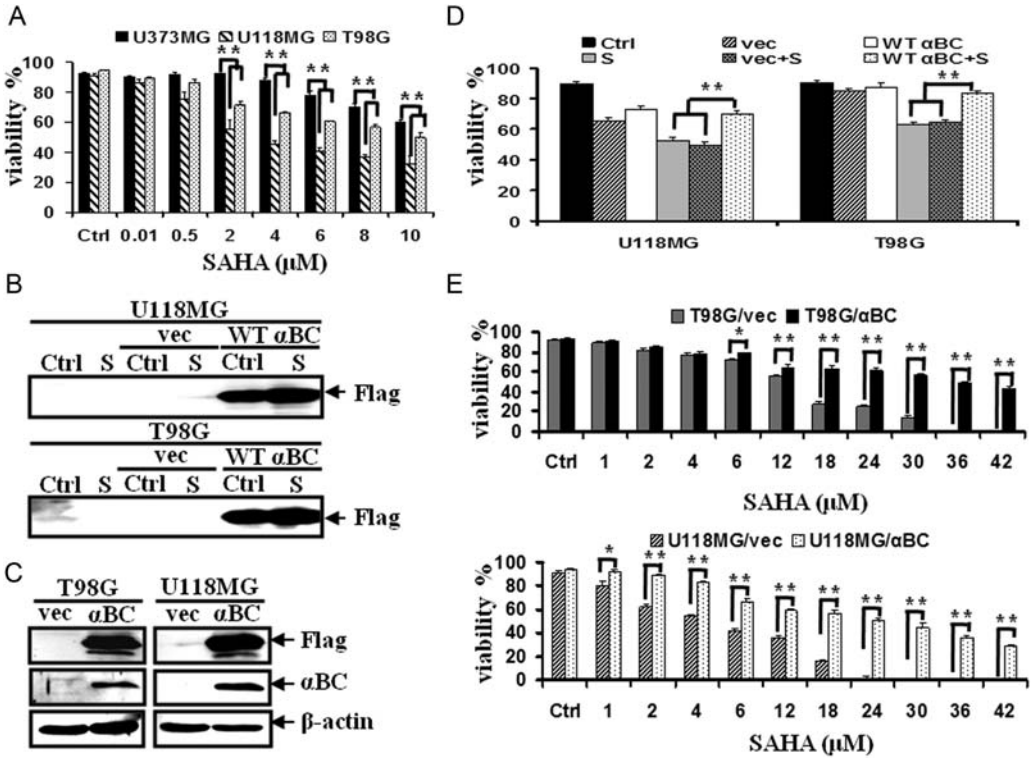

Fig. 3. αB-crystallin inhibits SAHA-induced cytotoxicity in glioma cells. (A) Viability as determined by the trypan blue exclusion assay. U373MG, black bar. U118MG, stripped bar. T98G, dotted bar. The cells were treated with different concentrations of SAHA for 48 h. $^{**}P < .01$. The asterisks indicate a significant difference compared with the untreated control. (B) Western blot analysis of exogenously over-expressed Flag-tagged αB-crystallin protein in U118MG and T98G cells. Empty 3x Flag-pcDNA vector (vec) served as a negative control. (C) The Western blot assay showing the over-expression of Flag-tagged αB-crystallin in the T98G and U118MG cells. β-actin, a loading control. (D) Viability was determined using the trypan blue exclusion assay. Ctrl, black bar. vec, stripped bar. WT αBC, white bar. S, shaded bar. vec + S, hatched bar. WT αBC + S, dotted bar. The cells were treated with 2 μM SAHA for 48 h. The transient over-expression of αB-crystallin prevented the reduction of viability by SAHA in both the U118MG and T98G cells. $^{**}P < .01$. The asterisks indicate a significant difference compared to each experimental control, containing empty 3x Flag-pcDNA (vec), treated with SAHA or SAHA alone. (E) Viability was determined using the trypan blue exclusion assay. T98G/vec, shaded bar. T98G/αBC, black bar. U118MG/vec, stripped bar. U118MG/αBC, dotted bar. Stable αB-crystallin-over-expressing cells, T98G/αBC and U118MG/αBC, were significantly resistant to SAHA treatment compared to each control cell line containing the empty vector. $^{*}P < .05$, $^{**}P < .01$. The asterisks indicate a significant difference compared to each control cell, containing the empty vector. See Fig. 1 for other definitions.

αB-crystallin enhanced the production of the cleavage products of caspase-3, -7, -8, and -12. Although the silencing of αB-crystallin augmented the degradation of XIAP, it did not substantially downregulate Hsp60 or Hsp70 (Fig. 2E). Flow cytometry also showed that the silencing of αB-crystallin enhanced the reduction of MMP (Fig. 2F). These data indicate that endogenously expressed αB-crystallin exerts an anti-apoptotic role in U373MG cells.

αB-Crystallin Inhibits the Cytotoxicity by SAHA in Glioma Cells

A viability assay using the 3 glioma cell lines treated with different concentrations of SAHA showed that the U373MG cells endogenously expressing αB-crystallin are more resistant to SAHA-induced cytotoxicity, as by SAHA compared with the other 2 glioma cell lines, U118MG and T98G, which did not endogenously express αB-crystallin (Fig. 3A). We next examined whether the ectopic expression of αB-crystallin in the U118MG and T98G cells protects them from the cytotoxicity by SAHA. αB-crystallin was transiently transfected into the U118MG and T98G cells, and the cells were then treated with 2 μM SAHA. Of note, 2 μM SAHA substantially induces cell death in the control cells containing empty vector, U118MG/vec and T98G/vec, but not in the typical U373MG cells. Of importance, the transient over-expression of αB-crystallin prevented the reduction of viability induced by SAHA in both of the U118MG/vec and T98G/vec cells (Fig. 3B–D). We next established αB-crystallin–over-expressing U118MG and T98G cells, and these cells were treated various doses of SAHA. Of note, the αB-crystallin–over-expressing U118MG and T98G cells were markedly resistant to SAHA when compared with each control cell containing the empty vector

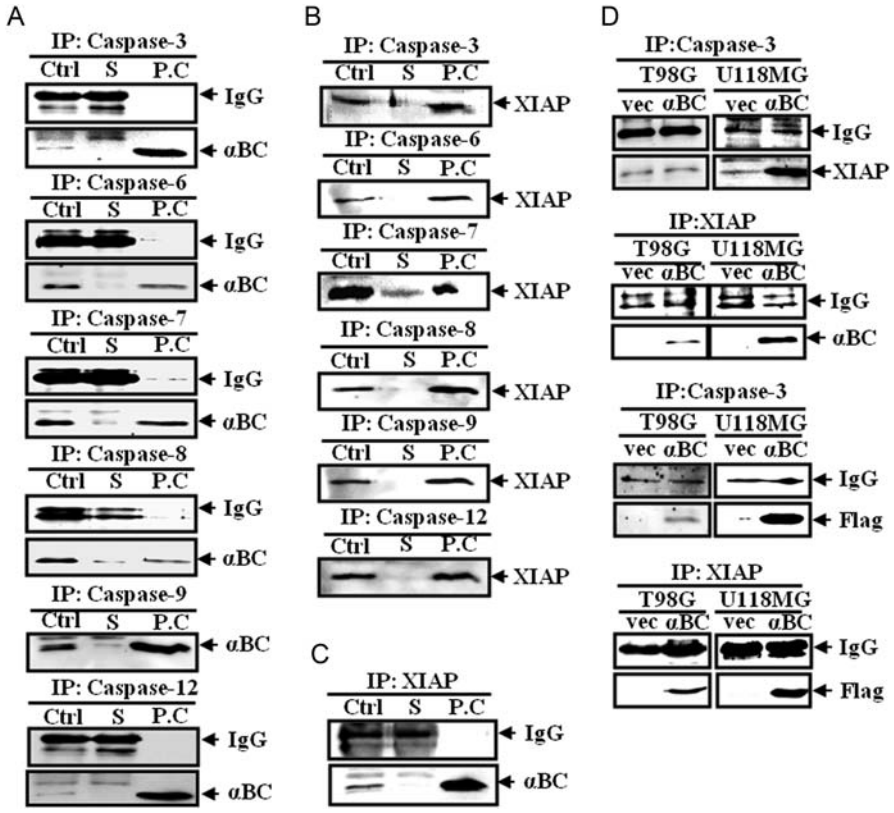

Fig. 4. Western blots showing interaction between caspase subtypes and αB-crystallin or XIAP. (A) Coimmunoprecipitation assay data showing the interaction of caspase subtypes and αB-crystallin in U373MG cells. (B) Co-immunoprecipitation assay data showing the interaction of each caspase subtype and XIAP in U373MG cells. (C) Co-immunoprecipitation assay data showing the interaction of XIAP and αB-crystallin in U373MG cells. (D) Co-immunoprecipitation assay data showing the interaction of caspase-3 and XIAP or the interaction of XIAP and αB-crystallin in untreated T98G and U118MG cells. See Fig. 1 for other definitions. Abbreviations: P.C, positive control; IP, immunoprecipitation; IgG, Immunoglobulin G.

(Fig. 3E). These results indicate that αB-crystallin inhibits the cytotoxicity induced by SAHA in glioma cells.

αB-Crystallin Associates with XIAP and Caspase Subtypes

Because previous reports showed that αB-crystallin regulates apoptosis through interactions with caspase-3, we next examined whether caspase subtypes associates with αB-crystallin in U373MG cells. We screened capases-3, -6, -7, -8, -9, and -12 in the control U373MG cells with use of a co-immunoprecipitation assay and found that αB-crystallin interacted with all of the caspase subtypes tested (Fig. 4A). Because XIAP is known to associate with these caspase subtypes, we examined whether XIAP interacts with these caspase subtypes in U373MG cells. As predicted, XIAP interacted with αB-crystallin and these caspase subtypes in U373MG cells (Fig. 4B and C). Furthermore, the SAHA treatment dissociated these caspase subtypes from αB-crystallin and XIAP and dissociated XIAP from αB-crystallin (Fig. 4A–C). αB-crystallin also physically interacted with all caspase subtypes and XIAP in stable, αB-crystallin–over-expressing glioma cells, U118MG/αBC and T98G/αBC cells (Fig. 4D, Western blot data on caspase-6, -7, -8, -9, and -12 are not shown).

αB-Crystallin Endogenously Expressed in U373MG Cells Overrides the Anti-Apoptotic Activity of XIAP

Previous studies reported that αB-crystallin exerts anti-apoptotic activity by binding and sequestering cell death–related factors. Because we observed that αB-crystallin is associated with XIAP in U373MG cells in which endogenously expressed αB-crystallin plays a pivotal anti-apoptotic role, we asked whether XIAP also functions as an anti-apoptotic factor in these cells. Because the silencing of αB-crystallin sensitized the U373MG cells to 2 μM SAHA and led to apoptosis (Fig. 2), we tested whether the silencing of XIAP sensitizes the U373MG cells to 2 μM SAHA and led to apoptosis. Of note, the silencing of XIAP did not significantly alter the amount of apoptosis induced by SAHA in the U373MG cells, as determined by various assays that evaluate apoptosis (Fig. 5A–D). Western blotting assay and confocal microscopy confirmed that the

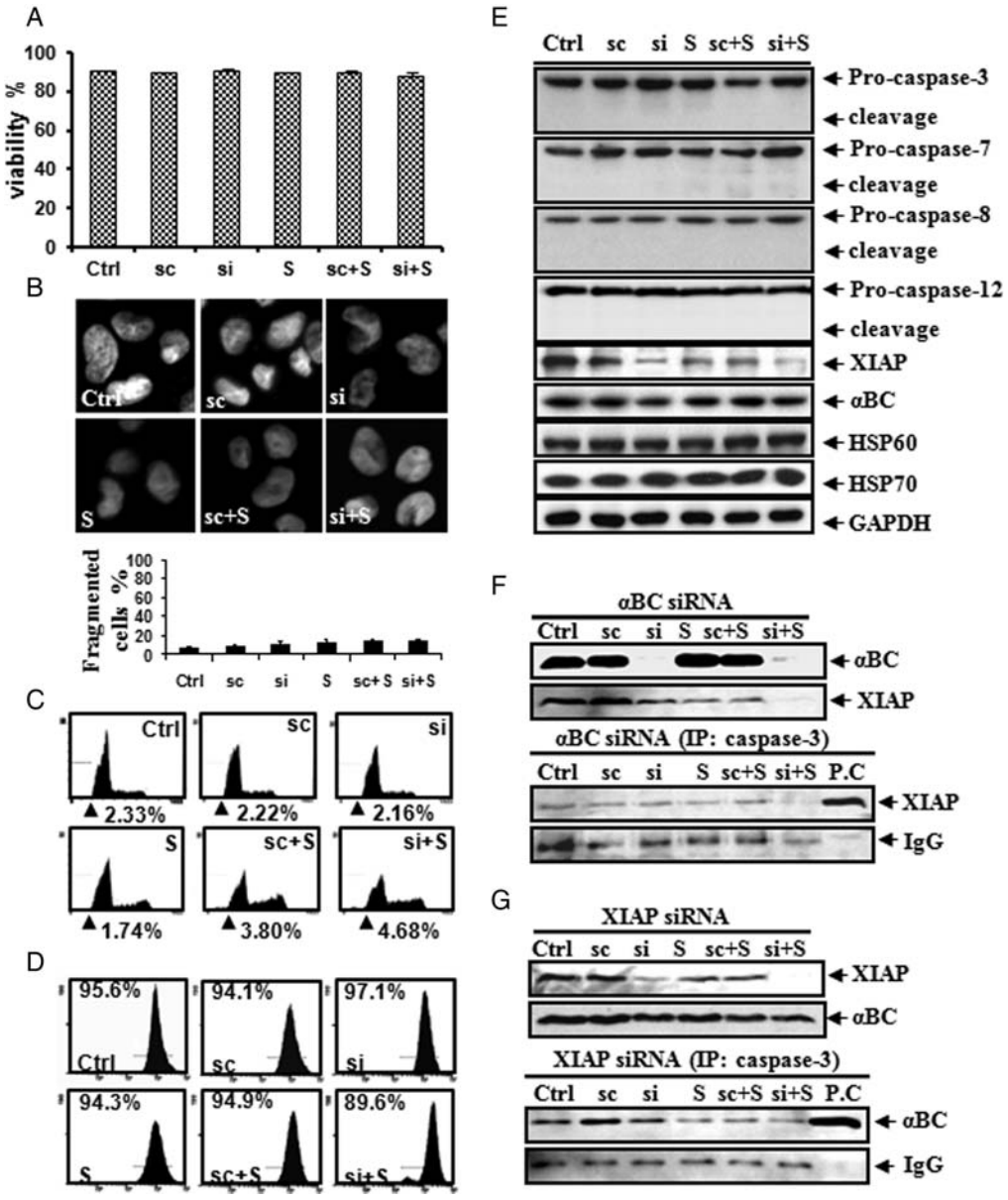

Fig. 5. Silencing of XIAP by siRNA did not alter the amount of cell death induced by SAHA in U373MG cells. Assays were performed on U373MG cells at 48 h after treatment with a sublethal dose of SAHA (2 μM), with or without XIAP siRNA or scrambled RNA. (A) Viability assay. XIAP siRNA did not alter the amount of cell death in the U373MG cells treated with SAHA. (B) Nuclear morphology after Hoechst staining (upper panel). Percentage of cells with condensed or fragmented nuclei is depicted in the diagram (lower panel). (C) Representative histograms showing the cell cycle progression and induction of apoptosis. (D) Flow cytometry showing the mitochondrial membrane potential. (E) Western blot assay on apoptosis-related proteins. GAPDH, a loading control. (F) Western blot and co-immunoprecipitation after the silencing of αB-crystallin in U373MG cells. (G) Western blot and co-immunoprecipitation after the silencing of XIAP in U373MG cells. See Fig. 1 for other definitions.

caspase subtypes are not activated in the U373MG cells treated with 2 μM SAHA, even though the XIAP protein was efficiently depleted by siRNA (Fig. 5E and Supplementary Fig. S1). Although the αB-crystallin siRNA substantially downregulated the XIAP protein expression and abolished the interaction between XIAP and caspase-3, the XIAP siRNA altered neither the expression level of the αB-crystallin protein nor the interaction between αB-crystallin and caspase-3 (Fig. 5F and G). We next examined functional hierarchy between αB-crystallin and XIAP. Of importance, transient overexpression of XIAP reversed neither the downregulation of αB-crystallin by SAHA treatment nor the cell death induced by αB-crystallin knockdown in presence of SAHA (Fig. 6A and B). These data indicate that, unlike αB-crystallin, XIAP does not function as a major anti-apoptotic factor in U373MG cells.

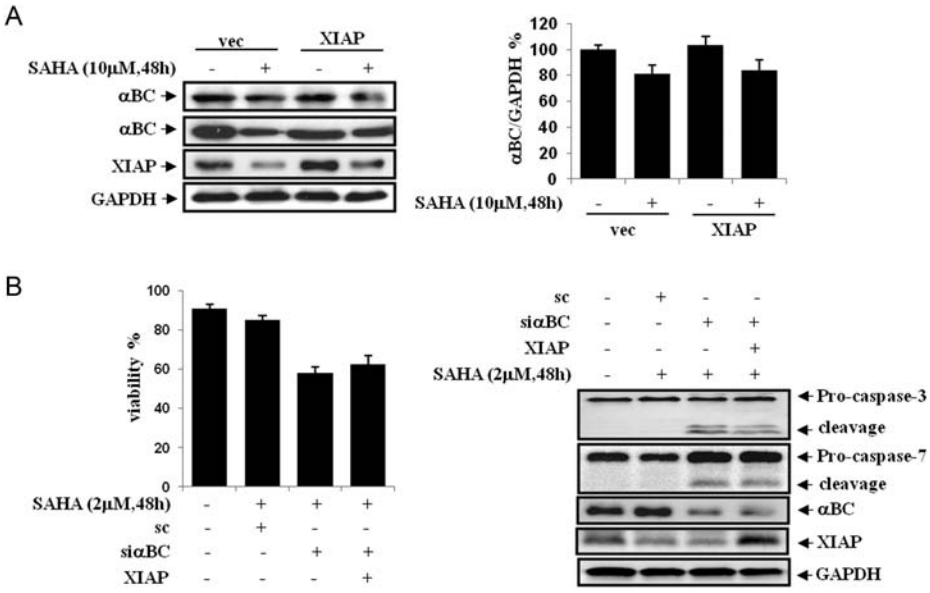

Fig. 6. Transiently over-expressed XIAP reversed neither the downregulation of αB-crystallin by SAHA treatment nor the cell death induced by αB-crystallin knockdown in presence of SAHA. (A) Transiently over-expressed XIAP did not reverse the downregulation of αB-crystallin by SAHA treatment. Western blot assay on the expression of αB-crystallin and XIAP after treatment with 10 μM SAHA for 48 h in U373MG cells. GAPDH, a loading control (left panel). The αB-crystallin were quantified and normalized relative to a GAPDH control band with ImageJ version 1.43u (right panel). (B) Transiently over-expressed XIAP did not reverse SAHA-induced cell death in αB-crystallin knockdowned U373MG cells. Viability assay (left panel). Western blot assay on apoptosis related factors (right panel). Cells were treated with a sublethal dose (2 μM) of SAHA for 48 h. GAPDH, a loading control.

Ectopic Expression of αB-Crystallin in Human Glioma U118MG and T98G Cells Overrides the Anti-Apoptotic Activity of XIAP

We next examined whether XIAP sustains its anti-apoptotic activity in the human glioma cells that are ectopically expressing αB-crystallin. We treated the cells with 2 μM SAHA, which slightly induced apoptosis in U118MG/vec and T98G/vec cells. We found that viability was slightly reduced and that the cleavage product of caspase-3 was produced in both the U118MG/vec and the T98G/vec cells. Furthermore, the XIAP siRNA augmented the level of the cleavage product of caspase-3 and the amount of cell death, indicating that XIAP exerts its anti-apoptotic activity (Fig. 7A and B). Of importance, the XIAP siRNA did not augment the level of the caspase-3 cleavage product or the amount of cell death in αB-crystallin–over-expressing T98G and U118MG cells (Fig. 7C and D). These data suggest that the ectopic expression of αB-crystallin in human glioma U118MG and T98G cells overrides the anti-apoptotic role of XIAP.

Endogenous or Ectopic Expression of αB-Crystallin Decreases the Sensitivity of Glioma Cells to Various Anti-Cancer Drugs

We examined whether αB-crystallin confers chemoresistance to various anti-cancer drugs in glioma cells. The 3 glioma cell lines were treated with 7 anti-cancer drugs, and a viability assay was performed. Our data show that the U373MG cells are more chemoresistant than are the other 2 cell lines (Fig. 8A). Of importance, we found that silencing of aB-crystallin but not XIAP sensitized U373MG cells to anti-cancer drug-induced cell death (Fig. 8B). We further examined the effect of the ectopic expression of αB-crystallin in the U118MG and T98G cells and found that the αB-crystallin–over-expressing U118MG and T98G cells were more chemoresistant than were the control cells containing the empty vector (Fig. 8C and D).

Discussion

Numerous HDACis have been characterized, and SAHA is an HDACis that is known as a therapeutic agent under various cell-proliferating conditions.[33] In preclinical studies, SAHA, an inhibitor of several members of the HDAC protein family,[34] has also been observed to have anti-glioma activity. SAHA causes glioma cells to accumulate in the G2-M phase of the cell cycle, decreases the levels of cyclin-dependent kinase, and inhibits glioma cell growth in orthotopic models.[36] The present study has also demonstrated that SAHA shows anti-glioma activity by causing apoptotic cell death in human glioma U373MG cells.

αB-crystallin is expressed in some glioma cells, such as U138MG, LN-319, and U373MG cells.[37] Furthermore, previous studies reported that αB-crystallin is mainly expressed in experimental xenograft models and in human gliomas in situ.[30] Although little is known about its functional role in tumor progression,[30] αB- crystallin has been

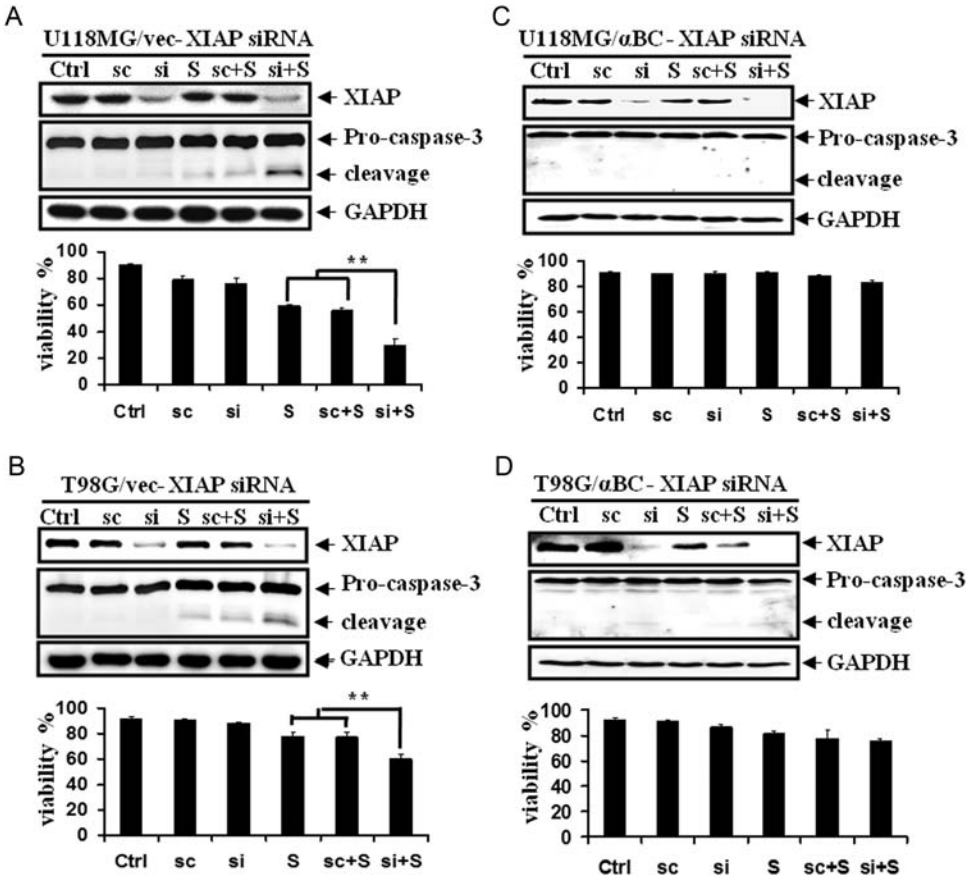

Fig. 7. Over-expressed αB-crystallin in human glioma cells overrides the anti-apoptotic activity of XIAP. Assays were performed with U373MG cells at 48 h after treatment with a sublethal dose of SAHA (2 μM), with or without XIAP or scrambled RNA. (A and B) Western blot and viability assays were performed with U118MG/vec (A) and T98G/vec (B) cells at 48 h after treatment with a sublethal dose (2 μM) of SAHA, with or without XIAP siRNA or scrambled RNA. GAPDH, a loading control. $^{**}P < .01$. The asterisks indicate a significant difference compared with the experimental control treated SAHA alone or scrambled RNA with SAHA. (C and D) Western blot and viability assays were undertaken on U118MG/αBC (C) and T98G/αBC cells (D) at 48 h after treatment with a sublethal dose (2 μM) of SAHA, with or without XIAP siRNA or scrambled RNA. GAPDH, a loading control. See Fig. 1 for other definitions.

shown to be expressed in high-grade gliomas.[38] The present work showed that αB-crystallin is expressed in U373MG glioma cells but not in T98G and U118MG glioma cells and that the expression of *CRYAB* was significantly up-modulated in the neural subtype.

On the basis of previous studies, the molecular role of αB-crystallin in glioma cells can be predicted. αB-crystallin is a member of the small heat shock protein family, which also includes Hsp27, whose members have a high degree of sequence homology.[39] Thus, αB-crystallin could exert its activity as molecular chaperone in glioma cells. Hsps function as stress-induced molecular chaperones to inhibit the aggregation of denatured proteins, thereby promoting cell survival.[40] Although both αB-crystallin and Hsp27 prevent cells from stress-induced apoptosis at various signaling steps, the exact mechanism or regulating targets seem to be different.[41–43]

Other studies showed that the ectopic expression of αB-crystallin in diverse cell types protected cells against diverse apoptotic stimuli, including TNF-α, TNF-related apoptosis-inducing ligand (TRAIL), etoposide, growth factor deprivation, and oxidative stress. In addition, the silencing of αB-crystallin expression by RNA interference (RNAi) sensitizes cells to apoptosis.[44] In the study presented here, we observed that the silencing of αB-crystallin by siRNA sensitized the U373MG cells to sublethal doses of SAHA, indicating that αB-crystallin caused the inhibition of toxicity by SAHA in the U373MG cells. A previous study demonstrated that αB-crystallin binds to both procaspase-3 and the partially processed procaspase-3 intermediate, preventing apoptosis.[45] The present study suggests that an entirely different mechanism underlies the anti-apoptotic activity exerted by αB-crystallin. According to the data obtained in the present study, αB-crystallin expressed endogenously or ectopically in human glioma cells overrides the anti-apoptotic role of XIAP.

Mammals have developed regulatory proteins, members of the IAP (inhibitor of apoptosis) family, which target a subset of the caspases.[46] The IAP family, including XIAP, cIAP1, and cIAP2, are capable of directly inhibiting the effector, caspase-3.[47] Among the IAP family members, the X chromosome-linked

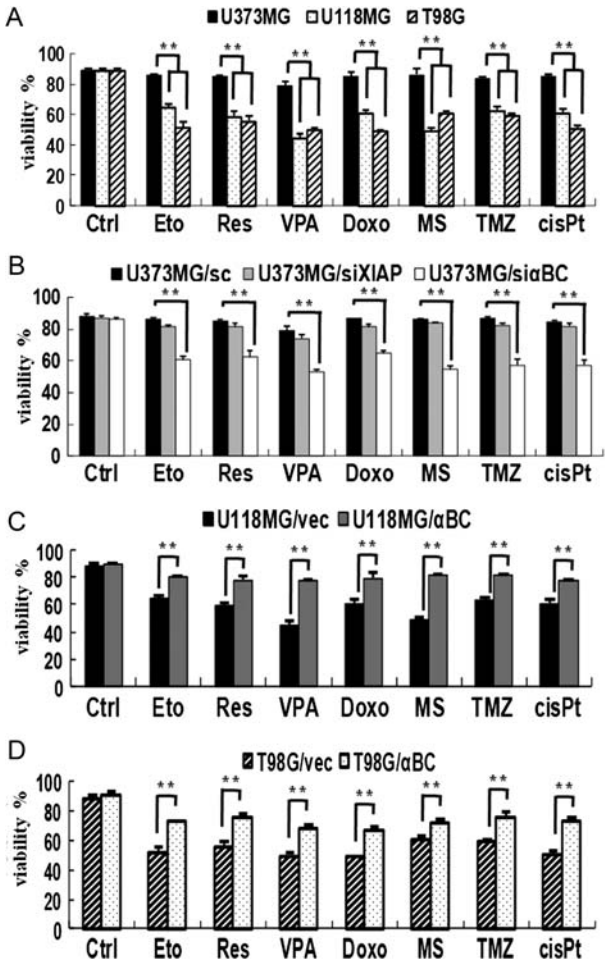

Fig. 8. Endogenous and ectopic expression of αB-crystallin decreases the sensitivity to various anti-cancer drugs. Assays were performed with U373MG, U118MG and T98G cells at 48 h after treatment with 40 μg/mL etoposide, 200 μM resveratrol, 30 mM valproic acid, 10 μg/mL doxorubicin, 11 μM MS-275, 25 μg/mL Temozolomide and 25 μM cisplatin. (A) Viability as determined by the trypan blue exclusion assay. U373MG, black bar. U118MG, dotted bar. T98G, stripped bar. Cells were treated with various anti-cancer drugs for 48 h. $^{**}P < .01$. The asterisks indicate a significant difference compared with the each experimental control depleted αB-crystallin. (B) Silencing of aB-crystallin but not XIAP sensitized U373MG cells to anti-cancer drug-induced cell death. U373MG/sc, black bar. U373MG/siXIAP, shaded bar. U373MG/siαBC, white bar. $^{**}P < .01$. The asterisks indicate a significant difference compared with the each experimental control. (C and D) U118MG/vec, black bar. U118MG/αBC, shaded bar. T98G/vec, stripped bar. T98G/αBC, dotted bar. Stably transfected cells over-expressing αB-crystallin, T98G/αBC and U118MG/αBC, substantially inhibited anti-cancer drug-induced cell death compared to the control cells containing the empty vector. $^{**}P < .01$. The asterisks indicate a significant difference compared to each experimental control. See Fig. 1 for other definitions. CisPt, cisplatin; Ctrl, control; Doxo, doxorubicin; Eto, etoposide; MS, MS-275; Res, resveratrol; TMZ, temozolomide; VPA, valproic acid.

inhibitor of apoptosis protein (XIAP) is considered as the most potent suppressor of cell death.[48] Previous studies showed that the knocking down of XIAP with siRNA or antisense oligonucleotides generally restores chemosensitivity to a variety of malignant cell lines.[49,50] Furthermore, some reports demonstrated that the down-regulation of XIAP induces apoptotic death in human glioblastoma cells.[51] However, XIAP functions as an anti-apoptotic factor in neither U373MG cells, which are endogenously expressing αB-crystallin, nor in U118MG and T98G cells ectopically over-expressing αB-crystallin. Accordingly, αB-crystallin expressed endogenously or ectopically in human glioma cells seems to override the anti-apoptotic activity of XIAP. This is supported by the data indicating that αB-crystallin confers glioma cells the chemoresistance to various anti-cancer drugs.

The combined treatment to reduce tumor formation in various cancer cells may represent an effective cancer treatment for the future.[52,53] Exogenous RNAi molecules (siRNA, oligodeoxynucleotides, micro-RNA, and short-hairpin RNA) also have the potential for therapeutic agents in human diseases,[53] and several factors that confer resistance to cancer cells are over-expressed in a broad range of tumors. Thus, combining the selective knockdown of these factors using RNAi tools with chemotherapeutic drugs could be an effective strategy for anti-cancer treatment.[54] Because the αB-crystallin expressed in glioma cells plays a pivotal anti-apoptotic role, the combined therapy of αB-crystallin siRNA with SAHA could be a therapeutic strategy to control αB-crystallin-expressing glioma cells.

In summary, αB-crystallin is expressed in glioma cells and overrides the anti-apoptotic activity of XIAP.

Supplementary Material

Supplementary material is available at Neuro-oncology Journal online (http://neuro-oncology.oxfordjournals.org/).

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