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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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ABSTRACT

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). Arsenite dose-dependently reduced cell growth, and low concentration of arsenite significantly induced γH2AX foci formation both in U87MG-neo and U87MG-E6 cells. However, senescence was induced by arsenite with senescence-associated β-galactosidase staining and dimethyl- and trimethyl-lysine 9 of histone H3 (H3DMK9 and H3TMK9) foci formation accompanied by p21 accumulation only in U87MG-neo but not in U87MG-E6 cells, suggesting that arsenite induces premature senescence as a result of DNA damage with heterochromatin formation through p53/p21 dependent pathway. Consistently, p21 and p53 siRNA decreased H3TMK9 foci formation in U87M G-neo but not inU87MG-E6 cells after arsenite treatment. Taken together, arsenite reduces cell growth p53-independently and induces premature senescence via p53/p21-dependent pathway following DNA damage. This finding explored new anti-tumor mechanisms induced by arsenite.

Keywords:

premature senescence, heterochromatin formation, glioma, arsenite, p53

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INTRODUCTION

Malignant gliomas, the most common primary brain tumors in adults, have a dismal prognosis, because even several combination therapies including surgery, radiation, and chemotherapy are not curative for most patients (1). Therefore, we need to find new therapeutic agents or alternative therapeutic approaches. Arsenite is a well known human carcinogen, but also can be used to treat some kinds of diseases as well as cancers (2). Recently, As_2O_3 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 blood-brain barrier (BBB) effectively. The penetrating ability of arsenite through BBB is an advantage for therapy 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 usability of arsenite for therapy of glioma, it is crucial to clarify mechanisms for cellular action, especially to determine whether arsenite induces premature senescence. In this study, we

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indicated 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 1.25 μM or higher concentration, a significant growth inhibition was observed after 3 days of post-incubation (Fig. 1A). The concentration of arsenite causing 10% colony-forming ability (IC_{10}) 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 IC_{10} induces premature senescence, we performed SA-β-gal staining, a classical marker of senescence. As ionizing radiation has been shown to induce premature senescence in U87MG cells (19), the cells irradiated with X-rays were used as positive control. The 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 5 Gy of X-rays (a representative image shown in Fig. 1D). These data demonstrate that arsenite induces premature senescence.

Arsenite induces premature senescence via p53-dependent pathway

To determine p53 dependency of 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-ray showed no SA-β-gal staining in the U87MG-E6 cells, whereas clearly positive staining was presented in the U87MG-neo cells (a representative image shown in Fig. 2A), indicating that arsenite induces senescence depending on p53. Recently, it was reported

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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 cellular premature 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 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 to 60 % approximately. Western blotting analysis also showed that protein levels of H3DMK9 and H3TMK9 were dose-dependently increased 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), so we thought 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 fraction of cells containing γH2AX foci reached 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

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arsenite. Co-existence of γH2AX foci with H3DMK9 or H3TMK9 foci was observed in U87MG-neo but not in U87MG-E6 cells. Fraction of cells with double positive for γH2AX and H3DMK9 foci was significantly increased from day 4 after arsenite treatment, while fraction of cells with double positive for γH2AX and H3TMK9 foci were significantly increased from day 6 (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. As U87MG lacks 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 increased in U87MG-neo cells on day 4 after treatment with arsenite at the concentration 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 knock-down p21 in U87MG-neo cells by siRNA treatment. Efficient knock-down by p21 siRNA treatment could be confirmed (Fig. 4B). As a result, we detected significant decrease in H3TMK9 expression level in U87MG-neo cells on day 6 after treatment with 1.25 μM arsenite similar to that after irradiation with X-rays both in p53-knocked-down and p21-knocked-down cells (Fig. 4C). These data suggest premature senescence induced by arsenite is via p53/p21 pathway.

DISCUSSION

Senescence is an anti-tumorigenic mechanism by which cells undergo growth arrest for long time period (30). However, mechanism for senescence of tumor cells are not well elucidated (23, 31). Cellular senescence in normal cells is mainly classified into two types: one involving p16 and the other involving p21 instead of p16 (28). It is known that methylation of histone H3 at

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lysine 9 occurs, following cell cycle arrest (21, 32). We demonstrated that premature senescence of U87MG cells, lacking a p16 gene (26, 27), after treatment with arsenite depended on p21 and required methylation of histone H3 at lysine 9 similarly to premature senescence of normal cells. These findings indicated a common mechanism of senescence in 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). SSB can be converted 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 in 1 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 the present study, we demonstrated that arsenite at low concentration induced premature senescence depending on p53 in accord with the previous research which showed 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 5 μM or higher concentrations of arsenite induce apoptosis in rat primary cortical neurons (38). Using human glioblastoma cell lines, Kanzawa et al (8, 12) reported that the arsenic concentration at 2 or 4 μM induced autophagy but not apoptosis, and Haga et al (9) reported that high concentration of arsenite (50 μM) can induce apoptosis. Recently it has been reported that arsenic at low concentration around 1 μM can induce premature senescence but not apoptosis in human mesenchymal stem cell and vascular smooth muscle cells (39, 40). Based on the above literature and our present study, it is likely the true that different concentration of arsenic induces different cell death type; a high concentrations of arsenite induces apoptotic cell death while a relatively low concentrations induces premature

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senescence and/or autophagy. The analysis focused on not only apoptosis and autophagy but also premature senescence may be necessary for study about new cancer therapy.

MATERIALS AND METHODS

Reagents

Sodium arsenite ($NaAsO_2$) was obtained from Wako Pure Chemical Industries. Primary antibodies of rabbit polyclonal anti-Histone H3 di- or tri-methylated on lysine 9 (Abcam), rabbit polyclonal anti-Histone H3 (Abcam), rabbit polyclonal anti-Histone H3 phosphorylated on serine 10 (Upstate) and mouse monoclonal anti-γH2AX (Ser 139) (Upstate), and secondary antibodies of Cy3-conjugated affinipure goat anti-rabbit IgG (Jackson ImmunoResearch), Cy2-conjugated affinipure goat anti-mouse IgG (Jackson ImmunoResearch) were used.

Cell lines

The human malignant glioblastoma cell line U87MG (wild type p53) was purchased from ATCC, USA. U87MG-neo (containing empty vector) and U87MG-E6 (containing viral oncoprotein E6 inactivating p53 by accelerating its degradation) cell lines were kindly provided by Dr. Mischel (17). The cells were cultured in Dulbecco's modified essential medium (Life Technologies) supplemented with 5% (v/v) of heat-inactivated fetal calf serum (Beit-Ha'Emek), 100 unit/mL penicillin and 100 μg/mL streptomycin (Invitrogen) in a 37°C with 5% CO_2-in-air. The medium was changed every other day. When arsenite was added into medium, the medium was replaced by fresh one without arsenite 4 days later. Cells were irradiated with X-rays from an X-ray generator operated at 200 kV and 20 mA with a 0.5 mm copper and 0.5 mm aluminum filter.

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Growth inhibition assay and cell viability assay

Growth inhibition by arsenite was assessed by either cell number counting or colony-forming assay. Cell viability was determined by staining cells with 0.2 % of trypan blue. For colony-forming assay, 300–3000 cells were plated per dish, and arsenite was added to the culture at the concentration of 0.625–2.5 μM 24 h later. The cells were incubated for 4 days in the presence of arsenite, followed by additional 10 days incubation in the absence of arsenite for colony formation. The cells were fixed and stained with solution containing 20 % methanol and 0.2 % crystal violet. Colonies containing more than 50 cells were counted as growing cells At least three parallel samples were scored in three to five repetitions performed for each treatment condition.

SA-β-gal. staining

The cells grown in dishes were washed with PBS, and fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS for 5 min at room temperature. Senescence-associated β-galactosidase (SA-β-gal) (pH 6.0) staining was carried out as described by Dimri *et. al* (18). The images were captured by using an Olympus DP71 microscope.

Immunofluorescence

The cells grown on cover slips were fixed with 4 % formaldehyde in PBS for 15 min and washed with PBS. Then, the cells were permeabilized with 0.2 % Triton X-100 for 5 min at 4°C, and blocked with PBS containing 10 % goat serum. Cells were incubated with the primary antibodies for 1 h and washed with PBS 3 times, each for 10 min. Cells were then incubated with secondary antibodies for 1 h and washed with PBS 3 times, each for 10 min. Cover slips were mounted in 4,6-diamidino-2-phenylindole (DAPI). Fluorescence images were captured by

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using an Olympus DP70 fluorescence microscope.

Western blotting

Cells were washed with PBS and lysed in lysis buffer containing 50 mM Tris-Hcl, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, 50 mM NaF, 25 mM β-glycerophosphate, 0.5 % Triton X-100, 0.1 % SDS, 0.1 mM sodium vanadate, 1 mM DTT, 0.1 mM, and Complete-Mini protease inhibitor (Roche Diagnostics). Protein concentrations were measured using Protein Assay Kit (Pierce). Total cellular lysates were loaded onto 10 % SDS-polyacylamide gels, separated by electrophoresis, and electro-transferred onto nitrocellulose membranes. Membranes were immunostained by following the manufacture's instruction. The blots were visualized by enhanced chemiluminescence method (GE Healthcare) with LAS-1000 (GE Healthcare).

siRNA treatment

p21 siRNAs (Hs_CDKN1A_6HP Validated siRNA, Qiagen), p53 siRNAs (Hs_TP53_9 HP Validated siRNA, Qiagen) and negative control siRNA (Allstar negative siRNA, Qiagen) were used. 2×10^5 cells were seeded into 35 mm dishes, and incubated 1 day before transfection with 20 nM siRNA using HiperFect transfection reagent (Qiagen) according to the instructions of the manufacturer. Transfected cells were then cultured in normal growth media for 48 h before treatment. Efficient knock-down was confirmed by Western blotting.

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FIGURE LEGENDS

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 x 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 arsenite or X-rays-irradiation (original magnification x 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, and confirmed that p53 expression exactly lost in U87MG-E6 cells. (D) H3DMK9 and H3TMK9 were analysed by western blotting on day 6 after treatment with arsenite in p53-wild-type U87MG-neo and p53-deficient U87MG-E6 cells. X-rays irradiation was used as positive control.

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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 dualstaining 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 analysed by Western blotting on day 4 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 analysed by Western blotting on day 2 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 analysed by Western blotting on day 6 after treatment with 1.25 μM arsenite or irradiation with 5 Gy of X-rays in p53-wild-type cells treated with negative control, 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.

UNCORRECTED PROOF

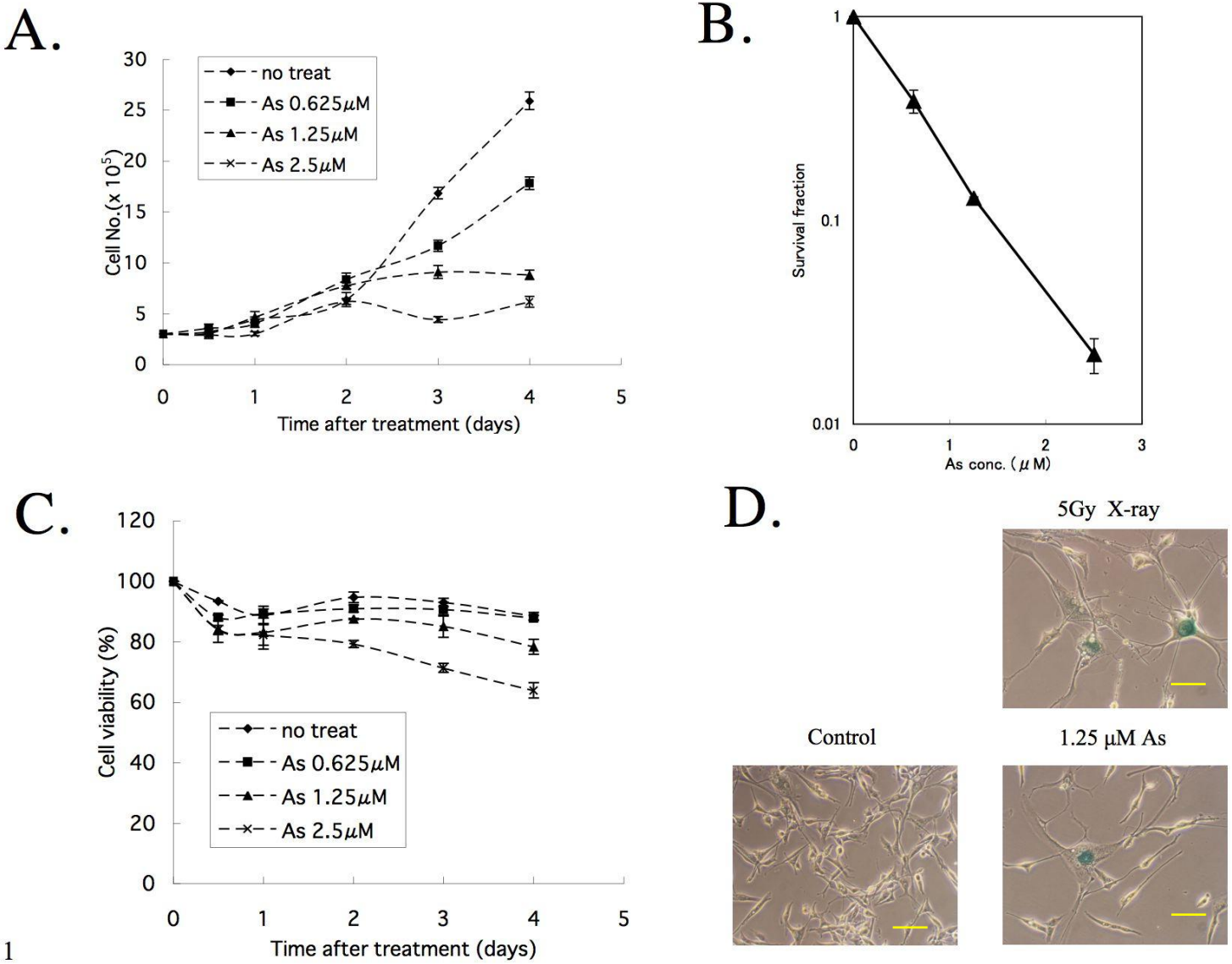

A.
no treat
As 0.625μM
As 1.25μM
As 2.5μM
Cell No.(x 10^5)
Time after treatment (days)
B.
Survival fraction
As conc. (μM)
C.
Cell viability (%)
no treat
As 0.625μM
As 1.25μM
As 2.5μM
Time after treatment (days)
D.
5Gy X-ray
Control
1.25 μM As

Fig. 1

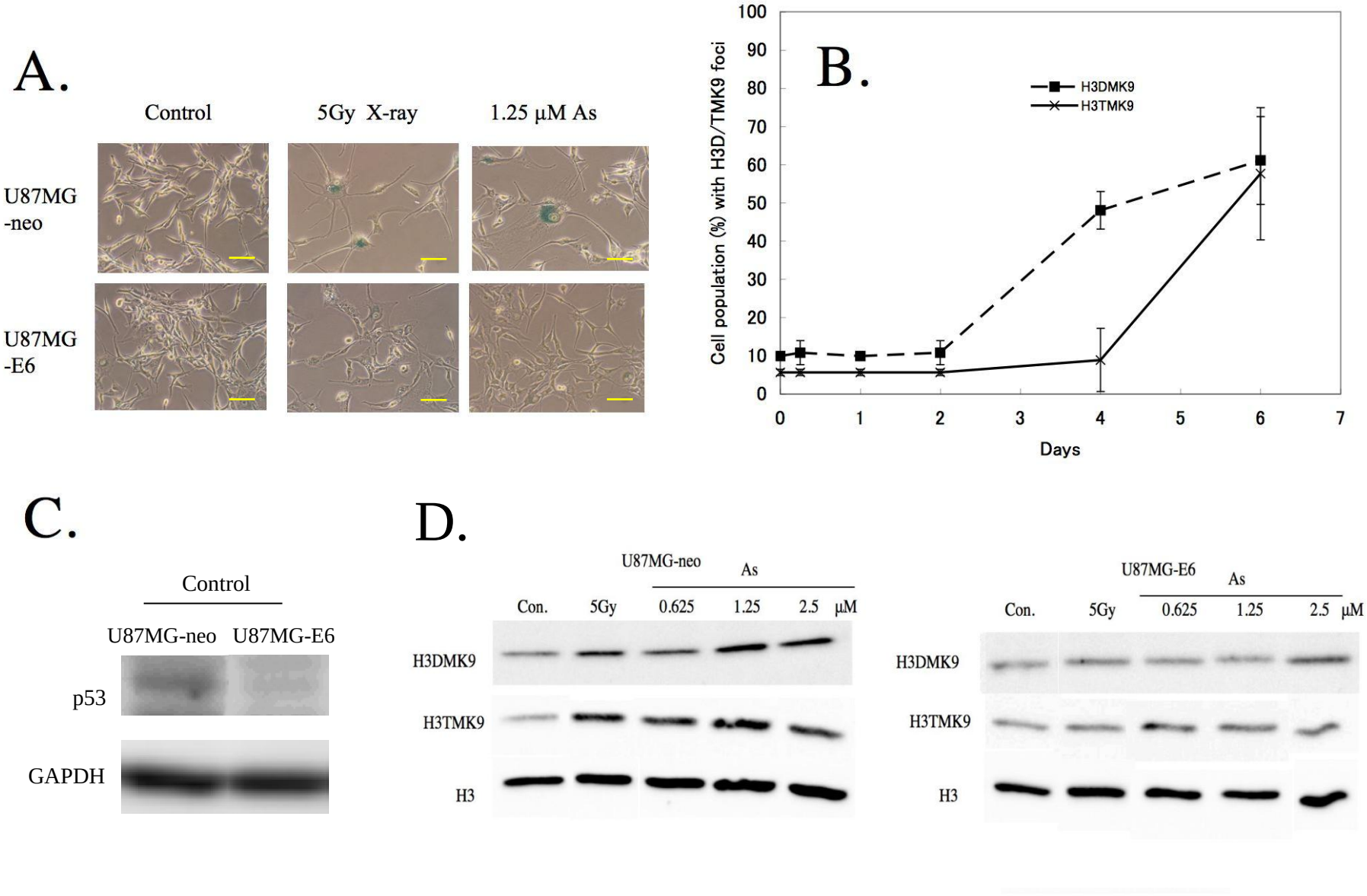
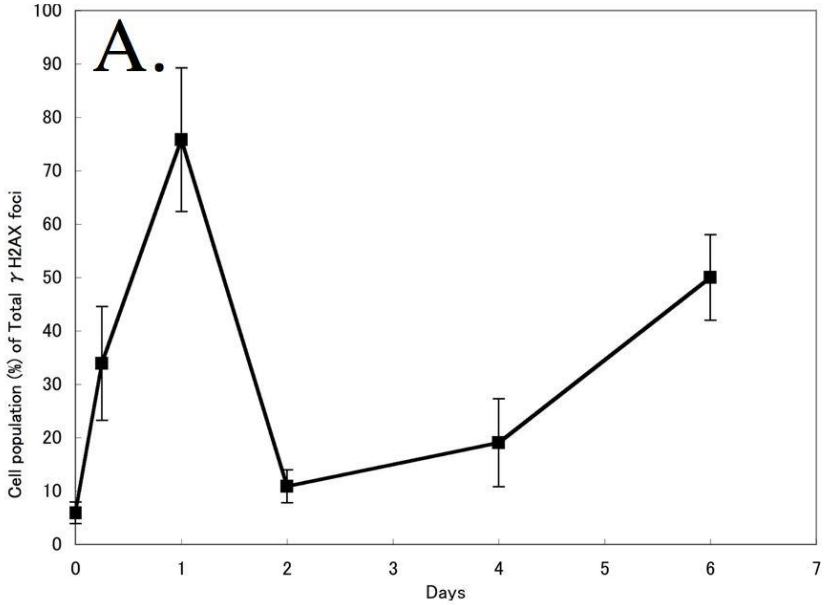
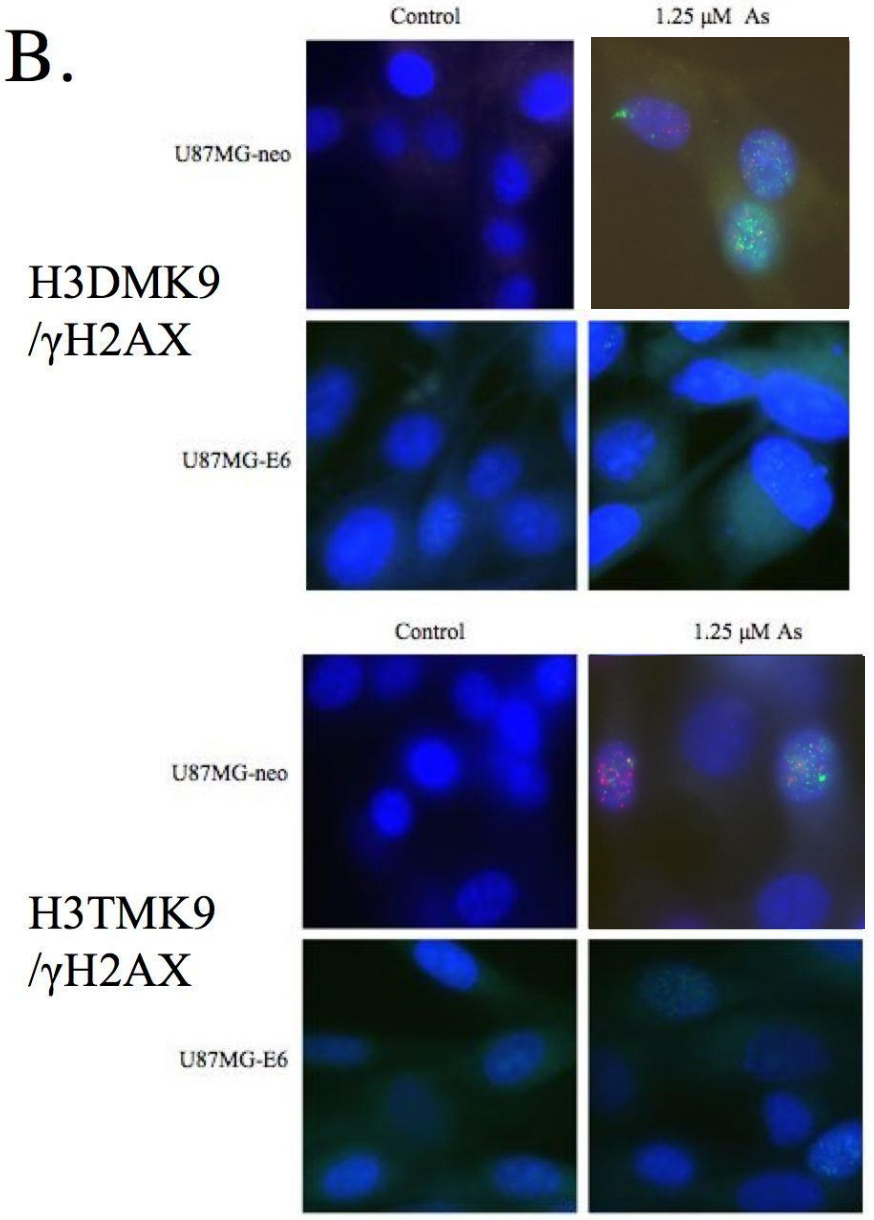

A.
Control
5Gy X-ray
1.25 μM As
U87MG -neo
U87MG -E6
B.
H3DMK9
H3TMK9
Cell population (%) with H3D/TMK9 foci
Days
C.
Control
U87MG-neo
U87MG-E6
p53
GAPDH
D.
U87MG-neo
As
Con.
5Gy
0.625
1.25
2.5 μM
H3DMK9
H3TMK9
H3
U87MG-E6
As
Con.
5Gy
0.625
1.25
2.5 μM
H3DMK9
H3TMK9
H3

Fig. 2


A.
Cell population (%) of Total γH2AX foci
Days


B.
Control
1.25 μM As
U87MG-neo
H3DMK9
/γH2AX
U87MG-E6
Control
1.25 μM As
U87MG-neo
H3TMK9
/γH2AX
U87MG-E6

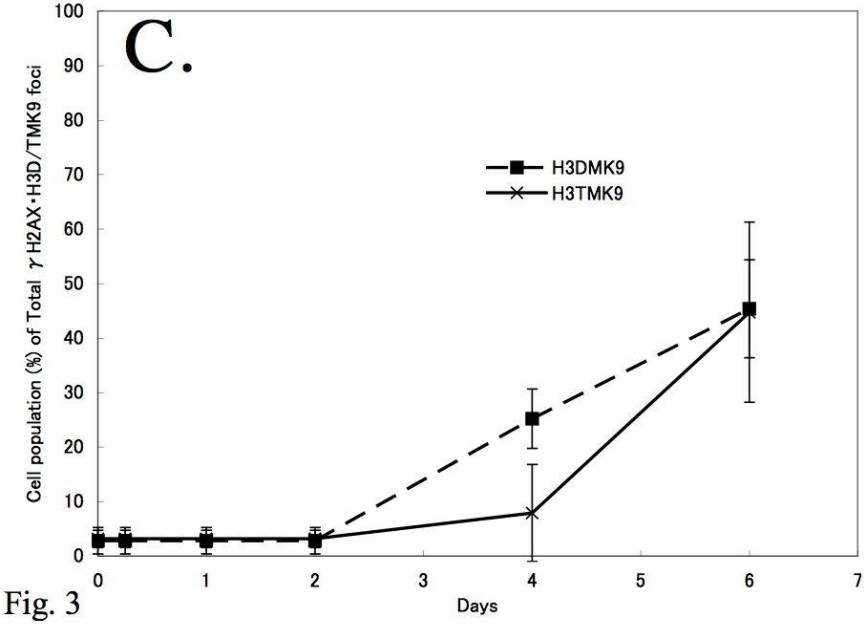

C.
H3DMK9
H3TMK9
Cell population (%) of Total γH2AX・H3D/TMK9 foci
Days

Fig. 3

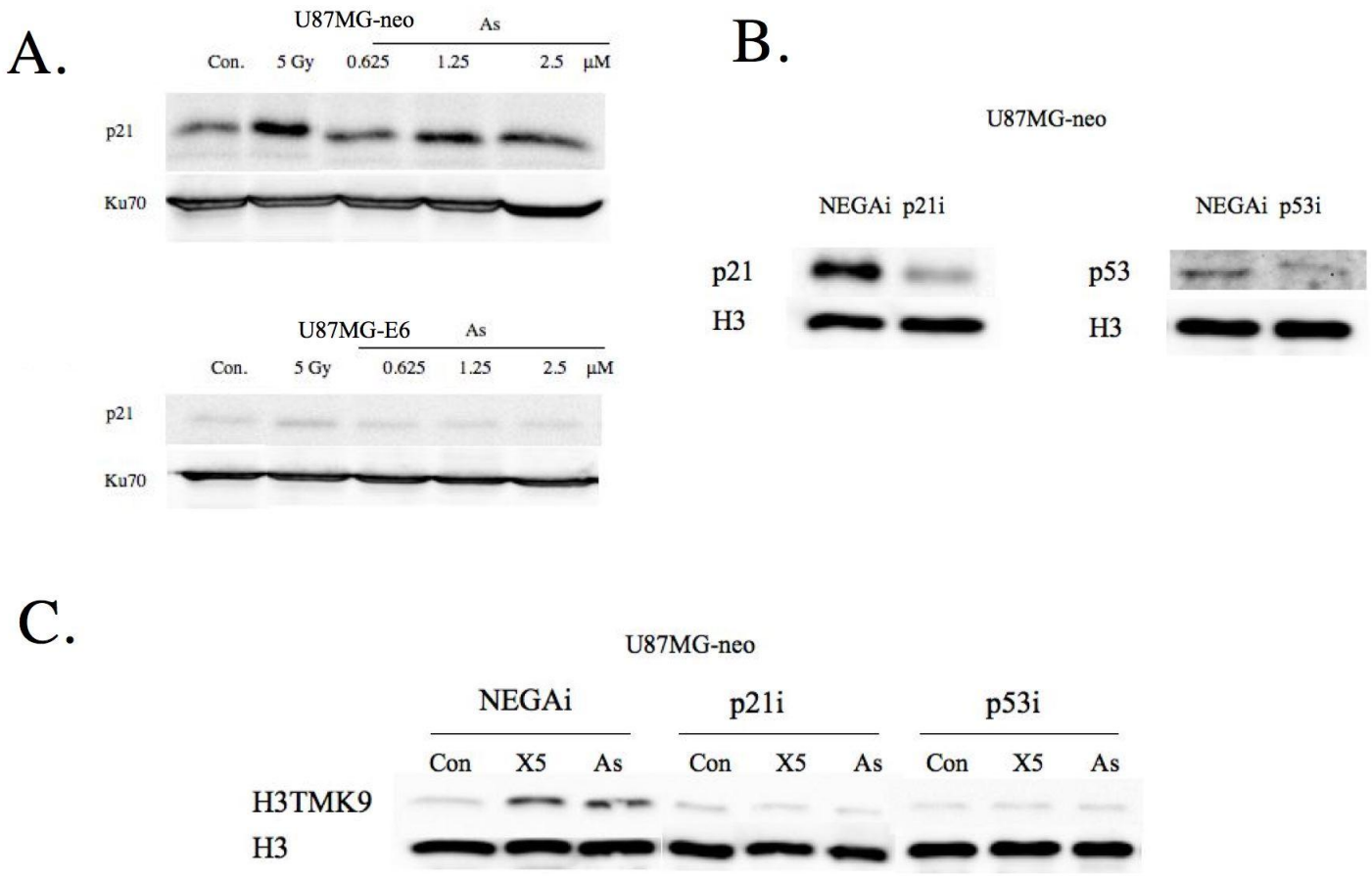

A.
U87MG-neo
As
Con.
5 Gy
0.625
1.25
2.5 μM
p21
Ku70
U87MG-E6
As
Con.
5 Gy
0.625
1.25
2.5 μM
p21
Ku70
B.
U87MG-neo
NEGAi p21i
NEGAi p53i
p21
H3
p53
H3
C.
U87MG-neo
NEGAi
p21i
p53i
Con
X5
As
Con
X5
As
Con
X5
As
H3TMK9
H3

Fig. 4