Lymphokine-Activated Killer T-Cell-Originated Protein Kinase Phosphorylation of Histone H2AX Prevents Arsenite-Induced Apoptosis in RPMI7951 Melanoma Cells

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Abstract

Purpose: Arsenic is a valuable therapeutic tool in cancer treatment. Lymphokine-activated killer T-cell-originated protein kinase (TOPK) is highly expressed in cancer cells, but its specific function is still unknown. We investigated the role of TOPK in arsenic-induced apoptosis in RPMI7951 human melanoma cells.

Experimental Design: Expression of TOPK was evaluated in different melanoma cell lines, and liquid chromatography-tandem mass spectrometry analysis was used to identify proteins binding with TOPK. Immunofluorescence, Western blot, and flow cytometry were used to assess the effect of arsenic on TOPK, histone H2AX, and apoptosis in RPMI7951 cells.

Results: Melanoma cell lines expressing high levels of TOPK were more resistant to arsenite (As3+) -induced apoptosis. As3+ treatment induced phosphorylation of TOPK and histone H2AX in RPMI7951 human melanoma cells. Liquid chromatography-tandem mass spectrometry results indicated that TOPK could bind with histone H2AX, and in vitro and in vivo assays confirmed that TOPK binds with and phosphorylates histone H2AX. As3+ treatment caused phosphorylation of TOPK, which colocalized with phosphorylated histone H2AX in the nucleus. TOPK small interfering RNA cells exhibited a decreased phosphorylation of histone H2AX with As3+ treatment. As3+-induced apoptosis was decreased in H2AX−/− cells but increased in TOPK small interfering RNA cells.

Conclusions: TOPK binds with histone H2AX and inhibits As3+-induced apoptosis through phosphorylation of histone H2AX. Melanoma cell lines with high levels of TOPK are more resistant to As3+-induced apoptosis. Therefore, inhibition of TOPK activity combined with As3+ treatment may be helpful in the treatment of melanomas.

Lymphokine-activated killer T-cell-originated protein kinase (TOPK) is a novel mitotic protein kinase that is highly expressed only in various cancers, such as leukemia, myeloma, and lymphoma, and its expression has been correlated with the malignant potential of these tumors (1–4). TOPK is phosphorylated and active only during mitosis (2) and has been shown to phosphorylate the p38 mitogen-activated protein kinase but not extracellular signal-regulated kinases or c-Jun NH2-terminal kinases (1N; ref. 1). Recent reports showed that TOPK is up-regulated in murine myeloma cells by an interleukin-6-mediated protein-protein interaction between TOPK and Raf-A (4, 5). In addition, TOPK is involved in cell cycle regulation and can be a substrate of cdc2/cyclin B (2, 6). TOPK expression has also been shown to be up-regulated during the G2 to M phase transition, where the cdc2/cyclin B complex plays an important role (6). Thr9 is an important phosphorylation site of TOPK because, when Thr9 was substituted to Ala, the binding ability of TOPK to cdc2/cyclin B was decreased (6). The cell cycle–specific transcription factors E2F and cyclic AMP-responsive element binding protein/activating transcription factor are critical regulators of TOPK expression during growth arrest in leukemia cells (7). Thus, TOPK may be a potential target for chemotherapeutic or chemopreventive compounds.

The effectiveness of many therapeutic approaches, including γ-irradiation and chemotherapeutic drug treatment, has been proposed to be associated with the reactivation of apoptosis in cancer cells. Arsenic is a paradoxical compound that induces global changes in gene expression and cell signal transduction pathways in different types of cells (8, 9). Dose-dependent effects of arsenic in a tissue-specific manner have enabled arsenic to be used for the effective treatment of certain types of cancers, including leukemia and myeloma, through its...
induction of apoptosis (10, 11). Arsenic treatment has been shown to induce the up-regulation of cyclin B1 and activates the cdc2/cyclin B1 complex in mitotic cells (12) and therefore could have an effect on TOPK and its potential binding partners.

Melanomas develop through well-defined morphologic and histologic stages that involve the loss of cell proliferation control, the acquisition of invasiveness, and, ultimately, the acquisition of metastatic potential (13). Melanoma cells generally express chromosome instability during their development (14). Histone H2AX has been shown to prevent aberrant repair of both programmed and general DNA breakage (15). H2AX deficiency decreases genomic stability and increases tumor susceptibility of normal cells and tissues (16). In addition, a lack of histone H2AX causes genomic instability in mice (17). Here, we have investigated a functional relationship and interaction of TOPK and histone H2AX in mediating arsenite (As3+)–induced apoptosis in melanoma cells. Results indicated that As3+–induced phosphorylation of TOPK and TOPK directly phosphorylated histone H2AX, resulting in the inhibition of As3+–induced apoptosis in RPMI7951 melanoma cells. This suggested that TOPK could be a potential target for chemotherapeutic treatment of melanomas.

Materials and Methods

Reagents and antibodies. As3+ and anti-β-actin were from Sigma (St. Louis, MO); NE-PER Nuclear and Cytoplasmic Extraction Reagents were from Pierce Biotechnology (Rockville, IL). Stock solutions of As3+ (10 μM/mL) in 0.1% DMSO were stored at −20°C. PDZ-binding kinase/TOPK, phosphorylated PDZ-binding kinase/TOPK (Thr2) antibodies, and active glutathione S-transferase-TOPK were from Cell Signaling Technology, Inc. (Beverly, MA). anti-histone H2AX, anti-phosphorylated H2AX, and histone H2AX (recombinant protein expressed in Escherichia coli) were from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-hemagglutinin (HA) probe (F-7) was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture. SK-MEL5 and SK-MEL28 human malignant melanoma and SK-MEL31 and RPMI7951 human malignant melanoma epithelial-like cell lines were from the American Type Culture Collection (Manassas, VA). Human melanoma cell lines were cultured in MEM containing 10% fetal bovine serum (FBS; 15% FBS for SK-MEL31), 2 mmol/L l-glutamine, and 25 μg/mL gentamicin, 0.1 mmol/L nonessential amino acids, and 1 mmol/L sodium pyruvate (SK-MEL31 also required 1.5 g/L sodium bicarbonate) at 37°C in humidified air with 5% CO2. Mouse epidermal JB6 promotion-sensitive C41 cells were cultured in MEM containing 5% FBS, 2 mmol/L l-glutamine, and 25 μg/mL gentamicin at 37°C in humidified air with 5% CO2. Mouse epidermal JB6 promotion-sensitive C41 cells were cultured in MEM containing 5% FBS, 2 mmol/L l-glutamine, and 25 μg/mL gentamicin at 37°C in humidified air with 5% CO2.

Generation of the TOPK-overexpressing cell line. pcDNA3, pcDNA3-HA-TOPK, or mutant pcDNA3-HA-TOPK-T9A (gifts from Dr. J. Abe, Department of Pathology, Division of Molecular Pathology, Ehime University School of Medicine, Toh-on, Ehime, Japan; ref. 6) was transfected into JB6 C41 cells using SuperFect (Qiagen, Valencia, CA). Transfected cells were selected in MEM containing 5% FBS and G418 (800 μg/mL) for 2 weeks. Stable cell lines were maintained in MEM containing 5% FBS and 200 μg/mL G418.

Small interfering RNA preparation and vector construction. Two pairs of hairpin small interfering RNA (siRNA) oligonucleotides, containing BamHI and HindIII sites, were designed as described previously (18). Hairpin siRNA template oligonucleotides were chemically synthesized, deprotected, and gel purified by Sigma-Genosys (Woodlands, TX). The TOPK siRNA sequence target sequences were aligned to the genome database in a BLAST search to ensure sequences without significant homology to other genes. The sense siRNA template sequence for TOPK was 5'-GATCGCCAGGGTTTGTCTCAATCTCCAAAGAGGAGAGATTGA-GCAAACCTCTTTTGGAAAA-3' and the antisense siRNA template sequence was 5'-AGCTTTCCTCAAAAAGAGTGTGTCTCAATTCTCCTCCTCTGAAAGGAGAATGAGACAAACCTCG-3'. The sense and antisense oligonucleotides were annealed and cloned into the psilencer 3.1-H1 neo vector (Ambion, Austin, TX) at the BamHI and HindIII sites as described by the manufacturer. A scrambled siRNA with a sequence lacking significant homology to the mouse, human, or rat genome database was used as the control or mock siRNA. The resulting psilencer 3.1-H1-siRNA plasmids were transfected into RPMI7951 cells, and the stable cell lines were obtained by G418 screening. These TOPK siRNA cell lines were cultured in MEM supplemented with 10% FBS, 2 mmol/L l-glutamine, 25 μg/mL gentamicin, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, and 200 μg/mL G418.

Identification of proteins binding with TOPK by liquid chromatography-tandem mass spectrometry analysis. The top gene was amplified by PCR and then cloned into pET-46 using a pET-46 EK/LIC kit (Novagen, Inc., Madison, WI). His-TOPK was purified from BL21 (DE3) cells (Novagen). His-TOPK (0.5 mg) was used for binding with 400 μL NiNTA agarose beads (Qiagen, Hilden, Germany). Then, a lysate (10 mg) of RPMI7951 cells was incubated with His-TOPK beads at 4°C overnight. The TOPK-binding proteins were eluted with 50% acetonitrile (Fisher Biotech, Fair Lawn, NJ). Approximately 12 μg of protein eluted from the His-TOPK beads were digested in solution with sequencing grade-modified trypsin (Promega, Madison, WI) according to the manufacturer’s protocol. The sample was purified using a C18 SepPak cartridge (Waters Institute, Milford, MA) according to the manufacturer’s directions and then speed vacuumed to dryness. The peptide mixture was reconstituted with loading buffer (98.2: H2O: acetonitrile, 0.1% formic acid) and analyzed by capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS; refs. 19, 20). Product ion mass spectra were searched against the nonredundant database4 of National Center for Biotechnology Information (April 20, 2005; total 1,182,676 protein sequences), interpreted using the Pro ID version 1.1 software (Applied Biosystems, Inc., Foster City, CA), which used the Interrogator algorithm for scoring peptide/protein candidates (21), and results were verified by manual interpretation.

SDS-PAGE and Western blotting. Cell lines (7×105) were cultured in their respective medium for 12 to 15 hours in 10-cm-diameter dishes to 70% to 80% confluence. Cells were treated with As3+ and harvested after 24 hours with 200 μL of radioimmunoprecipitation assay buffer (1× PBST, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L Na2VO4, 1 mmol/L aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride). The quantity of protein was determined by the Bradford method (22). The samples (30–50 μg protein) with 5× SDS were loaded into 10% to 15% SDS polyacrylamide gel for electrophoresis and subsequently transferred onto an Immobilon-P transfer membrane (Millipore, Chelmsford, MA). Antibody-bound proteins were detected by chemiluminescence (Enhanced Chemiluminescence, Amersham Pharmacia Biotech, Piscataway, NJ) and analyzed using the Storm 840 Scanner (Molecular Dynamics, Sunnyvale, CA). Untreated cell samples were used as negative controls.

In vitro kinase assays. Samples containing recombinant histone H2AX expressed in E. coli were incubated at 30°C for 30 minutes with active glutathione S-transferase-TOPK in 10× kinase buffer A [50 mmol/L Tris-HCl (pH 7.5), 10 mmol/L MgCl2, 1 mmol/L EGTA, 1 mmol/L DTT, 0.01% Brij 35; Cell Signaling Technology] containing 200 μmol/L ATP. The reactions were stopped by adding 5× SDS sample buffer (20 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 10% β-mercaptoethanol) was added to the samples, and they were boiled for 5 minutes prior to SDS-PAGE and Western blotting.
buffer. Then, phosphorylation of H2AX (Ser139) was analyzed by Western blot using a phosphorylated H2AX (Ser139) antibody. Phosphorylation of histone H2AX by JNK1 was used as a positive control. For some experiments, equal protein loading was verified by silver staining for histone H2AX.

**Immunofluorescence assay.** To determine the translocation ability of phosphorylated TOPK and phosphorylated H2AX, RPMI7951 melanoma cells (5 × 10^4) treated or not treated with 2.5 mmol/L As^3+ were incubated for 24 hours. Cells were fixed in 4% paraformaldehyde and incubated with anti-phosphorylated TOPK and anti-phosphorylated H2AX and then with either FITC-conjugated secondary antibody or Texas red–conjugated secondary antibody (Invitrogen, Carlsbad, CA). Samples were analyzed with a fluorescence microscope system (Leica, Mannheim, Germany).

**Isolation of histone H2AX.** Histones were extracted from As^3+-treated cells by disrupting cells with NETN buffer [150 mmol/L NaCl, 1 mmol/L EDTA, 20 mmol/L Tris (pH 8.0), 0.5% nonionic detergent Igepal CA630 (NP40); Sigma]. The insoluble fraction was pelleted for 5 minutes in a microcentrifuge (8,400 rpm). Nuclei were extracted with 0.1 N HCl to isolate total histones. Samples were precipitated with 1 mol/L Tris-HCl (pH 8.0) and then resuspended in double-distilled water (23).

**Flow cytometry analysis.** Apoptosis induced by As^3+ was determined using the Annexin V-FITC Apoptosis Detection kit (Medical & Biological Laboratories, Nagoya, Japan) according to the protocol provided. Briefly, cells were trypsinized, washed once with MEM containing serum, and incubated with Annexin V–conjugated FITC. Apoptosis was analyzed using a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ).

**Statistical analysis.** Comparisons were made using one-way ANOVA, and data are expressed as mean ± SD of three to four independent experiments. Differences were considered significant with P < 0.05.

**Results**

**TOPK expression in human melanoma cell lines.** TOPK is expressed in a wide range of cancers, including leukemia, myeloma, and lymphoma (1–4, 7). However, the expression of TOPK in melanoma cells is yet to be determined. Thus the expression of TOPK was compared in the mouse epidermal JB6 Cl41 skin cell line and in four different human malignant melanoma cell lines, SK-MEL28, SK-MEL31, SK-MEL5, and RPMI7951, which are routinely used in our laboratory. Results indicated that the expression of TOPK in the SK-MEL28 and RPMI7951 cell lines was highest compared with SK-MEL31, SK-MEL5, or JB6 Cl41 cells (Fig. 1). Based on these results, we chose the RPMI7951 melanoma cell line to study TOPK function and to identify potential binding partners.

**Identification of TOPK-binding partners by LC-MS/MS.** First, a His-TOPK protein was generated and was found to bind strongly with Ni-NTA agarose beads. His-TOPK was confirmed to remain bound to the beads even after elution with 50% acetonitrile (data not shown). Therefore, His-TOPK-Ni-NTA agarose (His-TOPK) beads could be used for immobilizing TOPK-binding proteins for subsequent identification. RPMI7951 cell lysate was incubated with Ni-NTA agarose beads as a negative control (Fig. 2A, lane 1) or Ni-NTA-His-TOPK beads (Fig. 2A, lane 2). After washing with PBS, proteins binding with His-TOPK beads were visualized with silver staining after elution with 50% acetonitrile (Fig. 2A). LC-MS/MS analysis (24) identified 26 individual proteins that could bind with TOPK (data not shown). A fragment composed of 19 amino acids, VTIAQGVLPNIQAVLPPK, was identified with a Pro ID confidence of 99% as a peptide from the family of H2A proteins (Fig. 2B). The human genome contains 16 genes that encode for H2A peptides classified as H2A variants (25). The identified peptide sequence was 100% homologous to the sequences of 12 genes of the 16 H2A histone family members, including the important H2A variant, H2AX. A role for H2AX phosphorylation has been shown in DNA repair, cell cycle checkpoint regulation, regulation of gene recombination events, and tumor suppression (26). The LC-MS/MS result suggested that TOPK could bind with histone H2AX. Thus, we focused on elucidating the function and the physiologic significance of the interaction of TOPK and histone H2AX.

**TOPK binds with and phosphorylates histone H2AX in vitro.** Histone H2AX was incubated with Ni-NTA agarose beads (as a negative control) or His-TOPK-Ni-NTA agarose beads at 30°C for 30 minutes. The binding interaction was analyzed by Western blot using an H2AX antibody. Results confirmed that TOPK binds with histone H2AX (Fig. 2C). Because TOPK is a kinase, we next determined whether TOPK can phosphorylate histone H2AX by using histone H2AX as substrate for active TOPK. The phosphorylation was visualized by autoradiography in the presence of [γ-32P]ATP (Fig. 2D) or by a phosphorylated H2AX (Ser139) antibody (Fig. 2E). Phosphorylation of H2AX by JNK1 was used as positive control (27). These results strongly indicated that TOPK binds with and phosphorylates H2AX at Ser139 in vitro. Silver staining of H2AX, TOPK, and JNK1 was used as an internal control to verify equal protein loading.

**As^3+ induces phosphorylation of TOPK and H2AX in RPMI7951 melanoma cells.** As^3+ is a toxin with multiple effects in animal and human populations. Low concentration doses of As^3+ have been reported to induce apoptosis of human melanoma cells (10, 11). The effect of As^3+ on TOPK (Thr7) or H2AX (Ser139) phosphorylation in RPMI7951 melanoma cells was investigated herein by using Western blot analysis. Results indicated that a strong phosphorylation of TOPK (Thr7) and H2AX (Ser139) was induced in a dose-dependent manner following a 24-hour As^3+ treatment of RPMI7951 cells (Fig. 3, top and middle top).
For further experiments, cells were treated with 2.5 μmol/L As\textsuperscript{3+} and harvested 24 hours after treatment. No change in the nonphosphorylated levels of TOPK or H2AX expression was observed in these cell lines under these conditions with As\textsuperscript{3+} treatment (Fig. 3, bottom and middle bottom).

Phosphorylated TOPK and phosphorylated H2AX colocalize in the nucleus after As\textsuperscript{3+} treatment. Yih et al. (28) previously reported that phosphorylated H2AX was clearly detectable in the interface nuclei of CGL-2 cells treated for 24 hours with 2 μmol/L As\textsuperscript{3+}. In the present study, RPMI7951 cells were

Fig. 2. TOPK binds with and phosphorylates histone H2AX \textit{in vitro}.

A, TOPK-binding proteins visualized by silver staining. Lane 1, silver staining of gel showing RPMI7951 cell lysate binding with Ni-NTA agarose beads; lane 2, silver staining of gel showing RPMI7951 cell lysate binding with His-TOPK-Ni-NTA agarose beads.

B, tandem mass spectrum of the VTIAGGGVLPNIQAVLLPK peptide. The gene sequence of this peptide was found to be 100% homologous to the sequences of 12 genes of the 16 H2A histone family members, including histone H2AX. The error in the experimental peptide MW (1930.140) was within 11 ppm of the theoretical MW. Diagnostic b-type and y-type fragment ions are labeled with the value and the ion type according to the fragment ion nomenclature of Biemann (24). The amino acid sequence is displayed above the spectrum. The experimentally measured y and b ions are written above and below the sequence, respectively. C, confirmation of the binding of TOPK with histone H2AX \textit{in vitro} using His-TOPK-Ni-NTA agarose beads. Histone H2AX was incubated with Ni-NTA agarose beads (as control, lane 2) or His-TOPK-Ni-NTA agarose beads (lane 5) at 30°C for 30 minutes. Their binding interaction was analyzed by Western blot using an H2AX antibody. H2AX only (lane 1), Ni-NTA agarose beads only (lane 2), and His-TOPK-Ni-NTA agarose beads only (lane 4) were used as controls. Bottom, the total levels of TOPK served to confirm the presence of TOPK bound to the beads. D, \textit{in vitro} kinase assay to determine the ability of TOPK to phosphorylate histone H2AX visualized by autoradiography in the presence of [γ\textsuperscript{32}P]ATP. E, \textit{in vitro} kinase assay to determine the ability of TOPK to phosphorylate histone H2AX visualized by Western blot using a phosphorylated H2AX (p-H2AX; Ser\textsuperscript{139}) antibody. The binding of JNK1 with H2AX was used as a positive control (unpublished data), and equal protein loading was verified by silver staining for H2AX. These data are representative of at least three independent experiments.
treated with 2.5 μmol/L As3+ for 24 hours and then cytoplasmic and nuclear proteins were extracted and TOPK and phosphorylated TOPK were detected by Western blot (Fig. 4A). The results indicated that TOPK is located mostly in the cytoplasm with very little protein in the nucleus in the absence of As3+ treatment (Fig. 4A). TOPK localization to the nucleus increased dramatically with As3+ treatment (Fig. 4A, top), and phosphorylated TOPK was located only in the nucleus (Fig. 4A, bottom). Immunocytofluorescence analysis was used to further examine the nuclear localization and interaction of TOPK and histone H2AX (Fig. 4B). Results indicated that phosphorylated TOPK (green) and phosphorylated H2AX (red) were both localized in the nucleus after As3+ treatment (Fig. 4B, top and middle right). The merged result confirmed that TOPK colocalized with histone H2AX in the nucleus with As3+ treatment (Fig. 4B, bottom right).

**TOPK siRNA-transfected cells inhibit the phosphorylation of histone H2AX.** RPMI7951 cells were transfected with pSilencer 3.1-H1-topk-siRNA and selected by G418. The expression of TOPK was verified by Western blot using anti-TOPK. Results indicated that TOPK inhibited As3+-induced phosphorylation of histone H2AX (Fig. 4B). The merged result confirmed that TOPK colocalized with phosphorylated TOPK (green) and phosphorylated H2AX (red) were both localized in the nucleus after As3+ treatment (Fig. 4B, top and middle right). The merged result confirmed that TOPK colocalized with histone H2AX in the nucleus with As3+ treatment (Fig. 4B, bottom right). TOPK siRNA-transfected cells inhibited the phosphorylation of histone H2AX. RPMI7951 cells were transfected with pSilencer 3.1-H1-topk-siRNA and selected by G418. The expression of TOPK was verified by Western blot using anti-TOPK. Results indicated that, in control siRNA mock-transfected cells, As3+ induced phosphorylation of TOPK (Thr9) and H2AX (Ser139; Fig. 5A). On the other hand, As3+-induced phosphorylation of histone H2AX (Ser139), TOPK (Thr9), and nonphosphorylated TOPK was dramatically decreased in TOPK siRNA-transfected cells (Fig. 5A). The expression of total H2AX or β-actin was not different between these cell lines (Fig. 5A, bottom and middle).

**Discussion**

TOPK is highly expressed in lymphoma cells, in myeloid leukemia cells, and in several other highly proliferative malignant cell lines derived from sarcomas, carcinomas, or myelomas of various tissue origins (3, 29). In the present study, we used LC-MS/MS analysis to confirm that His-TOPK could bind with histone H2AX. The in vitro kinase data indicated that TOPK directly interacted with and phosphorylated histone
H2AX. This is significant because H2AX has been designated as the histone guardian of the genome (26). Phosphorylation of H2AX has been suggested to have two important functions in DNA repair: to promote changes in the structural configuration of chromatin and to assist in chromatin binding of repair factors. These functions may or may not be related, but both are likely to be required for efficient synapses of broken chromosome ends (26, 30). Several reports have shown that As3+ can induce DNA damage (31, 32). Our laboratory has focused on the elucidation of mechanisms explaining how arsenic can act both as a carcinogen and as an effective chemotherapeutic agent (9, 33, 34). Accumulating data suggested that As3+ may specifically induce apoptosis in certain types of tumor cells, including megakaryocytic leukemia cell lines, human breast cancer cells (32), and human myeloma cells (11, 34, 35). Furthermore, mitogen-activated protein kinases have been shown to have a very important role in mediating As3+-induced apoptosis (36).

TOPK, a novel mitogen-activated protein kinase kinase–like protein kinase (6), is highly expressed in tumor cells but not in normal cells, and the function of this kinase is not clear. In this study, we showed that As3+ induced phosphorylation of both TOPK (Thr9) and histone H2AX (Ser139) and that TOPK directly phosphorylated histone H2AX (Ser139) in vitro and in vivo.
Fig. 5. TOPK inhibits As\(^{3+}\)-induced apoptosis through phosphorylation of histone H2AX at Ser\(^{139}\). A, effect of TOPK deficiency on H2AX phosphorylation. Control siRNA and TOPK siRNA cells were cultured and treated with 2.5 \(\mu\)mol/L As\(^{3+}\) for 24 hours. Phosphorylation of histone H2AX at Ser\(^{139}\) and TOPK at Thr\(^{9}\) was visualized by Western blotting using phosphorylated-specific antibodies. Total TOPK, H2AX, and \(\beta\)-actin were used as internal controls for confirmation of TOPK deficiency and equal protein loading. B, As\(^{3+}\)-induced phosphorylation of H2AX in H2AX\(^{+/+}\) cells but had no effect on phosphorylation of TOPK in H2AX\(^{+/+}\) or H2AX\(^{-/-}\) cells. Total TOPK, H2AX, and histone H3 were used as internal controls for confirmation of H2AX deficiency and equal protein loading. C, flow cytometry analysis of apoptosis in H2AX\(^{+/+}\) and H2AX\(^{-/-}\) cells. D, flow cytometry analysis of apoptosis in control siRNA or TOPK siRNA cells. Cells were incubated with Annexin V–conjugated FITC after 24 hours of treatment with 2.5 \(\mu\)mol/L As\(^{3+}\). Stained cells were analyzed by flow cytometry. Bottom right, percentage of early apoptotic cells (Annexin V–stained positive cells). These data are representative of at least four independent experiments.
Fig. 6. TOPK blocks As³⁺-induced apoptosis in RPMI7951 melanoma cells. A, As³⁺ does not induce apoptosis in cell lines that express TOPK compared (B) with cell lines that do not express TOPK. Columns, mean of four independent experiments; bars, SD. *, $P < 0.0001$, significant increase in apoptosis over time. C, JB6 cells were transfected with pcDNA3-HA-TOPK (JB6-pcDNA3-HA-TOPK), pcDNA3-HA-TOPK-T9A mutant (JB6-pcDNA3-HA-TOPK-T9A), or mock vector (JB6/Vector). Expression of HA-TOPK was detected with a HA antibody; β-actin was used as an internal control to verify equal protein loading. D, apoptosis is not induced in JB6 cells overexpressing TOPK but is restored in cells expressing mutant TOPK. JB6 cells were transfected as in (C) and cultured for 24, 48, or 72 hours with 2.5 μmol/L As³⁺. Apoptosis was determined by flow cytometry using Annexin V–conjugated FITC. Columns, mean of at least four independent experiments; bars, SD. *, $P < 0.0001$, significantly less apoptosis in JB6-TOPK-overexpressing cells compared with either JB6/Vector or JB6-TOPK-T9A mutant cells. E, in cells expressing TOPK and stimulated with As³⁺, TOPK is localized in the nucleus, where it phosphorylates histone H2AX resulting in decreased induction of apoptosis.
Further, Western blot and immunocytofluorescence analyses clearly showed that \( \text{As}^{3+} \) induced the accumulation of phosphorylated TOPK and phosphorylated H2AX in the nucleus of RPMI7951 cells. Another group has also shown that arsenic treatment induces the phosphorylation and nuclear accumulation of histone H2AX (28). However, our data indicated the colocalization and direct involvement of TOPK in \( \text{As}^{3+} \)-induced apoptosis. The accumulation of phosphorylated H2AX in the nucleus suggested that DNA damage occurred in \( \text{As}^{3+} \)-treated TOPK siRNA-transfected cells, \( \text{As}^{3+} \)-induced apoptosis was also markedly increased. These results suggested that TOPK phosphorylated histone H2AX is associated with an inhibition of \( \text{As}^{3+} \)-induced apoptosis in RPMI7951 melanoma cells.

Investigations using many different experimental systems suggested that arsenic may offer significant therapeutic benefits to patients across many neoplasms, and numerous clinical trials are under way in hematopoietic malignancies and solid tumors. Miller et al. (37) summarized reports of the activity of arsenic in animal models and in the clinic. Low doses of arsenic trioxide \((0.06-0.2 \text{ mg/kg of body})\) can induce complete remission in relapsed acute promyelocytic leukemia patients, and complete remissions occurred in 11 of the 12 patients (38). Low doses of \( \text{As}^{3+} \) induced apoptosis in some (11, 32–34), but not all, cancer cell types (39–41), suggesting that a mechanism exists that is responsible for resistance to \( \text{As}^{3+} \). Using different melanoma cell lines with different levels of TOPK expression, we found that \( \text{As}^{3+} \) induces apoptosis dependent on TOPK expression level. In melanoma cells without expression of TOPK, a low concentration of \( \text{As}^{3+} \) induces rapid apoptosis—more than 25% to 50% in 48 hours. In contrast, melanoma cells expressing high levels of TOPK showed full resistance to \( \text{As}^{3+} \) following 72 hours of exposure, suggesting that melanoma cells expressing TOPK are more resistant to \( \text{As}^{3+} \)-induced apoptosis. These various melanoma cells also display other abnormalities. For example, the \( p16 \) gene is deleted in SK-MEL5 (42) cells. Furthermore, B-raf is mutated (V599E) in SK-MEL28 and SK-MEL5 cells but not in SK-MEL31 cells (43, 44). Interestingly, the TOPK-expressing SK-MEL28 and RPMI7951 cells each express a mutant \( p53 \) (45, 46), whereas SK-MEL5 cells express wild-type \( p53 \) (45). However, additional studies are needed to specifically link the genetic background of these cell lines with resistance to \( \text{As}^{3+} \)-induced apoptosis. Our study indicated that this resistance seems to be related to the activation and phosphorylation of histone H2AX by activated TOPK (Fig. 6E). In this model, cells lacking TOPK expression readily undergo apoptosis induced by low levels of \( \text{As}^{3+} \). On the other hand, in cells expressing TOPK, \( \text{As}^{3+} \) induces its phosphorylation, which leads to phosphorylation of histone H2AX and resistance to apoptosis. Thus, discovery of a small-molecule inhibitor of TOPK may be very important in combination with low doses of \( \text{As}^{3+} \) in the treatment of melanomas and other TOPK-positive cancers.

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References

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