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Arsenic Trioxide (As_2O_3) Inhibits Invasion of HT1080 Human Fibrosarcoma Cells: Role of Nuclear Factor-κB and Reactive Oxygen Species

Myung-Jin Park,[1] Jae-Young Lee,[1] Hee-Jin Kwak,[1] Chang-Min Park,[1] Hyung-Chahn Lee,[1] Sang Hyeok Woo,[1] Hyun-Ok Jin,[1] Chul-Ju Han,[4] Sungkwan An,[5] Seung-Hoon Lee,[6] Hee Yong Chung,[7] In-Chul Park,[1] Seok-Il Hong,[1,3] and Chang Hun Rhee[1,2]*

[1]Laboratory of Cell Biology, Korea Institute of Radiological and Medical Sciences, Seoul, Korea
[2]Department of Neurosurgery, Korea Institute of Radiological and Medical Sciences, Seoul, Korea
[3]Laboratory Medicine and Clinical Pathology, Korea Institute of Radiological and Medical Sciences, Seoul, Korea
[4]Department of Internal Medicine, Korea Institute of Radiological and Medical Sciences, Seoul, Korea
[5]Laboratory of Molecular Cell Biology and Functional Genomics, Department of Microbial Engineering, Konkuk University, Seoul, Korea
[6]Center for Cancer of Special Organ, National Cancer Center, Goyang, Gyonggi, Korea
[7]Departments of Microbioloy, College of Medicine, Hanyang University, Seoul, Korea

Abstract In order to define the role of As_2O_3 in regulating the tumor cell invasiveness, the effects of As_2O_3 on secretion of matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA), and in vitro invasion of HT1080 human fibrosarcoma cells were examined. As_2O_3 inhibited cell adhesion to the collagen matrix in a concentration dependent manner, whereas the same treatment enhanced cell to cell interaction. In addition, As_2O_3 inhibited migration and invasion of HT1080 cells stimulated with phorbol 12-myristate 13-aceate (PMA), and suppressed the expression of MMP-2, -9, membrane type-1 MMP, uPA, and uPA receptor (uPAR). In contrast, As_2O_3 increased the expression of tissue inhibitor of metalloproteinase (TIMP)-1 and PA inhibitor (PAI)-1, and reduced the MMP-2, -9, and uPA promoter activity in the presence and absence of PMA. Furthermore, the promoter stimulating and DNA binding activity of nuclear factor-κB (NF-κB) was blocked by As_2O_3, whereas the activator protein-1 activity was unchanged. Pretreatment of the cells with *N*-acetyl-L-cysteine (NAC) significantly prevented suppression of MMPs and uPA secretion, DNA binding activity of NF-κB, and in vitro invasion of HT1080 cells by As_2O_3, suggesting a role of reactive oxygen species (ROS) in this process. These results suggest that As_2O_3 inhibits tumor cell invasion by modulating the MMPs/TIMPs and uPA/uPAR/PAI systems of extracellular matrix (ECM) degradation. In addition, the generation of ROS and subsequent suppression of NF-κB activity by As_2O_3 might partly be responsible for the phenomena. Overall, As_2O_3 shows potent activity controlling tumor cell invasiveness in vitro. J. Cell. Biochem. 95: 955–969, 2005.

Key words: arsenic trioxide; invasion; matrix metalloproteinases; urokinase plasminogen activator; nuclear factor-κB; reactive oxygen species

Abbreviations used: ECM, extracellular matrix; BM, basement membrane; PAs, plasminogen activators; MMPs, matrix metalloproteinases; MT-MMPs, membrane-type MMPs; TIMPs, tissue inhibitors of metalloproteinases; uPA, urokinase PA; uPAR, uPA receptor; PAI, PA inhibitor; APL, acute promyelocytic leukemia; ROS, reactive oxygen species; NF-κB, nuclear factor-kappa b; BSA, bovine serum albumin; H_2DCFDA, 2′, 7′-dichlorofluorescein diacetate; FBS, fetal bovine serum; PI, propidium iodide; PMA, phobol 12-myristate 13-acetate; AP-1, activator protein-1; EMSA, electrophoretic mobility gel shift assay; NAC, *N*-acetyl-L-cysteine.

Myung-Jin Park and Jae-Young Lee contributed equally to this work.

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*Correspondence to: Chang Hun Rhee, Department of Neurosurgery, Korea Institute of Radiological and Medical Sciences, 215-4 Gongneung-dong, Nowon-Ku, Seoul 139-706, Korea. E-mail: changhun@kcch.re.kr

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Tumor invasion is a multistep process in which cellular motility is coupled to proteolysis, which involves interaction with the extracellular matrix (ECM) [Johnsen et al., 1998]. During invasion, malignantly transformed cells detach from the primary tumor, migrate, and cross the structural barriers, including basement membrane (BM) and surrounding stromal ECM, consisting mainly of fibrillar collagens [Kahari and Saarialho-Kere, 1999]. This process depends on the ability of tumor cells to degrade the surrounding collagen matrix and then migrate through the matrix defects [MacDougall and Matrisian, 1995]. One of the early events in cancer cell invasion involves the proteolytic degradation of ECM components, and the invading tumor cells secrete several proteases such as serine proteases, plasminogen activators (PAs), and matrix metalloproteinases (MMPs) [Mignatti and Rifkin, 1993; Basset et al., 1997].

MMPs are a family of structurally related neutral metalloproteinases, which together can degrade all the components of the ECM proteins and are involved in tumor invasion, metastasis, and angiogenesis [Matrisian, 1992; Nagase and Fields, 1996; Vihinen and Kahari, 2002]. According to their structure and substrate specificity, they can be divided into five subgroups: collagenases, stromelysins, gelatinases, membrane type (MT)-MMPs, and others [Vihinen and Kahari, 2002]. Among the MMPs, the 72 kDa gelatinase A (or MMP-2) and 92 kDa gelatinase B (or MMP-9) are believed to be the critical enzymes for degrading type IV collagen, a major component of BM, thus playing a critical role in cancer cell invasion and metastasis [Liabakk et al., 1996; Johnsen et al., 1998; Vihinen and Kahari, 2002]. The recent cloning of several MT-MMPs has demonstrated that MMP-2 activation is mediated by MT-MMPs on the cell surface either as an activator or a receptor, and most MT-MMPs can degrade ECM components [Seiki, 1999]. The extracellular activity of these enzymes was inhibited when they formed complexes with specific inhibitors such as tissue inhibitor of metalloproteinase (TIMP)-1, -2, -3, and -4 [Brew et al., 2000]. Another important protease in cancer invasion and metastasis is urokinase PA (uPA). uPA binds to the uPA receptor (uPAR) and converts plasminogen to plasmin, which can either directly degrade BM and ECM or activate other zymogen proteases such as procollagenase [Wang, 2001]. This catalytic activity can be inhibited by the inhibitors such as PA inhibitor (PAI)-1 and -2 [Wang, 2001].

As_2O_3 is an active ingredient of a traditional Chinese medicine that has been used successfully for treating acute promyelocyte leukemia (APL) [Shen et al., 1997; Soignet et al., 1998], and has been shown to be an effective inducer of apoptosis in certain cancer cells, including APL, some myeloid leukemic cells, and esophageal, prostate, and ovarian carcinomas [Uslu et al., 2000; Maeda et al., 2001; Shen et al., 2002]. As_2O_3 has also an anti-vascular effect in well-established murine solid tumor models [Lew et al., 1999], and has been shown to prevent capillary tubule and branch formation in an in vitro endothelial cell differentiation assay by suppressing vascular endothelial growth factor production by the leukemic cell line, HEL [Roboz et al., 2000]. However, the effect of As_2O_3 on the tumor cell invasiveness has not yet been reported.

In the present study, we investigated the effect of As_2O_3 on the invasiveness of HT1080 cells stimulated with or without phorbol 12-myristate 13-acetate (PMA): although HT1080 cells have already invasive characters, in some experiments, we used PMA to enhance the invasive properties of the cells as previously reported [Simon et al., 1998; Park et al., 2000, 2002; Kobayashi, 2001] and to clearly demonstrate the anti-invasive activity of As_2O_3. The results showed that As_2O_3 decreased the invasiveness of HT1080 human fibrosarcoma cells in vitro, which was mediated by the inhibition of cell–matrix interaction, suppression of MMPs and uPA/uPAR expression, and up-regulation of TIMP-1. It was also demonstrated that the generation of reactive oxygen species (ROS), followed by the decreased DNA binding activity of the nuclear factor-kappa b (NF-κB), by As_2O_3 treatment might partly be responsible for these events. Based on these findings, As_2O_3 might be considered ad a possible agent for controlling the invasion of tumor cells and the clinical relevance of the agent should be further elucidated.

MATERIALS AND METHODS

Cell Culture

HT1080 (fibrosarcoma), HS683 (glioblastoma), MDA-MB-231 (breast adenocarcinoma), and NCI-H460 (lung carcinoma) cells were grown in DMEM (Gibco BRL, Grand Island,

NY) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL), and 10% heat inactivated FBS (Gibco BRL) in a humidified incubator containing 5% CO_2 at 37°C.

Materials

Human type I and IV collagens, bovine serum albumin (BSA), gelatin, casein, and As_2O_3 were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies against MT1-MMP, MMP-2, -9, TIMP-1, -2, and uPA were obtained from Calbiochem (La Jolla, CA), and uPAR antibody was from R&D Systems (Minneapolis, MN). Matrigel was purchased from Becton Dickinson (Bedford, MA), and NF-κB inhibitor, PPM-18, SN50, and SN50M, non-active analog of SN50, were purchased from Calbiochem, and H_2DCFDA (2′, 7′-dichlorofluorescein diacetate) was from Molecular Probes (Eugene, OR). 5×10^{-2}M As_2O_3 was prepared in 1N NaOH at as a stock solution and kept at −20°C. For in vitro use, the stock solution was diluted to an appropriate concentration with growth medium without fetal bovine serum (FBS). The highest concentration of NaOH used in culture had no influence on cell growth of these cell lines.

Cytotoxicity Assays

In vitro cytotoxic effect of As_2O_3 on HT1080 tumor cells was determined by using CellTiter 96 Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI). Briefly, cells growing in plates were dispersed in 0.05% trypsin solution and resuspended in DMEM containing 10% FBS. Approximately 5,000 cells were added to each well of 96-well plate and incubated for 18 h. After changing the media with or without 10% serum, various concentrations of As_2O_3 were added to each well. After 48 h of incubation, cell cytotoxicity was estimated by following the supplier's instructions.

Evaluation of Apoptosis

Apoptosis was evaluated by staining the cells with annexin V-FITC (Pharmingen, San Diego, CA) and PI according to manufacture's protocol. Cells were then analyzed by FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Zymography

Production of MMPs and uPA by HT1080 cells was analyzed by gelatin and casein zymography, respectively. HT1080 cells in subconfluent culture condition (about 70%–80% confluent) were washed and replenished with serum-free DMEM medium, and incubated with or without PMA (50 ng/ml) in the presence or absence of As_2O_3 for 18 h. Twenty microliters of serum-free conditioned medium were mixed with SDS sample buffer without heating or reduction and applied to 10% polyacrylamide gels copolymerized with 1 mg/ml gelatin or 1 mg/ml casein and 10 μg/ml plasminogen. After electrophoresis, gels were washed for 2 h at room temperature in 2.5% (v/v) Triton X-100 to remove SDS, rinsed twice with water, and then incubated in developing buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 0.1% NaN_3) for 18 h at 37°C. Subsequently, gels were fixed and stained with 10% 2-propanol and 10% acetic acid containing 0.5% Coomassie Blue R250. The protease activity was visualized as clear bands within the stained gel.

Cell Invasion Assays

Invasion assays were performed, using modified Boyden chambers with polycarbonate Nucleopore membrane (Corning, Corning, NY). Pre-coated filters (6.5 mm in diameter, 8 μm pore-size, Matrigel 100 μg/cm^2) were rehydrated with 100 μl of medium. Then, 2×10^5 cells in 100 μl of medium with or without various concentrations of As_2O_3 were seeded into the upper part of each chamber, whereas the lower compartments were filled with 1 ml of serum free DMEM supplemented with 0.1% BSA in the presence or absence of PMA (50 ng/ml). Following incubation for 18 h at 37°C, non-invaded cells on the upper surface of the filter were wiped off with a cotton swab, and the migrated cells on the lower surface of the filter were fixed and stained with Diff-Quick kit (Fisher Scientific, Pittsburgh, PA). Invasiveness was determined by counting cells in five microscopic fields per well, and the extent of invasion was expressed as an average number of cells per microscopic field.

Cell Migration Assays

To determine the effect of As_2O_3 on the migration of tumor cells, we performed a wound-migration assay, as described previously [Sato and Rifkin, 1998]. HT1080 cells were cultured on a 12-well plate. When cells were confluent, monolayers were wounded with a 200–1,000 μl micropipette tip, washed twice with serum-free medium, and incubated for 24 h in serum-free medium with PMA (50 ng/ml) in

the presence or absence of indicated concentrations of As_2O_3. After incubation, cells were washed twice with PBS and then fixed with Diff-Quick solution. Migration was quantified in duplicate cultures by counting the number of cells that migrated from the wound edge into the denuded area for a distance of 1 cm.

Cell–Matrix and Cell–Cell Adhesion Assays

HT1080 cells were grown on six-well plates and incubated at 37°C to 80% confluency. Cells were radiolabeled with [^{3}H] thymidine overnight and incubated with indicated concentrations of As_2O_3 for 2 h. Another set of 24-well plates were coated overnight at 37°C with 10 μg/ml type I collagen or type IV collagen. Nonspecific binding was blocked by incubating the plates with 0.1 mg/ml BSA containing PBS for 2 h at room temperature. After trypsinization, the cells were resuspended in DMEM with 10% FBS and plated on the collagen-coated 24-well plates. After 30 min of incubation, non-adherent cells were collected and discarded. Then, the plates were rinsed with PBS twice, and cells bound to the surface were trypsinized and collected in separated tube. Radioactivities of the attached cells were measured by liquid scintillation counter.

Cell–cell adhesion assays were performed by preparing 24-well plates covered with HT1080 cells in complete confluency. The radiolabeled, As_2O_3-treated HT1080 cells (5×10^4) were seeded additionally. After 3 h, the non-adherent cells were harvested, and the radioactivity was measured by liquid scintillation counter.

Luciferase Reporter Assays

pGL3 and pGL2 luciferase reporter vectors, containing MMP-2 and MMP-9, and uPA promoter regions, respectively, were kindly provided by Dr. Sang-Oh Yoon (Korea Advanced Institute of Science and Technology, Taejon, Korea) [Yoon et al., 2001]. NF-κB and activator protein (AP)-1 reporter constructs were purchased from Clontech Co. (Palo Alto, CA). β-galactosidase containing vector was used to evaluate transfection efficiency. HT1080 cells were seeded in 12-well plates and cultured until they reach 50%–60% confluency at 37°C. Plasmids were then transiently transfected into HT1080 cells by using Effecten reagents (Qiagen, Valencia, CA), according to the supplier's instructions. After 24 h, various concentrations of As_2O_3 were treated. The luminescence was measured by using a luminometer after 24 h of incubation. β-galactosidase activity was measured, using o-nitrophenyl β-galctopyranoside as a substrate.

Western Blot Analysis

HT1080 cells were lysed in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF). After a brief sonication, the lysates were clarified by centrifugation at $12{,}000 \times g$ for 15 min at 4°C, and protein content in the supernatant was measured by the Bradford's method. An aliquot (30–50 μg protein per lane) of the total protein was separated by 10% or 12% SDS–PAGE and blotted to nitrocellulose transfer membrane (0.2 μm) (Amersham, Arlington Heights, IL). The membrane was blocked with 5% non-fat skim milk in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.01% Tween-20) for 1 h at room temperature, followed by incubation with the antibodies against MT1-MMP or uPAR. After extensive washing with TBST, the membrane was reprobed with horseradish peroxidase-linked anti-rabbit immunoglobulin, at 1:3,000 diluted in 5% non-fat skim milk in TBST, for 40 min at room temperature. Immunoblots were visualized by enhanced chemiluminescence (Amersham) according to the manufacturer's protocol.

To measure the secreted uPA, MMP-2, -9, TIMP-1 and -2 proteins, the conditioned medium from each sample was also subjected to protein analysis. For this purpose, culture medium in each tissue culture dish was collected and concentrated ten-fold by using a Centricon 10 microconcentrator (Amicon, Beverly, MA). The concentrates were subjected to SDS–PAGE analysis.

Northern Blot Analysis

Total RNA was extracted from the cultured HT1080 cells by using RNeasy mini kit (Qiagen). RNA (20 μg/sample) was size fractionated through a 1% agarose–formaldehyde gel and transferred to nylon membrane (Schleicher and Schuell, Dassel, Germany). The cDNA probes for MT1-MMP, MMP-2, -9, uPA, uPAR, uPAI-1, TIMP-1, and TIMP-2 were labeled with [^{32}P]dCTP (ICN, Costa Mesa, CA) to high specific activity by Megaprime DNA Labeling System (Amersham). The filter was hybridized

for 24 h at 42°C, washed, and exposed to Kodak X AR 5 film (Eastman Kodak, Rochester, NY).

Electrophoretic Mobility Gel Shift Assay (EMSA)

EMSA was performed, as described previously with some modification [Huang et al., 2001]. Nuclear extracts were prepared from HT1080 cells with or without PMA (100 ng/ml) and As_2O_3 (2 μM). Synthetic oligonucleotides, consisting of consensus sequences of NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) or AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) were used as probes after annealing sense- and anti-sense fragments. The double strand oligonucleotides were then labeled with [γ-^{32}P]ATP. Ten micrograms of nuclear extracts were incubated with 50,000 cpm labeled probes for 30 min at 22°C and analyzed on 6% PAGE followed by authoradiography. The specificity of binding was confirmed by competition with unlabelled oligonucleotides. For supershift assays, nuclear extracts were incubated with antibody against p65 subunit of NF-κB for 30 min at 37°C before the complex was analyzed by EMSA.

Measurement of ROS

The intracellular accumulation of ROS was determined by using the fluorescent probe H_2DCFDA. Cells were plated in 12-well plates (1×10^5 cells/well) and incubated overnight in humidified chamber at 37°C. Cells were washed with PBS and loaded with 0.5 ml of 10 μM H_2DCFDA in PBS for 30 min. Unincorporated free H_2DCFDA was removed by washing twice with PBS. H_2DCFDA-loaded cells were treated with As_2O_3 (2 μM) in the presence or absence of NAC for 24 h. Fluorescence was measured, using a FACScan flow cytometer (Becton Dickinson).

Statistical Analysis

All results are expressed as mean ± standard deviation (SD) or SEM. Statistical significance was determined, using Student's *t*-test.

RESULTS

Effect of As_2O_3 on Cytotoxicity of HT1080 Cells

The human fibrosarcoma cells, HT1080, were cultured in serum-supplemented medium, and the effect of As_2O_3 on the cell cytotoxicity in the presence or absence of serum was measured by using a cell proliferation assay kit, as described in the "Materials and Methods." As shown in Figure 1A, significant cell cytotoxic effect was observed in the serum-supplemented media following the As_2O_3 treatment for 48 h. In the serum-supplemented condition, 2 μM As_2O_3

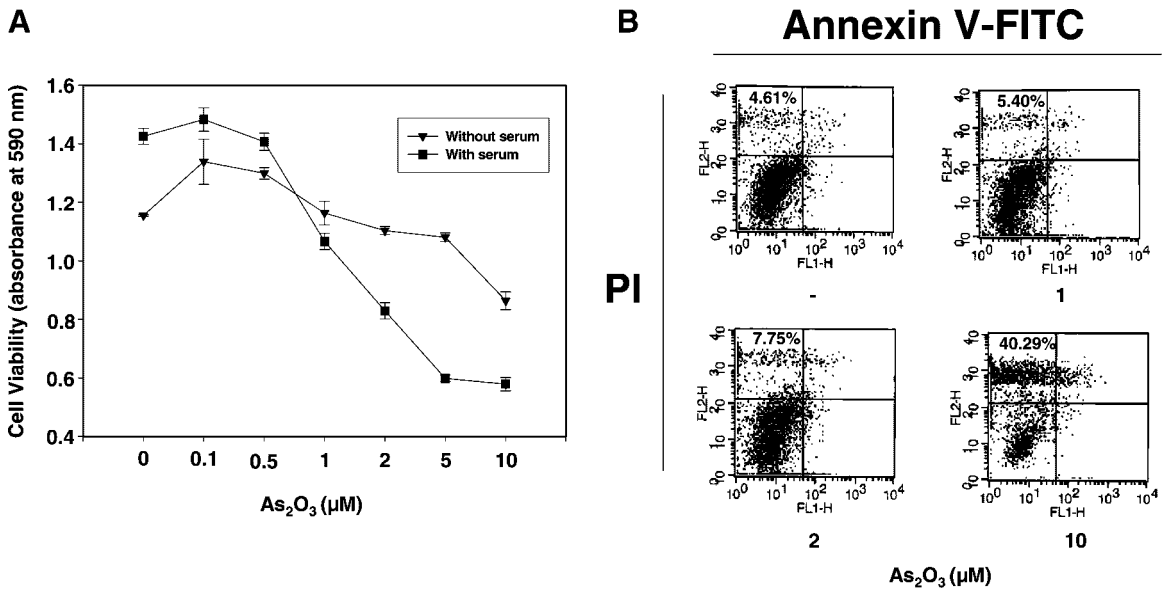

Fig. 1. Effect of As_2O_3 on the cytotoxidity of HT1080 cells. **A**: Exponentially growing cells were treated with indicated concentrations of As_2O_3 for 2 days in the presence or absence of serum. Cytotoxicity was determined by MTT based assay as described in Materials and Methods. Results represents mean ± SD of at least three independent experiments. **B**: Dual-parameter flow cytometric analysis of the As_2O_3-treated cells. Cells were treated with As_2O_3 for 2 days in serum free condition and were stained with annexin V-FITC and PI, as described in Materials and Methods. The result is a representative of three independent experiments with similar results.

exhibited approximately 50% growth inhibition (Fig. 1A). In the serum-free condition, 2 μM As_2O_3 did not affect the cell population, and significant reduction of the cell population was observed at higher than 10 μM concentrations (Fig. 1A). Also, Annexin V/PI staining did not detect any significant apoptotic cells in the serum-free condition at the concentration of 2 μM As_2O_3 (Fig. 1B). Significant cell death was observed at the concentration above 10 μM. These results show that As_2O_3 has more pronounced cytotoxic effect on the HT1080 cells in the presence of serum. Therefore, non-cytotoxic concentration of As_2O_3 (below 2 μM) in serum-free conditions was used in the following experiments.

Effect of As_2O_3 on the Migration and Invasion of HT1080 Cells

In order to evaluate the effects of As_2O_3 on cell migration in response to PMA, confluent monolayers of HT1080 cells were scraped with a pipette tip (200–1,000 μl) to remove a section of the monolayer, and the cells were cultured for 24 h with PMA (50 ng/ml) in the presence or absence of As_2O_3. The migration rate was assessed by counting the number of cells that migrated into the pre-defined denuded area of the cell monolayer. The results show that As_2O_3 dose-dependently inhibited the migration of PMA-stimulated HT1080 cells (Fig. 2A,B).

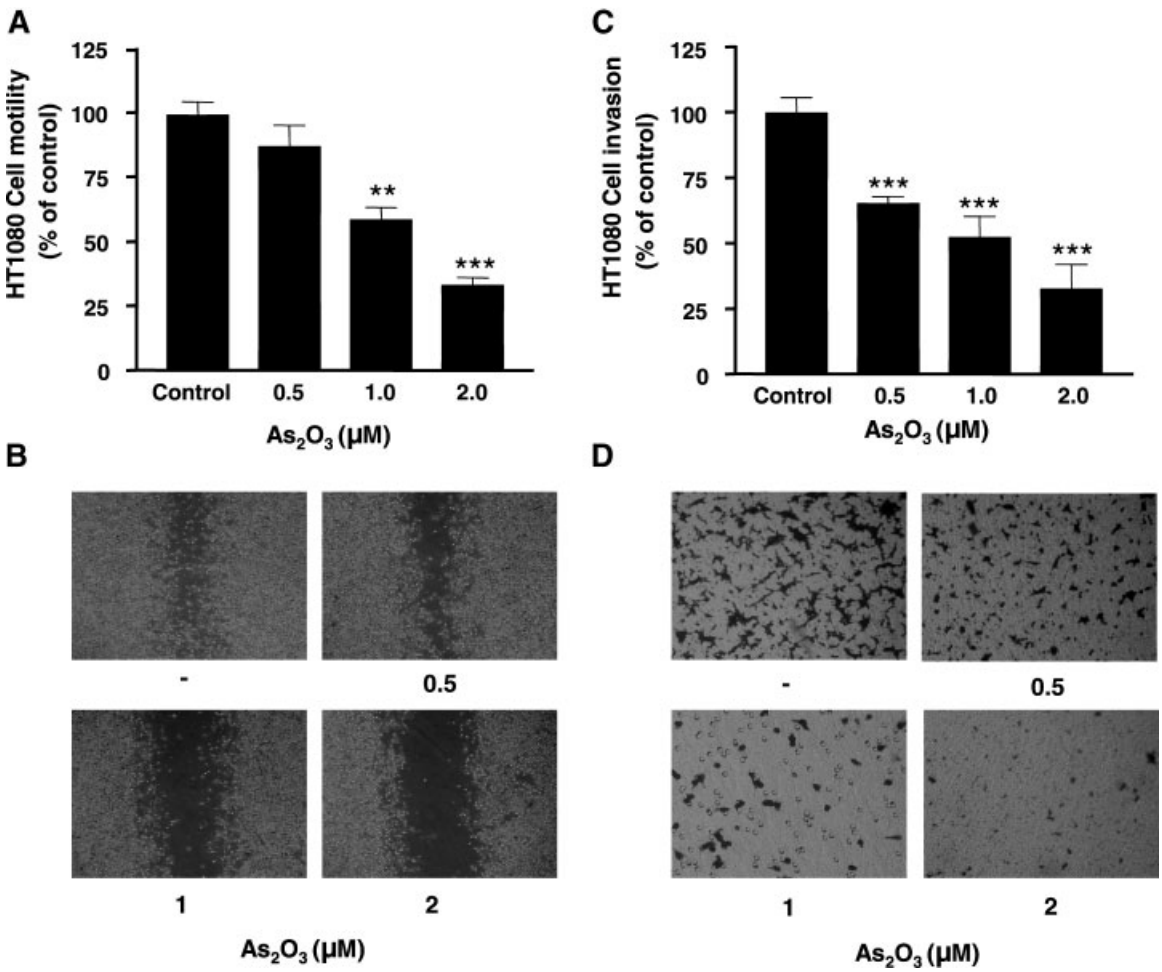

Fig. 2. Effects of As_2O_3 on the migration and invasion of HT1080 cells in vitro. **A**: Cell migration was assessed by counting the number of cells that migrated into the denuded area over a 1 cm distance along the wounded edge. Cells not exposed to As_2O_3 were used as positive controls in the presence of PMA (50 ng/ml). **B**: Photomicrographs of the HT1080 cells migrated into the wounded site (× 100). **C**: Invasion through a layer of Matrigel was determined by a Boyden chamber method. Cells not exposed to As_2O_3 were used as a control. **D**: Photomicrographs of HT1080 cells invaded under the membrane. Data represent mean ± SD from three independent experiments and are expressed as the percentage of control. Detailed experimental procedures are described under Materials and Methods. **, $0.005 < P < 0.01$; ***, $0.001 < P < 0.005$.

Next, the effects of As_2O_3 on the invasion of HT1080 cells were determined by using Boyden chamber assays by measuring the ability of the cells to pass through ECM layer on a Matrigel-coated filter. As seen in Figure 2C,D, PMA significantly stimulated the invasiveness of HT1080 cells. However, pretreatment of the cells with As_2O_3 inhibited the PMA-stimulated invasion in a dose dependent manner.

Effect of As_2O_3 on Cell–Cell and Cell–Matrix Adhesion

Cancer cells, invading the host tissue, break from their cell–cell contacts and make new contact with the ECM. Therefore, a low cell–cell adhesion and high cell–matrix adhesion is known to correlate well with a highly invasive phenotype [Bohle and Kalthoff, 1999]. Therefore, As_2O_3 was tested to determine if it affected the cell–matrix and cell–cell adhesion. As shown in Figure 3A, As_2O_3 significantly reduced the cell attachment to the type I and type IV collagen in a dose dependent manner, being more effective to the type I collagen. On the other hand, As_2O_3 treatment dose-dependently increased the cell–cell adhesion (Fig. 3B).

Effect of As_2O_3 on the Expression of Proteases and Their Inhibitors

In tumor invasion, tumor cells that escape from the tumor mass degrade the surrounding ECM. A variety of proteases produced by tumor cells are implicated in ECM degradation. To examine whether As_2O_3 could modulate the activities of the ECM degrading proteases, the effect of As_2O_3 on the secretion of MMPs and uPA were investigated by gelatin and casein zymography, respectively. As shown in Figure 4A,B, HT1080 cells constitutively secreted MMP-2, -9, uPA, and PMA (50 ng/ml) treatment increased the secretion of these proteases. However, pretreatment of HT1080 cells with As_2O_3 significantly reduced MMP-2, -9, and uPA secretion in a dose dependent manner, regardless of PMA treatment (Fig. 4A,B). The addition of 2 μM As_2O_3 to the zymogram reaction buffer had no effect on the proteases activities (data not shown), indicating that the inhibitory effect was not due to the direct inhibition of the protease activity by As_2O_3.

We further investigated the effect of As_2O_3 on the expressions and secretions of the proteases and their inhibitors. As shown in Figure 5, As_2O_3 decreased the MMP-2, -9, uPA, and uPAR protein levels in a dose dependent manner, and slightly decreased MT1-MMP and TIMP-2 protein levels. However, the same treatment increased TIMP-1 protein level (Fig. 5A). Northern blot analysis was performed to determine whether the proteases and their inhibitors were modulated by As_2O_3 at the mRNA mRNA level.

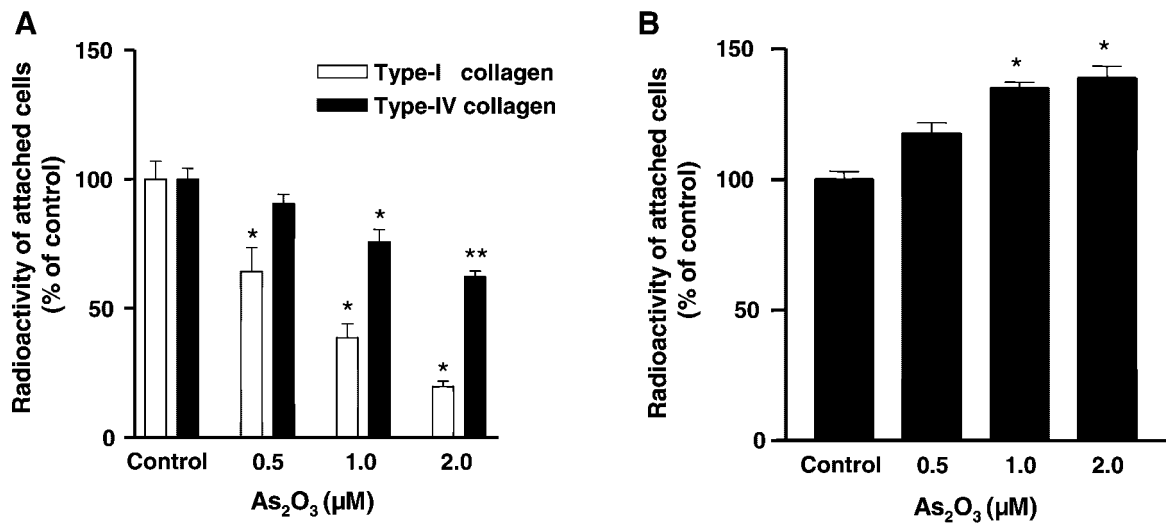

Fig. 3. Effects of As_2O_3 on the cell–matrix and cell–cell adhesion of HT1080 cells. **A**: Radiolabeled HT1080 cells incubated overnight with or without various concentrations of As_2O_3 were seeded onto the 24-well plates coated with type I or type IV collagen. After 30 min, attached cells were trypsinized, collected, and radioactivity was measured by a scintillation counter. **B**: 24-well plates were covered with confluent monolayer of HT1080 cells prior to seeding the As_2O_3 treated, radiolabeled HT1080 cells. After 3 h, non-adherent cells were collected, and the radioactivity was measured by liquid scintillation counter. Data are presented as percentages of control and given as means ± SD of three separate experiments. *, $0.01 < P < 0.05$; **, $0.005 < P < 0.01$.

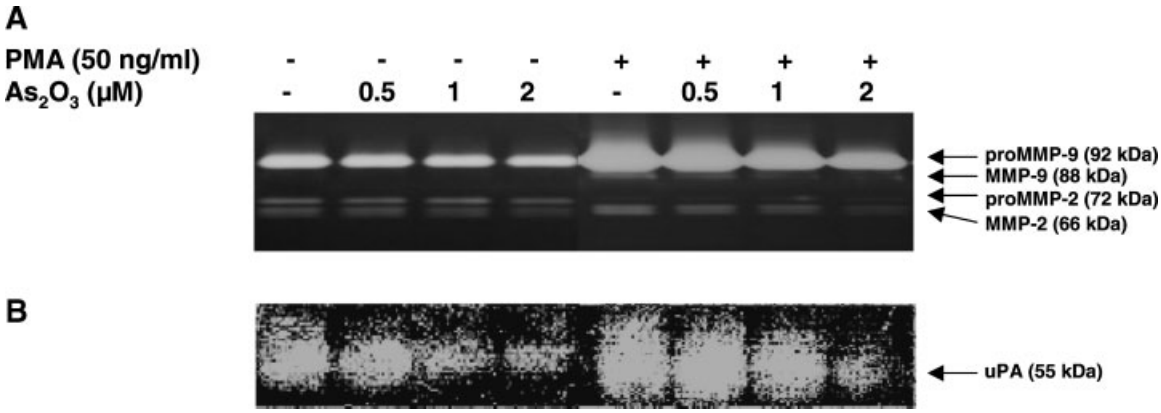

Fig. 4. Effects of As_2O_3 on the secretion of MMP-2, -9, and uPA in HT1080 cells. Cells were incubated in serum-free medium containing various concentrations of As_2O_3 and/or PMA (50 ng/ml) for 18 h. Conditioned medium was collected and subjected to gelatin (**A**) (for MMP-2 and -9) or casein (**B**) (for uPA) zymography for the detection of secreted proteases, as described in Materials and Methods.

It was found that As_2O_3 significantly reduced the mRNA levels of MMP-2, -9, uPA, MT1-MMP, and TIMP-2, but it increased TIMP-1 and uPAI-1 levels in a dose dependent manner (Fig. 5B). These results are in good agreement with the results from Western blot and zymographic analysis.

Since As_2O_3 significantly decreased the MMP-2, -9, and uPA mRNA levels, As_2O_3 was further tested to determine if it affects the promoter activities of these genes, using the luciferase assay system. Similar to the Northern blot analysis, As_2O_3 markedly dose-dependently reduced the activities of the promoters

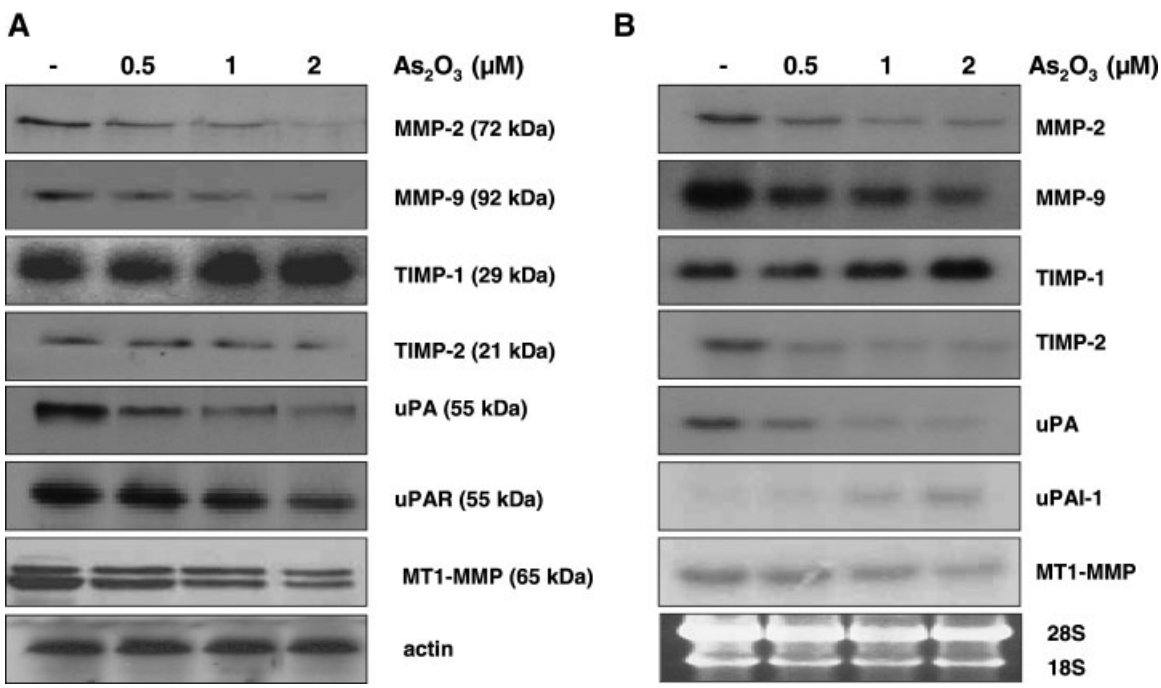

Fig. 5. Effects of As_2O_3 on the expression of various proteases and their inhibitors in HT1080 cells. **A**: Western blot analysis was performed with concentrated culture media (MMP-2, -9, TIMP-1, -2, and uPA) and cell lysates (MT1-MMP, uPAR, and actin) of the cells treated with indicated concentrations of As_2O_3 for 24 h. **B**: Northern blot analysis of the cells treated with indicated concentrations of As_2O_3 for 24 h. Detailed experimental procedures are described under Materials and Methods. kDa, M_r in thousands.

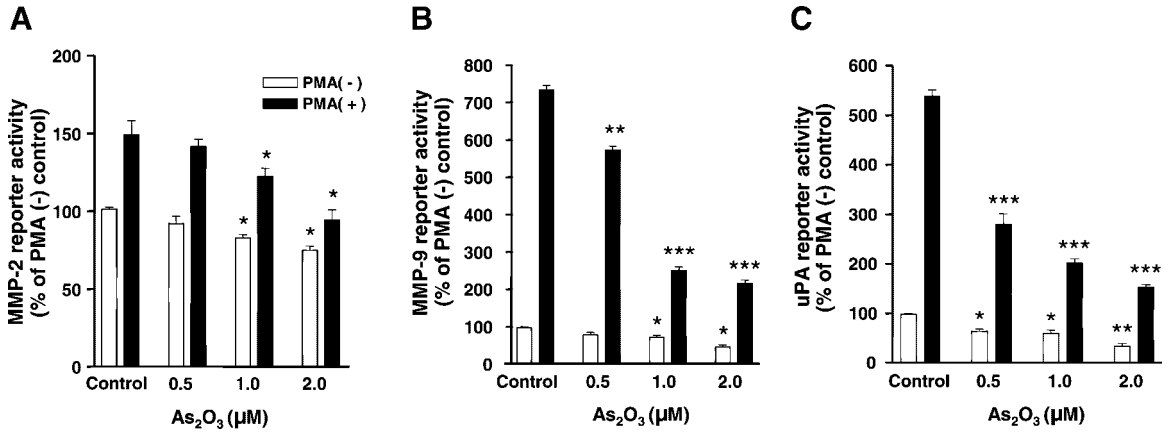

Fig. 6. Effects of As_2O_3 on the promoter activities of MMP-2, -9, and uPA in HT1080 cells. Cells were transfected with reporter vectors containing MMP-2 (**A**), -9 (**B**), and uPA (**C**) promoter regions for 24 h and treated with or without indicated concentrations of As_2O_3 in the presence (black bar) or absence (white bar) of PMA for 18 h. The cells were then lysed, and the extract was analyzed for luciferase activity. Data are presented as percentage of control and given as means ± SD of the results from three separate experiments. *, $0.01 < P < 0.05$; **, $0.005 < P < 0.01$; ***, $0.001 < P < 0.005$.

tested, regardless of the PMA treatment (Fig. 6A–C). However, As_2O_3 suppressed more potently the MMP-9 and uPA promoter activities than the MMP-2 promoter activity. These results suggest that As_2O_3 regulates the expressions of ECM degrading proteases and their inhibitors at the transcriptional level.

NF-κB Activity Plays a Major Role in the Regulation of Proteases Expression by As_2O_3

The expressions of ECM degrading proteases and their inhibitors are regulated by several transcription factors, such as AP-1 and NF-κB [Westermarck and Kahari, 1999]. In order to examine weather the inhibitory effect of As_2O_3 on MMPs and uPA expression was linked to NF-κB and/or AP-1 activities, NF-κB, and AP-1 responsive reporter vectors were transiently transfected into HT1080 cells, and it was found that As_2O_3 reduced NF-κB, but not AP-1-dependent promoter activity (Fig. 7A,B). Subsequently, EMSA was performed to further confirm these results, and it was found that pre-treatment of the cells with As_2O_3 suppressed the NF-κB binding activity to the oligonucleotides, containing its consensus responsive element. However, the same treatment had no effect on the AP-1 binding activity (Fig. 7C,D). Finally, the cells were treated with the specific NF-κB inhibitors, PPM18 and SN50, to determine if the inhibition of NF-κB binding activity was actually affected during the production of proteases, and it was found that PPM18 and SN50, but not SN50M, which is an inactive form of SN50, significantly inhibited MMP-2, -9, and uPA secretion, revealed by zymographic analysis (Fig. 7E). Overall, these results suggest that NF-κB inhibition may be the major target of the anti-invasive activity of As_2O_3.

Suppression of MMPs and uPA Secretion, DNA Binding Activity of NF-κB and Invasion of HT1080 Cells by As_2O_3 Appears to be Dependent of ROS Formation

Several studies have shown that the generation of ROS regulates As_2O_3-induced apoptosis [Jing et al., 1999; Chun et al., 2002; Woo et al., 2002]. To examine the relationship between ROS production by As_2O_3 and suppression of MMPs and uPA production and invasion of HT1080 cells, we used a ROS-sensitive dye, H_2DCFDA. Treatment of the cells with As_2O_3 increased the H_2DCFDA-derived fluorescence, and this increase of fluorescence was significantly inhibited by *N*-acetyl-L-cysteine (NAC), an anti-oxidant (Fig. 8A). In zymographic analysis, NAC treatment was found to block suppression of MMP-2, -9 and uPA secretion by As_2O_3 (Fig. 8B). In addition, treatment restored the DNA binding activity of NF-κB, which was suppressed by As_2O_3 (Fig. 8C). Finally, As_2O_3 induced suppression of invasion through Matrigel also blocked by NAC treatment (Fig. 8D). All these data indicate that ROS generated by As_2O_3 regulates invasiveness of HT1080 cells

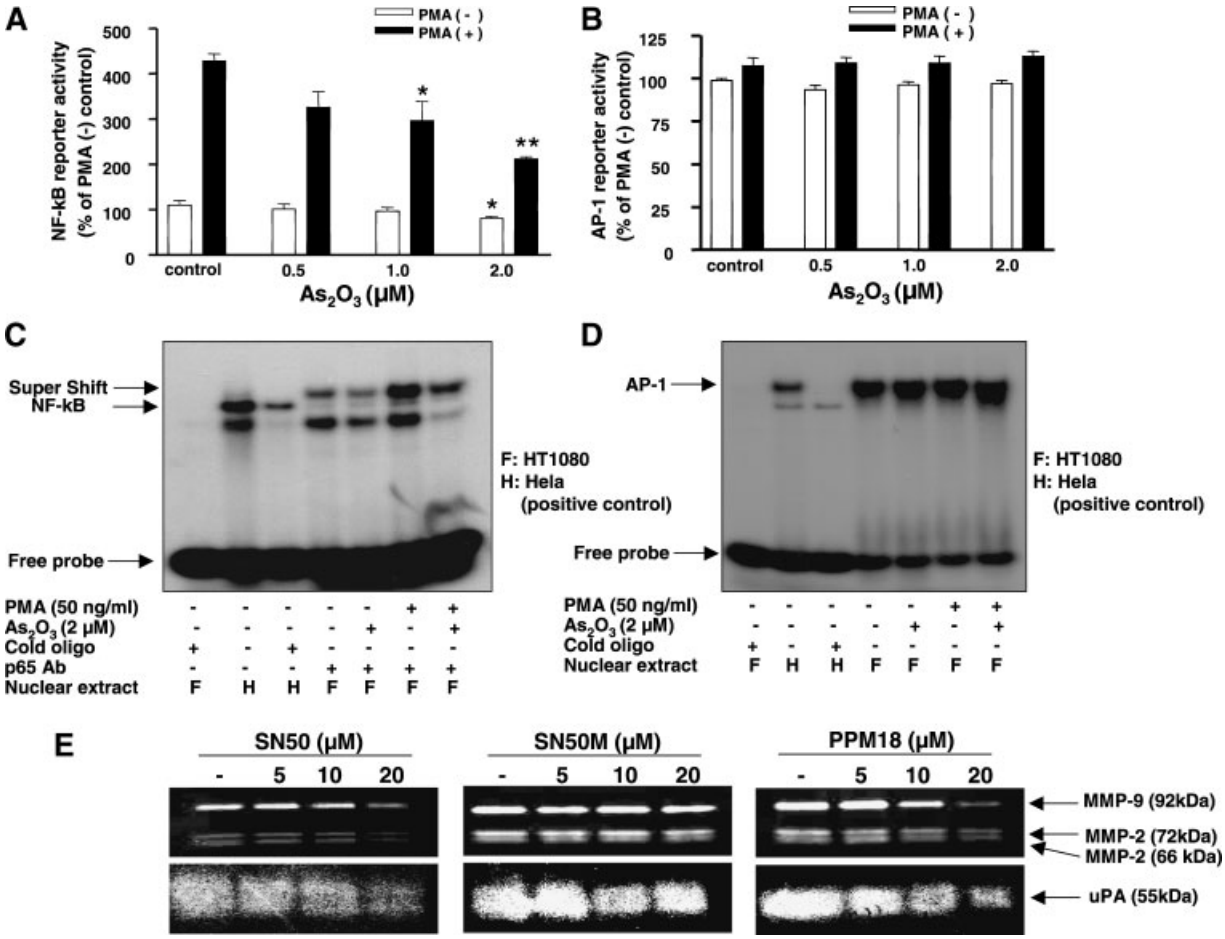

Fig. 7. Effects of As_2O_3 on the promoter and DNA binding activities of NF-κB and AP-1 in HT1080 cells. Cells were transfected with reporter vectors containing NF-κB (**A**) or AP-1 (**B**) responsive elements. After 24 h, cells were treated with various concentrations of As_2O_3 in the presence (black bar) or absence (white bar) of PMA for additional 18 h. The cells were then lysed and analyzed for luciferase activity. Data are presented as percentages of control and given as means ± SD of three independent experiments. *, $0.01 < P < 0.05$; **, $0.005 < P < 0.01$. For EMSA, cells were pretreated with As_2O_3 (2 μM) for 24 h and then stimulated with or without PMA (50 ng/ml) for further 4 h. The nuclear extracts were subjected to gel shift assay with NF-κB (**C**) and AP-1 (**D**) consensus oligonucleotides as described in Materials and Methods. Also, for supershift and specificity analysis of NF-κB activation, nuclear extracts were incubated for 30 min with p65 antibody, and then analyzed for NF-κB binding activity by EMSA. Nuclear extracts of Hela cells were used as a positive control for DNA binding activity of NF-κB and AP-1. **E**: Gelatin (**upper**) and casein (**lower**) zymographic analysis of conditioned medium of HT1080 cells treated with SN50 (cell-permeable inhibitor peptide of NF-κB), SN50M (inactive control peptide), and PPM18 (NF-κB inhibitor) at the indicated concentrations. A representative result from three independent experiments with highly similar results is shown.

through regulation of DNA binding activity of NF-κB.

As_2O_3 Inhibits MMPs Secretion and Invasion in Various Tumor Cell Lines

Similar experiments were performed, using MDA-MB231 (human breast adenocarcinoma cells), HS683 (human glioblastoma cells), and NCI-H460 (human lung carcinoma cells) cell lines in order to determine if the anti-invasive activity of As_2O_3 can generally be applicable to other types of tumor. As seen in Figure 9A, gelatin zymography and Matrigel invasion analysis clearly showed that As_2O_3 inhibited the secretion of MMP-2 (HS683 and NCI-H460) or MMP-9 (MDA-MB231) in a dose dependent manner, and was clearly inhibited the invasion of these cancer cells, thus indicating that As_2O_3 might have anti-invasive properties against a broad spectrum of cancer cells.

DISCUSSION

Invasion of tumor cells through the ECM and BM barriers is a crucial step in tumor dissemination and metastasis [Birkedal-Hansen, 1995; Johnsen et al., 1998]. The present study

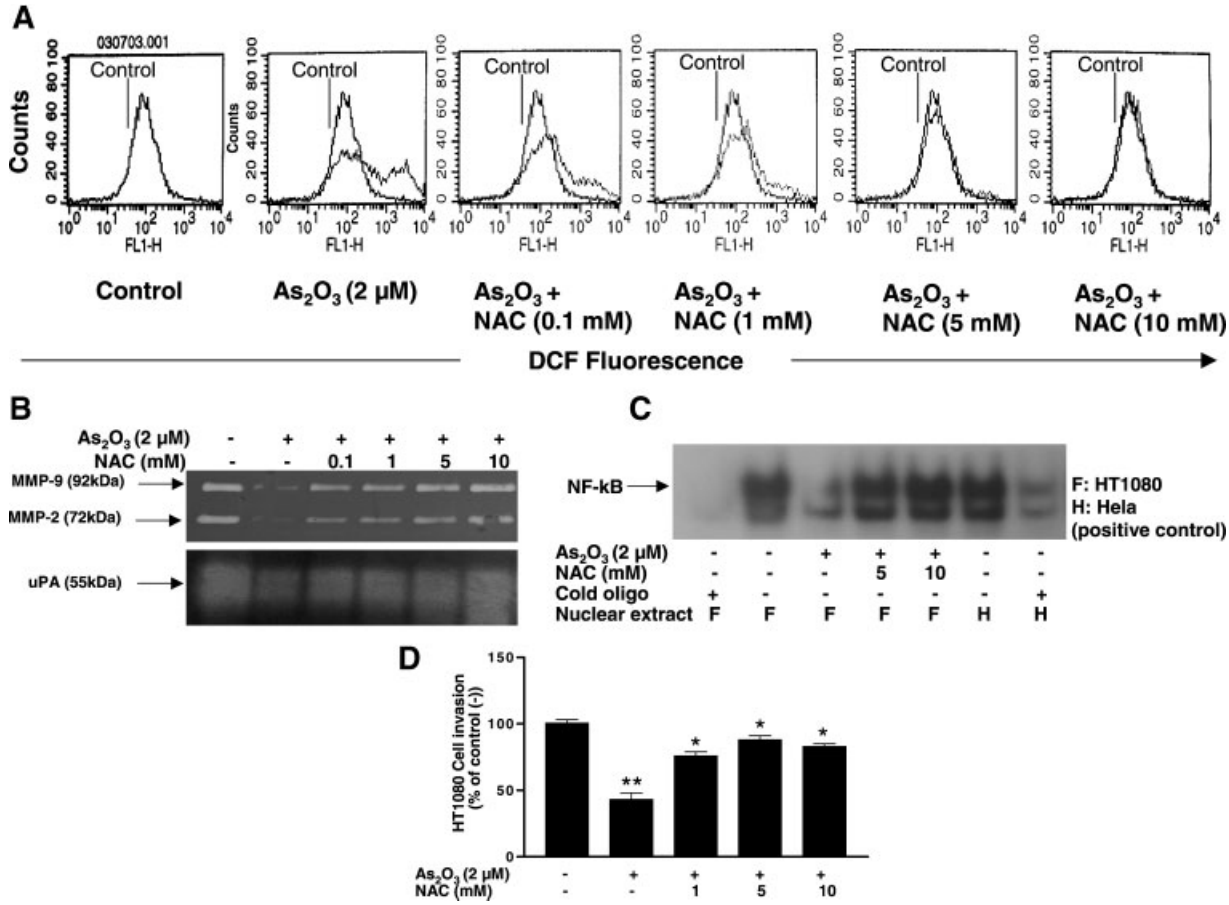

Fig. 8. Role of ROS generated by As_2O_3 on the invasiveness of HT1080 cells. **A**: HT1080 cells were loaded with H_2DCFDA and stimulated with As_2O_3 (2 μM) in the presence or absence of NAC at the indicated concentrations. After 24 h, fluorescence was measured, using flow cytometry. **B**: Gelatin (**upper**) and casein (**lower**) zymographic analysis of conditioned medium of HT1080 cells treated with As_2O_3 in the presence or absence of NAC at the indicated concentrations. **C**: EMSA assay of the cells treated with As_2O_3 (2 μM) for 24 h in the presence or absence of NAC at the indicated concentrations. The nuclear extracts were subjected to gel shift assay with NF-κB consensus oligonucleotides, as described in Materials and Methods. Nuclear extracts of Hela cells were used as a positive control for DNA binding activity of NF-κB and AP-1. **D**: The effects of As_2O_3 and NAC on in vitro invasion (**lower**) through Matrigel on Transwell filters were analyzed as described before. Cells not exposed to As_2O_3 or NAC were used as a control. A representative result from three independent experiments with highly similar results is shown. Data are presented as percentages of control and given as means ± SD of three independent experiments. *, $0.01 < P < 0.05$; **, $0.005 < P < 0.01$.

demonstrated that As_2O_3 suppressed the migration and invasion of HT1080 cells by modulating the production of ECM degrading proteases and their inhibitor proteins. In addition, As_2O_3 inhibited the DNA binding activity of NF-κB, but not AP-1, which might be the major pathway to regulate the proteases secretion and invasion of the cells. Moreover, ROS generated by As_2O_3 appeared to play crucial role in the anti-invasive action of the agent. Finally, this study showed that As_2O_3 could inhibit MMPs secretion and invasion in various tumor cell lines, suggesting that the inhibition of tumor cell invasion by As_2O_3 is a general phenomenon.

Cell migration plays a key role in a wide variety of normal and pathological phenomena, such as inflammatory immune responses, wound healing, angiogenesis, and cancer cell invasion and metastasis [Lauffenburger and Horwitz, 1996]. Cellular movement requires reorganization of actin cytoskeleton, and distinct patterns of actin reorganization are needed, as cells establish leading edge and then generate contractile force to migrate forward [Lauffenburger and Horwitz, 1996]. Our results showed that As_2O_3 potently inhibited the PMA-stimulated migration of HT1080 cells. The inhibitory action of As_2O_3 on tumor cell migration might occur through the inhibition of either

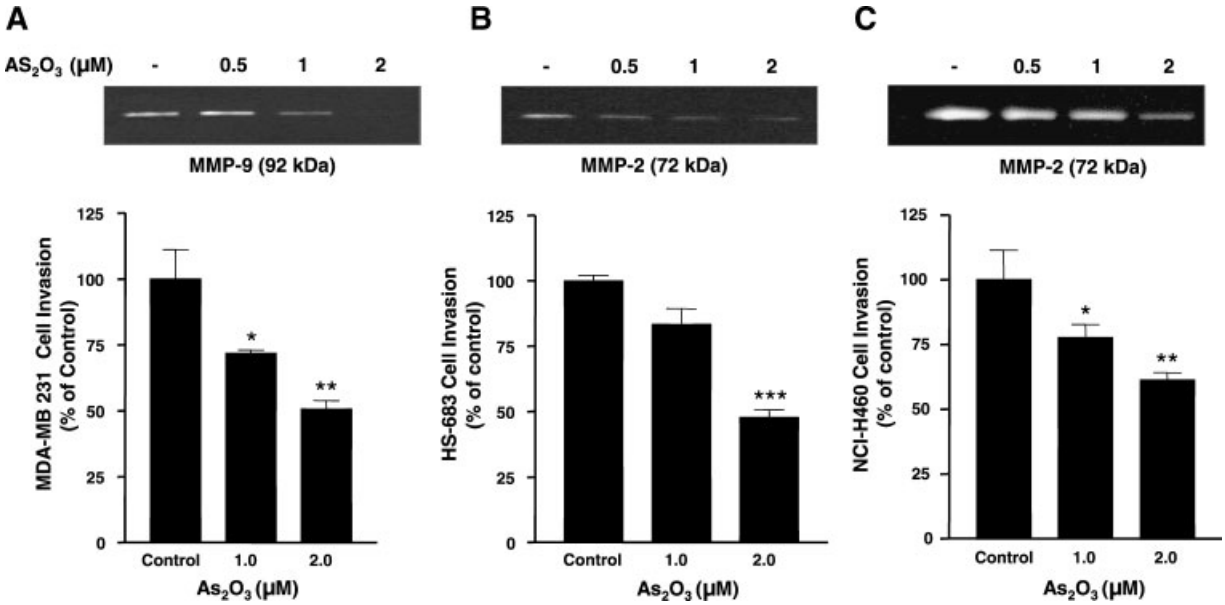

Fig. 9. Effects of As_2O_3 on MMPs secretion and in vitro invasion in various cancer cells. MDA-MB 231 (human breast adenocarcinma cells) (**A**), HS-683 (human glioblastoma) (**B**), and NCI-H460 (human lung cancer) (**C**) cells. The effects of As_2O_3 on MMPs secretion were analyzed by gelatin zymography (**upper**). The effects of As_2O_3 on in vitro invasion (**lower**) through Matrigel on Transwell filters were analyzed as described before. Data are means ± SD from three independent experiments, each carried out in triplicate. *, $0.01 < P < 0.05$; **, $0.005 < P < 0.01$.

the cellular motility machinery or a signal transduction cascade necessary for effective cell migration. It has been reported that As_2O_3 markedly inhibits GTP-induced polymerization and microtubule formation in vitro in myeloid leukemia cells [Li and Broome, 1999]. In addition, there are reports to suggest that microtubule-affecting agents, such as taxol, vincristine, and vindesine at non-cytotoxic doses can significantly reduce the level of tumor cell migration [Hayot et al., 2002]. Overall, these studies indicate that the anti-migratory effect of As_2O_3 may occur through a similar pathway; via modifications of actin cytoskeleton organization.

In many cases, the spreading of tumor cells from the primary site to a distant organ has been characterized as an active process involving the loss of cell–cell adhesion and gain of cell–matrix interaction. Cell–cell adhesion is mediated for the most part by the E-cadherin/catenin complex [Takeichi, 1993; Hsu et al., 2000], and expression of E-cadherin, the most abundant adhesion molecule in adherent junctions of epithelium, is down-regulated in most epithelial cancers [Hirohashi, 1998]. In most metastasizing tumors, cellular interactions with the ECM, which promotes adhesion and migration, are believed to be essential for primary tumor invasion, migration, and metastasis [Werb, 1997]. Our present study showed that As_2O_3 significantly inhibited the attachment of HT1080 cells to type I and IV collagen, and increased the intercelluar interaction in a dose dependent manner. These results suggest that down- and up-regulation of cell–matrix and cell–cell interaction, respectively, by As_2O_3 may be a crucial event in the As_2O_3-induced suppression of invasion and migration of HT1080 cells.

The secretion of extracellular proteases plays an important role in cancer cell migration and invasion [Mignatti and Rifkin, 1993; MacDougall and Matrisian, 1995; Johnsen et al., 1998; Kahari and Saarialho-Kere, 1999]. Of these proteases, the MMPs, a family of zinc-dependent ECM-degrading enzymes, are involved in tumor invasion, metastasis and angiogenesis in cancer. There have been reports to suggest that the expression of MMPs correlates with the progression of various types of tumors [Mignatti and Rifkin, 1993; MacDougall and Matrisian, 1995; Basset et al., 1997; Kahari and Saarialho-Kere, 1999]. In addition to MMPs, the serine proteases such as uPA can catalyze the formation of plasmin from plasminogen to initiate the

proteolytic cascade that contributes to the degradation of ECM, which is a fundamental step in cancer metastasis [Wang, 2001]. Therefore, uPA is also believed to be a key player in cancer invasion and metastasis along with MMPs [Rabbani, 1998]. This study demonstrated that As_2O_3 inhibits the invasion of HT-1080 cells in a Matrigel model and this inhibitory activity appeared to be dependent on the decrease in MMPs and uPA production in these cells. In addition, As_2O_3 exerts its action by up-regulating TIMP-1, which is endogenous inhibitor of MMP, and the augmentation of TIMP-1 expression results in the inhibition of tumor invasion both in vivo and in vitro by decreasing the overall MMP-9 activity [Kruger et al., 1998]. Interestingly, these results showed that As_2O_3 increased the TIMP-1 expression level, but it decreased the TIMP-2 expression level. Although both TIMP-1 and TIMP-2 are inhibitors of MMP-9 and MMP-2, respectively, the expressions of these inhibitors are differentially regulated in vivo as well as in vitro [Stetler-Stevenson et al., 1990; Nii et al., 2000]. Therefore, these observations suggest that the anti-invasive action of As_2O_3 may be attributable not only to down-regulation of MMP-2, -9, and uPA levels, but also to up-regulation of TIMP-1 level.

In many cases, MMPs, TIMPs, and uPA expressions are independently regulated by the transcriptional activation of their genes, since the promoter sequences contain the putative binding sites for the transcription factors such as, NF-κB, AP-1, and PEA3 [Jing et al., 1999]. There are reports to suggest that the promoter activity of *MMP-9*, *uPA*, and *MT1-MMP* genes can be regulated by AP-1 and/or NF-κB [Guerrini et al., 1996; Gum et al., 1996; Yokoo and Kitamura, 1996; Jing et al., 1999; Han et al., 2001; Yoon et al., 2002]. Furthermore, it is well known that both NF-κB and AP-1 transcription factors are associated with inflammation, cell adhesion, cell invasion, metastasis, and angiogenesis [Jing et al., 1999; De Martin et al., 2000; Baldwin, 2001], and that suppression of any of these transcription factors is potentially an effective means to block tumor migration and invasion [Murono et al., 2000; Huang et al., 2001; Sato et al., 2002]. It has been reported that arsenite inhibits IκB kinase, which is essential for NF-κB activation, and As_2O_3 prevents the tumor necrosis factor α-induced NF-κB activation [Miller et al., 2002]. In our studies, As_2O_3 was found to significantly suppress the luciferase activity of the reporter constructs containing NF-κB, MMP-2, -9, and uPA promoters in HT1080 cells, regardless of PMA treatment. However, it did not affect AP-1 dependent promoter. In addition, As_2O_3 effectively inhibited the DNA binding activity of NF-κB, but not AP-1, and treatment of the cells with selective NF-κB inhibitors effectively inhibited the secretion of MMP-2, -9, and uPA, as shown by zymographic analysis. Although MMP-2 promoter itself does not contain a NF-κB binding site, several reports show that NF-κB can augment MT1-MMP and MMP-2 activation [Kim and Koh, 2000; Han et al., 2001; Yoon et al., 2002]. Therefore, As_2O_3 suppresses the transcription of *MMP-2*, *-9*, and *uPA* genes by interfering with the NF-κB binding activity, thereby reducing the invasiveness of HT1080 cells.

Recent reports suggest that the generation of ROS is one of the major mediators of apoptosis of tumor cells by As_2O_3 [Jing et al., 1999; Chun et al., 2002; Woo et al., 2002]. In this study, we examined whether the ROS was involved in the suppression of MMPs and uPA secretion and invasion of the HT1080 cells by As_2O_3, and showed that antioxidant NAC prevented the inhibition of MMP-2, -9, and uPA secretion of the cells by As_2O_3, and that NAC treatment blocked the suppression of DNA binding activity of NF-κB by As_2O_3. Finally, suppression of invasion by As_2O_3 was also reverted by NAC treatment. Taken together, our data suggest that the generation of ROS by As_2O_3 is partly at least responsible for the inhibition of invasion by suppression of MMPs and uPA production, followed by down-regulation of NF-κB activity.

In summary, this study showed that As_2O_3 inhibits the invasion of HT1080 human fibrosarcoma cells mainly by suppressing MMP-2, -9, MT1-MMP, and uPA expression, and down-regulation of NF-κB activity, and that As_2O_3 also up-regulated TIMP-1 expression and further down-regulated the protease activity. As_2O_3 induced ROS might ultimately be responsible for these phenomena. Therefore, As_2O_3 is suggested to a possible candidate for controlling tumor invasion and deserves further investigation.

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