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Arsenic Trioxide Promotes Histone H3 Phosphoacetylation at the Chromatin of *CASPASE-10* in Acute Promyelocytic Leukemia Cells*

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Arsenic trioxide (As_2O_3) is highly effective for the treatment of acute promyelocytic leukemia, even in patients who are unresponsive to all-*trans*-retinoic acid therapy. As_2O_3 is believed to function primarily by promoting apoptosis, but the underlying molecular mechanisms remain largely unknown. In this report, using cDNA arrays, we have examined the changes in gene expression profiles triggered by clinically achievable doses of As_2O_3 in acute promyelocytic leukemia NB4 cells. *CASPASE-10* expression was found to be potently induced by As_2O_3. Accordingly, caspase-10 activity also substantially increased in response to As_2O_3 treatment. A selective inhibitor of caspase-10, Z-AEVD-FMK, effectively blocked caspase-3 activation and significantly attenuated As_2O_3-triggered apoptosis. Interestingly, the treatment of NB4 cells with As_2O_3 markedly increased histone H3 phosphorylation at serine 10, an event that is associated with acetylation of the lysine 14 residue. Chromatin immunoprecipitation assays revealed that As_2O_3 potently enhances histone H3 phosphoacetylation at the *CASPASE-10* locus. These results suggest that the effect of As_2O_3 on histone H3 phosphoacetylation at the *CASPASE-10* gene may play an important role in the induction of apoptosis and thus contribute to its therapeutic effects on acute promyelocytic leukemia.

Acute promyelocytic leukemia (APL)[1] accounts for ~10–15% of adult myeloid leukemias with 3,500–5,500 new cases diagnosed annually (1, 2). The vast majority of APL patients harbor the chromosomal translocation t(15,17)(q22;q21) involving the retinoic acid receptor α (*RARα*) gene on chromosome 15 and the promyelocytic leukemia (*PML*) gene on chromosome 17, generally giving rise to two fusion genes, *PML-RARα* and *RARα-PML* (1, 3). Studies using transgenic mice have demonstrated that the protein product of the *PML-RARα* fusion gene is primarily responsible for the leukemogenic property of this characteristic translocation (4, 5). All-*trans*-retinoic acid (ATRA), a physiologically active derivative of vitamin A, can induce complete remission in most APL patients associated with an enhancement of differentiation pathways (6). Recent studies (7–9) have provided strong evidence that the induction of leukemia by the PML-RARα protein relies on its ability to repress gene transcription by recruiting transcription repressor complexes. Pharmacological doses of ATRA stimulate the release of the transcription repressor complexes from PML-RARα, thereby activating the transcription of genes critical for normal granulocytic differentiation. However, ATRA is not curative, and resistance rapidly develops usually within 10 months of therapy (2, 6). Therefore, alternative therapies are necessary.

Recently, As_2O_3 was identified as a potent anti-leukemic agent for treating not only newly diagnosed but also relapsed APL patients (2, 10–13), and it is remarkably effective in ATRA-refractory patients (2, 12). However, the mechanisms underlying its therapeutic effects are not well understood (2, 11, 13). As_2O_3 induces a drastic reorganization of the nucleus characterized by restoration of intact PML nuclear bodies followed by a progressive degradation of the PML-RARα fusion protein (14, 15). Although As_2O_3 induces partial differentiation of leukemic cells (13), the apoptosis-promoting effect of As_2O_3 is believed to play a prominent role in the remission of APL as large numbers of apoptotic myelocytes have been documented in bone marrow and peripheral blood of As_2O_3-treated APL patients (2, 11, 13). As_2O_3 has been shown to trigger the early collapse of mitochondrial transmembrane potential (16, 17), the release of cytochrome *c* (10), and the activation of caspases-1, -2, -3, and -8 (2, 16, 18).

To elucidate the key molecular events involved in mediating As_2O_3-induced apoptosis, we have employed the APL-derived NB4 cell line exposed to clinically relevant concentrations of As_2O_3 and analyzed the changes in gene expression profiles using cDNA array. *CASPASE-10* was among the genes upregulated with this treatment. Interestingly, treatment with As_2O_3 also resulted in a significant increase in histone H3 phosphoacetylation, an event that is associated with altered gene transcription (19–21). Importantly, selective inhibition of caspase-10 substantially abolished As_2O_3-triggered caspase-3 activation and significantly reduced As_2O_3-induced apoptosis. Chromatin immunoprecipitation (ChIP) assays revealed that As_2O_3 potently enhances histone H3 phosphoacetylation at the *CASPASE-10* locus. These results suggest that As_2O_3-induced histone H3 phosphoacetylation may be implicated in altering gene transcription profile and apoptosis and play an important role in the therapeutic effect of As_2O_3 on APL.

EXPERIMENTAL PROCEDURES

Cell Culture, Treatment, and Assessment of Apoptosis—NB4 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum, 10 mM HEPES, and 2 mM glutamine. As_2O_3 was from Sigma, and Z-AEVD-

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[1] The abbreviations used are: APL, acute promyelocytic leukemia; *PML*, promyelocytic leukemia gene; *RARα*, retinoic acid receptor α gene; ChIP, chromatin immunoprecipitation; MES, 4-morpholineethanesulfonic acid; ATRA, all-*trans*-retinoic acid; As_2O_3, arsenic trioxide; MAPK, mitogen-activated protein kinase; C_T, number of PCR cycles required to reach a threshold arbitrarily set as 0.5.

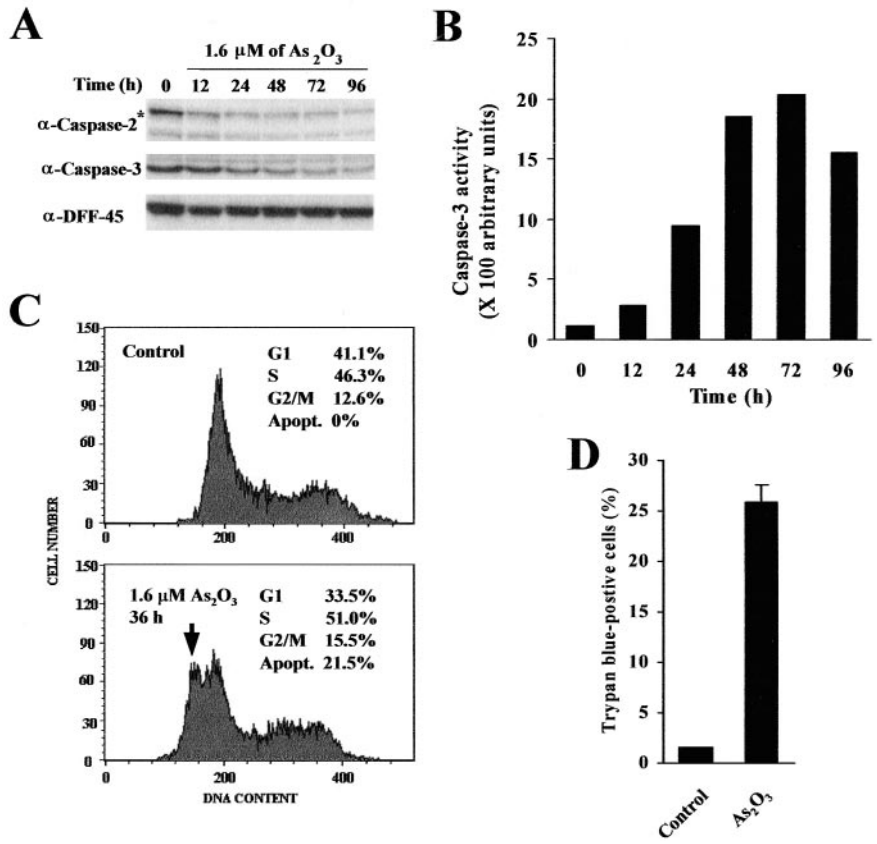

FIG. 1. **Induction of apoptosis by As_2O_3 in NB4 cells.** *A*, Western blot analysis of caspase-2, caspase-3, and DEF-45 expression. *Asterisk*, 48-kDa caspase-2. *B*, caspase-3 activity in NB4 cells treated with 1.6 μM As_2O_3 detected by fluorometric assays. *C*, FACS analysis of control and As_2O_3-treated NB4 cells. *Arrow* indicates the apoptotic (*Apopt.*) population. *D*, assessment of cell death by trypan blue staining after As_2O_3 treatment (1.6 μM, 72 h).

FMK was from Enzyme Systems (Livermore, CA). Flow cytometry and estimation of sub-G_1 apoptotic populations were performed as described previously (22). Where indicated, percentages of dead cells were assessed by direct cell counting after trypan blue staining using a hemacytometer. For caspase activity determinations, 100-μg aliquots of cell lysate were prepared and used following the manufacturers' recommendations to assay caspase-2 (BioVision, Mountain View, CA), caspase-3 (Molecular Probes, Eugene, OR), or caspase-10 activity (R&D, Minneapolis, MN) by fluorometry.

Northern and Western Blotting—Total RNA was isolated using a NucleoSpin RNA II isolation kit (Clontech, Palo Alto, CA), and Northern blot analysis was performed as described previously (23). *Caspase-10* mRNA was detected through the simultaneous use of two radiolabeled oligonucleotides (5′-CAGGAATACTGTCCTGCAGGGAAGTGGGTGCCTGCTCAGGGTTCAGA-3′ and 5′-TGCCGTATGATATAGAGGAGTTCTGCCAGGAAGAAAGTTCCTCACT-3′) as probes. Signals were quantitated with a PhosphorImager (Amersham Biosciences).

Caspases-2, -3, and DNA fragmentation factor-45 were detected by Western blot analysis using monoclonal antibodies (Transduction Laboratories, Lexington, KY). Caspase-8 was detected by Western blotting using a rabbit polyclonal antibody (BD Biosciences). Active caspase-3 was detected by Western blotting using a rabbit polyclonal antibody that recognizes the active/cleaved caspase-3 (Asp-175) (Cell Signaling, Beverly, MA). Modified histone H3 was detected by Western blotting as described previously (23). Crude histone proteins were extracted as described previously (23) and separated through electrophoresis using 10% NuPAGE gels and MES buffer (Invitrogen). Phosphorylation of histone H3 at serine 10 was detected by Western blotting using an antibody specific for Ser-10-phosphorylated histone H3 (Cell Signaling). Histone H3 phosphoacetylation was detected by Western blotting using an antibody, which is specific for histone H3 phosphorylated at Ser-10 and acetylated at Lys-14 (Clontech). Histone H3 hyperacetylation was detected by Western blotting using an antibody specific for histone H3 that is acetylated at both Lys-9 and Lys-14 (Clontech).

cDNA Array—DNase-treated total RNA (5 μg), prepared from either untreated or As_2O_3-treated cells, was used to synthesize cDNA probes using reverse transcriptase, [α-^{33}P]dATP, and a specific CDS primer mix for genes represented on the Atlas human cancer 1.2 array (Clontech) containing 1,176 human cDNAs. Arrays were hybridized and washed according to the manufacturer's instructions and were visualized and quantitated using a PhosphorImager. Signal intensities were normalized to the signals of housekeeping genes in each membrane, and simple ratios (treated relative to untreated) were calculated. The array analysis was performed at least twice for each time point.

ChIP and Real-time PCR—ChIP was performed using 1.5×10^8 cells as described previously (23). Ten percent of the chromatin solution was used for isolating input DNA, and the remainder was used for performing ChIP assays. ChIP procedures were carried out either using 10 μl of rabbit pre-immune serum or 100 μl of purified antibody specifically recognizing phosphoacetyl-histone H3 (p-Ser-10/Ac-Lys-14) (Clontech). Immunoprecipitated DNA was heated at 65 °C for ≥6 h to reverse the formaldehyde cross-links, extracted with phenol/chloroform, precipitated, and dissolved in water. Real-time PCR was performed using SYBR® Green PCR Master Mix and GeneAmp® 5700 Sequencing Detection System (Applied Biosystems, Foster City, CA). Primers 5′-TGCTTACCAGCGGCTACAC-3′ and 5′-CCCTCCCTACCCCAGAAAC-3′ were used to amplify the *CASPASE-10* genomic DNA spanning a 151-bp region in the second intron of *CASPASE-10* (24). Human *β-GLOBIN* was amplified using primers 5′-GGCAAGGTGAACGTGGATGAAGTTGGTG-3′ and 5′-GGAGTGGACAGATCCCCAAAGGACTCAAAG-3′, which span a 234-bp region from the first to the second exon of the human *β-GLOBIN* gene (25). PCR amplification was carried out using these parameters: 10 min at 95 °C followed by 45 cycles of denaturation (95 °C, 15 s), annealing (60 °C, 30 s), and extension (72 °C, 30 s). Upon the completion of the PCR reactions, PCR products were resolved on 2% agarose gels and visualized under UV light to confirm the quality of the PCR amplification.

RESULTS

As_2O_3 Stimulates Apoptosis and Induces CASPASE-10 Expression in NB4 Cells—Treatment of NB4 cells with As_2O_3 led to a time-dependent cleavage of caspase-2 and caspase-3 as indicated by the progressive decrease in the unprocessed caspases. However, it did not affect the expression of another apoptosis-related protein, DNA fragmentation factor-45 (Fig. 1*A*). Confirming the previous report that As_2O_3 activates caspase-3 in APL cells *in vivo* (12), we observed a substantial increase in caspase-3 activity after 24 h in As_2O_3-treated NB4 cells. The activity of caspase-3 reached its maximal levels at 48–72 h (Fig. 1*B*). In addition, treatment with As_2O_3 caused

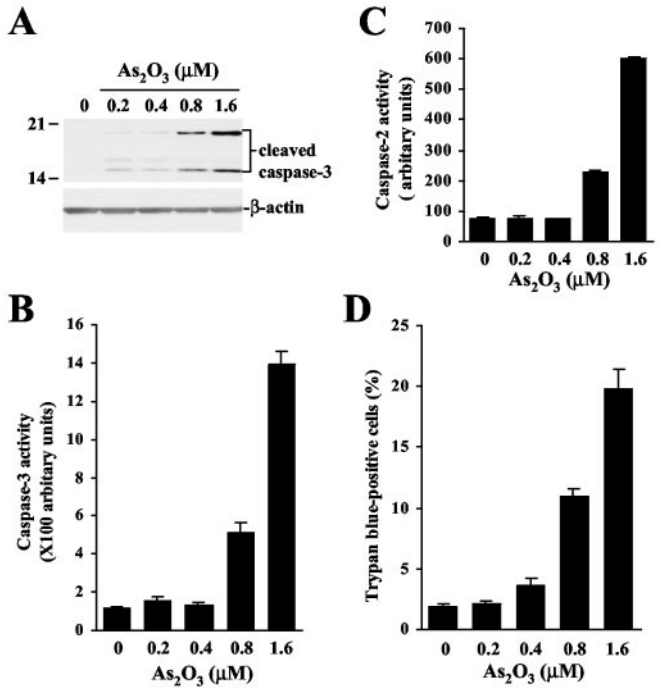

FIG. 2. **Dose response of caspase activation and apoptosis induced by As_2O_3.** NB4 cells were treated with the indicated concentration of As_2O_3 for 48 h. *A*, Western blot analysis to assess the levels of active/cleaved caspase-3 in cell lysates (*upper panel*). β-actin signals served as loading controls (*lower panel*). Results from a representative experiment are shown. *B*, caspase-3 activation detected by fluorometric assays. *C*, caspase-2 activation detected by fluorometric assays. *D*, assessment of cell death by trypan blue staining. Results in *B*, *C*, and *D* represent the mean ± S.E. from three independent experiments.

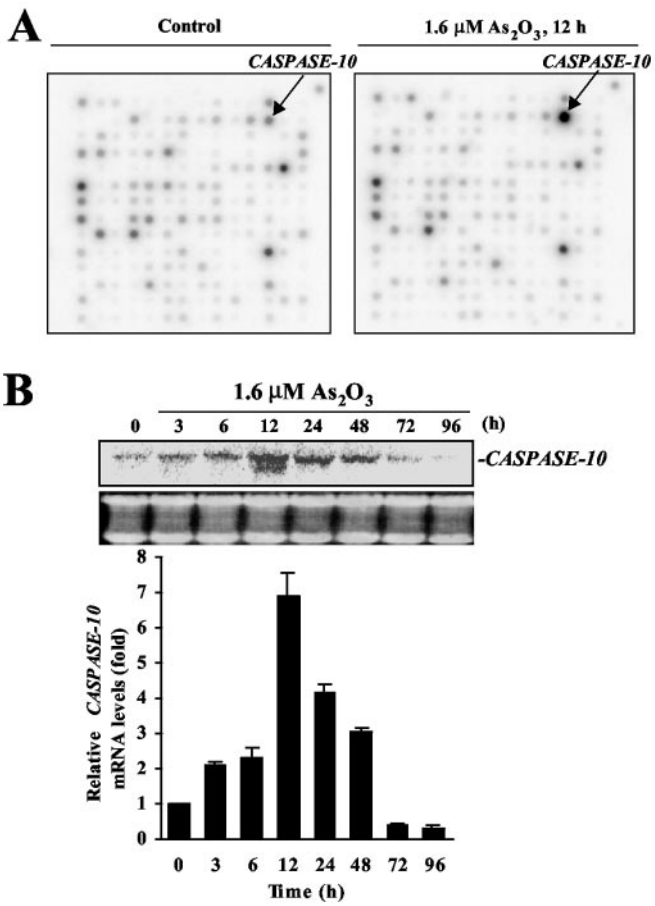

FIG. 3. **Effect of As_2O_3 on *CASPASE-10* gene expression.** *A*, cDNA array analysis of cells that were either left untreated or treated with As_2O_3. Representative paired array fields are shown to illustrate the up-regulation of *CASPASE-10*. *B*, representative results from Northern blot analysis of *CASPASE-10* mRNA. Ethidium bromide staining of total RNA is shown. *Caspase-10* mRNA levels after treatment with 1.6 μM As_2O_3 were quantitated, normalized to 18 S signals (data not shown), and presented as relative *Caspase-10* mRNA levels compared with those in untreated cells. Data represent the mean ± S.E. from three independent experiments.

the appearance of an apoptotic sub-G_1 population (Fig. 1*C*) and a marked increase in cell death (Fig. 1*D*). It has been demonstrated that that As_2O_3 at concentrations above 0.5 μM induces apoptosis in APL cells (10). Consistent with this report, we also observed a substantial increase in caspase-3 activity in cells treated with 0.8 and 1.6 μM As_2O_3 but not in cells treated with lower doses of As_2O_3 (0.2 and 0.4 μM). The increase was detected by both Western blotting using an antibody specific for the active/cleaved caspase-3 (Fig. 2*A*) and fluorometric assays (Fig. 2*B*). Similarly, a significant increase in caspase-2 activity was detected by fluorometric assays in cells treated with 0.8 and 1.6 μM As_2O_3 but not in cells treated with lower doses (0.2 and 0.4 μM) (Fig. 2*C*). The substantial increases in caspase-2 and caspase-3 activity in cells treated with 0.8 and 1.6 μM As_2O_3 were reflected in a significant increase in trypan blue-positive cells (Fig. 2*D*). It has been reported that in patients receiving daily intravenous As_2O_3 infusion, the plasma As_2O_3 concentration stays within a range between 0.5 and 3 μM for the most time with a peak level between 4.2 and 6.7 μM shortly after infusion (10). Based on this report, we chose a highly effective and clinically relevant dose of 1.6 μM for most of the subsequent analyses.

To understand the molecular basis for the As_2O_3-induced apoptosis, we performed a large-scale gene expression analysis to identify genes regulated by As_2O_3. Total RNA was isolated from either untreated or As_2O_3-treated cells, and radiolabeled reverse transcripts were used to hybridize cDNA arrays containing 1,176 human gene transcripts closely related to a variety of cancers (Atlas Human Cancer Arrays, Clontech). As_2O_3 consistently caused a ≥2-fold change in the expression of 25 genes on the DNA array, representing ~2% of genes analyzed. Among the most interesting genes up-regulated by As_2O_3 was *CASPASE-10* (Fig. 3*A*), which has been shown to play an important role in apoptosis (26, 27).

To further characterize the effect of As_2O_3 on *CASPASE-10* expression, Northern blot analysis was performed. Consistent with the cDNA array results, *CASPASE-10* mRNA levels increased markedly with the As_2O_3 treatment, reaching its highest expression (~7-fold) after 12 h of treatment and gradually declining thereafter (Fig. 3*B*).

The effect of As_2O_3 on caspase-10 activity was examined using fluorometric assays. As_2O_3 treatment resulted in a time-dependent increase in caspase-10 activity, which peaked between 48 and 72 h after the addition of As_2O_3 to the medium, and decreased abruptly thereafter, possibly because of protein degradation during the late stages of apoptosis (Fig. 4*A*). The activation of caspase-10 by As_2O_3 was dose-dependent. Significant activation of caspase-10 only occurred in cells treated with 0.8 and 1.6 μM As_2O_3 (Fig. 4*B*). Importantly, treatment with the caspase-10 inhibitor Z-AEVD-FMK led to a significant reduction of the apoptosis triggered by As_2O_3 as indicated by the considerable decrease in cell death (Fig. 4C). These observations demonstrate the important role of caspase-10 in As_2O_3-mediated cytotoxicity.

To further address the role of caspase-10 in the apoptosis cascade, the effect of the selective caspase-10 inhibitor Z-AEVD-FMK on caspase-3 activation was examined (Fig. 5).

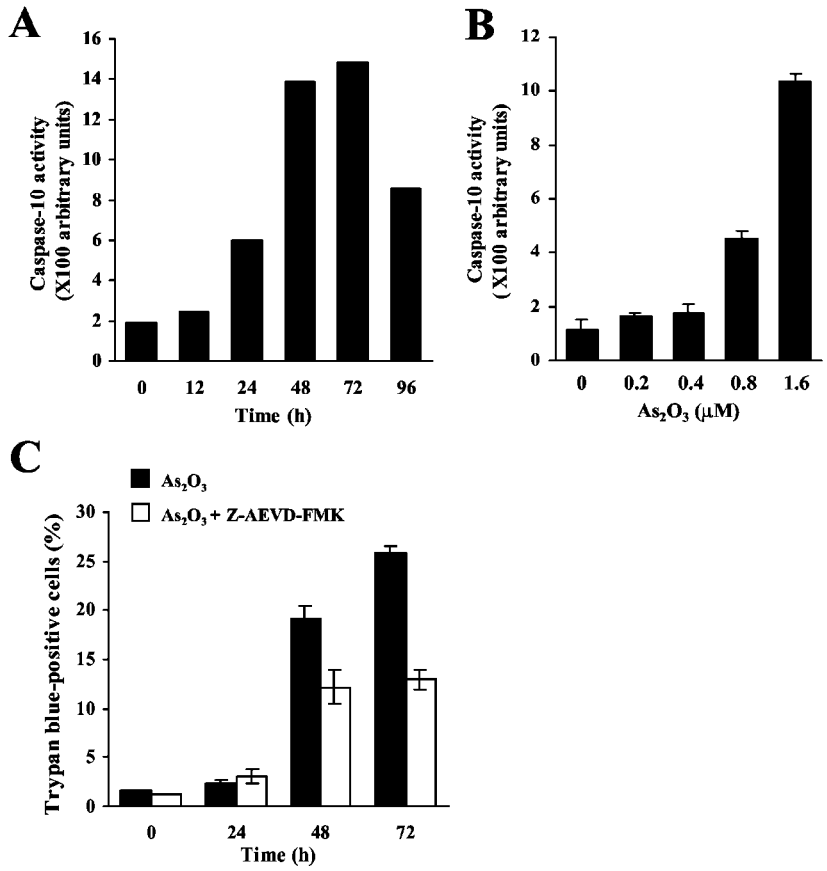

FIG. 4. **Activation of caspase-10 in As_2O_3-treated cells.** *A*, kinetics of caspase-10 activation in response to As_2O_3 (1.6 μM) determined by fluorometric assays. Data from a representative experiment are shown. *B*, dose-response of caspase-10 activation by As_2O_3. Cells were treated with As_2O_3 at the indicated concentration for 48 h. *C*, the effect of caspase-10-selective inhibitor Z-AEVD-FMK on As_2O_3-induced cell death determined by trypan blue staining. NB4 cells were treated with 1.6 μM As_2O_3 in the absence or presence of 2 μM Z-AEVD-FMK. Data in *B* and *C* represent the means ± S.E. from three assessments.

In cells treated with As_2O_3, an ~20-fold increase in caspase-3 activity was detected after 48 h using fluorometric assay. Importantly, the caspase-10 inhibitor Z-AEVD-FMK at a very low concentration of 2 μM substantially attenuated the As_2O_3-induced caspase-3 activation, decreasing caspase-3 activity by 75% at the 48-h time point (Fig. 5*A*). The partial inhibition of caspase-3 by Z-AEVD-FMK was confirmed by Western blot analysis using an antibody that specifically recognizes the active/cleaved caspase-3 (Fig. 5*B*). It is worth noting that although higher concentrations of Z-AEVD-FMK further decreased caspase-3 activity, even at a high concentration of 50 μM, the caspase-10 inhibitor was unable to completely abolish the As_2O_3-induced caspase-3 activation (Fig. 5*C*). Likewise, the high concentrations of Z-AEVD-FMK (50 μM) only partially blocked the apoptotic process triggered by As_2O_3 (Fig. 5*D*). These observations suggest that in addition to caspase-10, other early caspase(s) may also contribute to the activation of caspase-3 by As_2O_3. Indeed, a cleaved form of caspase-8 was detected by Western blotting in cells treated with As_2O_3 (Fig. 5*B*), confirming the earlier report by Kitamura *et al.* (18) that caspase-8 is rapidly activated in response to As_2O_3 treatment and plays a significant role in As_2O_3-induced apoptosis.

As_2O_3 Induced Phosphorylation of Serine 10 and Acetylation of Lysine 14 on Histone H3—We have previously shown that arsenite-stimulated MAPK phosphatase-1 induction is associated with an increase of phosphoacetyl-histone H3 on its chromatin (23). To assess whether similar chromatin changes were implicated in the As_2O_3-mediated induction of apoptosis, we examined histone H3 modification in NB4 cells treated with different doses of As_2O_3 using antibodies specific for various modified histone H3 molecules (Fig. 6*A*). As shown in Fig. 5*A*, lower doses of As_2O_3 (0.2 and 0.4 μM) did not significantly alter the level of either phospho-histone H3 (p-Ser-10) or phosphoacetyl-histone H3 (p-Ser-10/Ac-Lys-14). However, at higher doses (0.8 and 1.6 μM), As_2O_3 induced a significant increase in both the phospho-histone H3 (p-Ser10) and phosphoacetyl-histone H3 (p-Ser10/Ac-Lys14) levels. The effect of As_2O_3 on histone H3 phosphoacetylation did not appear to be caused by a nonspecific inhibition of the histone deacetylase activity, because As_2O_3 treatment did not result in a significant increase in either Lys-14-acetylated histone H3 or hyperacetylated histone H3 (Ac-Lys-9/Ac-Lys-14) (Fig. 6*A*). Histone H3 phosphorylation at Ser-10 and dual modification (phosphorylation at Ser-10 and acetylation at Lys-14) occurred with similar kinetics in As_2O_3-treated cells (Fig. 6*B*), supporting the notion that acetylation at the Lys-14 residue and phosphorylation at the Ser-10 residue are coupled events (19). Comparable loading of the crude histone proteins among the various samples was verified by Western blotting using a monoclonal antibody recognizing total histone H3.

As_2O_3 Stimulates the Phosphoacetylation of Histone H3 at the Chromatin of the CASPASE-10 Locus—To examine whether histone H3 modification occurs *in vivo* at the chromatins of *CASPASE-10*, ChIP assays were performed using an antibody specifically against dually modified histone H3 (23). To ascertain that equal amounts of starting chromatin material were used, DNA amplifications were carried out by real-time PCR using input chromatin DNA (not subject to immunoprecipitation) from both untreated and As_2O_3-treated cells and a pair of primers specific for *CASPASE-10* (Fig. 7*A*, *left panel*). The very similar *CASPASE-10* amplification curves and C_T values, which represent the number of PCR cycles required to reach a threshold set arbitrarily at 0.5, from both the untreated and As_2O_3-treated input DNA indicated that the starting chromatin materials used in the subsequent ChIP assays were comparable. To detect histone H3 phosphoacetylation, chromatin solutions were incubated with a rabbit polyclonal antibody specifically recognizing phosphoacetyl-histone H3 (phospho-

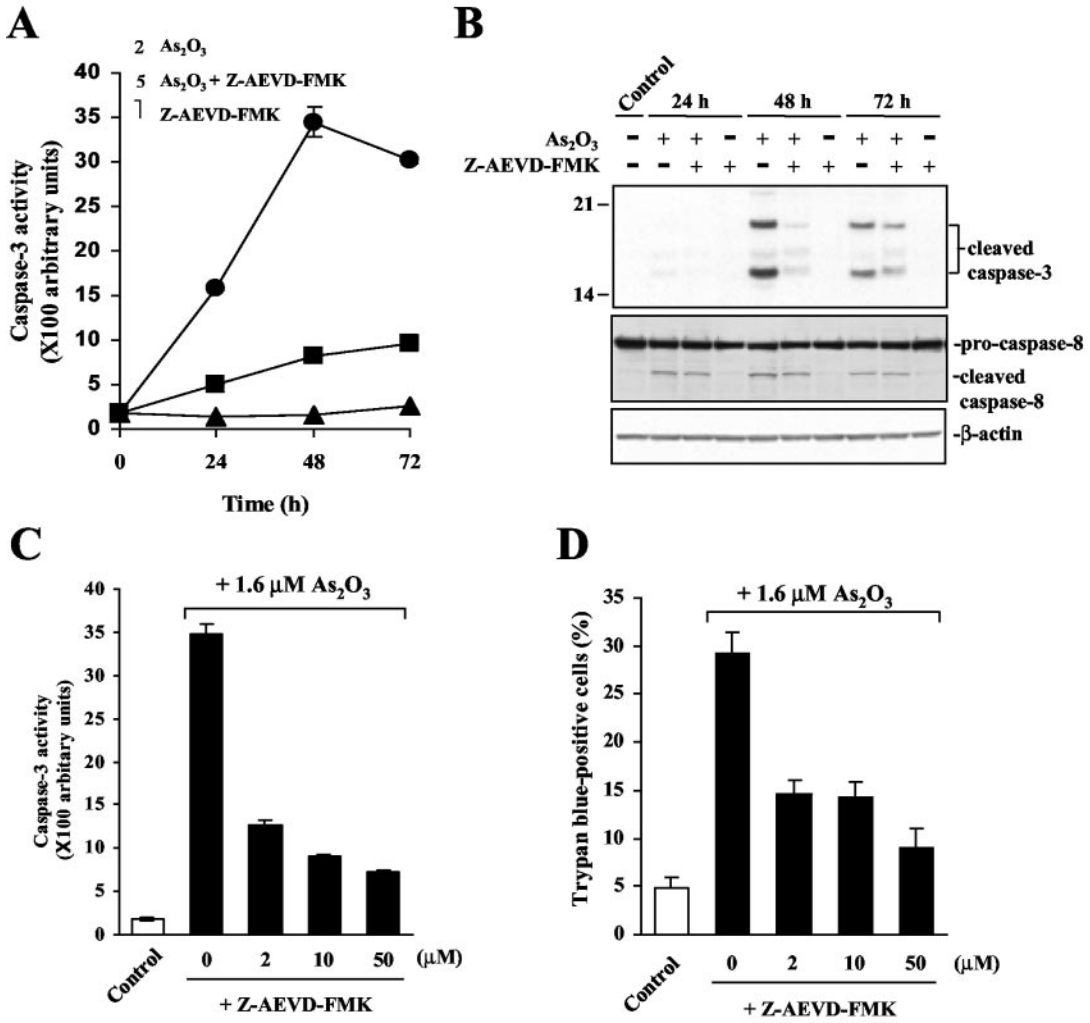

FIG. 5. **The role of caspase-10 in mediating caspase-3 activation induced by As_2O_3.** Caspase-10-selective inhibitor Z-AEVD-FMK was added to the medium 15 min before adding As_2O_3 (1.6 μM). *A*, caspase-3 activity induced by As_2O_3 in the absence or presence of 2 μM Z-AEVD-FMK determined by fluorometric assays. *B*, Western blotting analysis either using an antibody specific for cleaved caspase-3 (*upper panel*) or using an antibody recognizing both pro-caspase-8 and cleaved caspase-8 (*middle panel*). Comparable protein loading was verified by Western blotting using a monoclonal antibody against β-actin (*lower panel*). Z-AEVD-FMK concentration was 2 μM. *C*, effect of various concentrations of Z-AEVD-FMK on As_2O_3-induced caspase-3 activity determined by fluorometric assays. *D*, effect of various concentrations of Z-AEVD-FMK on As_2O_3-induced cell death determined by trypan blue staining. Data presented in *A*, *C*, and *D* represent the means ± S.E. from three assessments.

Ser-10/Ac-Lys-14) and protein A. Genomic DNA present in the immunoprecipitates was extracted and analyzed by real-time PCR using primers specific for *CASPASE-10* (Fig. 7*A*, *right panel*). A substantial enhancement in histone H3 phosphoacetylation at the *CASPASE-10* gene was detected after As_2O_3 treatment. The C_T value was reduced from 32.6 (untreated) to 28.7 (As_2O_3-treated), corresponding to a 15-fold increase in chromatin-bound phosphoacetyl-histone H3. The specificity of the PCR reaction was indicated by the presence of a single band of the anticipated size on agarose gel after separating the final PCR products (Fig. 7*B*, *upper panel*). To test the specificity of the immunoprecipitation assay procedures, mock ChIP assays were carried out using rabbit pre-immune serum. The *CASPASE-10* sequence was virtually undetectable in the mock immunoprecipitates (Fig. 7*B*, *upper panel*). Importantly, As_2O_3 appeared to stimulate histone H3 phosphoacetylation only at the chromatin of a small subset of genes[2] including *CASPASE-10* (Fig. 7). Phosphoacetyl-histone H3 was absent at the transcriptionally inactive *β-GLOBIN* chromatin in both untreated and As_2O_3-treated cells (Fig. 7*B*, *lower panel*), further illustrating the specificity of such modification in As_2O_3-stimulated cells.

DISCUSSION

As_2O_3 has been shown to constitute effective therapy for APL patients relapsing from or refractory to ATRA treatment (10, 13). As_2O_3 and ATRA appear to exert their anti-leukemic effects through partially overlapping mechanisms including those leading to the restoration of granulocyte differentiation (6, 10, 12, 13, 28). However, As_2O_3 and ATRA therapies exhibit important differences. For example, As_2O_3 can induce complete remission in t(15,17)-positive APL patients, regardless of their sensitivity to ATRA. In addition, As_2O_3 can induce a high rate of molecular conversion from positive to negative for t(15,17) translocation. This is indicative of the elimination of the neoplastic progenitors that is unusual after treatment with ATRA alone (10, 12, 13). Recent studies (2, 12, 13, 29) have provided considerable support for the notion that the anti-leukemic effect of As_2O_3 lies primarily in its ability to induce apoptosis. Both *in vivo* and *in vitro*, As_2O_3 has been found to induce apoptosis of APL cells at clinically achievable concentrations (10–13). Here, we have confirmed that As_2O_3-induced apoptosis is associated with activation of the late caspases includ-

[2] J. Li and Y. Liu, unpublished observations.

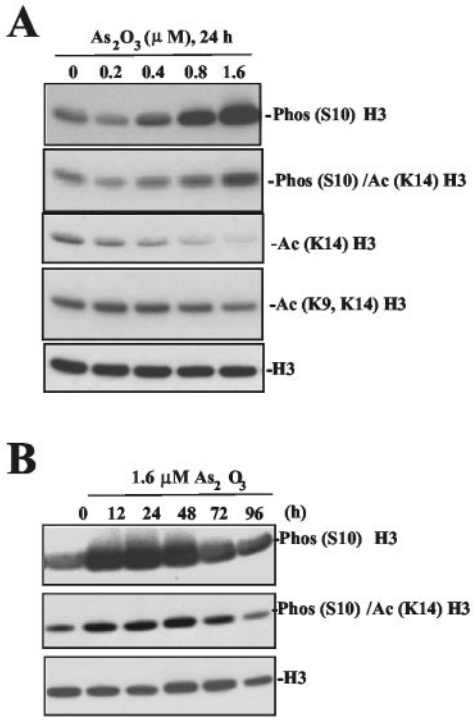

FIG. 6. **Histone H3 phosphorylation and acetylation induced by As_2O_3.** NB4 cells were treated with As_2O_3. Crude histone proteins were extracted and separated on NuPAGE gel using MES buffer. Histone H3 modification was examined via Western blotting using antibodies specific for the modified histone H3. *A*, dose-response of histone H3 modification at 24 h. *B*, time course of histone H3 phosphorylation and phosphoacetylation (p-Ser-10/Ac-Lys-14) in NB4 cells treated with 1.6 μM As_2O_3. Total histone H3 was detected using a histone H3 monoclonal antibody.

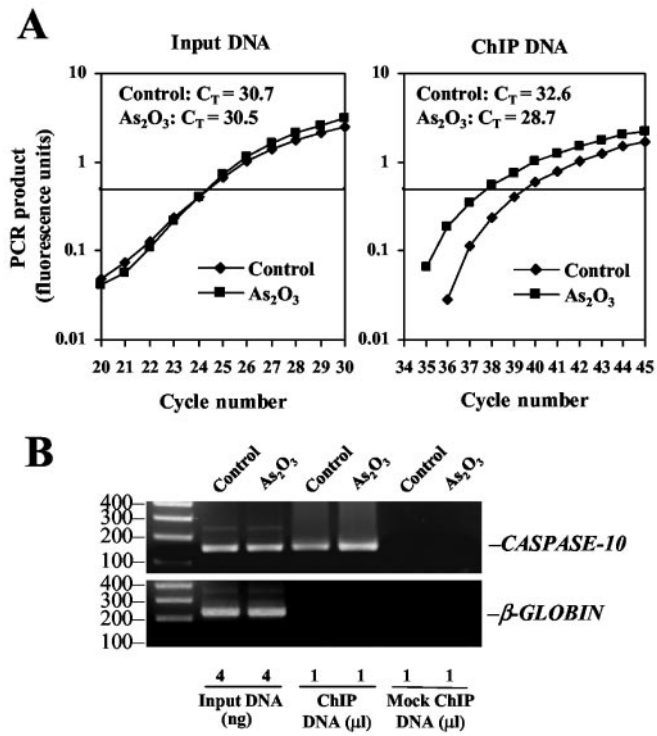

FIG. 7. **Analysis of histone H3 phosphoacetylation at the chromatin of the *CASPASE-10* gene.** NB4 cells were treated with As_2O_3 (1.6 μM) for 48 h or left untreated, whereupon chromatin was prepared after cross-linking using formaldehyde. ChIP assays were performed using either rabbit pre-immune serum or an antibody specific for phosphoacetyl-histone H3. *A*, real-time PCR amplification of *CASPASE-10* from template DNA isolated from the input chromatins (*left graph*) or from template DNA isolated from immunoprecipitated chromatins using an antibody against phosphoacetyl-histone H3 (p-Ser-10/Ac-Lys-14) (*right graph*). C_T values representing the cycle number required to reach the threshold 0.5 are indicated in the graphs. *B*, final products of *CASPASE-10* (*upper panel*) and *β-GLOBIN* (*lower panel*) obtained after the completion of the real-time PCR (45 cycles) using either *Input DNA*, *ChIP DNA*, or *Mock ChIP DNA* (no antibody) as templates.

ing caspases-2 and -3 (Figs. 1, 2, and 5). Through the use of large-scale gene expression analysis, we have shown that As_2O_3 treatment of NB4 cells rapidly induced the expression of *caspase-10* (Fig. 3). The marked As_2O_3-triggered elevation in caspase-10 activity (Fig. 4) appeared to be critical for the activation of caspase-3 (Fig. 5). This is evidenced by the observation that the caspase-10-selective inhibitor Z-AEVD-FMK at a very low concentration (2 μM) markedly attenuated As_2O_3-induced caspase-3 activation. Nevertheless, it is important to note that caspase-10 is probably only partially responsible for executing As_2O_3-induced apoptosis, because Z-AEVD-FMK even at a high concentration of 50 μM only partially inhibited the apoptotic process (Figs. 4*B* and 5*D*). This observation is consistent with our results that Z-AEVD-FMK did not totally abolish caspase-3 activation, although it significantly delayed caspase-3 activation (Fig. 5). Indeed, it has been reported that caspase-8 is activated in APL cells in response to As_2O_3 treatment and plays a role in As_2O_3-induced apoptosis (18). It is possible that both caspase-10 and the structurally related caspase-8 contribute to the activation of caspase-3. In the presence of Z-AEVD-FMK, caspase-10 activity was inhibited, whereas caspase-8 was still active, resulting in attenuated caspase-3 activation (Fig. 5, *A–C*) and decreased apoptosis (Figs. 4*C* and 5*D*).

A critical role for histone deacetylase-dependent transcriptional repression by PML-RARα has recently been established in the pathogenesis of APL (7–9, 30). Indeed, the stimulation of histone modification is emerging as a common property shared by many effective APL therapeutic regimens including ATRA and various histone deacetylase inhibitors (11, 31–33). Here, we have examined the effect of As_2O_3 on histone H3 modification. Clinically relevant doses of As_2O_3 were found to potently enhance histone H3 phosphorylation and phosphoacetylation (Fig. 6), modifications that are similar to those reported to occur following treatment with mitogens and stress agents (20, 23, 34). The role that histone H3 phosphorylation and acetylation plays in activating gene transcription has recently been documented (20, 34, 35). Here, we show that As_2O_3 strongly increases histone H3 phosphoacetylation at the *CASPASE-10* chromatin (Fig. 6). Thus, enhanced histone phosphoacetylation at the *CASPASE-10* chromatin is likely to contribute to the observed increase in *CASPASE-10* expression, caspase-10 activation, and subsequent apoptosis.

As_2O_3 may promote apoptosis through multiple mechanisms. Caspase-10 activation is probably only part of the apoptotic execution machinery, because As_2O_3 causes $\geq$2-fold change in the expression of only ~2% of genes on the DNA array (25 genes of 1,176). In this regard, As_2O_3 enhances gene expression in a relatively selective manner. This finding is consistent with our observation that neither As_2O_3 significantly altered the levels of hyperacetylated (Ac-Lys-9/Ac-Lys-14) at the global levels (Fig. 5), nor did it induce histone H3 acetylation at the *β-GLOBIN* locus (Fig. 6*B*). Although it is unclear how the relative specificity of As_2O_3 is achieved, we can speculate that it may involve the degradation of the PML-RARα and a protein kinase that can phosphorylate histone H3 at Ser-10. Interestingly, it has been shown that As_2O_3 induces the phosphorylation of the SMRT protein, the dissociation of SMRT from its nuclear receptor partner including PML-RARα, and the trans-

port of SMRT from the nucleus into the cytoplasm through a MAPK-mediated mechanism (36). Moreover, it has been reported that As_2O_3-induced apoptosis of NB4 cells is partially blocked by a pharmacological inhibitor of MAPK kinase; thus, further supporting a significant role of MAPK in the process (36). MAPK can activate two downstream kinases, MSK1 and $p90^{Rsk2}$ (37, 38), which are shown to directly phosphorylate histone H3 at Ser-10. It has been shown that both epidermal growth factor and stress stimulate histone H3 phosphoacetylation through a MAPK-mediated pathway (19, 23). Thus, one possible mechanism that As_2O_3 induces apoptosis in APL cells is by promoting histone H3 phosphoacetylation and degradation of PML-RARα at the promoters of ATRA-regulated genes through a process regulated at least in part by MAPKs. Further characterization of the mechanisms involved in this process may aid in the development of more effective less toxic therapeutic regimens for APL.

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MOLECULAR BASIS OF CELL AND DEVELOPMENTAL BIOLOGY:

Arsenic Trioxide Promotes Histone H3 Phosphoacetylation at the Chromatin of *CASPASE-10* in Acute Promyelocytic Leukemia Cells

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