Short Communication

Ataxia telangiectasia and rad3 related (ATR)-promyelocytic leukemia protein (PML) pathway of the DNA damage response in the brain of rats administered arsenic trioxide

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Abstract: To examine the *in vivo* responses of promyelocytic leukemia protein (PML) to arsenic, rats (male, 6 weeks old, Sprague Dawley) were administered a single intraperitoneal dose of 5 mg/kg arsenic trioxide (ATO). The protein was examined in the heart, lung, liver, and brain 6 and 48 hours after administration: a significant response of PML was observed in the brain. Oxidative DNA modification was also observed in the brain as revealed by increased immunoreactivity to anti-8-hydroxy-2'-deoxyguanosine (8-OHdG) antibody. In contrast, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) stain reactivity was only slightly increased, suggesting oxidative cellular stress without apoptotic cell death in the ATO-administered rat brain. Among the DNA damage response pathways, the ATR-Chk1 axis was activated, while the ATM-Chk2 axis was not, implying that the PML response is associated with activation of the ATR-Chk1 DNA repair pathway in the brain. (DOI: 10.1293/tox.2017-0020; J Toxicol Pathol 2017; 30: 333–337)

Key words: arsenic trioxide, brain, DNA damage, promyelocytic leukemia protein, ataxia telangiectasia and rad3 related

Arsenic is an environmental risk factor for humans as it is a frequent contaminant in drinking water, especially in developing countries[1–3]. Arsenic trioxide (ATO) is one of the most toxic species among arsenic substances, as trivalent arsenic (As^{3+}) acts as a bridge between vicinal cysteine residues within and/or between proteins.

The promyelocytic leukemia (PML) protein is a multifunctional protein involved in inflammation, aging, and cancer[4]. PML is a highly cysteine-rich protein and, therefore, is extraordinarily sensitive to ATO[5]. The *in vivo* response of this protein is the basis for the efficacy of ATO as a medication for the treatment of acute promyelocytic leukemia (APL), which is caused by the generation of the oncogenic PML-retinoic acid receptor (RAR) fusion protein[6, 7]. Binding to ATO causes PML to undergo conformational changes that lead to its degradation by cellular proteases.

PML undergoes a complicated posttranscriptional modification process that includes the addition of small ubiquitin-like modifier (SUMO) and SUMO-dependent ubiquitination by RING finger protein 4[4]. The addition of SUMO to PML is necessary not only for its degradation but also for its oligomerization and the subsequent formation of PML-nuclear bodies, which are implicated in the cellular response against oxidative stresses[8]. Both the PML-RAR oncogenic fusion protein and the wild type PML protein have been shown to be extremely sensitive to arsenic *in vitro*[5], but their sensitivity *in vivo* has so far received little attention in the literature.

Oxidative DNA damage is a form of oxidative cellular stress and leads to two distinct, but mutually interacting, cellular responses: 1) when the DNA damage is relatively serious, such as a double-strand DNA break, ATM (ataxia telangiectasia mutated) and its downstream target Chk2 (check point kinase2) are activated, thus leading to apoptosis; 2) when the DNA damage is relatively mild, such as an oxidative DNA modification or nick formation, ATR (ATM and rad3 related) and its downstream Chk1 are activated to repair the DNA damage[9]. In the response to double-strand DNA break, histone H2AX is phosphorylated by ATM[10]. The aim of this study was to examine the *in vivo* effect of ATO on the PML protein and the possible pathophysiological implication of the PML response to ATO.

The anti-PML antibody was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and the antibodies against ATR, p-ATR (Ser428-phosphorylated), p-ATM (Ser1981-phosphorylated), p-Chk1 (Ser345-phosphorylated), p-Chk2 (Thr68-phosphorylated), p-H2AX (Ser139-

phosphorylated), and cleaved caspase-3 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-actin and anti-8-hydroxy-2'-deoxyguanosine (8-OHdG) antibodies were from Sigma-Aldrich (St. Louis, MO, USA) and JaICA (Shizuoka, Japan), respectively. Animal experiment protocols were approved by the Institutional Animal Care and Use Committee of the Tokyo Medical and Dental University. Sprague-Dawley rats (male, six-weeks-old, $n = 4$/group) received a single intraperitoneal injection of ATO (Sigma, St. Louis, MO, USA; 1 mg/mL solution prepared in PBS, pH 7.4) at a dose of 5 mg/kg body weight (ATO group) or PBS (control group). The rats were sacrificed 6 or 48 hours after injection, the organs were extracted, and the tissues were fixed immediately in formalin and embedded in paraffin for histological and immunohistochemical analysis. Tissue lysates were also extracted and stored at −80°C until used for western blot analysis. Western blot analysis was performed as described previously[11, 12]. In brief, tissue lysates were electrophoresed, blotted to PVDF membranes (Millipore), and probed with the abovementioned primary antibodies, followed by incubation with appropriate secondary antibodies conjugated with horseradish peroxidase. For immunohistochemical analysis, paraffin-embedded tissue sections were deparaffinized, rehydrated, and retrieved with their antigenicity by microwave exposure in 10 mM sodium citrate buffer. The samples were then incubated with anti-8-OHdG antibody overnight at 4°C, and the antigens were visualized using a Histofine Simple Stain MAX PO kit (Nichirei Biosciences, Tokyo, Japan) with diaminobenzidine (DAB) as a substrate and examined under a light microscope (Olympus AX80). For terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis, an Apoptosis *in situ* detection kit (Wako, Osaka, Japan) was used.

We first examined the responses of the PML protein to ATO administration in the major organs of rats. The rats were treated with ATO or PBS (control), and western blot analysis was performed to examine the status of the PML protein in the heart, lung, liver, and brain (Fig. 1A). In accordance with the presence of various PML isoforms that are generated from a single gene through tissue-dependent alternative splicing[13], western blot analysis showed different band patterns among the organs: while no apparent changes in the PML proteins were observed in the heart or liver, numerous changes were observed in the lung and brain (Fig. 1A). In the ATO-treated lung, ~55 kDa and 100 kDa species of PML seemed to be upregulated and downregulated, respectively (Fig. 1A). In the brain, two bands (bands A and B) showed clear and statistically significantly different patterns between ATO-treated and control samples (Fig. 1 A and B). *In vitro* experiments have indicated that prior to PML degradation, SUMO- and/or ubiquitin-modified high molecular weight PML species accumulate transiently in ATO-treated cells[13]. Thus, this high molecular PML (band A) might be

SUMO/ubiquitin-modified PML, but for the time being, we cannot draw a definitive conclusion concerning the exact nature of this PML species. Since the changes in the brain seem to be the most obvious response of PML, we further examined the brains of ATO-administered rats.

Although PML has been implicated in various cellular activities, it has been stressed that PML plays a crucial role in the DNA damage response[8, 14]. As arsenic causes oxidative cellular stress, including oxidative DNA damage, we performed an immunohistochemical analysis of 8-OHdG, a marker of DNA oxidation. Immunoreactivity against an anti-8-OHdG antibody was observed even in the cerebral cortex of rats in the control group, suggesting the development of 8-OHdG during tissue fixation in formalin (Fig. 2A). However, substantially increased immunoreactivity was observed 48 hours after ATO treatment compared with in the case of no treatment, showing the development of oxidative DNA damage during ATO treatment (Fig. 2A). We further checked the status of DNA damage pathway molecules in the ATO-administered rat brain. Western blot analysis indicated the activation of ATR as revealed by the phosphorylation of ATR (Fig. 2 B and E). ATR activation was also supported by the phosphorylation of Chk1, a direct target of ATR (Fig. 2 C and E). In contrast to the activation of the ATR-Chk1 axis, neither phosphorylated ATM nor phosphorylated Chk2 was detected (Fig. 2D). The absence of ATM-Chk2 axis activation was also supported by the constant phosphorylation levels of H2AX, which is generally activated in parallel with ATM-Chk2[9] (Fig. 2C).

We finally evaluated apoptosis in the ATO-treated rat brain. TUNEL-positive nuclei were scarcely observed in the ATO-treated rat brain (Fig. 3A). Only a subtle increase in the levels of cleaved-caspase-3, the activated form of caspase-3, was observed in the ATO-treated brain as compared with the control brain (Fig. 3B). Taking the results shown in Figs. 2 and 3 together, we conclude that ATO at this dose activates the ATR-Chk1 axis of the DNA damage response, which might lead to DNA repair in neuronal cells rather than apoptosis.

In the current study, we observed the response of PML to ATO in the brain but not in the liver or heart (Fig. 1A). The apparent lack of a PML response in the liver is somewhat surprising, but this might be due to the ability of this organ to efficiently detoxify ATO[15–17]. It has been suggested that ATO might penetrate the blood-brain barrier and reach the central nervous system: it has been reported that ATO concentrations in the cerebrospinal fluid of humans treated orally with ATO reach approx. 17–18% of that of the blood concentration[18]. Indeed, there are some reports of coma and encephalopathy arising from ATO[19]. A report has suggested oxidative DNA damage in the brain of mice administered ATO orally[20].

To the best of our knowledge this is the first report demonstrating the *in vivo* response of PML to ATO in the
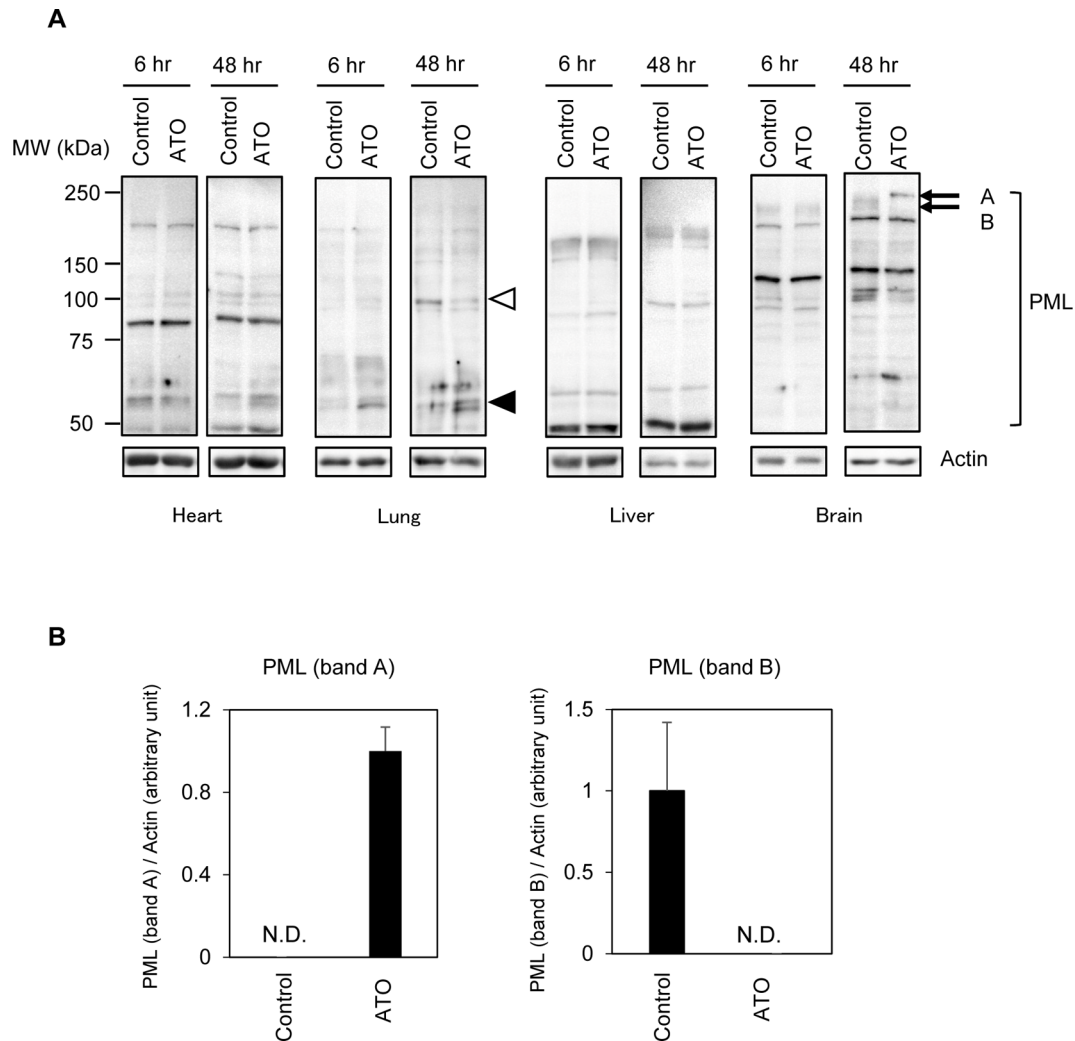
Fig. 1. (A) Western blot analysis of PML protein in the heart, lung, liver, and brain of rats at the indicated times following the administration of arsenic trioxide (ATO). White and black arrowheads indicate protein bands that decreased and increased, respectively, in response to ATO treatment in the lung. (B) Bands A and B indicate protein bands that increased and decreased, respectively, in response to ATO treatment in the brain. Representative western blot images and a densitometric analysis of the levels of bands A and B relative to actin are shown (n = 4). N.D., not detected.

brain. Activation of the ATR-Chk1 system in response to ATO was also demonstrated. PML is one of the targets of ATR, and the nucleolar localization of PML is dependent on its phosphorylation by ATR[21]. On the other hand, PML participates in the activation of Chk1 in response to DNA damage, at least in a certain situation[22]. Thus, ATO might activate ATR-Chk1 in an oxidative DNA damage-dependent manner. ATO-dependent changes in PML dynamics might be also involved in the Chk1 activation. Although at present we cannot draw a definitive conclusion concerning the precise cause-and-effect relationship among them, all of these pathways should converge at the activation Chk1. Given the established importance of PML in oxidative stress and the DNA damage response in cells, the ATR-PML response to

ATO in the brain demonstrated in this study should be important for understanding the pathophysiology of arsenic intoxication.

Disclosure of Potential Conflicts of Interest: The authors declare that there are no conflicts of interest.
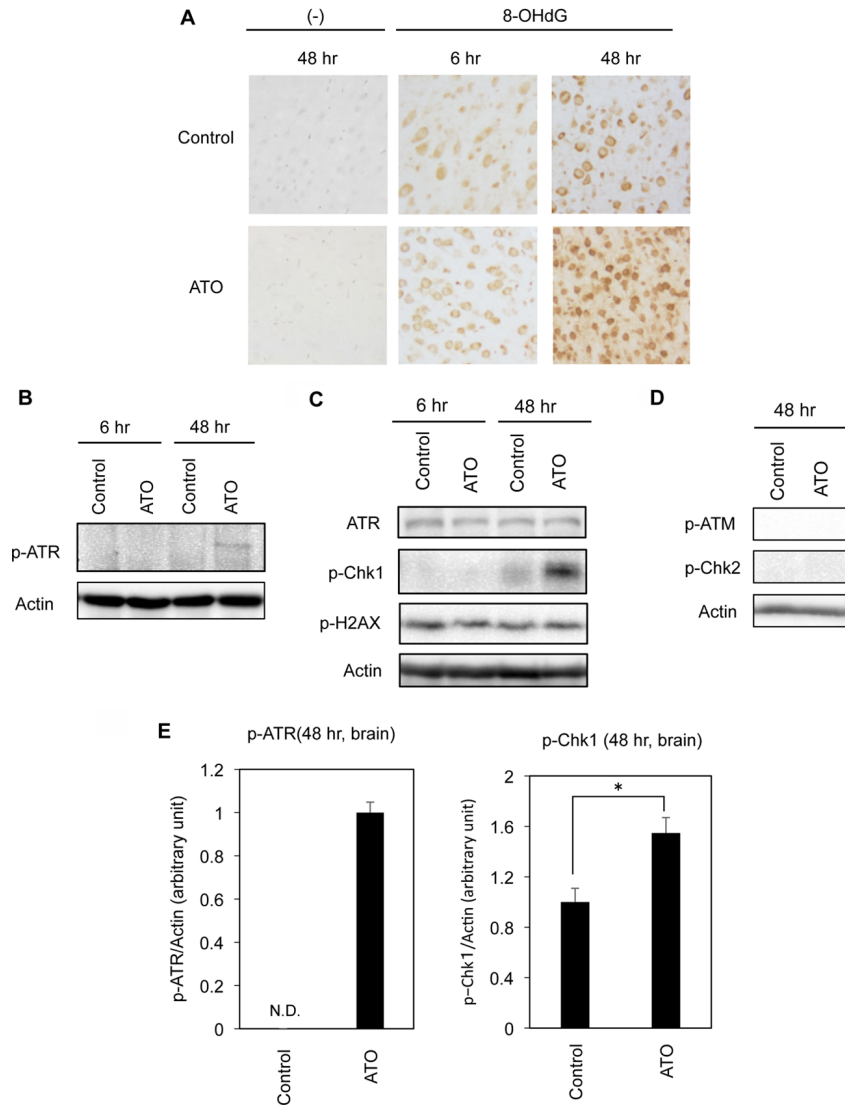
Fig. 2. Oxidative DNA damage and the activation of the ATR-Chk1 axis of the DNA damage response pathway in the brain. Whole brains were excised 6 or 48 hours after the administration of ATO. (A) Immunohistochemical analyses of 8-OHdG in the brain. Immunoreactivities to 8-OHdG in the cerebral cortex are shown. (−) Tissue sections stained without anti-8-OHdG antibody (negative control). (B–E) Western blot analyses for p-ATR, ATR, p-Chk1, p-H2AX, p-ATM, p-Chk2, and actin. Representative results (n = 4) are shown. Densitometric analysis of the levels of p-ATR and p-Chk1 relative to actin are shown (n = 4). *$p<0.05$.
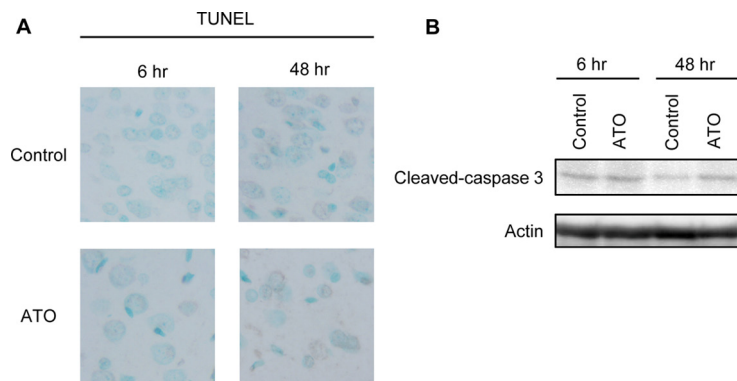
Fig. 3. Neuronal cell death is scarcely observed in the cerebral cortex of the ATO-administered rat brain. Whole brains were excised 6 or 48 hours after the administration of ATO. (A) TUNEL analysis in the cerebrum cortex. Nuclei were also counterstained with methylene blue. Representative images (n = 4) are shown. (B) Cell lysates extracted from whole brain were subjected to western blot analysis using anti-cleaved-caspase-3 and actin antibodies. Representative results (n = 4) are shown.

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