

Up-regulation of telomere-binding TRF1, TRF2 related to reactive oxygen species induced by As₂O₃ in MGC-803 cells

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Abstract

In this work, our study focused on As₂O₃ action in view point of telomere. Results showed that treatment of human gastric cancer MGC-803 cells with arsenic trioxide could cause up-regulation of telomeric repeat binding factor TRF1 and TRF2 mRNA and protein levels, and induced G2/M phase arrest and cell apoptosis. At the same time, telomere length shortening and telomerase inhibitory were not obvious. Flow cytometry measurements indicated that the increase of TRF1 and TRF2 proteins is related to oxidative stress by arsenic trioxide. Results also indicate that after arsenic trioxide treatment, p53 protein levels increased significantly and also could bind directly at the telomere t-loop junction. These findings demonstrate arsenic trioxide-induced cell cycle arrest and apoptosis might involve a novel pathway related to TRF1, TRF2 protein.

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1. Introduction

Telomeres, the specialized nucleoprotein complexes at the ends of eukaryotic chromosomes, have important functions for maintaining chromosome stability and regulating the replicative life-span of somatic cells (Klappe et al., 2001). The DNA sequence at telomeres typically consists of tandem GT-rich repeats, which is TTAGGG in human. The 3' end of each telomere is a single stranded G-rich overhang that has been proposed to stabilize a loop structure at chromosome ends (Griffith et al., 1999; Chen et al., 2001a,b). Telomeres protect chromosome ends from recombination, fusion, and from being recognized as damaged DNA. In addition, they allow access to the telomerase enzyme to add telomere repeats in order to maintain telomere length (Cech, 2004). Telomerase activity has been found in 85% of human cancers, including

stomach, breast, colon, and in immortalized cell lines, but low levels of telomerase activity have been detected in resting lymphocytes (Sheng et al., 2003; Bodnar et al., 1996).

The length and structure of telomeres are controlled by a variety of proteins, some of which function exclusively at the telomere, others of which also participate in DNA repair. The major protein components of mammalian telomeres are the duplex TTAGGG repeat-binding factors (TRF) that are localized at telomeres (Zhong et al., 1992). Overexpression of wild-type and dominant-negative TRF1 induces progressive telomere shortening and elongation in human cells (Smogorzewska et al., 2000). In addition, recent evidence shows that TRF1 interacts with other telomere-binding molecules and integrates into the functional telomere structure (Iwano et al., 2004). TRF2 is nuclear protein that coats the length of all human telomeres at all stages of the cell cycle. Similar to the phenotype observed with TRF1, overexpression of TRF2 results in the progressive telomere shortening, too. But, the primary role of TRF2 is to protect chromosome ends from end-to-

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end fusion by promoting formation of large duplex loop, which is closed telomeric state (capping state) (Karlseder et al., 2002). What's more, increasing evidence showed TRF2 could remodel linear telomeric DNA into large duplex loop in vitro by electron microscopy (Stansel et al., 2001). Recently, it reported that TRF2 has a novel function of neurite formation that is not related to the telomere protecting function (Jung et al., 2004).

Arsenic trioxide (As_2O_3) is an interesting anticancer drug for application in tumor. Last decade, the efficiency of As_2O_3 in both newly diagnosed and relapsed patients with acute promyelocytic leukemia has been established. In present days, numerous clinical trials are under way in hematopoietic malignancies and solid tumors, and great success in cultured cell lines has been received. Fully understanding the mechanism of As_2O_3 would promote its application in clinical. As_2O_3 showed a high binding affinity to proteins containing the vicinal sulfhydryl(SH) groups, which are found in many enzymes such as protein kinase, tyrosine phosphatase, glutathione system, etc. Intracellular environments, that is oxygen-redox status, might affect the action of As_2O_3 (Dai et al., 1999). Oxidative damage has been postulated to be a key mechanism by which arsenic initiates the apoptotic process. A number of studies have examined the relationships between arsenic, reactive oxygen species and telomerase in human. The recent evidence causally linking reactive oxygen species with telomere is derived from experiments showing telomere attrition, chromosome instability and apoptosis subjected to oxidative stress induced by arsenic (Gao et al., 2004).

It is argumentative whether the pathway by which arsenic promotes apoptosis is dependent of p53. Huang et al. (1999) revealed that arsenic exposure of embryo fibroblasts with either active or inactivated p53 induced apoptosis, while our and other groups found activation of p53 by As_2O_3 exposure (Zhang et al., 2002; Yeh et al., 2003). Many studies also showed that telomeric repeats could activate p53 protein (Li et al., 2003). Up to date, Stansel et al. (2002) observed firstly that p53 could bind telomeric single strand overhangs and t-loop junctions in vitro. To gain insight into the relationships between telomere proteins, arsenic stress, and cell fate, we researched As_2O_3 -mediated telomere proteins response. Our results revealed a possible role of TRF1 and TRF2 that are different from protection of t-loop previously.

2. Materials and methods

2.1. Materials

Arsenic trioxide (As_2O_3), propidium iodide, *N*-acetylcysteine were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Dihydroethidium was from Molecular Probes. Oligodeoxynucleotides were obtained from SBS Genetech. Co., Ltd.

Purification and desalination were performed by reverse high performance liquid chromatography (C-18).

2.2. Cell culture and treatment

Human gastric cancer MGC-803 cells (obtained from Beijing Medical University) were seeded in 35-mm tissue culture dishes (Costar, New York, New York, USA) and were cultured by RPMI 1640 medium (Gibco-BRL, Grand Island, New York, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 μ g/ml streptomycin, and 100 U/ml penicillin. Cells were incubated at 37 °C in a humidified atmosphere containing 95% air/5% CO_2 . Exponentially growing cells were used for experiments. Immediately prior to experimental treatment, medium was replaced; 5 μ M As_2O_3 was added to medium for 24 h or 48 h. Antioxidant, *N*-acetylcysteine, was administered in a 24 h pretreatment.

2.3. Flow cytometry analysis of apoptosis and cell cycle

Flow cytometry (FCM) measurements were carried out according to the methods described by Ishibashi and Lippard (1998). About 1×10^6 cells from control and treatment were collected, washed twice with cold phosphate-buffered saline (PBS), and fixed in 70% ethanol overnight. Then the cells were incubated in 1 ml PBS contained 50 μ g/ml propidium iodide and 250 μ g/ml RNase at 37 °C for 30 min to stain the DNA and eliminate RNA. The fluorescence intensity was measured using Coulter EPICS XL Flow cytometer. Percentage of cells in sub-G1 phase was regarded as apoptotic percentage. The cell cycle distribution was also determined by the fluorescence of individual cells measured by flow cytometry (FACSS Vantage, BD Inc., USA).

2.4. Determination of reactive oxygen species

Superoxide production was determined by measuring the conversion of dihydroethidium to ethidium (Huang et al., 2000). Briefly, cells were collected at indicated times after treatment and washed in PBS. Dihydroethidium was added to each sample (final concentration 2 μ M). Samples were immediately vortexed and incubated at 37 °C in the dark for 30 min, and then analyzed by FACS. Excitation peak of ethidium is at 518 nm, fluorescence emission peak is at 605 nm.

2.5. RT-PCR

Total RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction. The first-strand cDNA was synthesized using M-MLV reverse transcriptase. Briefly, 2 μ g of total RNA was transcribed using Oligod (T)₁₈ primer in a reaction mixture with a total volume of 25 μ l. PCR was done using 2 μ l of first-strand cDNA. The reaction sequence was at 94 °C, for 30 s, 55 °C for 60 s, 72 °C for 90 s (35 cycle for TRF1, TRF2, 22 cycle for actin). The primer sequences were as follows: 5'-TGG CTG CCG GCT GGA TGC TG-3', 5'-TTATTA AGG TCT TGT TGC TG-3' for TRF1, 5'-CGC TGG GTA CTC AGG TTC TA-3', 5'-CTG GTG CTG GCT GTT TAT CT-3' for TRF2, 5'-GTG GGG CGC CCC AGG CAC CA-3', 5'-GTC CTT AAT GTC ACG CAC GAT TTC-3' for β -actin. 8 μ l of PCR product was loaded in 1.5% agarose gel. The gels were

stained with ethidium bromide, and the DNA fragments were visualized using ultraviolet light and photographed.

2.6. Western blotting

Western was conducted as described previously (Steensel et al., 1998). Cells were lysed and boiled for 5 min. The same loading was analyzed by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to nitrocellulose by semidry blotting. The membrane were blocked with 5% non-fat milk in PBS and 0.1% Tween-20, then incubated with antibodies to TRF1, TRF2, p53, actin (Santa Cruz Biotechnology, Santa Cruz, California, USA). This initial incubation was followed by incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Antibody binding was visualized by enhanced chemiluminescence kit (Amersham Life Science, Amersham, UK).

2.7. Telomere length measurement

Telomere length was determined by Telomere Length Assay kit (Roche) protocol. Briefly, treated and untreated genomic DNA was isolated as described previously, and then cleaned with *Hinf*I and *Rsa*I overnight at 37 °C. Following DNA digestion, approximately 5 µg DNA fragments were separated on 0.8% agarose and transferred to positive charged nylon membranes by capillary transfer using 20× SSC transfer buffer. The blotted DNA fragments are hybridized to a digoxigenin-labeled probe specific for telomeric repeats and incubated with a digoxigenin-specific antibody covalently coupled to alkaline phosphatase. Finally, the immobilized telomere probe was visualized by virtue of alkaline phosphatase metabolizing CDP-Star, a highly sensitive chemiluminescence substrate. The average telomere length was determined by comparing the signals relative to a molecular weight standard.

2.8. Telomerase activity assay

Telomerase activity was assayed using polymerase chain reaction (PCR)-based telemetric repeat amplification protocol (TRAP) assay as previously described. The cells were collected as pellets after centrifuging at 1000×g for 5 min at 4 °C. The pellets then were washed and lysed in 1, 3-(3-cholamidopropyl) dimethylammonio)-1-propanesulfonic acid (CHAPS, Sigma) buffer, incubated on ice for 30 min, the lysates were centrifuged at 16,000×g for 20 min at 4 °C. The supernatants were collected and their protein concentrations were measured according to Lowry method. Each of TRAP reactions contained 2 µg of total protein, 10 pmol forward TS primer (5'-AATCCGTCGAGCAGAGTT-3'), 10 mM dNTPs, 1U Taq DNA polymerase, 10× buffer, 20 mM MgCl₂ to final volume of 25 µl, the complex was incubated 30 min at 37 °C. Then 10 pmol reverse CX primer (5'-CCCTTACCCT-TACCCTTACCCTAA-3') was added before PCR. PCR was performed by repeat of 30 cycles (94 °C, for 30 s, 55 °C for 60 s, 72 °C for 90 s). 10% polyacrylamide gel electrophoresis and stained by AgNO₃.

2.9. Determination of 8-hydroxydeoxyguanosine

Determination of 8-hydroxydeoxyguanosine (8-OH-dG) was determined using an HPLC system consisting of a pump

(ESA delivery system) and an electrochemical detector (ESA, Inc., Chelmsford, MA, USA). The extracted DNA from cells was dissolved in 500 µl of 1 mM ethylenediaminetetra acetate and was digested with 20 µl of nuclease P1 (5 mg/ml) and 10 µl of acid phosphatase (47 mg/ml) suspension in 1.8 M (NH₄)₂SO₄ in the presence of 20-mM sodium acetate buffer at pH 4.5 at 37 °C for 2 h in a water bath. The digested DNA was further incubated for 1 h after adding 40 µl of 80 mM Tris-base and 20 µl of alkaline phosphatase (0.1 U/µl). Aliquots (75 µl) of the DNA hydrolyzed were injected into an HPLC column (Partisil 5 µm ODS-3 reverse-phase analytical column, 25 cm×4.6 mm i.d., Whatman, Clifton, NJ), that was maintained at 25 °C. The mobile phase contained 4 mM citric acid, 8 mM ammonium acetate, pH 4.0, 5% methanol. The flow rate was 1 ml/min. 8-OH-dG was eluted with a retention time of 16.5 min. The peak area was used to calculate the concentration. A standard curve was generated by using different concentrations of an 8-OH-dG standard (Sigma Chemical Co., St. Louis, MO) for quantification. The level of oxidative DNA damage was expressed as 8-OH-dG per 10⁵ dG.

2.10. Atomic force microscopy visualization

Atomic force microscopy observation: A drop of the sample (5 µl) was distributed on freshly cleaved mica compressed nitrogen gas was applied to the sample in such a way that the liquid spread out on the mica surface, and immediately dried under an infra-red lamp prior to the atomic force microscopy observation. Tapping mode images were obtained on a nanoscope III with a multimedia. Microscopic analysis was carried out by using a commercial atomic force microscope (Digital instruments, Santa Barbara, CA). All Atomic force microscopy images were obtained in air at room temperature by using contact mode with a spring contact of 0.12 nN/nm.

2.11. Statistical analysis

The data are expressed as means±standard deviation, statistical significance was assessed by the Student's *t*-test. All experiments performed at least three times independently.

3. Results

3.1. Up-regulation of telomere-binding TRF1, TRF2 by As₂O₃

Cells were treated with 5 µM As₂O₃ for 24 h or 48 h. Then expression of TRF1, TRF2 mRNA level and protein level were detected using RT-PCR and western blotting. Fig. 1A showed expression of TRF1, TRF2 mRNA after As₂O₃ treatment was up-regulated. Fragments of TRF1, TRF2 and β-Actin are 733 bp, 628 bp, 539 bp, respectively. And this accumulation was dependent of time of treatment. Similarly, both proteins increased significantly when cells were exposed to 5 µM As₂O₃ for 24 h or 48 h (Fig. 1B) compared with untreated group. Molecular weights of both proteins were 50 kDa and 54 kDa, respectively.

3.2. Telomere length shortening and telomerase inhibitory were not obvious

As shown in Fig. 2A, untreated cells had longer telomeres, telomeres in cells exposed to 5 μM As_2O_3 for 24 h or 48 h had a little shortening, and further analysis showed arsenic stress acted in some shorter telomere individuals, not influencing the longer individuals, suggesting a mild telomere shortening by As_2O_3 .

Telomerase, a factor contributed to maintenance of telomere, was positive in most malignant tumor, MGC-803 cells had a strong telomerase activity as shown in Fig. 2B. 5 μM As_2O_3 inhibited the telomerase mildly in a time-dependent manner.

3.3. Up-regulation of TRF1, TRF2 involved reactive oxygen species

Our previous work identified that reactive oxygen species was an important signal in As_2O_3 -induced apoptosis (Zhang et al., 2003a,b). To determine the role of reactive oxygen species in arsenic-mediated TRF1, TRF2 response, we detected the reactive oxygen species produced by 5 μM As_2O_3 for 24 h with flow cytometry. Fig. 3 showed that percentage of reactive oxygen species of treated group increased to 14.5 ± 1.13 compared with control group. An antioxidant, *N*-acetylcysteine could decrease reactive oxygen species of normal cells and counteract reactive oxygen species produced by As_2O_3 . Percentage of reactive oxygen species in As_2O_3 group and As_2O_3 plus *N*-acetylcysteine group had significant difference as shown in Fig. 3B ($p < 0.05$). Fig. 4 revealed the effects of reactive oxygen species on TRF1, TRF2. Obviously, both protein levels decreased when reactive oxygen species produced by As_2O_3 was inhibited by *N*-acetylcysteine. This means that reactive oxygen species maybe play an important role in regulating expression of telomere binding proteins.

p53 is one of the key molecules in apoptosis or senescence pathway. Our previous work identified that p53 protein levels increased with As_2O_3 dose increase in As_2O_3 -treated cells (Zhang et al., 2002). Fig. 4 also showed that compared with control groups, p53 protein levels increased significantly exposed to 5 μM As_2O_3 for 24 h. When cells were pretreated

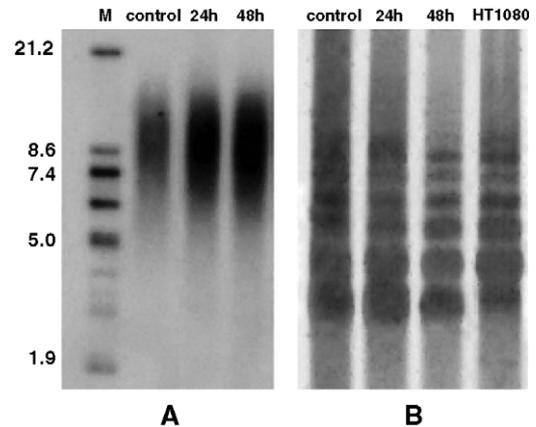


Fig. 2. Telomere length analysis and telomerase activity. (A) Telomere Southern blot of MGC-803 cells. (B) Measurement of telomerase activity. HT-1080 tumor cells acted as inner standard. One typical experiment out of three is shown.

with 10 mM *N*-acetylcysteine for 20 h s before As_2O_3 treatment, 24 h s later, total protein was examined by Western blot analysis using DO-1 anti-p53 monoclonal antibody. As_2O_3 -induced activation of p53 protein was inhibited by *N*-acetylcysteine (Fig. 4).

3.4. Cell cycle arrest in G2/M phase

Ohki et al. found that TRF proteins inhibited the replication of telomeric repeats in vitro, and this effect is relevant in vivo. It evidenced that TRF1, TRF2-overexpression leads to cell cycle arrest at G2/M phase in HeLa cells (Rieko and Fuyuki, 2004). We found significantly (1) larger fractions of cells in S phase at exposure of 5 μM As_2O_3 for 24 h and 48 h (26.6% and 30.6% in TRF1, TRF2-exposure cells versus 15.5% in control cells, respectively); (2) G2/M phase arrest, percentage of G2/M phase increased from 9.53 ± 0.76 to 27.03 ± 1.1 ($p < 0.01$) after 24 h treatment, to 21.9 ± 1.67 ($p < 0.01$) after 48 h treatment; (3) smaller fractions of cells in G1 phase at 24 and 48 h post-exposure (45.2% and 42.4% in TRF1, TRF2-expressing cells versus 75.1% in control cells, respectively). The results suggest that cell cycle progression was delayed or arrested during S-G2/M phases in TRF1, TRF2-expressing cells. Fig. 5A showed that distribution of cell cycle. Similar observations of TRF1-induced apoptosis were reported previously (Kishi et al., 2001). Flow cytometric analysis also showed that percent of sub-G1 phase increased with time of As_2O_3 treatment (Fig. 5A), and thereafter underwent apoptosis.

3.5. The increase of 8-OH-dG formation

Cell DNA was extracted as described in Materials and Methods. The 8-OH-dG formed was analyzed using HPLC analysis. The mean (\pm S.D., $N=5$) levels of 8-OH-dG were 5.8 ± 0.38 , 10.8 ± 0.94 and 12.5 ± 0.89 per 10^5 dG in samples exposed to 5 μM As_2O_3 for 0, 24 and 48 h, respectively. The 8-OH-dG levels increased with increasing time exposed to As_2O_3 and the difference was significant (p value < 0.05). It was suggested that the formation of the 8-OH-dG in cells may be associated with reactive oxygen species induced by As_2O_3 .

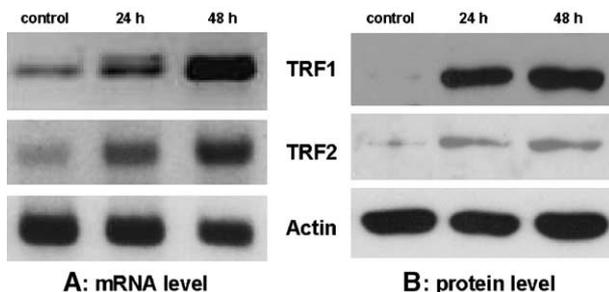


Fig. 1. Expression of TRF1, TRF2 mRNA and protein levels in cells treated with As_2O_3 . (A) Isolated RNA from untreated and treated group was reverse transcribed with specific primers of TRF1 and TRF2. β -Actin was internal standard. (B) Cells were harvested at indicated times after incubation with 5 μM As_2O_3 , 20 μg of total protein was loaded to 10% SDS-PAGE, transferred to nitrocellulose membrane and protein levels were analyzed by western blotting using anti-TRF1 and TRF2 antibodies, and then normalized to actin. One typical experiment out of three is shown.

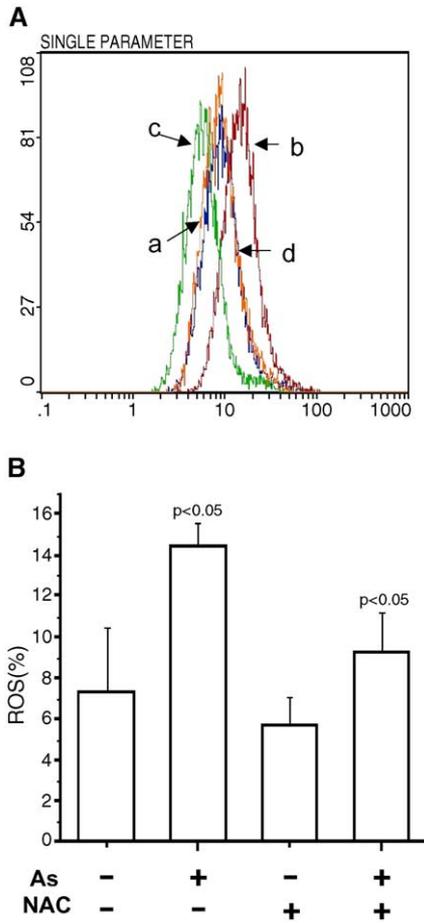


Fig. 3. *N*-acetylcysteine inhibited reactive oxygen species production by As_2O_3 in MGC-803 cells. Cells were treated with 5 μM As_2O_3 for 24 h, in presence or absence of *N*-acetylcysteine, and then were stained with dihydroethidium and analyzed by FACS. The data were presented as means \pm S.D. of dihydroethidium fluorescence from three experiments. (A) Fluorescence intensity for dihydroethidium in As_2O_3 -treated MGC-803 cells with and without *N*-acetylcysteine pretreatment. (B) Single parameter in As_2O_3 -treated MGC-803 cells with and without *N*-acetylcysteine pretreatment.

3.6. Interaction of p53 proteins with telomere

The high resolution and cross sectioning measurement function of Atomic force microscopy make it convenient for

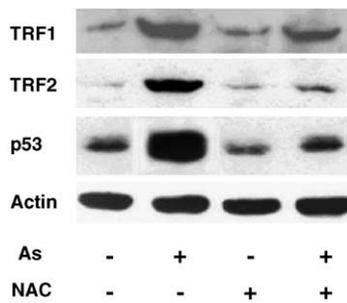


Fig. 4. Antioxidant decreased the expression of TRF1, TRF2 and p53. Cells were pretreated with 10 mM *N*-acetylcysteine for 20 h before As_2O_3 treatment, and then detected expression of three proteins using Western blot.

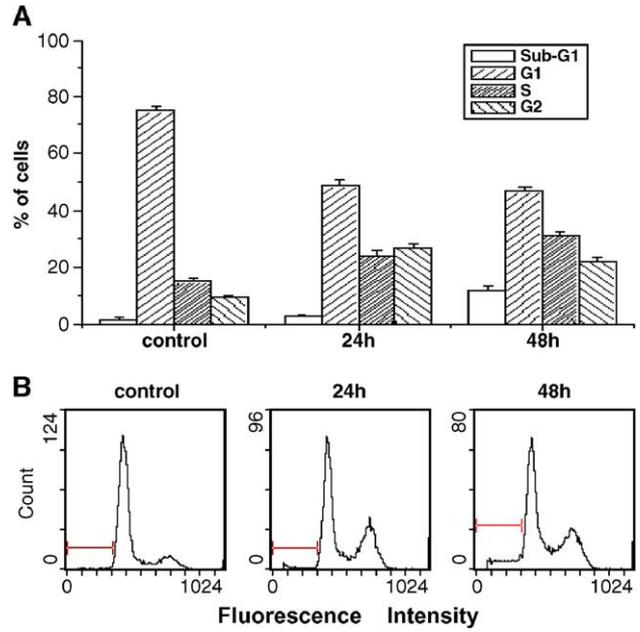


Fig. 5. As_2O_3 induced cell cycle arrest and apoptosis in MGC-803 cells. (A) Distribution of cells was determined by propidium iodide staining. (B) Data were presented as the means \pm S.D. of the percentage of cell cycle in three independent experiments.

us to observe the interaction of telomere DNA and proteins. The telomeric DNA isolated from the psoralen photo-cross cells according to a previous method (Griffith et al., 1999; Chen et al., 2001a,b) showed a typical t-loop structure (Fig. 6A). According to their higher, the loop circle portion was considered as duplex DNA and the tail-loop junction as triplex (type I). Compared with untreated group, a t-loop structure with tetra-stranded junction (type II) was observed when cells were exposed to 5 μM As_2O_3 for 48 h (Fig. 6B). It was about 9% of total t-loops ($N=40$) suggesting that the formation of tetra-stranded structure might be the result of telomeric DNA damage such as a single-stranded overhang loss. In vitro, p53 protein had a high affinity for above type I loop junction (Fig. 6C). It was about 81% of total t-loops ($N=40$), but a lower affinity for the type II loop junction, possibility due to the telomeric overhang loss. However, the interaction of TRF2 and P53 proteins was not observed. The sufficient TRF2 might bind other protein, such as ATM kinase, to repress ATM by this pathway increased. TRF2 can protect critically short telomeres and block a DNA damage response at telomeres (Karlseder et al., 2004).

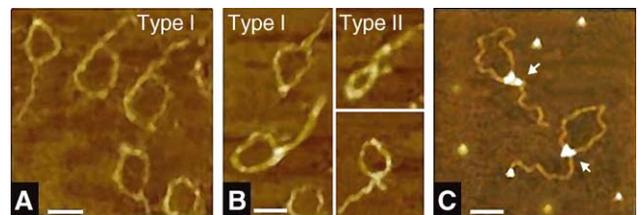


Fig. 6. The looped structures in cells. A, cells without As_2O_3 treatment; B, cells with As_2O_3 treatment; C, p53 protein binding sites (arrow). Scale bars: 200 nm.

4. Discussion

Telomeres play an important role in maintaining chromosome stability and regulating the replicative lifespan of somatic cells. Telomere dysfunction leads to genetic instability at the chromosome end, and such instability is associated with the initiation of carcinogenesis. Hence, adequate telomere length, telomerase activity, and t-loop formation are required for maintenance of telomere function. Our previous studies primarily identified increasing sensitivity to As_2O_3 through altering telomere state (Zhang et al., 2003a,b). In this study, we further focused on telomere length, telomerase and telomere binding protein in arsenic stress.

Arsenic had multiple effects on different cells. Very-low dose arsenic induced differentiation and promoted proliferation. Low-dose As_2O_3 induced cell apoptosis with different cell sensitivity. And high dose arsenic produced toxic effects on cells and animals (Zhang et al., 1999; Chen et al., 2001a,b). Sensitivity to As_2O_3 varied in different cells. Our group scanned As_2O_3 -sensitive MGC-803 (human gastric carcinoma cells) in previous work. In order to research the mechanism of As_2O_3 , we used the apoptosis model induced by As_2O_3 in MGC-803. We detected effects of very-low and high dose As_2O_3 . Cells treated with 0.25 μM As_2O_3 for 2 weeks presented genome instability, i.e. chromosome end fusions. Although 50 μM As_2O_3 induced more apoptosis cells, necrosis cells increased correspondingly (unpublished data).

The length and structure of telomeres are controlled by a variety of proteins, some of which function exclusively at the telomere, others of which also participate in DNA repair. TRF1 and TRF2 play an important role in telomere function. The expression of TRF1 and TRF2 has been examined in a limited number of tumors and cell culture experiments. The expression of both has been reported to be increased, decreased or unchanged in tumors (Nakanishi et al., 2003; Narayan et al., 2001; Yamada et al., 2002). But relationship between both protein and arsenic is not clear. To research telomeric protein in arsenic stress, we detected expression of TRF1 and TRF2 mRNA and protein levels. It was unexpected that both proteins' expressions were significantly elevated in MGC-803 cell line treated with As_2O_3 . Recent evidence linked oxidative stress with telomere attrition (Tchirkov and Lansdorp, 2003). Our results revealed that reactive oxygen species increased significantly in As_2O_3 treatment group. Antioxidant, *N*-acetylcysteine, inhibited production of reactive oxygen species, and decreased expression of TRF1 and TRF2 induced by As_2O_3 correspondingly. It is obvious that As_2O_3 -induced up-regulation of TRF1, TRF2 is related to reactive oxygen species produced by As_2O_3 in MGC-803 cells.

Telomeres are highly sensitive to reactive oxygen species in vitro assay. In fact, our results indicated that telomere length was not sharply shortened and telomerase was mild

inhibited when cells were treated with 5 μM As_2O_3 . There is controversy about what the key sensor of cell apoptosis or senescence is. Some researchers considered that loss of telomere lead to cells undergoing apoptosis (Ramirez et al., 2003; Ijima and Greider, 2003). Telomere length has frequently been used as a means to predict the future life of cells. Recent studies show that telomere length does not seal the fate of a cell (Blackburn, 2002). Our results support this view. Many studies identified inhibitory effects of arsenic on telomerase. One group found that low-dose As_2O_3 activated telomerase, while high-dose As_2O_3 inhibited telomerase (Zhang et al., 2003a,b). Recent studies revealed that telomerase had a binary switch between two states, extendible and inextendible (Loayza and de Lange, 2004; Teixeira et al., 2004). This means that telomerase preferentially acts on shorter telomere. Our experiments also showed that arsenic stress acted in some shorter telomere individuals, not influencing the longer individuals. Our results showed that MGC-803 cells had a strong telomerase activity. However, telomerase might be mildly inhibited by 5 μM As_2O_3 for 24 h. 50 μM of As_2O_3 had obviously inhibitory effects on cells (unpublished). It indicated that the effect of telomerase on telomere binary switch between two states is not obvious at low-dose As_2O_3 , suggesting that this inhibitory may not contribute to telomere attrition in our study.

In general, TRF1 and TRF2 are negative regulators of telomere length, and play important role in maintaining capping state of telomere. Especially, TRF2 promoted formation of t-loop by binding the junction. Negative-mutant TRF2 induced senescence by altering telomere state (Karlsson et al., 2002). TRF1 and TRF2 are believed to promote the formation of the closed state, abundance of TRF1 and TRF2 proteins seems to be prone to maintain capping state instead of uncapping state, thus cells should be protected from apoptosis fate. Why did MGC-803 cell go into apoptosis fate in spite of abundance of TRF1 and TRF2 in arsenic stress? Possible explanations are as follows.

One is, abundant TRF1 and TRF2 proteins might stall the replication fork by assembling to telomeric repeats. The replication could be stalled under various circumstances. For example, d(TC)_n-d(GA)_n, known to assume a triplex structure in vitro, stalls the replication fork progression both in vivo and in vitro (Baran et al., 1991). In other cases, the replication fork progression is stalled by protein-DNA complexes, a very strong and stable DNA-protein interaction appears to interfere with the progression of the replication fork (Kamada et al., 1996). TRF1 is unique in that the two Myb-like domains of the TRF1 homodimer bind to two AGGGTT sites independently and with extreme flexibility in terms of the spatial arrangement of the two target sites (Bianchi et al., 1999). This unique binding mode enables TRF1 to form string, loop and synaptic structures within or between telomeric repeats. Ohki et al. found that replication fork stall at telomeric repeats happens when TRF1 and TRF2 proteins are added

to the in vitro system or overexpressed in vivo and cells with 4 N DNA content were accumulated (Rieko and Fuyuki, 2004). However, the DNA damage response to uncapped telomeres induces phosphorylation of DNA damage response proteins, including H2AX, SMC1, Rad17, CHK1, and CHK2, and up-regulation of p53, p21, and p16, resulting in a G1 arrest (d'Adda di Fagagna et al., 2003; Karlseder et al., 1999; Smogorzewska and de Lange, 2002). In this work, it was observed that cells resulted in G2/M arrest following treatment of 5 μ M As₂O₃ for 48 h. And a few apoptotic cells appeared within 24 h and percent of apoptotic cells increased significantly following increase of As₂O₃-treated time. It seems that abundant TRF1 and TRF2 might stall the replication fork by binding telomeric repeats, lead to G2/M arrest and apoptosis, and not respond to uncapped telomeres.

Another possible explanation is, abundant TRF1 and TRF2 proteins bind with the telomeric repeats in the damaged telomere or with other proteins such as ATM kinase, to inhibit the DNA damage response. Stansel et al. demonstrated that TRF2 protein has been implicated in remodeling telomeres into t-loops in vitro and, 19±8% had one end folded back into a loop with TRF2, at the loop junction. 32±19% had a TRF2 particle bound at one end of the DNA (presumably the end with the telomeric repeats). 11±5% had a TRF2 particle bound along the telomeric repeats tract and the remainder (39%) were scored as being protein free (Stansel et al., 2001). Abundant TRF2 might bind with the end of the single-stranded overhang with the telomeric repeats in the damaged telomere; therefore concealed damage in telomere repeat array, blocked access of DNA repair enzyme, such as telomerase, was thought to enhance DNA repair capacity by recruiting other proteins (Shin et al., 2004). It reported TRF2 functions as a core promoter-selectivity factor and directs transcription (Hochheimer et al., 2002). TRF2 interacts with DNA replication-related element binding factor (DREF) and several components of the nucleosome remodeling factor/chromatin remodeling complex. TRF2 regulates the DREF-responsive promoter of the PCNA gene. TRF2 may direct the expression of a subset of DREF dependent genes. In this work, TRF2 bound directly with the loop junction were also observed, which could promote the formation of the closed state. Recently we found that TRF2 in cytoplasm increased obviously after As₂O₃ treatment by confocus microscopy (unpublished). Those abundant TRF2 might bind other protein, such as ATM kinase, which is an important transducer of the DNA damage signal, to repress ATM, by this pathway abundant TRF2 can protect critically short telomeres and block a DNA damage response at telomeres (Karlseder et al., 2004), which is consistent to above-observed results of a mild telomere shortening and G2/M arrest. With results, damaged telomere could not be repaired immediately, single-break accumulated could specifically block a DNA damage response at telomeres

without affecting the surveillance of chromosome internal damage.

In addition, the attack of reactive oxygen species might make normal substrate of TRF1 and TRF2 change, that is, modified or mutated telomere became ineffective substrate. In vitro assays showed that nitric oxide (NO) free radical caused base alteration at the 5' site of the 5'-GGG-3' in the telomere sequence. It was observed the formation of nitric oxide (NO) free radical in cells with As₂O₃ treatment (Shen et al., 2002). And oxygen free radical (O₂⁻, H₂O₂) induced 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodG) formation in telomere sequences more efficiently than that in non-telomere sequence. And heterogenous telomeric sequences, such as double-strand arrays TTGGGG, TTAGGC, TTTAGGG, TTAGGGG and TAGGG repeats are not effective substrate of TRF1 and TRF2. That might mean that much more GGG sequence was attacked by reactive oxygen species (Rubio et al., 2004). From the above results, we also observed that number of 8-OH-dG formation increased when cells were exposed to As₂O₃. We thought abnormal telomere could not bind TRF1 and TRF2 in spite of abundance so that telomeric t-loop presented an opening state. Subsequently, cells went into apoptosis.

The effects of As₂O₃ on p53 varied according to different cell types. Some studies showed that As₂O₃ could activate p53, and others had little influence on the expression of p53. Because of a wide range of targets of As₂O₃, cell cycle arrest or apoptosis also were presented in a p53-dependent or -independent manner. p53^{wt} cells could induce cell cycle arrest at G1 or G2/M phase (Chow et al., 2004; Zhang et al., 2002). On the other hand, p53 has been proposed to be an important mediator of telomere dysfunction. Some data showed that telomere repeats were important constituents of a novel pathway leading to p53 protein activation, and telomere shortening triggered p53-dependent apoptosis or growth arrest (Milyavsky et al., 2001) In MGC-803(p53^{wt}) cell line, our results demonstrated that As₂O₃ induced G2/M phase arrest and apoptosis and that p53 protein had a high affinity for the t-loop junction. Interestingly, telomere structure state might signal p53 protein directly through association with the t-loop junction or extrication in cells. And up-regulation of TRF1 and TRF2 may link to arsenic treatment, and not be related to p53 status (unpublished data).

5. Conclusion

Treatment of human gastric cancer MGC-803 cells with As₂O₃ caused up-regulation of TRF1, TRF2 mRNA and protein levels, and increased cell cycle arrest in G2/M phase, and therefore, underwent apoptosis, but had no obvious effects on telomere length and telomerase. At the same time, compared with control group, percentage of reactive oxygen species and 8-OH-dG products of treated group increased in cells. P53 also could bind directly at the t-loop junction.

These findings demonstrate that the increase of TRF1 and TRF2 proteins is related to oxidative stress. Arsenic-induced cell cycle arrest and apoptosis involve a novel pathway leading to p53 protein activation directly by altered telomeric states, which differed from the protection of t-loop previously.

Arsenic exposure influences transcription of a wide range of genes that affect cell cycle progression and apoptosis. Future investigation may determine whether these different effects vary according to cell type, or arsenic form, or other factors.

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