Arsenic Trioxide Compound Modulates Multiple Myeloma Phenotypes: Assessment on Cell Line Models

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Abstract

Recent evidences suggest that multiple myeloma phenotypes (MMPs) are involved in the infiltration of multiple myeloma-affected marrow foci. In this study, the effects of arsenic trioxide on the invasive and angiogenic phenotypes of multiple myeloma (MM) cell line were assessed on a dose-response and time-course basis. Multiple myeloma cell line, Karpas 707, was treated with step-wise elevated concentrations of arsenic trioxide compound at 24, 48, and 72 h intervals. Cytotoxicity was assessed with a colorimetric assay. Potential antiinvasive phenotype was analyzed with MMP-2 zymography. To verify directly the anti angiogenic effect, F1 endothelial cell line was also treated with arsenic and the dose-dependent cytotoxicity was assessed with a colorimetric assay. Apoptotic properties of arsenic trioxide compound were investigated using TUNEL assay. The significant dose-dependent inhibitory effects of arsenic trioxide on MMP-2 were seen at given concentrations. Cytotoxicity analysis revealed much higher cell death than untreated cells ($P< 0.01$), both in Karpas 707 and F1 endothelial cell lines. Colectively, this study showed that arsenic trioxide might potentially elicit anti-invasive anti-angiogenesis properties in the treatment of myeloma dissemination process. In addition, the concurrent inhibition of MMPs activity and endothelial cell proliferation could compose the scenario of neoangiogenesis inhibition in the marrow-infiltrated foci.

Keywords: *Multiple myeloma, Karpas 707, MMP-2, Angiogenesis, Apoptosis, Cytotoxicity, Arsenic trioxide*

Introduction

Arsenic is a naturally occurring substance that has been used as a medicinal agent for more than 2 400 years (1). From two past centuries, arsenic was a mainstay in the treatment of leukemia (2-4). Several reports from China about the use of arsenic in hematological malignancies have caused resurgence in the investigation of this medicinal agent in management of these disorders, including multiple myeloma (5). Multiple myeloma (MM) is a B-cell neoplasia due to the proliferation of clonal plasma cells. Remarkable progress has been made recently in the understanding of the pathophysiology of this disease. Myeloma cells derive from B cells rescued in germinal centers by an antigen selection process before homing in the BM. Their survival and/or expansion depend on interactions with the BM environment and involve various cytokines, the most important of which is IL 6 (6).

Multiple myeloma remains an incurable disease, with median survival duration of 4 to 6 yr, even with high-dose chemotherapy, bone marrow transplantation, and intensive supportive care. Moreover, MM is primarily a disease of

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the elderly, many of whom cannot tolerate aggressive chemotherapy. Thus, newer treatments with good safety profiles are needed to improve the quality of responses, prolong time to disease progression, and extend overall survival. The arsenic compounds are considered as one of the new introducing medicinal agents proposed for treatment of this malignancy (7). It has been suggested that As_2O_3 might induce apoptosis selectively in acute promyelocytic leukemia (APL) cells. Recent preliminary reports suggest that the apoptotic effect of As_2O_3 is not specific for APL cells but can also be observed in various cell lines of either myeloid or lymphoid origin such as APL, CML, CLL, ALL, MM and some solid tumors (8-10). Kelly et al. (2000) have considered the potential roles for MMPs in the pathogenesis of MM. They provided evidences suggesting that matrix metalloproteinases (MMPs) were involved in the infiltration of myeloma affected marrows and foci of myeloma cells in the bone marrow were nourished by neoangiogenic blood vessels that infiltrated into the marrow (11). In this study, the potential inhibitory effect of As_2O_3 on the invasive phenotype of KARPAS 707, a typical MM cell line was assessed through a dose-response and time-course fashion. The potential anti-angiogenic effect of As_2O_3 was also analyzed using F1 endothelial cell line as a model.

Materials and Methods

Arsenic trioxide was purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals used were of analytical grade. Serial concentrations of 1, 5, and 10 μmol As_2O_3, dissolved in a total volumes of 100μl full cell culture medium, were freshly prepared for treatments of cells.

The Multiple myeloma cell line (Karpas 707) and F1 endothelial cell line were purchased from National Cell Bank of Tehran, Iran.

The cell lines, either Karpas 707 or F1 endothelial, were seeded at an initial density of 3×10^4 cells/well in 96-well tissue culture plates

in triplicate repeats. Cells were maintained in 100 μl RPMI-1640 medium supplemented with 10% fetal calf serum, penicillin at 100 units/ml, and streptomycin at 100 μg/ml, with 5% CO_2, 37 °C and saturated humidity.

Dose-response and time-course treatments
Overnight cell culture media were changed immediately before treatments. Then, triplicate arsenic trioxide preparations were transferred to cultured Karpas 707 cells. Non-treated cells were used as controls. Cells were cultured for 24 h, 48 h, or 72 h and then subjected to colorimetric cytotoxicity assay. A sample of the each media was also used for gelatinase zymography. To analyze anti-angiogenic potential of As_2O_3, Sub-confluent F1 endothelial cells were treated with arsenic trioxide preparations at concentrations of 1, 5 and 10 μmol, incubated for 24 h, and then subjected to colorimetric cytotoxicity assay.

Colorimetric cytotoxicity assay After each experiment, cells were washed three times with ice-cold phosphate-buffered saline (PBS), followed by 10 min fixation in a 5% formaldehyde solution. Fixed cells were washed three times and stained with 1% crystal violet. Stained cells were washed, lysed and solublised with 33.3% acetic acid solution. The density of developed purple color was read at 580 nm.

Gelatinase zymography This technique was used for determining gelatinase (collagenase type IV or matrix metalloproteinase type 2, MMP-2) activity, in conditioned media according to a modified method previously published by the authors (12). Briefly, triplicate 10 μl aliquots of conditioned media were subjected to electrophoresis in (2mg/ml) gelatin containing 7% polyacrylamide gels, in the presence of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. The gels underwent electrophoresis for 3 h 34 at a constant voltage of 80 volts.

After electrophoresis, the gels were washed and gently shaken in three consecutive 10 min washings of 2.5% Triton X100 solution to re-

move SDS. The gel slabs were then incubated at 37 °C overnight in 0.1 M Tris HCl gelatinase activation buffer (pH 7.4) containing 10 mM $CaCl_2$ and were subsequently stained with 0.5% Coomassie blue. After intensive destaining, proteolysis areas appeared as clear bands against a blue background. A representative zymogram is depicted (Fig. 1). Using a UVI Pro gel documentation system (GDS-8000 System), quantitative evaluation of both surface and intensity of lyses bands, based on grey levels, were compared relative to non-treated control wells and expressed as "Relative Expression" of gelatinolytic activity.

Cell apoptosis assay Overnight cultured Karpas 707 cells were treated with arsenic trioxide at concentration of 1, 5 and 10µmol, for 24, 48 and 72 h and were then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay for detecting DNA fragmentation was performed by flowcytometric analyze as indicated by kit instructions. (APO-BRDU, Roche, CA, USA). The cells' nuclei were stained with Fluorescein and Propidium iodide. Apoptotic and total cells were then counted by flowcytometry instrument (FACS Calibar Becton Dickinson, USA). The results were expressed as percentage of apoptotic cells.

Statistical analyses The differences in cell proliferation and gelatinase activity were compared using the Student's *t* test. *P* <0.05 were considered significant.

Results

The effects of As_2O_3 at different concentrations on proliferation of multiple myeloma cell line, Karpas 707. As_2O_3 inhibited the growth of MM cells in a dose-dependent as well as time-course fashion (Fig. 2).

Dose-response analysis of As_2O_3 effects on MMP-2 activity of multiple myeloma cell line, Karpas 707, is presented in Fig. 3. Significant differences at given doses were observed between treated and untreated groups ($P< 0.01$).

As_2O_3 imposed a dose-dependent anti-proliferative effect on F1 endothelial cell line (Fig. 4). Significant differences at given doses were observed between treated and untreated groups ($P< 0.01$).

Effect of as₂o₃ on apoptosis As depicted in panels A and B in Fig. 5, the rate of apoptosis for arsenic trioxide at concentrations of 1, 5, and 10 µmol was 3.62%, 5.32%, and 6.20%, respectively. These results show that apoptosis occurred significantly higher in As_2O_3-treated cells, as compared to un-treated ones ($P< 0.01$).
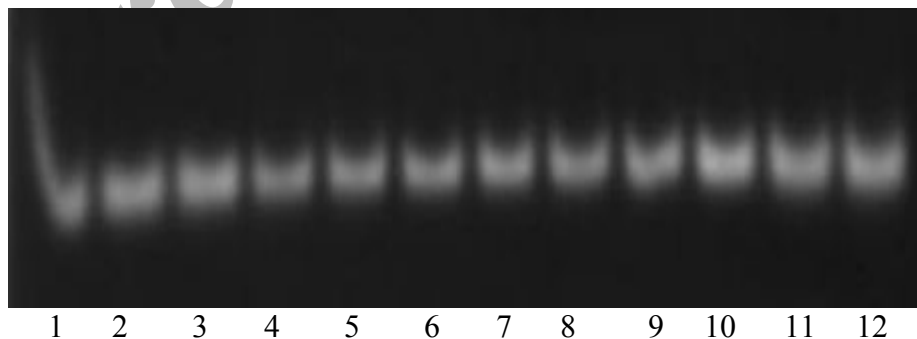
Fig. 1: Representative zymogram gel. Triplicate 10µl supernatant aliquot of cultured Karpas 707 cultured cells, already treated with As_2O_3, was subjected to zymography as described in Materials and Method. Lanes 1-3 untreated cells, lanes 4-6 10µl As_2O_3 treated cells, lanes 7-9 5µl treated cells, and lanes 10-12 1µl treated cells. A gradual decrease in MMP-2 activitity is seen as the concentration of As_2O_3 is increased in the culture media. This zymogram is an example of the many repeated ones and shows only 24 hours As_2O_3 treatments.
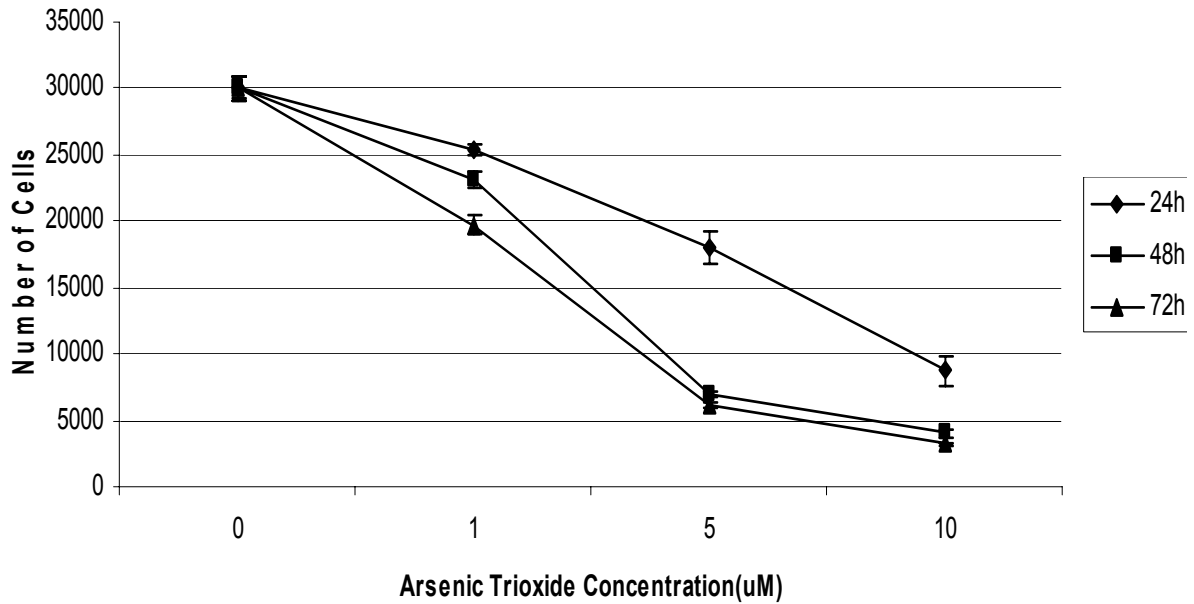
Fig. **2: Cytotoxic effect of As$_2$O$_3$ on multiple myeloma cell line.** (Karpas 707) cells were subjected to As$_2$O$_3$ at different doses (1, 5, and 10 μM) and cytotoxicity was analyzed at 24, 48, and 72 hours, as indicated in Materials and Method.
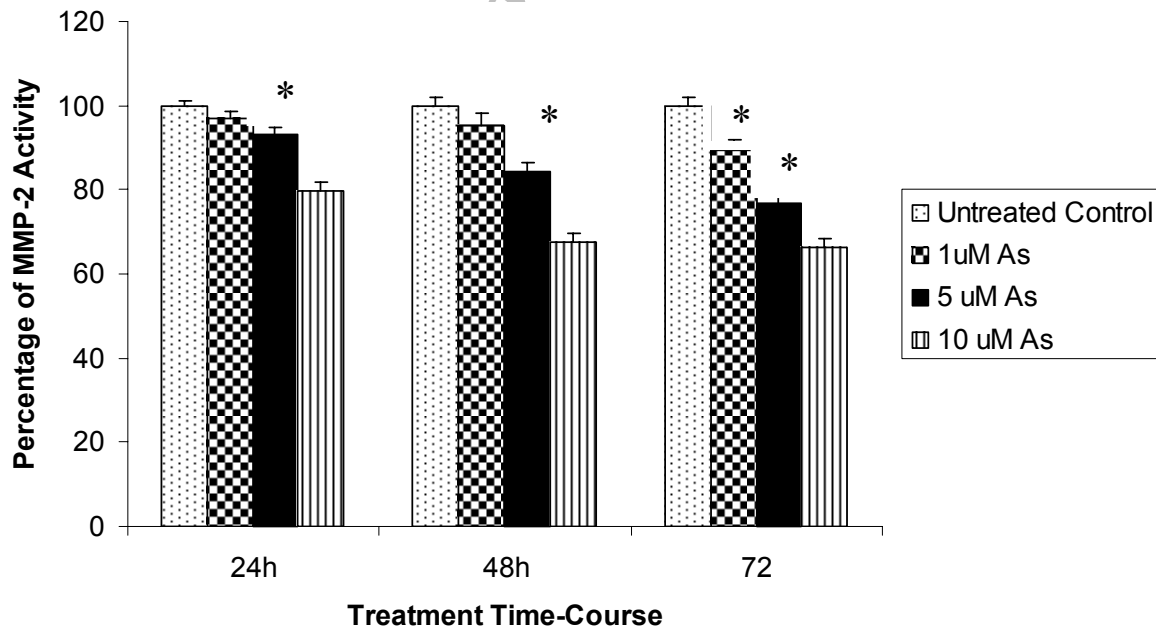

Fig. 3: Dose response and time course analysis of As$_2$ O$_3$ effect on MMP-2 activity.
KARPAS 707 multiple myeloma cells (3x10^{4}cell/well) were incubated for overnight with increasing dose of arsenic as described in Materials and Methods. Arsenic treated and non-treated cells were investigated in triplicate. Asterisks indicate a statistically significant difference.
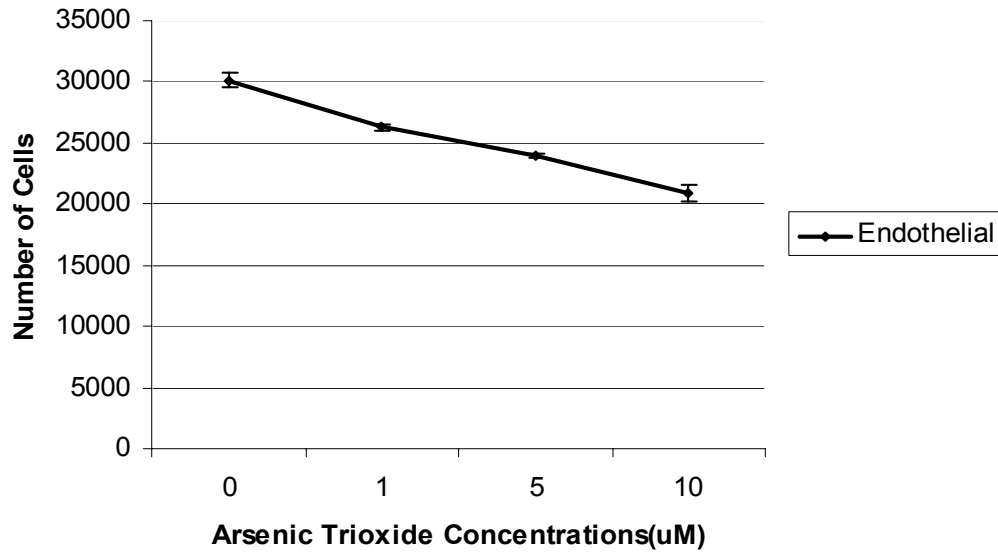
Fig. 4: Cytotoxic effect of As₂O₃ on endothelial cell line. F1 endothelial cells $(3 \times 10^4$ cell/well) were subjected to As₂O₃ at different doses (1, 5, and 10 µM). Arsenic treated and non-treated cells were investigated in triplicate.
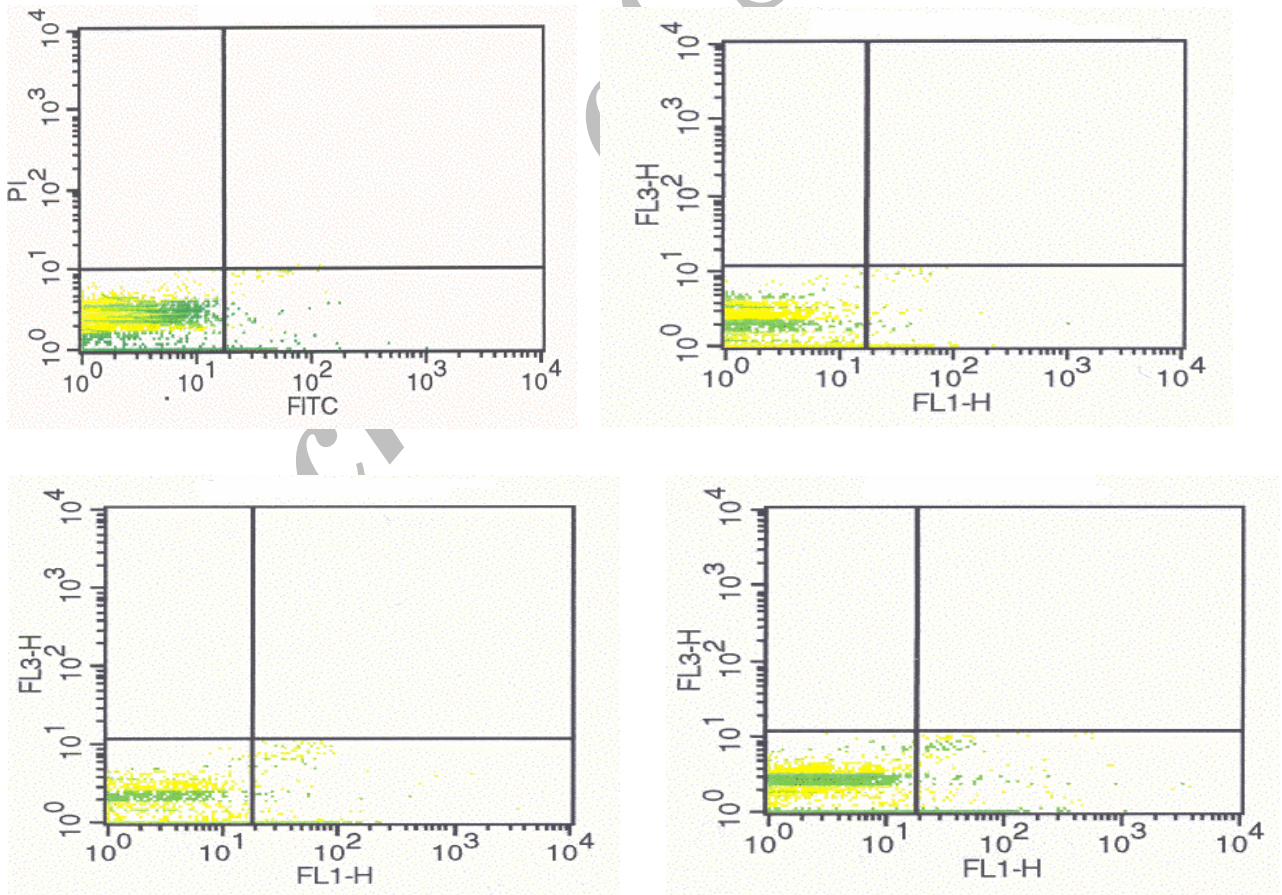
Fig. 5 Panel A: Flowcytometry of Arsenic-induced Apoptosis: Panel A. The TUNEL Assay. X axis represents FITC and Y axis represents Propidium Iodide arbitrary fluorescence units, respectively. Upper left, negative control (untreated cells). Upper right, cells treated with 1µ M As₂O₃. Lower left, cells treated with 5µM As₂O₃. Lower right, cells treated with 10µM As₂O₃.
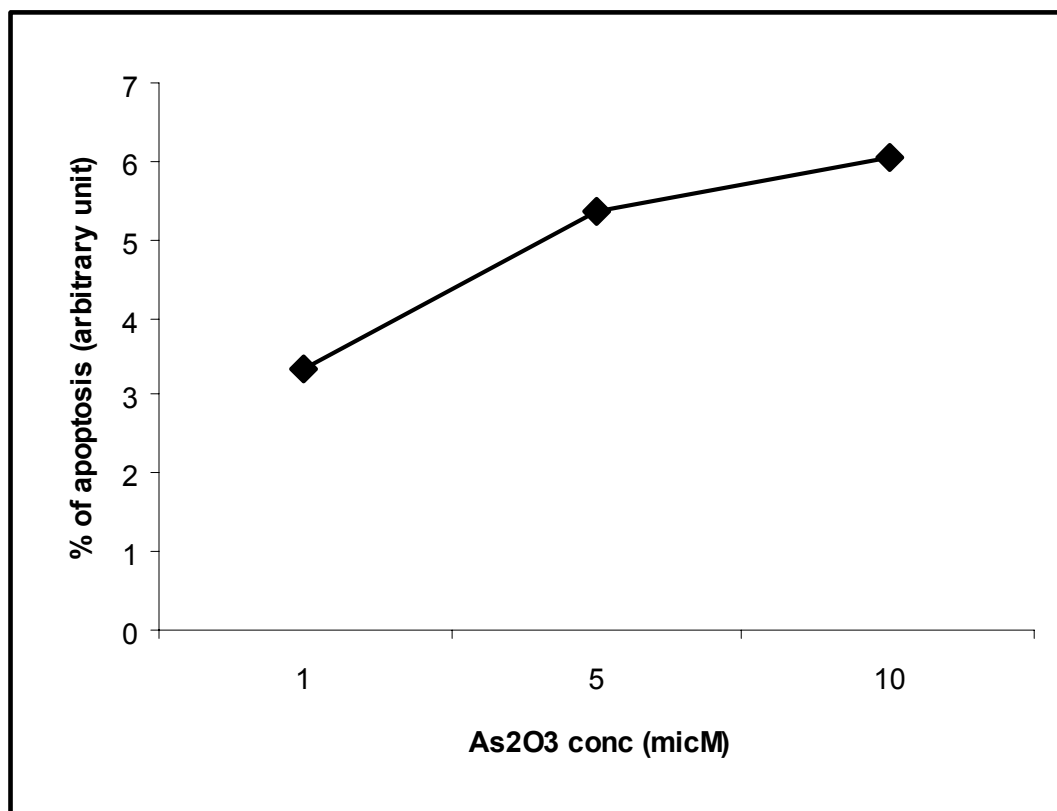
Fig. 5 Panel B: Flowcytometry of Arsenic-induced Apoptosis: Panel B. Apoptosis rate. The rate of apoptosis as compared to positive, negative and non-treated cells for arsenic was 3% and 5.33%, 6.02 % at concentrations of 1, 5, and 10 µM of arsenic-treatments, respectively. The number of apoptotic cells was demonstrated as "percentage of apoptosis" in a given gated area.

Discussion

Inorganic arsenic, a fashionable compound in hematological therapies at the end of the last century (13, 14), was rediscovered by Chinese hematologists in the last two decades (15, 16). Since then, extraordinary results have been obtained using this drug in the therapy of APL. As_2O_3 exerts apoptotic and differentiative effects through various mechanisms in these cells. In the present study, we show that As_2O_3 proceeds directly on MM cells, inhibiting the proliferation of a defined MM cell line, Karpas 707. Cytotoxicity analysis test for arsenic treated cells revealed that this compound was able to exert its cytotoxic effect at employed concentrations (1, 5, and 10µM). Moreover, As_2O_3-induced apoptosis was approximately paralleled with cytotoxic patterns. These results

were, to some extent, similar to previous investigation (17).

In addition, MMP activity was also assessed in this study. Destruction of extracellular matrix, a characteristic indication of tumor invasion, metastasis, and angiogenesis, is catalyzed mainly by the MMPs (18-21). As observed in transformed cells derived from invasive and metastatic solid tumors, the MM cells express and secrete high levels of MMP-2. Our zymogeraphy analysis test of arsenic trioxide showed a significant decrease of MMPs activity in a dose response fashion (Fig. 3). Reduction of expression of MMPs in KARPAS 707 cell line is associated with increasing amount of arsenic trioxide concentrations. Indeed, a concomitant reduction of MMPs activity and increasing level of cytotoxicity and apoptosis could be deduced

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when one looks at the presenting data alto-gether. Hence, MMP activity assessment might be applied as an indication of the cell death process. The cytotoxicity results on F1 endothe-lial cell line showed that arsenic trioxide treatment could hinder angiogenesis process. In line with this speculation, Roboz et al. sug-gested that activated endothelial cells release cytokines that may stimulate cell growth. Based on this observation, he proposed that As_2O_3 might interrupt a reciprocal loop between leu-kemic cells and the endothelium by direct ac-tion on both cell types by causing apoptosis of both cell types and by inhibiting leukemic cell VEGF production (22).

Collectively, the applicability of arsenic com-pound to inhibit MMP activity could be prom-ising to prevent tumor invasion, metastasis, and neovascularization. Further studies might then be prompted for the clinical efficacy of the As_2O_3 in patients with MM, as is the case for As_2O_3 in APL.

Acknowledgements

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