ATR, PML, and CHK2 Play a Role in Arsenic Trioxide-induced Apoptosis

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Arsenic trioxide (ATO) is a potent anti-leukemic chemotherapeutic agent for acute promyelocytic leukemia (APL) that results from a t (15, 17) chromosomal translocation that produces PML-RARα, a fusion protein between a tumor suppressor PML and the retinoic acid receptor RARα. APL patients are initially treated with retinoic acid, but most develop resistance and relapse. In contrast, ATO induces prolonged remissions even in the relapsed cases. However, the molecular mechanisms by which ATO kills the leukemic cells are not fully understood. We find that ATO induces apoptosis, at least in part, by activating proapoptotic kinase Chk2. ATO does this by stimulating ATR (ataxia telangiectasia mutated and Rad3-related) kinase, a Chk2-activating kinase. In conjunction, ATO degrades PML-RARα, resulting in the restoration of PML, which is required for autophosphorylation and full activation of Chk2. As a result, the p53-dependent apoptosis pathway is activated. Based on this, we propose that a pathway composed of ATR, PML, Chk2, and p53 plays a role in ATO-mediated apoptosis, a notion that is consistent with the observation that Chk2 is genetically intact and mutations in the p53 gene are extremely rare in APL.

Most acute promyelocytic leukemia (APL) cases have t (15, 17) chromosomal translocation between a proapoptotic tumor suppressor PML (1, 2) and the retinoic acid receptor α (RARα) (3). The resulting PML-RARα fusion protein functions as a constitutive transcriptional silencer in the retinoic acid signaling pathway, thereby inducing a differentiation block. Two commonly used treatments for APL include ATRA (all-trans retinoic acid) and arsenic trioxide (ATO). ATRA functions by degrading PML-RARα and releasing the differentiation block. ATO also degrades PML-RARα and restores the level of PML expressed from the unaffected allele, but unlike ATRA, ATO induces a potent apoptosis of the APL cells in culture. ATO-induced apoptosis is thought to play a major role for the therapeutic effects of ATO (4–6). In addition, ATO induces pleiotropic effects including oxidative stress and, at a low dose, partial differentiation (7, 8).

ATO is known to activate JNKs, p38 kinase, and ERKs in the mitogen-activated protein kinase pathway (9–11). The mitogen-activated protein kinase pathway was shown to enhance the proapoptotic effects of the PML protein after ATO treatment. ATO was shown to induce phosphorylation of PML through the ERK pathway, and this increased phosphorylation was associated with increased sumoylation and increased apoptotic activity of PML (9). Apoptosis was decreased by expression of the PML mutants that contain mutations in all of the sumoylation sites or ERK phosphorylation sites. However, these PML mutants still exhibited a considerable amount of apoptosis compared with the control empty vector, suggesting that PML could contribute to ATO-induced apoptosis by additional mechanisms.

Our previous work suggested that PML functions upstream and downstream of the proapoptotic Chk2 kinase in the p53-dependent and -independent apoptotic pathways (12, 13). DNA damage-induced Chk2 directly phosphorylates p53 and is essential for transcriptional and apoptotic activities of p53 (14, 15). In our previous study (13), we have shown that PML plays an important role in Chk2 activation by facilitating autophosphorylation after γ irradiation and that in APL-derived NB4 cells PML-RARα suppresses Chk2 by dominantly inhibiting autophosphorylation, which is required for full activation. Because Chk2 is critical for p53 apoptotic activity and mutations in the p53 gene are extremely rare in APL (16), these results suggested that a chemotherapeutic agent that induces PML-RARα degradation and concomitantly stimulates DNA damage response may restore the PML-Chk2 pathway to induce apoptosis in APL cells.
In this study, we have shown that PML potentiates ATO-induced apoptosis, at least in part, by activating Chk2 kinase. ATO modulated ATR kinase activity for Chk2 activation and induction of apoptosis, and PML contributed to ATO-induced apoptosis by facilitating Chk2 autophosphorylation. By demonstrating that ATR, Chk2, and PML play a role in ATO action, this study adds new molecular insights into the ATO-induced apoptosis.

EXPERIMENTAL PROCEDURES

Mice and Cells—129Sv PML−/− mice (17) (P. P. Pandolfi, Sloan-Kettering) and 129Sv/C57BL/6 Chk2−/− mice (18) (N. Motoyama, Aichi, Japan), ATM−/−, ATR+/−, (19), p53−/−, and SCID mice were used in this study. Mouse embryonic fibroblasts (MEFs) were prepared from embryos at day 13.5 of development. Early passage MEFs (<5) were used in all experiments. Splenocytes or thymocytes were prepared from 6–8-week-old mice.

Co-Immunoprecipitation Assay and Immunoprecipitation Kinase Assay—Assays were performed as described previously (13). For detection of γ-H2AX phosphorylation, harvested cells were resuspended in urea-containing lysis buffer (9 M urea, 75 mM Tris-HCl, pH 7.5, 0.15 M β-mercaptoethanol) and sonicated for 10–15 s. Supernatants after centrifugation were used for immunoblotting analysis with anti-Ser-139 phospho-γ-H2AX antibody.

Confocal Microscopy—NB4 cells were treated with ATO and harvested on coverslips. The cells were washed in PBS and fixed for 30 min with 4% formaldehyde in PBS with 0.1% Triton X-100. After five PBS washes, the cells were permeabilized with 0.05% saponin at room temperature for 30 min, followed by two PBS washes. The cells were blocked with 1% chick egg albumin (A-5503; Sigma) in PBS, 0.5% Nonidet P-40 at room temperature for 30 min, followed by two PBS washes. After the wash, the cells were incubated with monoclonal anti-human PML (PG-M3, SC-966, 1:100 dilution; Santa Cruz Biotechnology) and polyclonal anti-Chk2 raised against glutathione S-transferase-tagged recombinant Chk2 protein (20) at 4 °C overnight. After PBS washes, the cells were incubated with Texas Red-labeled anti-mouse IgG (1:75 dilution; Vector Laboratories) or fluorescein isothiocyanate-labeled anti-rabbit IgG (1:75 dilution; Vector Laboratories) for 30 min. After washing extensively in PBS and 0.5% Nonidet P-40, cells were further washed in water and mounted in Vectashield mounting medium (Vector Laboratories). Images were captured using a Leica SP confocal microscope.

Small Interference RNA Experiments—The small interference RNA constructs targeting human ATR, human Chk1, and GFP were generated by inserting cDNA sequences of ATR (5′-GGGAGCCCTGTGAGACAGATTA-3′), Chk1 (5′-GGGAGAAGGTTGCTATGGAGAAA-3′), or GFP (5′-GGGAGTGCCACCTACGGCAAGA-3′) into REP4-Puro episomal vector under the control of U6 promoter (21). These plasmids were transfected into HeLa cells with Effectene transfection reagent (Qiagen, Valencia, CA). Puromycin (20 μg/ml) was added to the culture after 24 h of transfection, and cells were cultured in the presence of Puromycin for 1–2 weeks. Culture media were changed every 2–3 days and Puromycin-resistant cells were used to examine the ATO effect.

Real-time PCR Analysis—RNAs were isolated from splenocytes or thymocytes from various null mice and HeLa cells expressing ATR siRNA and the control siRNA. Puma induction was examined by reverse transcription PCR. A pair of primers specific to each gene was purchased from Applied Biosystems, Inc.: Inv Puma (Mm 00519268_m1); glyceraldehyde-3-phosphate dehydrogenase (internal control, Mm 99999915-g1). The relative amounts of specifically amplified cDNA were calculated using glyceraldehyde-3-phosphate dehydrogenase as an internal control. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase level.

Apoptosis and Cell Death Analysis—Splenocytes or thymocytes derived from 6–8-week-old ATM−/−, ATR+/−, PML−/−, Chk2−/−, p53−/− mice and the corresponding wild-type mice were treated with ATO for the indicated hours and stained with Annexin V and propidium iodide (Roche Applied Science). Apoptotic cells were detected by flow cytometric analysis. Values were normalized to the number of apoptotic cells remaining in untreated cultures derived from the same animal.

RESULTS AND DISCUSSION

In our previous study, we have shown that PML and Chk2 interact in vivo and some Chk2 exist in the PML-containing nuclear foci termed PML nuclear bodies (NB) (12). In APL-derived NB4 cells, PML NBs are disrupted and PML is despersed in microspeckles. Because ATO treatment restores PML NBs upon PML-RARα degradation and PML interacts with Chk2, it was expected that ATO would induce relocalization of Chk2 to the NBs upon PML-RARα degradation and PML interacts with Chk2, it was expected that ATO would induce relocalization of Chk2.
a subpopulation of Chk2 into the PML NBs in NB4 cells. Indeed, Chk2 staining in PML NBs was observed in NB4 cells starting after 6–8 h of ATO treatment (supplemental Fig. S1). Longer exposure of ATO (36–48 h) caused disappearance of PML NBs in NB4 cells as reported in other studies (22); however, Chk2 degradation was not observed as detected by the anti-Chk2 immunoblotting experiment (not shown). We were then curious to know whether ATO affects Chk2 activation (Fig. 1).

Activation of Chk2 requires phosphatidylinositol 3-kinase-like kinases ATM and ATR, which sense DNA damage (15). Another upstream phosphatidylinositol 3-kinase-like kinase, DNA-dependent protein kinase, is also known to regulate Chk2 after γ irradiation (23). Once activated by γ irradiation, Chk2 activity may be sustained up to 24 h (12). Phosphorylation of threonine 68 by ATM/ATR or DNA-dependent protein kinase leads to autophosphorylation of threonines 383 and 387 (Thr-383/387) in the activation loop, a required step for full activation of Chk2 (15).

In Fig. 1, a and b, ATO treatment alone was sufficient to cause Thr-68 and Thr-383/387 phosphorylation in NB4 cells and 293T cells. Chk2 activation was also observed in U937 cells.
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DNA double strand breaks without causing PML-RARα degradation induced Thr-68 phosphorylation but no autophosphorylation (phosphorylation of Thr-383 and Thr-387) in NB4 cells. These results demonstrated that ATO activates Chk2, and this was also confirmed with the immune complex kinase assay (Fig. 1c).

In Fig. 2a, ATO induced H2AX phosphorylation (Ser-139) in NB4 cells with similar kinetics to that of Chk2 Thr-68 phosphorylation (Chk2 level, not shown). Because ATM or ATR rapidly phosphorylates H2AX after DNA double strand breaks (25, 26), ATO-induced Chk2 activation and H2AX phosphorylation suggested the possibility that ATO might generate DNA lesions. Alternatively, ATO may activate Chk2 or ATM/ATR through the inhibition of phosphatases for these kinases. We then thought that ATM, which phosphorylates Chk2 Thr-68 and H2AX after γ irradiation, may be one of the upstream kinases that activate Chk2 in response to ATO. However, in NB4 cells ATO did not induce ATM autophosphorylation, a hallmark of activated ATM, at the same dose and time points that it induced H2AX phosphorylation and Chk2 Thr-68 phosphorylation (Fig. 2a, ATM autophosphorylation after γ irradiation, not shown). Consistent with this, ATO treatment resulted in phosphorylation of H2AX and Chk2 in both AT<sup>−/−</sup> and AT<sup>+</sup> cells (Fig. 2b, upper panel and lower left panel) and lymphocytes derived from ATM<sup>−/−</sup> and ATM<sup>+/+</sup> mice (not shown). In AT<sup>+</sup> cells, ATM autophosphorylation after ATO treatment was negligible compared with what occurred after 2 Gy γ irradiation.

Similarly, ATO-induced phosphorylation of H2AX and Chk2 was also observed in splenocytes derived from DNA-dependent protein kinase-deficient SCID mice (severe combined immunodeficiency) (27) (Fig. 2b, lower right panel). These results indicated that ATO-stimulated phosphorylation of H2AX and Chk2 is mediated by a kinase(s) other than ATM or DNA-dependent protein kinase. One possibility was that ATR, which is known to activate Chk2 in response to UV irradiation or hydroxyurea (28), plays a role in ATO action.

ATR mediates cell cycle checkpoint by phosphorylating (serine 345) and activating the effector kinase Chk1 in response to stalled replication forks, UV damage, single strand DNA breaks,
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and double strand DNA breaks (29–31). Although ATR is the main kinase responding to replication stress after the treatment with hydroxyurea or UV irradiation (32), UV-induced ATR activation was observed in non-replicating human fibroblasts, indicating that ATR activation can occur independent of replication stress (33).

To examine whether ATR is involved in ATO action, we tested whether ATO induces ATR-dependent phosphorylation of Chk1. As shown in Fig. 2c, ATO induced Chk1 phosphorylation (Ser-345) in NB4 cells and 293 T cells, suggesting that ATR may be involved in ATO action. The level of Chk1 or ATR (not shown) did not change significantly within 24 h of treatment, and ATRA treatment in NB4 cells did not cause Chk1 activation (not shown). As expected, ATO induced Chk1 phosphorylation in AT+/+, AT−/− cells (not shown). Interestingly, Chk1 phosphorylation by ATO occurred much earlier than phosphorylation of H2AX or Chk2(Thr-68) in NB4 cells (Fig. 2c, the level of Chk2 and H2AX, not shown), suggesting that ATO activates ATR prior to H2AX phosphorylation. At this point, we do not understand how ATO activates ATR, Chk1, or Chk2. One possibility is that oxidative stress induced by ATO may lead to the formation of DNA damage lesions either directly or through replication stress to activate DNA damage signaling. Alternatively, ATO may activate these kinases through the inhibition of phosphatases.

We next sought to examine whether ATR activity is required for ATO-induced phosphorylation and activation of Chk2. In splenocytes derived from ATR+/+ mice or HeLa cells expressing ATR siRNA, ATO-induced Chk2 phosphorylations (Thr-68, Thr-383/387) were significantly reduced (Fig. 2d). H2AX phosphorylation was also inhibited in cells expressing ATR siRNA (not shown). As expected, Chk1 phosphorylation was inhibited in cells transfected with ATR siRNA vector. Similar inhibition of the Chk2 and Chk1 phosphorylation was observed with another ATR siRNA (5'-AACCTCCGTTAGGTGCTGA-3', not shown). Chk2 or Chk1 phosphorylation in the absence of ATO treatment but after transfection with siRNA vectors (Fig. 2, d and e) may be due to the basal level activation of these kinases triggered by transfected DNA. The results in Fig. 2d suggest that ATO-induced Chk2 activation requires ATR. These results, however, do not necessarily indicate that ATO-induced Chk2 activation is carried out solely by ATR. For example, other upstream kinases such as DNA-dependent protein kinase, which is known to regulate Chk2 and phosphorylate H2AX after γ irradiation (23, 34), may also contribute to Chk2 activation in collaboration with or in parallel to ATR.

Because ATO activates Chk1 before Chk2, we examined whether Chk2 activation after ATO treatment is Chk1 dependent (Fig. 2e). ATO-induced Chk2 Thr-68 phosphorylation (not shown) and autophosphorylation were still observed in HeLa cells transfected with Chk1 siRNA vector (Chk2 level, not shown). As expected, Chk1 activation after hydroxyurea treatment was not detected in cells expressing Chk1 siRNA. These results indicated that ATO-induced Chk2 activation is Chk1 independent.

In Fig. 2f, Chk1 phosphorylation occurred efficiently in Chk2+/+, Chk2−/− splenocytes as well as in PML+/+, PML−/− splenocytes (not shown). Although we currently do not understand the mechanism of how ATO induces Chk1 phosphorylation in splenocytes, these results suggested that the ATR-Chk1 pathway is not responsible for the differential apoptotic effects of the wild-type and various null lymphocytes derived from the Chk2 or PML mice in Fig. 4.

Chk2 autophosphorylation, which follows Thr-68 phosphorylation by ATM or ATR, is an essential step for Chk2 activation and is PML dependent after γ irradiation (13). Previously, ATO-induced apoptosis was shown to be mediated by both PML-dependent (9) and PML-independent mechanisms (35). ATO treatment induces phosphorylation and sumoylation of PML through a mitogen-activated protein kinase pathway, and these posttranslational modifications of PML were shown to be important for the proapoptotic effects of ATO (9). In other studies, however, PML−/− MEFs were as sensitive as PML+/+ MEFs to the growth inhibitory or apoptotic effects of ATO, suggesting that PML-independent mechanism may also contribute to ATO-induced cell death under certain conditions (35). We then tested whether the PML status is important for ATO-induced Chk2 activation.

Phosphorylations of H2AX, Chk2(Thr-68), and Chk1 were not significantly compromised by the PML deficiency as shown in ATO-treated PML−/− splenocytes (Fig. 3a). Chk2 autophosphorylation was observed in PML+/+ cells starting after 4–6 h of ATO treatment (not shown). However, ATO-induced Chk2 autophosphorylation was impaired in PML−/− cells as shown by the results obtained after 8–16 h of treatment (Fig. 3b, Thr-68 phosphorylation of Chk2 in the PML+/+ and PML−/− cells is not shown in lower panel). Similar results were obtained using 1–2 μM concentrations of ATO for 24 h (not shown).

Together with the results shown in our previous study (13), these observations suggest that regardless of the initiating source to stimulate DNA damage signaling (γ irradiation or ATO) or the identity of the upstream kinase (ATM or ATR), Chk2 autophosphorylation and activation require PML. To date, we have not observed any direct interaction between ATR and PML (endogenous PML or ectopically expressed PML IV) either before or after ATO treatment (0–24 h, 0–10 μM ATO), suggesting that Chk2 activation may not require protein-protein interaction of ATR with PML (not shown).

DNA damage-induced apoptosis requires Chk2, p53, and the p53-induced apoptotic protein PUMA. We investigated the role of ATR and the Chk2-p53 pathway in ATO-mediated induction of p53-responsive apoptotic gene Puma (36) in ATO-treated cells. Although ATO treatment increased the level of p53, the induction of p53 was not significantly affected by PML or Chk2 deficiency (Fig. 3c). On the other hand, ATO-induced Puma induction was inhibited in HeLa cells transfected with

FIGURE 4. Involvement of ATR, PML, and Chk2 in ATO-induced apoptosis. a–e, splenocytes or thymocytes from various null mice were subjected to ATO treatment. Cells were collected after the indicated hours and stained with Annexin V and propidium iodide. Values were normalized to the number of apoptotic cells remaining in untreated cultures derived from the same animal. f, diagram of involvement of ATR, PML, and Chk2 in ATO-induced apoptosis.
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*ATR* siRNA vector and in *Chk2*−/− and *PML*−/− splenocytes (Fig. 3d). Therefore, ATO-induced Chk2 appears to increase PUMA expression by increasing the transcriptional activity of p53 rather than its level. This is consistent with the previous finding that Chk2 increases p53 activity more than its level after γ irradiation (18).

To demonstrate the role of Chk2 in ATO-induced apoptosis, we measured apoptosis in ATO-treated lymphocytes from mice deficient in ATM, ATR, DNA-dependent protein kinase (not shown), PML, Chk2, or p53 (Fig. 4). Consistent with the observation that Chk2 activation by ATO is not impaired in ATM−/− cells or SCID cells (Fig. 2b), ATM−/− lymphocytes (Fig. 4a) or SCID splenocytes (not shown) were sensitive to ATO-induced apoptosis. ATM−/− lymphocytes showed even a higher rate of apoptosis than ATM+/− lymphocytes (Fig. 4a). Similar results were obtained after 12 h of treatment in ATM−/− lymphocytes (not shown). The levels of ATO-induced apoptosis in the wild-type and SCID splenocytes were similar (not shown). Although the mechanism for the higher level of apoptosis in ATM−/− lymphocytes is not understood, these results indicated that ATM or DNA-dependent protein kinase is dispensable for ATO-induced apoptosis.

In contrast, ATR+/+, PML−/−, Chk2−/−, and p53−/− lymphocytes were less sensitive to ATO-induced apoptosis compared with the wild-type cells, supporting a role of the Chk2 pathway in the ATO-induced apoptosis (Fig. 4, b–e). The decreased apoptosis in PML−/− cells (Fig. 4c), however, may not be solely due to the impaired PML-Chk2 pathway, because PML receives ATO-induced mitogen-activated protein kinase signaling to enhance apoptosis (9) and PML may influence multiple p53 activities (1, 2) in response to ATO.

This study provides new molecular insights for ATO-induced apoptosis by demonstrating that it is mediated, at least in part, by the DNA damage pathway composed of ATR, PML, and Chk2. Although it remains to be seen whether the ATR/PML/Chk2 pathway indeed plays a role in the therapeutic action of ATO in APL patients, it is possible to speculate that in APL cells, where Chk2 and its downstream apoptotic effectors are functionally suppressed by PML-RARα, ATO may create two conditions required for Chk2 activation: activation of DNA damage signaling and restoration of PML by causing PML-RARα degradation (Fig. 4f). The ATR/PML/Chk2 pathway shown in this study could function cooperatively with the ERK, JNK, and p38 pathways or other unidentified signaling pathways to enhance sensitivity to apoptosis. Whereas DNA damage signaling may play a role in ATO-induced apoptosis, our results suggest that anti-cancer chemotherapeutics that merely activate the ATR/ATM pathway in the absence of PML-RARα degradation would not be as effective as ATO for APL treatment. By inducing PML-RARα degradation in APL cells where the wild-type p53 is expressed, ATO may be able to activate Chk2. This view is also consistent with the clinical findings that virtually all treatments that cause remissions in APL restore PML or PML NBs, underscoring an important role of PML for APL treatment (9).

PML protein is frequently lost in various human cancers (37), and PML gene variants are detected in non-APL hematopoietic malignancies and retinoic acid-resistant APL cases (38). PML loss was also reported to be highly associated with tumor progression and metastatic status in several cancers. Our results suggest the danger of using cancer chemotherapeutics that induce DNA damage response for PML-disrupted tumors: inflicting DNA damage response in Chk2-suppressed cells may confer survival of genomically unstable cells and cause increased incidence of secondary therapy-induced malignancies.

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