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Arsenite induces premature senescence via p53/p21 pathway as a result of DNA damage in human malignant glioblastoma cells

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In this study, we investigate whether arsenite-induced DNA damage leads to p53-dependent premature senescence using human glioblastoma cells with p53-wild type (U87MG-neo) and p53 deficient (U87MG-E6). A dose dependent relationship between arsenite and reduced cell growth is demonstrated, as well as induced γH2AX foci formation in both U87MG-neo and U87MG-E6 cells at low concentrations of arsenite. Senescence was induced by arsenite with senescence-associated β-galactosidase staining. Dimethyl- and trimethyl-lysine 9 of histone H3 (H3DMK9 and H3TMK9) foci formation was accompanied by p21 accumulation only in U87MG-neo but not in U87MG-E6 cells. This suggests that arsenite induces premature senescence as a result of DNA damage with heterochromatin forming through a p53/p21 dependent pathway. p21 and p53 siRNA consistently decreased H3TMK9 foci formation in U87MG-neo but not in U87MG-E6 cells after arsenite treatment. Taken together, arsenite reduces cell growth independently of p53 and induces premature senescence via p53/p21-dependent pathway following DNA damage. [BMB Reports 2014; 47(10): 575-580]

INTRODUCTION

Malignant gliomas, the most common primary brain tumors in adults, have a dismal prognosis. Even several combination therapies including surgery, radiation, and chemotherapy are not curative for most patients (1). Therefore, new therapeutic agents or alternative therapeutic approaches are need. Arsenite is a well known human carcinogen, but also can be used to treat some kinds of diseases as well as cancers (2). Recently, As2O3 has shown considerable efficacy in treating patients with acute promyelocytic leukemia (APL) by activating numerous intracellular signal transduction pathways, resulting in induction of apoptosis, promotion of differentiation, and autophagy (3, 4). It has also been demonstrated that not only APL but also solid tumor cells derived from several tissues, such as liver (5), prostate (6), lung (7), and brain (8-12) are susceptible to arsenite.

Chemotherapy by use of arsenite needs to have a strategy to deliver drugs effectively. As for the drug delivery, Au, *et al*. (13) indicated that the arsenic concentration in cerebrospinal fluid (CSF) is about a half of that in plasma of APL patient after oral administration of the drug. Thus, arsenite was able to enter CSF overcoming the blood-brain barrier (BBB) effectively. The penetrating ability of arsenite through the BBB is an advantage for the treatment of glioblastoma.

Arsenite produces DNA damage (14) and induces cell death in glioblastoma (8). DNA damage induces not only cell death but also cellular senescence (15). Cellular senescence is mainly classified into two types: replicative senescence and premature senescence. Replicative senescence is triggered by telomere-shortening, and premature senescence is telomere-independently induced by cellular stress (16). In order to evaluate the potential for arsenite use to treat glioma, it is crucial to clarify mechanisms for cellular action, especially to determine whether arsenite induces premature senescence. In this study, we determine that arsenite induces premature senescence in human glioma cell line U87MG through the pathway involving DNA damage, p53 and p21.

RESULTS

Arsenite reduces cell growth and induces premature senescence

By treatment with arsenite at a concentration of 1.25 μM or higher, significant growth inhibition was observed after 3 days of post-incubation (Fig. 1A). The concentration of arsenite that

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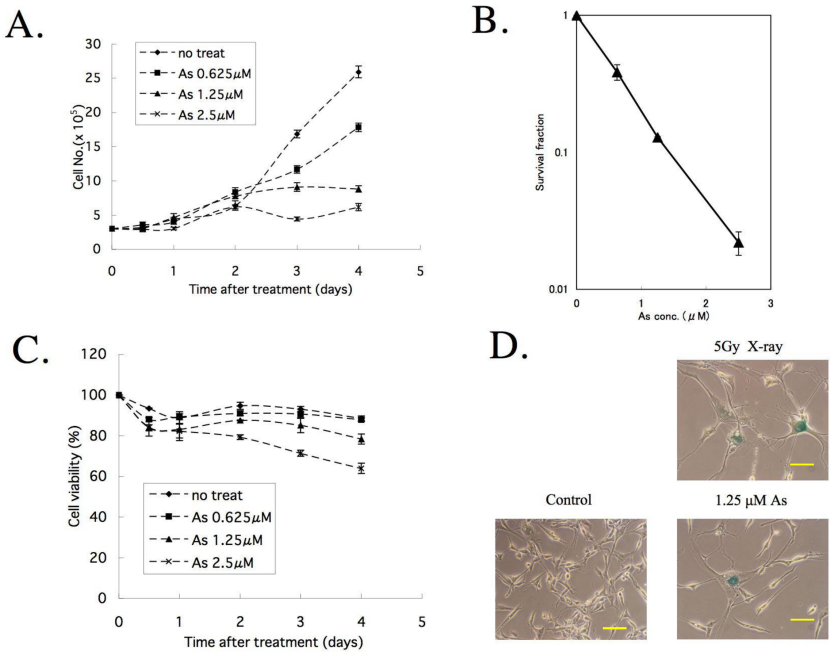

Fig. 1. Effects of arsenite on growth, viability and senescence of U87MG human glioma cells. (A) Time course change of cell number after treatments with arsenite was determined. (B) The dose effect of arsenite on growth inhibition of U87MG cells was determined by colony-forming assay. (C) Time course change of proportion of viable cells after treatments with arsenite was determined. The data points are the means and standard deviations from at least two independent experiments. (D) Senescence of U87MG cells was examined by SA-β-galactosidase staining 4 days after arsenite treatment or X-ray irradiation. The microphotographs were taken at 200 × magnification. SA-β-galactosidase positive cells are stained blue. Scale bar = 100 μm.

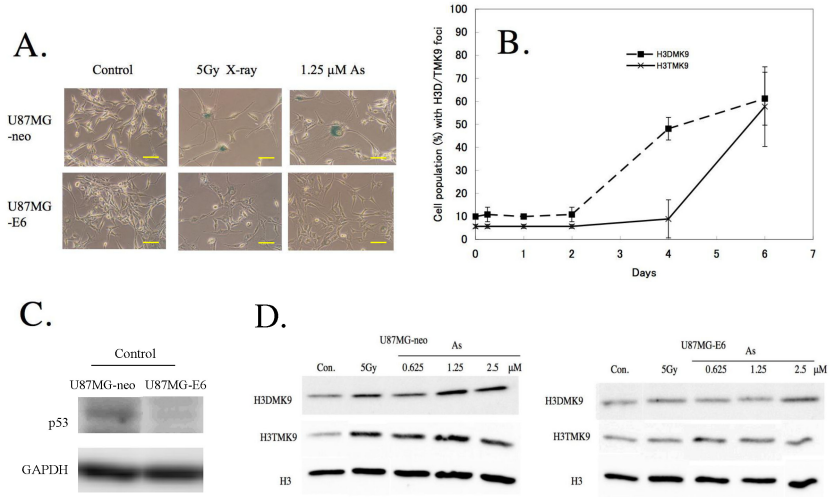

Fig. 2. Arsenite-induced senescence in glioblastoma cells with p53-wild-type U87MG-neo and p53-deficient cells (U87MG-E6). (A) SA-β-gal expression was examined 4 days after treatment with either arsenite or X-ray-irradiation (original magnification × 200). Scale bar = 100 μm. (B) Time course changes in proportion of cells containing senescence-associated foci of dimethylated (H3DMK9) or trimethylated (H3TMK9) lysine 9 of histone H3 were determined in p53-wild-type U87MG-neo cells treated with 1.25 μM arsenite. (C) Expression of p53 was determined by western blotting in p53-wild-type U87MG-neo and p53- deficient U87MG-E6 control cells confirming that p53 expression exactly lost in U87MG-E6 cells. (D) H3DMK9 and H3TMK9 were analyzed by western blotting six days after treatment with arsenite in p53-wild-type U87MG-neo and p53-deficient U87MG-E6 cells. Cells irradiated with X-rays werei used as positive control.

causes 10% colony-forming ability (IC10) was 1.25 μM (Fig. 1B). At all concentrations of arsenite used in this study, cell viability was higher than 60%, and notable cell death was not observed by trypan-blue-staining during the time period examined (Fig. 1C). To test whether treatment of arsenite at IC10 induces premature senescence, we performed SA-β-gal staining, a classical marker of senescence. Because ionizing radiation has been shown to induce premature senescence in U87MG cells (19), cells irradiated with X-rays were used as positive control. An X-ray dose causing 10% colony-forming ability (5 Gy, data not shown) was used. We observed that U87MG cells treated with arsenite showed positive for SA-β-gal staining to a similar extent in cells irradiated with X-rays (a representative image shown in Fig. 1D), demonstrating that arsenite induces premature senescence.

Arsenite induces premature senescence via p53-dependent pathway

To determine the dependency of p53 on premature senescence induced by arsenite, we used p53-wild-type U87MG-neo and p53 deficient U87MG-E6 cells. The cells treated with arsenite or irradiated with X-rays showed no

SA-β-gal staining in the U87MG-E6 cells, whereas clearly positive staining was observed in the U87MG-neo cells (a representative image shown in Fig. 2A), indicating that arsenite induces senescence depending on p53. Recently, it was reported that Rb mediates silencing of E2F target genes by causing heterochromatin formation through di-methylation (H3DMK9) and tri-methylation (H3TMK9)) of lysine 9 of histone H3K9, resulting in premature cellular senescence (20-23). We examined heterochromatin formation in U87MG-neo cells after treatment with 1.25 μM arsenite by an immunofluorescence analysis. The percentage of cells with H3DMK9 and H3TMK9 foci was significantly increased from day 2 and day 4 after arsenite treatment, respectively (Fig. 2B). In contrast, arsenite treatment changed neither the fraction of cells containing H3DMK9 nor H3TMK9 foci when p53-deficient U87MG-E6 cells were used (data not shown). On day 6, the fraction of cells with H3DMK9 or H3TMK9 foci reached approximately 60%. Western blotting analysis also showed that protein levels of H3DMK9 and H3TMK9 were dose-dependent, increasing on the day 6 after treatment with arsenite in p53-wild-type U87MG-neo but not in p53-deficient U87MG-E6 cells. X-rays-induced H3DMK9 and H3TMK9 were used as the positive control (Fig. 2C). These results further support the idea that arsenite induces premature senescence in U87MG cells depending on p53.

Arsenite induces DNA damage and premature senescence via p53/p21 pathway

It was previously reported that arsenite can induce DNA damage (24, 25), it was hypothesized that the premature senescence induced by arsenite might be triggered by DNA damage. In order to ascertain the involvement of DNA double strand break (DSB) in arsenite-induced senescence, we examined γH2AX foci formation. Analysis of time course changes of γH2AX foci formation after treatment with arsenite showed that the fraction of cells containing γH2AX foci reached its peak on day 1 and sharply reduced on day 2 followed by a gradual increase until day 6 in U87MG-neo cells (Fig. 3A). Fig. 3B shows simultaneous staining of γH2AX foci with H3DMK9 or H3TMK9 foci in U87MG-neo and U87MG-E6 cells 6 days after treatment with arsenite. Co-existence of γH2AX foci with H3DMK9 or H3TMK9 foci was observed in U87MG-neo but not in U87MG-E6 cells. The fraction of cells that are double positive for γH2AX and H3DMK9 foci significantly increased from the fourth day after arsenite treatment, while fraction of cells that are double positive for γH2AX and H3TMK9 foci significantly increased after the sixth day (Fig. 3C). The observed co-existence of γH2AX with either H3DMK9 or H3TMK9 foci suggests that DNA damages trigger premature senescence.

Cyclin dependent kinase inhibitors p21 and p16 are involved in premature senescence through regulation of Rb (16). It has been reported that malignancy of glioma correlates with p16 deficiency. Because U87MG lacks the p16 gene (26, 27), p53 and its transcriptional target p21 were thought to play a pivotal role in cellular senescence (28, 29). Western blotting analysis showed that p21 levels increased in U87MG-neo cells four days after treatment with arsenite at concentrations of 0.625 μM or higher, but not in U87MG-E6 cells (Fig. 4A). To examine the functional involvement of p21 in premature senescence, we knocked-down p21 in U87MG-neo cells by siRNA treatment. Efficient knock-down of p21 by siRNA treatment was confirmed (Fig. 4B). As a result, significant decrease in H3TMK9 expression levels in U87MG-neo cells were detected six days after treatment with 1.25 μM arsenite, similar to that after irradiation

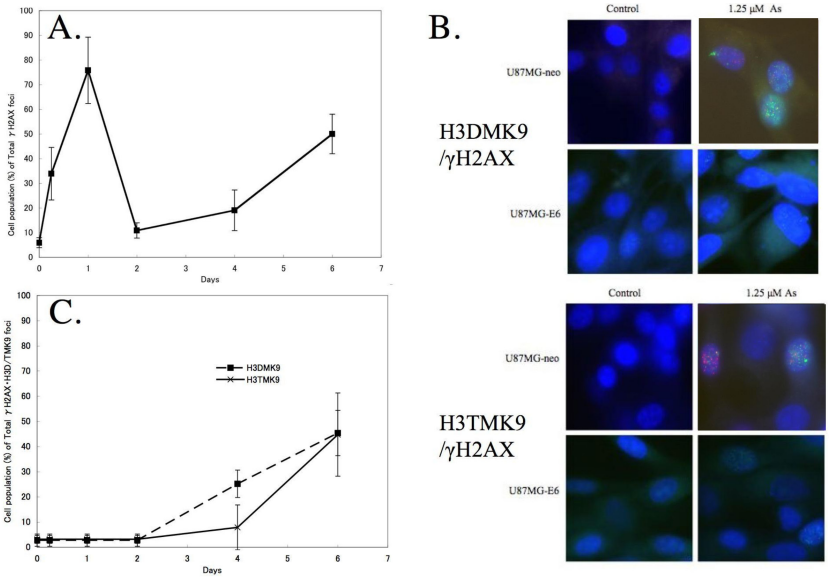

Fig. 3. γH2AX, H3DMK9 and H3TMK9 foci formation after treatment with arsenite. (A) Time course changes of fraction of cells containing γH2AX foci were determined after arsenite treatment in p53-wild-type U87MG-neo cells. (B) Representative microphotographs of dual staining of H3DMK9 or H3TMK9 (red foci) with γH2AX (green foci) in p53-wild-type U87MG-neo and p53-deficient U87MG-E6 cells treated with arsenite for 4 days are shown. (C) Time course changes in proportion of U87MG-neo cells with double positive for γH2AX and H3DMK9 or H3TMK9 foci after treatment with 1.25 μM arsenite are shown.

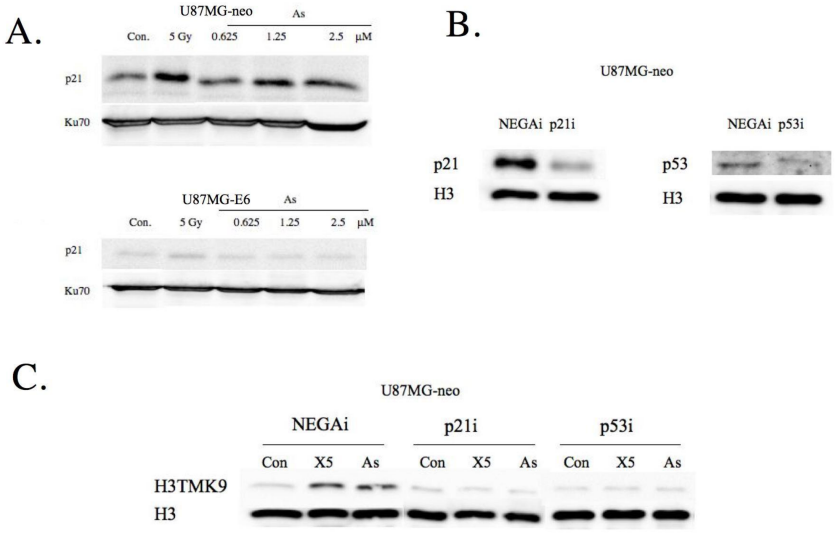

Fig. 4. Arsenite-induced premature senescence via p53/p21 pathway. (A) p21 expression levels were analyzed by Western blotting four days after treatment with 1.25 μM arsenite or 5 Gy of X-rays in p53-wild-type U87MG-neo and p53-deficient U87MG-E6 cells. (B) p21 or p53 expression levels were analyzed by Western blotting two days after irradiation with 5 Gy of X-rays in p53-wild-type cells treated with negative control or p21 or p53 siRNA. (C) H3TMK9 expression levels were analyzed by Western blotting six days after treatment with 1.25 μM arsenite or irradiation with 5 Gy of X-rays in p53-wild-type cells treated with negative control (i.e. p21 or p53 siRNA). p21i and p53i designate cells treated with siRNA for p21 and p53, respectively. NEGAi designates cells treated with a negative control siRNA.

with X-rays both in p53-knocked-down and p21-knocked-down cells (Fig. 4C). This data suggests premature senescence is induced by arsenite via p53/p21 pathway.

DISCUSSION

Senescence is an anti-tumorigenic mechanism by which cell growth is arrested for long periods of time (30). However, mechanisms for senescence in tumor cells have not been sufficiently elucidated (23, 31). Cellular senescence in normal cells is mainly classified into two mutually exclusive types: one involves p16 and the other involves p21(28). It is known that methylation of histone H3 at lysine 9 occurs following cell cycle arrest (21, 32). We demonstrated that premature senescence of U87MG cells (i.e. cells that lack a p16 gene) (26, 27) after treatment with arsenite depends on p21 and requires methylation of histone H3 at lysine 9. This criteria for premature senescence is similar in normal cells indicating a common mechanism of senescence in both human glioma cells and normal cells. DNA damage has been shown to induce senescence (15). Arsenite is thought to be able to produce oxidative DNA damage such as single strand break (SSB) via reactive oxygen species (ROS) (3, 33, 34) leading to DSB during DNA replication (35). It was reported that toxicity of arsenite depends on DNA replication (36). Based on these facts, it may be speculated that DSB's were generated in glioblastoma cells one day after arsenite treatment through the mechanism involving ROS production and conversion of ROS-induced SSB's to DSB's during DNA replication (Fig. 3A). Irreparable DSB remaining on day 6 may have finally induced premature senescence. In this study, we demonstrate that low concentrations of arsenite induce premature senescence depending on p53 in agreement with previous research showing p53-dependent stress-induced senescence in tumor cells (29).

It has been reported that 15 μM arsenite induces apoptosis in rat tumor cells (37). Namgung, *et al*. reported that arsenite concentrations of 5 μM or higher induce apoptosis in rat primary cortical neurons (38). Using human glioblastoma cell lines, Kanzawa, *et al*. (8, 12) reported that the arsenic concentrations of 2 or 4 μM induced autophagy but not apoptosis, and Haga, *et al*. (9) reported that high concentrations of arsenite (50 μM) can induce apoptosis. Recently it has been reported that arsenic at concentrations around 1 μM can induce premature senescence but not apoptosis in human mesenchymal stem cells and vascular smooth muscle cells (39, 40). Based on the above literature and our present study, it is likely that different concentrations of arsenic induces different types of cell; a high concentration of arsenite induces apoptotic cell death while a relatively low concentrations induces premature senescence and/or autophagy. Analysis focusing not only on apoptosis and autophagy but also on premature senescence is needed to aid the development of new cancer therapies.

MATERIALS AND METHODS

Material and Methods are described in the online data supplement, available at http://www.bmbreports.org/jbmb_by_volume.html?vol=47.

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