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Arsenite retards the cardiac differentiation of rat cardiac myoblast H9c2 cells

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ABSTRACT

It is well known that exposure to inorganic arsenic through groundwater leads not only to cancer and cardiovascular disease, but also to detrimental effects on development. In this study, we investigated the effects of arsenite on the cardiac differentiation of rat myoblast H9c2 cells. The cardiac differentiation of H9c2 cells cultured in media containing 1% fetal bovine serum and all-*trans* retinoic acid was confirmed by enhanced expression of cardiac troponin T (cTnT), the appearance of multinucleated cells, and cell cycle arrest at G0/G1 phase. Exposure of H9c2 cells to inorganic arsenite (As(III)) during cardiac differentiation suppressed the appearance of the morphological and biological characteristics observed in the cardiac phenotype of H9c2 cells. In addition, As(III) inhibited PKCδ phosphorylation, which is detected in early-stage differentiation. These results suggest that As(III) retards the cardiac differentiation of H9c2 cells, at least partly, via the inhibition of PKCδ phosphorylation.

1. Introduction

Epidemiological studies have indicated that chronic exposure to arsenic via the drinking of well water results in diverse clinical conditions such as cancer of the liver, skin, lung, and bladder; peripheral vascular disorders; and cardiotoxicity, as detected by QT-interval prolongation on electrocardiogram [1–4]. On the other hand, arsenic trioxide is used to treat acute promyelocytic leukemia (APL). The most critical adverse effect that limits the application of this effective agent in APL patients is severe cardiotoxicity. To explore the heart-specific mechanisms involved in protection against arsenic toxicity, several animal studies have been conducted to investigate the modifying factors that are responsible for the cardiotoxicity of arsenicals [5–7]. In addition, our previous study indicated that rat H9c2 cardiac myoblasts were more sensitive to sodium arsenite (As(III)) than rat liver-derived TRL1215 cells; this effect was due to the modest activation of transcription factor Nrf2, which plays an important role in the protection of cells from arsenicals [8].

Several studies have provided evidence that arsenic is a developmental toxicant. Hopenhayn-Rich et al. [9] showed that the risk of late fetal, neonatal, and postneonatal mortality was associated with the arsenic concentration in the drinking water in human populations in Chile. Furthermore, Ahmed et al. revealed that maternal exposure to arsenic through drinking water caused adverse pregnancy outcomes such as spontaneous abortion, still birth, and preterm birth [10]. In a experimental model, exposure of pregnant mice to As(III) through drinking water resulted in lower fecundity due to the impairment of placental vasculogenesis [11]. In addition, Li et al. [12] indicated that exposure of Zebrafish during embryogenesis to As(III) resulted in abnormal ventricular phenotype. However, the mechanisms underlying cardiac abnormality induced by arsenic exposure remain unclear.

When exposed to a low concentration of serum (1% FBS) and all-*trans* retinoic acid (ATRA, 10^{-6} M), H9c2 cells have been reported to differentiate into cardiac myocytes, as determined by the appearance of multinucleated cells and cardiac troponin T (cTnT) expression [13–15]. Although the factors involved in the cardiac differentiation of H9c2 cells are still unknown, it has been reported that PKCδ is involved in the differentiation of leukemia cells induced by ATRA [16,17]. Giacomo et al. [18] demonstrated that the transfection of PKCδ siRNA to H9c2 cells retards the differentiation of H9c2 cells, suggesting that PKCδ plays an important role in H9c2 cell differentiation.

In this study, we utilized this differentiation model to examine whether arsenicals affect cardiac differentiation in mammals. The results indicated that the cardiac differentiation of H9c2 cells was retarded by exposure to As(III), at least in part by the inhibition of PKCδ phosphorylation.

Abbreviations: As(III), inorganic arsenite; ATRA, all-*trans* retinoic acid; cTnT, cardiac troponin T.

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2. Materials and methods

2.1. Materials

Sodium arsenite (As(III)) was purchased from Wako Pure Chemicals (Osaka, Japan). Antibodies for PKCδ, phospho-PKCδ, and anti-β-actin were purchased from Cell Signaling (Beverly, MA). Anti-cTnT was purchased from the Abcam (Cambridge, MA). All other reagents and chemicals used were of the highest grade available.

2.2. Cell culture and cardiac differentiation

Rat H9c2 cardiac myoblasts were obtained from DS Pharma Biomedical (Osaka, Japan). The H9c2 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 using Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemicals) containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) (normal media). The day before differentiation, 1×10^6 cells were seeded onto 10-cm dishes. Cardiac differentiation was initiated by changing the medium to that containing 1% FBS and 10^{-7} or 10^{-6} M ATRA (differentiation media). To improve the cardiac myocyte yield, supplementation with ATRA was carried out daily. The culture medium was replaced every 2 days. In case of exposure to As(III), As(III) was added at the same time as the medium change. The duration of continuous culture in 1% FBS containing ATRA plus As(III) has been described in the Results.

2.3. Western blotting

The total cell lysates were used for Western blotting of all the proteins. Samples for each analysis were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Gels were transferred to an Immun-Blot PVDF membrane and then placed in a blocking solution consisting of TBST (10 mM Tris [pH 8.0], 150 mM NaCl, and 0.05% Tween 20) and 5% skim milk for 1 h. The blotted membranes were incubated with the appropriate antibody, washed with TBST, and incubated with HRP-conjugated secondary antibody. The bound IgG was visualized with Luminata detection reagents (Merck Millipore, Billerica, MA) according to the manufacturer's protocol. The band intensities were measured by ImageJ software (1.44).

2.4. Counts of multinucleated cells

The differentiated cells were washed with PBS followed by fixation for 20 min with 4% paraformaldehyde in PBS. Cells were washed twice with PBS and stained with Hoechst 33342 (Invitrogen, Carlsbad, CA) for 1 h at room temperature in the dark. After two washes in PBS, fluorescent and phase-contrast images were captured with a BIOREVO microscope (Keyence, Osaka, Japan). The numbers of multinucleated cells were calculated from four random fields per treatment.

2.5. Cell cycle analysis

For the cell cycle analysis, 5×10^5 trypsinized cells were stained with 200 μl of Guava Cell Cycle Reagent (Merck Millipore) for 30 min at room temperature. Cell cycle profiles (5×10^3 cells) were analyzed with Guava easyCyte Flowcytometry (Merck Millipore). The data were analyzed using Guava Cell Cycle software.

2.6. Statistical analysis

Data were obtained from three or four separate experiments. The values are shown as means ± SEM. Statistical significance was assessed with ANOVA followed by Tukey–Kramer post hoc testing. Differences between groups were considered statistically significant at $p < 0.05$.

3. Results

To investigate whether As(III) affects cardiac differentiation in rat H9c2 cardiac myoblasts, we first confirmed the differentiation of H9c2 cells into a cardiac muscle phenotype using low-serum media and the daily addition of ATRA [13,15]. As shown in Fig. 1A, cTnT expression was detected in H9c2 cells when the cells were cultured in media containing 1% FBS + ATRA (10^{-7} or 10^{-6} M) (differentiation media) for 4 or 6 days. Treatment of H9c2 cells with differentiation media for 4 or 6 days produced an increase in the number of multinucleated cells of different sizes compared to that of H9c2 cells cultured in normal media (DMEM + 10% FBS) (Fig. 1B). These findings demonstrated that H9c2 cells, when cultured in differentiation media for 4 to 6 days, did express the cardiac phenotype.

In order to examine the effects of As(III) on the cardiac differentiation of H9c2 cells, the H9c2 cells were exposed to 0.1, 0.5, and 1 μM of As(III) added to the differentiation media for 6 days. As shown in Fig. 2A and B, the expression of cTnT significantly decreased with exposure of the cells to 1.0 μM As(III) for 6 days. Next, the number of multinucleated cells was quantified as an indicator of cardiac differentiation. As shown in Fig. 2C, the percentage of multinucleated cells in a field had increased from 1.7 to 10.4 by the time of differentiation. However, exposure to As(III) attenuated the appearance of multinucleated cells in a concentration-dependent manner.

It has been demonstrated that cell proliferation and DNA synthesis are reduced in differentiated H9c2 cells [18]. Thus, we conducted a cell cycle analysis of H9c2 cells cultured in normal or differentiation media in the absence or presence of As(III) (Table 1). The results revealed that the cell populations in G0/G1 phase increased, and those in the S and G2/M phases decreased among H9c2 cells cultured in differentiation media. Exposure of H9c2 cells to As(III) in differentiation media attenuated the

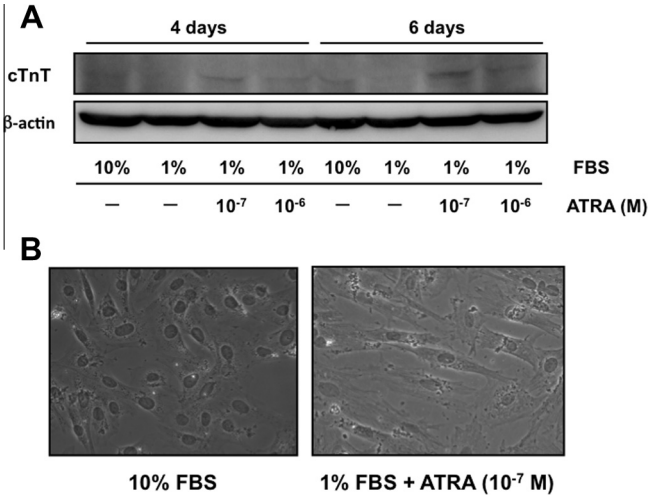

Fig. 1. Cardiac differentiation in H9c2 cells. (A) Cells were cultured with 1% FBS and ATRA (10^{-7} and 10^{-6} M) for 4 and 6 days. Western blot analysis was performed for cTnT. (B) Cells were cultured with 1% FBS and ATRA (10^{-7} M) for 6 days. Phase-contrast images were captured by microscope.

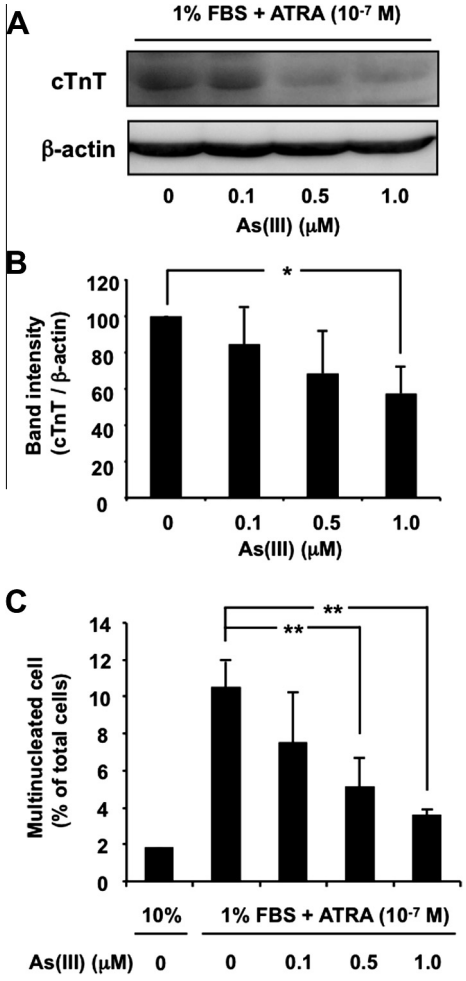

Fig. 2. Effect of As(III) on cTnT expression and the formation of multinucleated cells induced by cardiac differentiation in H9c2 cells. (A) Cells were exposed to the indicated concentrations of As(III) in DMEM with 1% FBS and ATRA (10^{-7} M) for 6 days. Western blot analysis was performed for cTnT. (B) Band intensities were quantified with ImageJ software. Values are relative band intensities of cTnT protein normalized to those of β-actin. (C) Cells were stained with Hoechst 33342. Fluorescent and phase contrast images were captured by microscope. The number of multinucleated cells was calculated from four random fields per treatment. Each value is the mean ± SEM of three individual determinations. $^{*}p < 0.05$, $^{**}p < 0.01$.

Table 1
Effect of As(III) on cell cycle at cardiac differentiation in H9c2 cells.

	G0/G1 phase	S phase	G2/M phase
10% FBS	55.4 ± 1.3	13.2 ± 0.3	22.2 ± 2.7
1% FBS + ATRA	71.3 ± 0.9[a]	8.3 ± 0.3[a]	15.3 ± 0.1[a]
1% FBS + ATRA + 0.1 μM As(III)	68.9 ± 0.8	8.9 ± 0.5	16.2 ± 1.1
1% FBS + ATRA + 0.5 μM As(III)	63.1 ± 0.9[b]	9.2 ± 0.4	18.9 ± 0.9
1% FBS + ATRA + 1.0 μM As(III)	61.8 ± 1.0[b]	9.6 ± 0.2	19.5 ± 1.0

[a] $P < 0.05$ vs. 10% FBS.
[b] $P < 0.05$ vs. 1% FBS + ATRA.

increase of cell populations in the G0/G1 phase in a concentration-dependent manner, suggesting that As(III) inhibits the cardiac differentiation of H9c2 cells by disturbing the cell cycle.

To explore the mechanisms underlying this attenuation of cardiac differentiation of H9c2 cells by exposure to As(III), we examined the effects of As(III) on the phosphorylation of PKCδ. As shown in Fig. 3A and B, H9c2 cells cultured in differentiation media for 6 days showed marked phosphorylation of PKCδ compared to that of cells cultured in normal media. However, the phosphorylation of

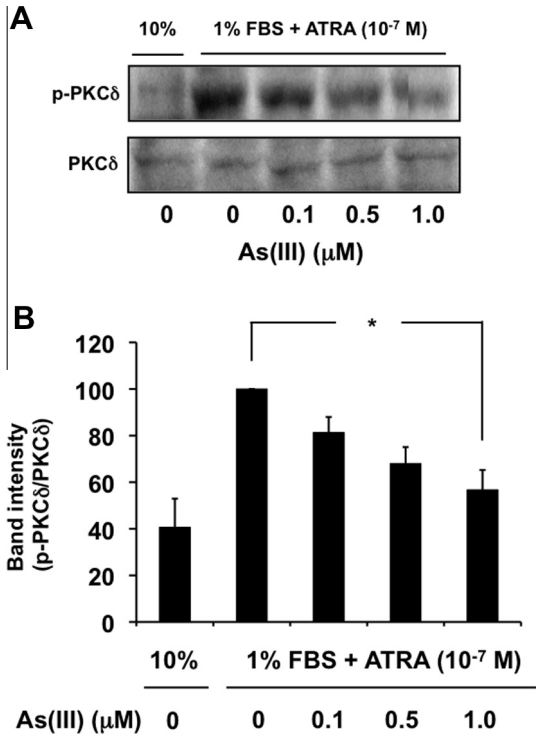

Fig. 3. Effect of As(III) on PKCδ phosphorylation induced by cardiac differentiation in H9c2 cells. (A) Cells were exposed to indicated concentrations of As(III) in DMEM with 1% FBS and ATRA (10^{-7} M) for 1 day. Western blot analysis was performed for p-PKCδ and PKCδ. (B) Band intensities were quantified with ImageJ software. Values are relative band intensities of p-PKCδ normalized to those of PKCδ. Each value is the mean ± SEM of three individual determinations. $^{*}p < 0.05$.

PKCδ in cells in the presence of differentiation media was inhibited by As(III) in a concentration-dependent manner.

4. Discussion

Arsenic is a known environmental pollutant worldwide, and a great number of people are regularly exposed to arsenic-polluted underground water sourced from wells. People with sufficient exposure have been shown to suffer from chronic arsenic poisoning, which includes cardiotoxicity [4]. Previous investigations have revealed an association between chronic exposure to arsenic and QT-interval prolongation representative of cardiotoxicity in human populations in Inner Mongolia, Taiwan, and Bangladesh [4,19,20]. Likewise, APL patients treated with arsenic trioxide exhibit adverse outcomes such as QT-interval prolongation [21–23]. Although certain modifying factors related to QT-interval prolongation due to arsenic exposure have been described in the literature, the underlying pathogenic mechanisms of cardiotoxicity are still unknown [5–7]. Thus, it is remains very important to elucidate the mechanisms of cardiotoxicity induced by chronic exposure to arsenic in terms of both environmental factors and clinical issues.

Accumulating evidence has suggested that in addition to its cardiotoxicity, arsenic is also a developmental toxicant. Maternal exposure to arsenic has been reported to have toxic effects on offspring via maternal-fetal transmission [9,10,24,25]. *In vitro* studies have also investigated the effects of arsenicals on differentiation stages of various types of cells. Wang et al. [26] showed that arsenic trioxide inhibits the differentiation of 3T3-L1 pre-adipocytes through the disruption of the interaction between PPARγ and RXRα. Yen et al. [27] examined whether skeletal muscle differentiation was inhibited by exposure to As(III) *in vitro* and *in vivo*. When

C2C12 cells, which have the ability to differentiate into skeletal muscle, were exposed to As(III), skeletal differentiation was inhibited, as evidenced by the appearance of multinucleated myotube formation. In addition, arsenic trioxide administration in mice led to the inhibition of muscle regeneration after glycerol injury *in vivo* [27]. On the other hand, Steffens et al. [28] showed that exposure of C2C12 cells to As(III) alters the methylation patterns of the transcription factor myogenin, leading to the delay of differentiation of C2C12 cells. Taken together, these studies suggest that arsenic affects pre-adipocyte and skeletal muscle differentiation; however, until the present study, it has remained unknown whether arsenic exposure affects cardiac differentiation. In a previous study, we investigated the sensitivity of rat cardiac myoblasts H9c2 cells to As(III), because H9c2 cells are often used as a model for cardiac cells in an undifferentiated state [8]. Thus, we investigated here whether As(III) affects cardiac differentiation in H9c2 cells.

The results of the present study showed that As(III) does indeed inhibit the low-serum and ATRA-induced differentiation of H9c2 cells to a cardiac phenotype, as evidenced by a decrease in the level of expression of cTnT and in the number of multinucleated cells (Figs. 2 and 3). To the best of our knowledge, this is the first report showing that As(III) inhibits the differentiation of cardiac cells. During the differentiation of H9c2 cells, cell proliferation and DNA synthesis have been shown to be reduced [18]. We therefore conducted a cell cycle analysis of H9c2 cells. As shown in Table 1, cell populations in the G0/G1 phase were increased by exposure to As(III) as compared to those of H9c2 cells incubated with differentiation media. These results suggest that As(III) disturbs the H9c2 cell cycle during cardiac differentiation. Numerous factors are known to be involved in the regulation of the cell cycle; however, there is little known about factors related to the differentiation of H9c2 cells. Au et al. [29] showed that the overexpression of calcyclin binding protein (CacyBP) promoted the differentiation of H9c2 cells. Prior to that study, CacyBP had already been identified as one of the proteins that interact with calcyclin, which plays an important role in the cell cycle [30]. Thus, we it is possible that As(III) impairs calcyclin and CacBP function during the cardiac differentiation of H9c2 cells.

It has been reported that PKCδ is involved in the differentiation of leukemia cells induced by ATRA [16,17]. In addition, di Giacomo et al. [18] showed that PKCδ plays an important role in H9c2 cell differentiation. Thus, we focused on PKCδ activation in order to explore the mechanisms underlying the inhibition of cardiac differentiation of H9c2 cells by As(III). Our results indicated that PKCδ phosphorylation was inhibited by As(III) (Fig. 3). Although the mechanisms remain unclear by which PKCδ phosphorylation is induced by differentiation media in the H9c2 cell line, most PKCs have been shown to be phosphorylated by phosphoinositide-dependent protein kinase (PDK) [31]. Paul et al. [32] showed that As(III) inhibits glucose uptake induced by insulin in 3T3-L1 adipocytes via the inhibition of PDK1 and 2. Thus, it is likely that As(III) inhibits the activity of both PDK1 and 2 in H9c2 cells.

Compared to the less well-understood mechanisms of cardiac differentiation, those governing the skeletal differentiation of cells have been well investigated. Pagano et al. [33] showed that cAMP-elevating agents such as 8-Br-cAMP, forskorin, and IBMX inhibit skeletal muscle differentiation. In addition, they revealed that levels of cAMP were decreased during skeletal muscle differentiation. These findings suggested that a decrease in cAMP triggers in cells skeletal muscle differentiation. Hunter et al. [34] also demonstrated suppressed skeletal muscle differentiation in a stable clone of H9c2 cells in which the apoptosis repressor with caspase recruitment domain (ARC) was overexpressed. To date, it remains unknown whether these factors are also involved in the cardiac differentiation of H9c2 cells. Further studies will be necessary to determine the effects of As(III) on the concentration of cAMP and the expression of ARC during cardiac differentiation.

The present study was the first to show that As(III) retards the cardiac differentiation of H9c2 cells, as determined by morphological and biochemical assessments. In addition, we found that PKCδ phosphorylation, induced by a low serum concentration and ATRA, was inhibited by As(III). Although further *in vivo* studies will still be necessary, our findings suggest that the effects of arsenicals on cardiac differentiation may be related to certain abnormalities in cardiac function observed in populations living in arsenic-contaminated areas.

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