Arsenic Trioxide Inhibits Growth of Human Multiple Myeloma Cells in the Bone Marrow Microenvironment[1]

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Abstract

Multiple myeloma (MM) remains incurable with current therapies, and novel biologically based therapies are urgently needed. Thalidomide and its analogues, as well as proteasome inhibitors, are examples of such novel agents that target both the myeloma cell and its microenvironment and can overcome classical drug resistance. In this study we demonstrate that arsenic trioxide (As_2O_3) mediates anti-MM activity both directly on tumor cells and indirectly by inhibiting production of myeloma growth and survival factors in the bone marrow (BM) microenvironment. Specifically, As_2O_3 at clinically achievable levels (2–5 μM) induces apoptosis even of drug-resistant MM cell lines and patient cells via caspase-9 activation, enhances the MM cell apoptosis induced by dexamethasone, and can overcome the antiapoptotic effects of interleukin 6. As_2O_3 also acts in the BM microenvironment to decrease MM cell binding to BM stromal cells, inhibits interleukin 6 and vascular endothelial growth factor secretion induced by MM cell adhesion, and blocks proliferation of MM cells adherent to BM stromal cells. These studies provide the rationale for clinical trials of As_2O_3, either alone or together with dexamethasone, to overcome classical drug resistance and improve outcome in patients with MM.

Introduction

MM[3] remains an incurable plasma cell malignancy, despite the use of conventional or high-dose chemotherapy with stem cell support (1, 2). However, recent data suggest that drugs that target both the tumor cell and its microenvironment can overcome drug resistance. For example, thalidomide can achieve responses in 30% of patients whose disease is refractory to all other therapies (3) and appears to have multiple mechanisms of action: direct induction of G_1 growth arrest or apoptosis in MM cells (4); inhibition of secretion of IL-6, the major MM growth and survival factor, triggered by tumor cell binding to BMSCs (5); inhibition of VEGF-induced angiogenesis in the BM (6) as well as tumor cell proliferation and migration (7); and stimulation of natural killer cell anti-MM immunity (8). Proteasome inhibitors also directly induce apoptosis even of drug-resistant MM cells and act in the microenvironment to block MM cell binding to BMSCs, IL-6 secretion, and angiogenesis (9). These and other drugs offer great potential to improve outcome in patients with MM.

As_2O_3 is an old drug that achieves remarkable clinical responses in patients with APL (10), both by causing tumor cell differentiation at low concentrations (0.1–0.5 μM) and by inducing apoptosis at relatively high concentrations (0.5–2 μM; Ref. 11). Because the latter effect is not specific to APL (12, 13), preclinical studies and early clinical testing of As_2O_3 is ongoing in other malignancies, including myeloid leukemia (14), chronic B-cell leukemia (15), MM (16), and some solid tumors. In MM cells, recent evidence suggests that As_2O_3 induces apoptosis associated with caspase-3 cleavage (16, 17). Our prior studies have shown that γ-irradiation triggers MM cell apoptosis dependent on mitochondrial cytochrome *c* release and mediated via caspase-8, whereas Fas- and Dex-induced apoptosis occurs independent of cytochrome *c* release (18). Most recently, we showed that Dex-induced MM cell apoptosis is associated with second mitochondria-derived activator of caspases (Smac) release from mitochondria and complex formation of Smac with X-linked inhibitor of apoptosis protein, with resultant caspase-9 activation, downstream caspase-3 cleavage, and apoptosis (19). However, the pathway mediating As_2O_3-induced apoptosis in MM cells is not yet defined.

The BM microenvironment plays a crucial role in the pathogenesis of MM by influencing tumor growth, survival, and drug resistance (20, 21). Adhesion of MM cells to fibronectin confers protection from apoptosis (22), whereas binding of MM cells to BMSCs induces the production of cytokines including IL-6, VEGF, and TNF-α (5, 20, 23). IL-6 is the major known growth and survival factor for MM cells (24, 25), VEGF triggers growth and migration of MM cells (7) and stimulates

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[3] The abbreviations used are: MM, multiple myeloma; IL, interleukin; BM, bone marrow; BMSC, BM stromal cell; VEGF, vascular endothelial growth factor; APL, acute promyelocytic leukemia; TNF, tumor necrosis factor; MAPK, mitogen-activated protein kinase; MC, mononuclear cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; JAK, Janus kinase; STAT, signal transducer and activator of transcription; EMSA, electrophoretic mobility shift assay; NF, nuclear factor; ICAM, intercellular adhesion molecule; DC, dendritic cell; ERK, extracellular signal-regulated kinase; Dex, dexamethasone.

BM angiogenesis (6), and TNF-α alters the adhesion molecule profile of MM cells and BMSCs to increase adhesion of MM cells to BMSCs and triggers IL-6 transcription and secretion in BMSCs (23). The proliferative effect of these cytokines in MM cells is mediated via Ras/Raf/mitogen-activated protein/ERK kinase/MAPK signaling (20, 25), whereas migration induced by VEGF is protein kinase C dependent (7). Importantly, adhesion of MM cells to BMSCs confers protection against drug-induced apoptosis (22), and IL-6 mediates drug resistance against Dex, but not irradiation (26, 27). We and others have begun to demonstrate the molecular mechanisms whereby both adherence to BMSCs and IL-6 protect against apoptosis (28). To date, however, whether As_2O_3 can overcome these MM survival advantages in the BM microenvironment is not known.

In the present study, we demonstrate that As_2O_3, like other novel biologically based agents, acts both directly on MM cells and in the BM microenvironment. As_2O_3 induces apoptosis even of drug-resistant MM cells, inhibits MM cell to BMSC binding, and abrogates IL-6 and VEGF production in the BM milieu. These studies provide the framework for clinical trials of As_2O_3 in MM.

Materials and Methods

Reagents. As_2O_3 (5 mM in PBS) was provided by Cell Therapeutics Inc. (Seattle, WA) and stored at room temperature, and it was diluted in culture media (0.01–100 μM) just before use. Recombinant human IL-6 (Genetics Institute, Cambridge, MA) and TNF-α (R&D Systems, Minneapolis, MN) were reconstituted with sterile PBS containing 0.1% BSA and stored at –20°C.

Cell Lines and Cell Culture. Human MM cell lines RPMI 8226 and U266 were obtained from the American Type Culture Collection (Manassas, VA). Dex-sensitive (MM.1S) and Dex-resistant (MM.1R) human MM cell lines were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). Melphalan-resistant (LR5) and doxorubicin-resistant (Dox_{40}) RPMI 8226 cells were kind gifts from Dr. William Dalton (Moffitt Cancer Center, Tampa, FL). All cell lines were cultured in RPMI 1640 (Mediatech, Herndon, VA) containing 10% fetal bovine serum (Harlan, Indianapolis, IN), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc., Grand Island, NY). Drug-resistant cell lines were cultured with Dex, melphalan, or doxorubicin to confirm their lack of drug sensitivity.

MM Cells and BMSCs from MM Patients. BM specimens were acquired from patients with MM after obtaining informed consent. Primary MM cells (96% CD38 positive and CD45RA negative) were purified from BM specimens, as described previously (29). MCs separated by Ficoll-Paque (Pharmacia, Piscataway, NJ) were used to establish long-term BMSC cultures, as in our prior studies (9). Briefly, BM MCs were cultured in Iscove's modified Dulbecco's medium (Sigma, St. Louis, MO) containing 20% fetal bovine serum, 8 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Growth Inhibition Assay. The inhibitory effect of As_2O_3 on growth of MM cell lines, MM patients' cells, normal lymphocytes, and BMSCs from MM patients was assessed by MTT (Chemicon International, Temecula, CA) assay. In brief, 3×10^4 cells were cultured in 96-well cell culture plates (Corning Inc., Corning, NY) for 48 h, and 10 μl of 5 mg/ml MTT reagent were then added to each well for 4 h. After 100 μl of isopropanol containing 0.04 N HCl was added, absorbance was measured at 570 nm using a spectrophotometer (Molecular Devices Corp., Sunnyvale CA).

Cell Proliferation Assay. Cell proliferation was measured by [^{3}H]thymidine (NEN Life Science Products, Boston, MA) incorporation. MM cells (2×10^4 cells/well) were incubated in 96-well culture plates at 37°C with or without As_2O_3, Dex, and IL-6. [^{3}H]Thymidine (0.5 μCi) was then added to each well for the last 8 h of 48-h cultures. Cells were harvested onto glass filters with an automatic cell harvester (Cambridge Technology, Cambridge, MA) and counted using a Micro-Beta Trilux counter (Wallac, Gaithersburg, MD).

Evaluation of Apoptosis. Dual staining with annexin V-FITC and PI was used to detect apoptosis. After treatment with 2 μM As_2O_3 for 0, 6, 12, 24, and 36 h, 1×10^6 MM cells were washed with PBS and resuspended in 100 μl of HEPES buffer containing annexin V-FITC and PI (annexin V-FLUOS staining kit; Roche Diagnostics, Mannheim, Germany). Cells were analyzed by Coulter Epics XL flow cytometry (Beckman Coulter, Fullerton, CA) for the annexin V-FITC-positive and PI-negative apoptotic cell population. Apoptosis was also confirmed by caspase cleavage, as described below.

Immunoblotting. MM cells were cultured with As_2O_3, harvested, washed, and lysed using lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 2 mM Na_3VO_4, 1 mM NaF, and protease inhibitor mixture (Complete; Roche Diagnostics). Total cell lysates were subjected to SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membranes were immunoblotted with antibodies against caspase-3 (PharMingen, San Diego, CA), caspase-8, cleaved caspase-9 (Asp315; Cell Signaling Technology, Beverly, MA), caspase-9, Bcl-2, Bcl-x_L, Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and α-tubulin (Sigma). Anti-Fas antibody (CH-11; Medical and Biological Laboratories, Nagoya, Japan) was used as a positive control for induction of caspase-8-dependent apoptosis.

To characterize inhibition of constitutive or IL-6-induced growth signaling by As_2O_3, immunoblotting was also done with anti-phosphorylated JAK1, anti-phosphorylated JAK2 (Biosource International, Camarillo, CA), anti-phosphorylated STAT3, anti-phosphorylated ERK (Santa Cruz), or anti-phosphorylated Akt (Cell Signaling Technology) antibodies, as well as antibodies against their nonphosphorylated counterparts. The immunoblots were visualized using the enhanced chemiluminescence detection system (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). In our studies of signaling cascades, we used higher concentrations (25 μM) of As_2O_3 and IL-6 (10–25 ng/ml) than are clinically achievable, as well as short time (1 h) exposures, as in previous reports (30).

Cell Adhesion Assay. Cell adhesion assays were performed as described previously (9), with some modifications. Briefly, 5×10^4 BMSCs were seeded in each well of 96-well plates and cultured for 24 h. After confirming the develop-

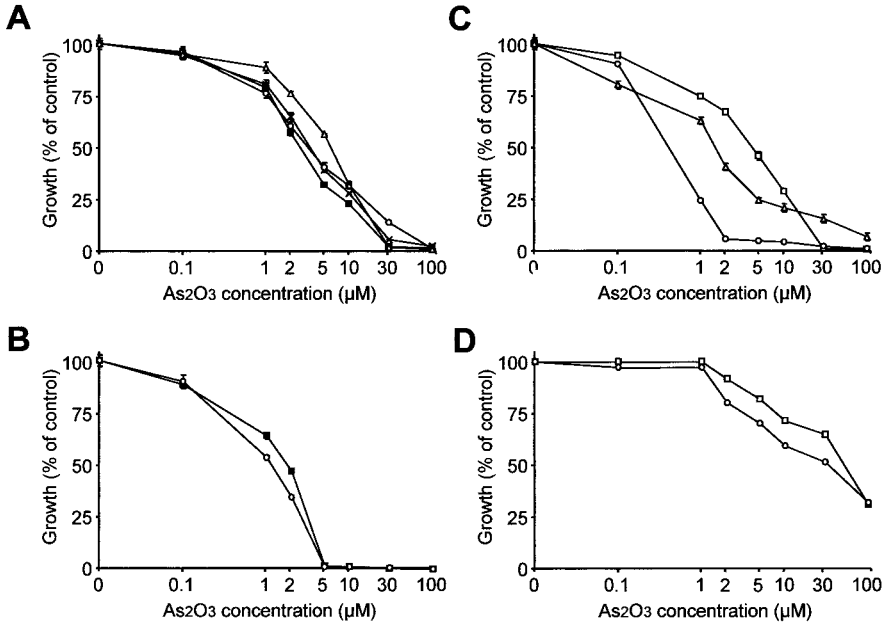

Fig. 1. As_2O_3 inhibits proliferation of MM cell lines and MM patients' cells. Cells were cultured with control media alone or As_2O_3 (0.1–100 μM) for 48 h, and MTT reagent was then added for 4 h. Mean ± SD absorbance relative to control for triplicate cultures is shown. *A,* U266 (x), RPMI 8226 (■), doxorubicin-resistant RPMI 8226/DOX_{40} (○), and melphalan-resistant RPMI 8226/LR5 (△). *B,* Dex-sensitive MM cell line MM.1S (■) and Dex-resistant MM cell line MM.1R (○). *C,* MM cells from patients 1 (□), 2 (○), and 3 (△). *D,* peripheral blood MCs from normal donors 1 (□) and 2 (○).

ment of a confluent adherent monolayer of BMSCs, then As_2O_3 (0, 1, or 10 μM) was added to each well for 12 h, followed by TNF-α (5 ng/ml) for 2 h. After replacing culture media, 5×10^4 MM cells labeled with Na_2CrO_4 (NEN Life Science Products) were added. After incubation for 1 h, each well was washed twice with media, and cells were lysed with 0.5% NP40. Radioactivity in supernatants was counted using a Wizard 3 gamma counter (Wallac).

Measurements of Cytokine Levels. MM cells (5×10^4 cells/well) were cultured with MM patient-derived BMSC monolayers and As_2O_3 (0, 1, and 10 μM) in 96-well plates for 24 h. IL-6 and VEGF levels in culture supernatants were measured by ELISA (DuoSet; R&D Systems).

Flow Cytometric Analysis. Flow cytometric analysis using the Coulter Epics XL (Beckman Coulter) was performed to determine the expression profile of adhesion molecules. BMSCs treated with As_2O_3 (10 μM for 12 h) and TNF-α (5 ng/ml for 4 h) were washed with PBS and incubated with FITC-conjugated anti-CD54 (Beckman Coulter) antibody for 20 min on ice. Cells were then washed, fixed with 1% paraformaldehyde, and evaluated by flow cytometry.

Preparation of Nuclear Extract and EMSA Analysis. BMSCs were preincubated with As_2O_3 (25 μM) at 37°C for 1 h, and TNF-α (5 ng/ml) was then added. After incubation for 0, 10, or 20 min, BMSCs were harvested, and nuclear extracts were prepared by suspension in 1.0 ml of cold buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP40, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin] on ice for 15 min. The lysate was vortexed for 10 s and centrifuged for 10 min at $850 \times g$. The sediment was resuspended in 25 μl of cold buffer B [20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin]. Samples were vigorously rocked for 30 min on ice, followed by centrifugation at 12,000 $\times$ *g* for 15 min at 4°C. Supernatants were stored at −70°C until use.

EMSA was carried out using nuclear extracts, as described previously (23). Double-stranded NF-κB consensus oligonucleotide probe (5′-GGGGACTTTCCC-3′; Santa Cruz Biotechnology) was end-labeled with [γ-^{32}P]ATP (50 μCi at 222 TBq/mM; NEN Life Science Products) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA) in kinase buffer [70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 5 mM DTT] at 37°C for 1 h. Binding reactions containing 1 ng of oligonucleotide and 6 μg of nuclear protein were conducted at room temperature for 20 min in a total volume of 25 μl of binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol, and 0.5 μg of poly(dI-dC) (Pharmacia, Peapack, NJ)]. The samples were loaded on a 4% polyacrylamide gel, transferred to filter paper (Whatman International, Maidstone, United Kingdom), and visualized by autoradiography.

Statistical Analysis. Statistical significance of differences observed in drug-treated *versus* control cultures was determined using the Mann-Whitney U test. The minimal level of significance was $P < 0.05$.

Results

Effects of As_2O_3 on Proliferation of MM Cell Lines and MM Patients' Cells. We first investigated the effects of As_2O_3 at various concentrations on proliferation of MM cell lines. As_2O_3 inhibited the growth of MM cells in a dose-dependent (Fig. 1, *A*–*C*) as well as time-dependent (data not shown) fashion. Fifty percent growth inhibition (IC_{50}) in U266 cells and RPMI 8226 cells at 48 h was observed at 3.2 and 2.4 μM As_2O_3, respectively (Fig. 1*A*). To examine whether there was cross-resistance between As_2O_3 and conventional therapies, we similarly studied drug-resistant MM cell lines.

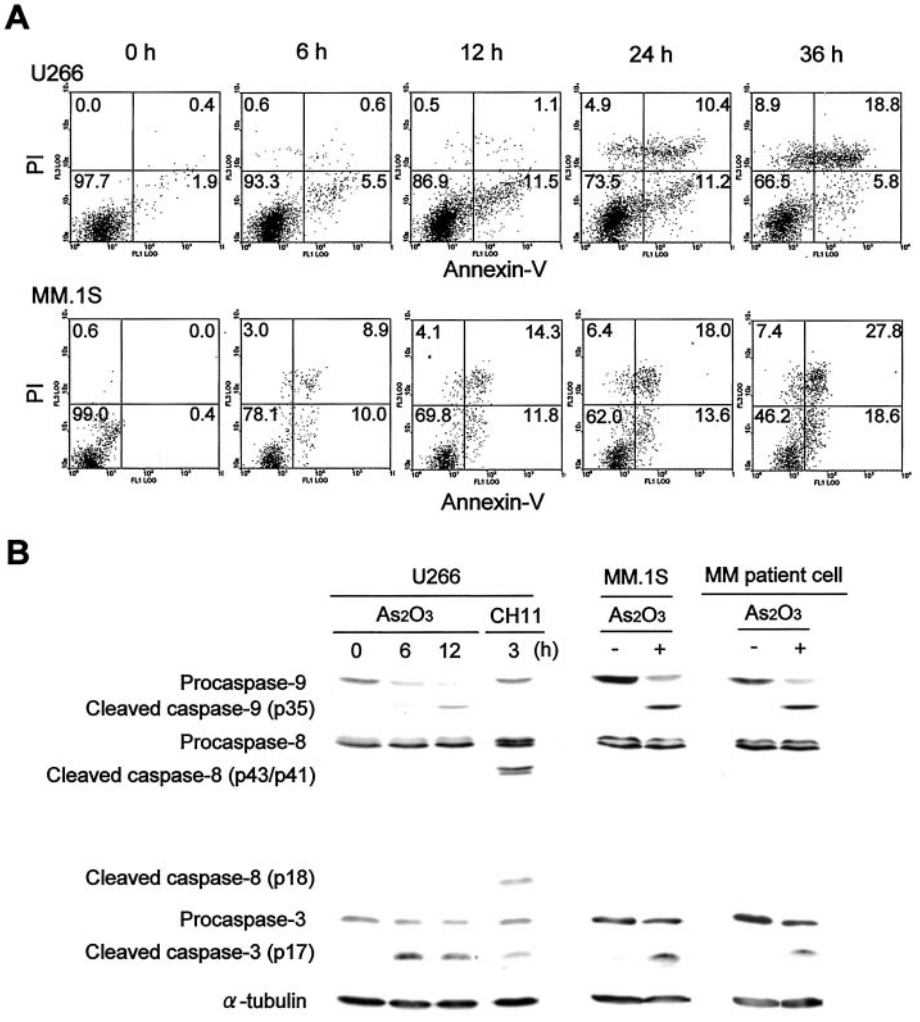

Fig. 2. As_2O_3 induces apoptosis via caspase-9 signaling in MM cell lines and MM patients' cells. *A,* U266 and MM.1S cells were incubated with As_2O_3 (2 μM) for 0–36 h and then stained with annexin V-FITC and PI to determine annexin V-FITC-positive and PI-negative apoptotic fraction. *B,* U266 cells were cultured with As_2O_3 (2 μM) for 0–12 h, and cell lysates were then immunoblotted with anti-procaspase-9, anti-cleaved caspase-9, anti-caspase-8, and anti-caspase-3 antibodies. Treatment with Fas-activating antibody CH-11 (250 ng/ml, for 3 h) was used as a positive control for caspase-8-dependent apoptosis. MM.1S cells were cultured with (+) or without (−) As_2O_3 (2 μM) for 4 h, and then cell lysates were detected in the same way. MM patients' cells were similarly analyzed after incubation with As_2O_3 [0 (−) or 5 (+) μM] for 8 h. The results are representative of three patient tumor samples. Reprobing with anti-α-tubulin antibody confirmed equal protein loading.

Proliferation of RPMI 8226/DOX_{40} was not inhibited by doxorubicin (400 nM), whereas RPMI 8226/LR5 cells could be maintained in the presence of melphalan (5 μM; data not shown). Importantly, these cell lines retained sensitivity to As_2O_3, with an IC_{50} of 3.1 μM for RPMI 8226/Dox_{40} and 5.8 μM for RPMI 8226/LR5 (Fig. 1*A*), within the range of clinically achievable concentrations (5–7 μM) after i.v. administration (10). As_2O_3 also suppressed the proliferation of both Dex-sensitive (MM.1S) and Dex-resistant (MM.1R) MM cell lines with IC_{50} values of 1.8 and 1.1 μM, respectively (Fig. 1*B*). These data demonstrate that As_2O_3 effectively inhibits the growth of drug-resistant MM cells at clinically achievable levels and can overcome resistance to doxorubicin, melphalan, and Dex.

We next investigated the effects of As_2O_3 on patients' MM cells. As_2O_3 inhibited the proliferation of MM cells from three patients in a dose-dependent manner, with an $IC_{50} < 5$ μM (Fig. 1*C*). In contrast, peripheral blood MCs from two normal donors were less sensitive ($IC_{50} > 30$ μM) than MM cell lines and tumor cells from MM patients (Fig. 1*D*). These results demonstrate enhanced sensitivity of MM cells *versus* normal cells to As_2O_3.

As_2O_3 Induces Apoptosis in MM Cells via Caspase-9 Signaling. To investigate the mechanism of cytotoxic effects caused by As_2O_3, we next performed dual staining with annexin V-FITC and PI. MM cells were incubated with As_2O_3 (2 μM), stained with annexin V-FITC and PI, and examined using flow cytometry. As shown in Fig. 2*A,* the annexin V-FITC-positive and PI-negative cell fraction gradually increased, with transition to annexin V/PI double-positive component. All MM cells, including drug-resistant cell lines, showed similar profiles (data not shown). Because this result indicated that As_2O_3 induced apoptosis in MM cells, we next investigated the activation of caspases mediating apoptosis (31, 32). Moreover, in both MM cell lines and MM patients' cells, we observed the cleavage of procaspase-9, but not procaspase-8, followed by cleavage of procaspase-3 (Fig. 2*B*), suggesting that As_2O_3 caused activation of caspase-3 mainly via caspase-9.

As_2O_3 Inhibits Constitutive Activation of STAT3 in MM Cells. We next investigated the effects of As_2O_3 on constitutive activation of STAT3, which mediates MM cell survival (33). As shown in Fig. 3*A,* both STAT3 and ERK are constitutively activated in U266 cells, and As_2O_3 inhibited the ac-

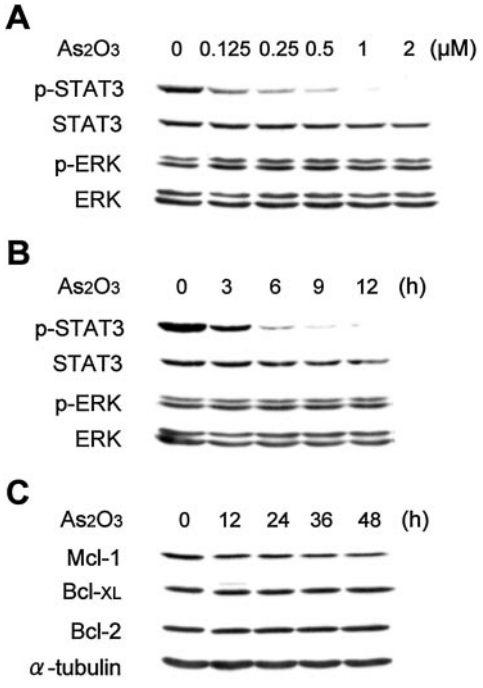

Fig. 3. As_2O_3 inhibits constitutive activation of STAT3 in MM cells. *A,* U266 cells were cultured with As_2O_3 (0, 0.125, 0.25, 0.5, 1, and 2 μM) for 12 h. Total cell lysates were electrophoresed on 7.5% polyacrylamide gel, transferred to nitrocellulose membranes, and then immunoblotted with phosphorylated tyrosine-specific anti-STAT3 (*p-STAT3*) and anti-ERK (*p-ERK*) antibodies. The blot was stripped and reprobed with anti-STAT3 and anti-ERK1 antibodies to confirm equal protein loading. *B,* U266 cells were incubated with As_2O_3 (2 μM) for 0, 3, 6, 9, and 12 h. Cells were then harvested, lysed, electrophoresed, and immunoblotted as described above. *C,* U266 cells were incubated with As_2O_3 (2 μM) for 12, 24, 36, and 48 h. Whole cell lysates were immunoblotted with antibodies specific for Mcl-1, Bcl-x_L, and Bcl-2. Blots were stripped and reprobed with anti-α-tubulin antibody to assure equivalent loading.

tivation of STAT3 in a dose-dependent manner (Fig. 3*A*). This effect was also dependent on the duration of As_2O_3 treatment, with almost complete abrogation of constitutive STAT3 activation at 12 h (Fig. 3*B*). In contrast, activation of ERK was not affected by As_2O_3. This selective inhibition of STAT3 persists for at least 48 h (data not shown).

Because activation of STAT3 mediates up-regulation of Bcl-x_L and Mcl-1 that confer survival in MM cells (33, 34), we next examined the effects of As_2O_3 on members of Bcl-2 family of proteins. As shown in Fig. 3*C,* As_2O_3 decreased the expression level of Mcl-1, with little, if any, effect on Bcl-x_L or Bcl-2.

Dex Enhances the Effect of As_2O_3 on Proliferation of MM Cells. Because Dex is one of the most common therapeutic agents for MM, we next determined whether Dex enhanced the inhibitory effect of As_2O_3 on proliferation of MM cells. Dex-sensitive MM.1S cells were incubated with various concentrations of As_2O_3 and Dex for 48 h. The addition of Dex (0.01–0.1 μM) to As_2O_3 (0.01–1 μM) significantly enhanced its inhibitory effect on the growth of MM cells in a dose-dependent fashion (Fig. 4, $P = 0.007$ at 0.01 M Dex). Although As_2O_3 inhibited proliferation of Dex-resistant MM.1R cells, it did not reverse resistance of MM.1R cells to Dex (data not shown).

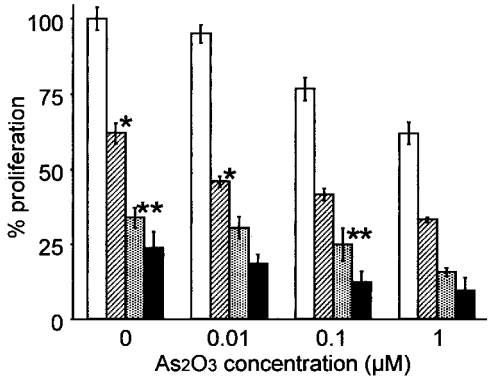

Fig. 4. Dex enhances the inhibitory effect of As_2O_3 on proliferation of MM cells. MM.1S cells were cultured with As_2O_3 (0, 0.01, 0.1, or 1 μM) and Dex [0 (□), 0.01 (▨), 0.05 (▩), or 0.1 (■) μM]. [^{3}H]Thymidine uptake was measured during the last 8 h of 48-h cultures. Values represent the mean ± SD of triplicate cultures. Statistically significant increased inhibition in MM cell proliferation (*, $P = 0.007$; **, $P = 0.03$) was observed with addition of Dex to As_2O_3.

As_2O_3 Inhibits IL-6-induced JAK-STAT Signaling in MM Cells. IL-6 is a major growth factor for MM cells (24) and protects against Dex-induced apoptosis (28, 35); therefore, we next examined the effects of As_2O_3 in the presence of IL-6. Although IL-6 (50 ng/ml) induced proliferation (stimulation index 1.8) in MM.1S cells, it did not rescue MM cells from apoptosis triggered by As_2O_3. Specifically, As_2O_3 overcomes the protective effects of IL-6 in a dose-dependent manner (Fig. 5*A*).

Having shown that As_2O_3 can overcome the effects of IL-6, we next investigated the effect of As_2O_3 on IL-6-induced signaling cascades in MM.1S cells. IL-6 (25 ng/ml) was added to MM.1S cells pretreated with As_2O_3 (25 μM) for 1 h, and total cell lysates were prepared at 0, 10, 30, and 60 min. To define the effects on signaling pathways in viable cells, we used higher concentrations of IL-6 and As_2O_3 and shorter time exposures. Trypan blue dye exclusion confirmed >90% viability of treated cells (data not shown). As shown in Fig. 5*B,* IL-6 triggered phosphorylation of STAT3 in MM.1S cells, and pretreatment of MM.1S cells with As_2O_3 inhibited this effect of IL-6. Because IL-6 activates STAT3 via the gp130 receptor complex and protein tyrosine kinases (25, 36), we next examined whether As_2O_3 pretreatment also inhibited IL-6-induced activation of JAKs. As_2O_3 inhibited IL-6-induced phosphorylation of JAK1 and JAK2 at early time points (1 and 5 min; data not shown), but this inhibitory effect did not persist beyond 10 min (Fig. 5*B*). Although IL-6 also stimulated MAPK and AKT signaling, As_2O_3 did not inhibit the activation of these pathways (Fig. 5*B*). These studies suggest selective effects of As_2O_3 on IL-6-triggered signaling cascades.

As_2O_3 Inhibits MM Cell Growth in the BM Microenvironment. We next examined the effects of As_2O_3 on the BM microenvironment, where MM cells bind to extracellular matrix proteins and BMSCs, thereby enhancing their growth and survival due to both adhesion and resultant cytokine secretion (20, 22, 37, 38). We first examined the cytotoxic effects of As_2O_3 on BMSCs. As shown in Fig. 6*A,* BMSCs

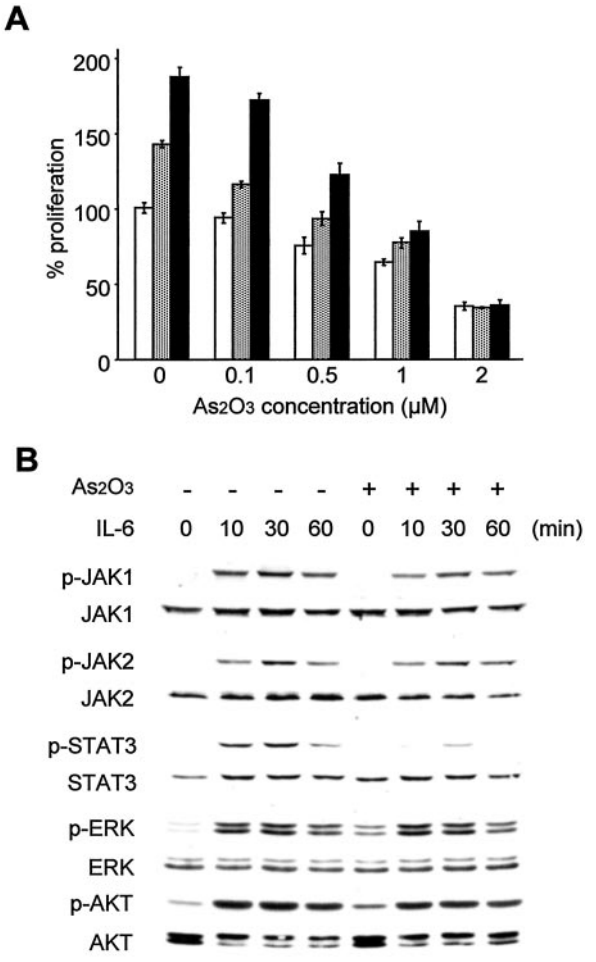

Fig. 5. As_2O_3 inhibits IL-6-induced MM cell growth and STAT3 activation. *A,* MM.1S cells were cultured with 0 (□), 5 (▨), or 50 (■) ng/ml IL-6 and As_2O_3 (0, 0.1, 0.5, 1.0, or 2.0 μM). [^{3}H]Thymidine uptake was measured during the last 8 h of 48-h cultures. Values represent the mean ± SD of triplicate cultures. *B,* after 12 h of serum starvation, MM.1S cells with (+) or without (−) As_2O_3 pretreatment (25 μM, 1 h) were stimulated with IL-6 (25 ng/ml) for 0, 10, 30, and 60 min. Whole cell lysates were immunoblotted with antibodies specific for phosphorylated JAK1 (*p-JAK1*), JAK2 (*p-JAK2*), STAT3 (*p-STAT3*), ERK (*p-ERK*), and AKT (*p-AKT*). Blots were stripped and reprobed with antibodies against their nonphosphorylated proteins.

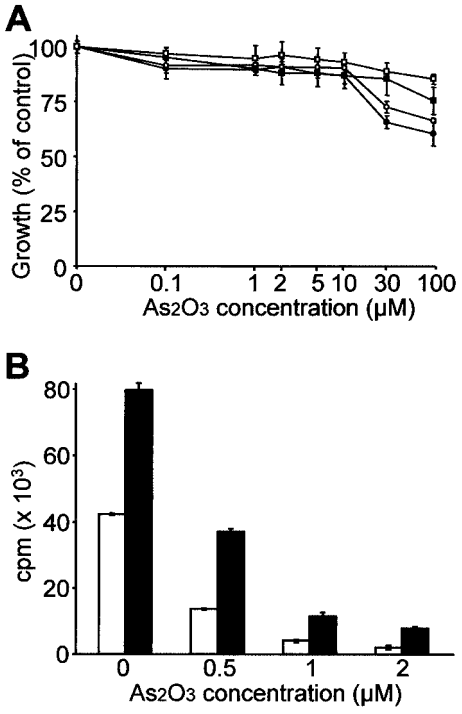

Fig. 6. As_2O_3 inhibits MM cell growth in the BM microenvironment. *A,* growth of BMSCs from two patients with MM cultured with As_2O_3 (0–100 μM) was assessed by MTT assay at 48 h (patient 1, □; patient 2, ○) and 72 h (patient 1, ■; patient 2, ●). Values represent the mean relative absorbance ± SD relative to control of triplicate cultures. *B,* proliferation of MM.1S cells was assessed by [^{3}H]thymidine incorporation during the last 8 h of 48-h cultures with 0, 0.5, 1.0, or 2.0 μM As_2O_3 in the presence (■) or absence (□) of BMSCs. [^{3}H]Thymidine uptake by BMSC alone was less than 500 cpm. Values represent the mean ± SD in triplicate cultures.

from two MM patients were resistant to As_2O_3, assayed by MTT at 48 and 72 h in the presence of high concentrations (100 μM) of As_2O_3. To assess whether As_2O_3 inhibits the growth of MM cells adherent to BMSCs, we measured [^{3}H]thymidine uptake of MM cells adherent to BMSCs in the presence of increasing concentrations (0–2 μM) of As_2O_3. As can be seen in Fig. 6*B,* adherence of MM cells to BMSCs triggered a 1.8-fold increase in MM cell proliferation relative to MM cells alone; importantly, however, this increased proliferation of adherent MM cells was completely inhibited by As_2O_3.

As_2O_3 Inhibits Cytokine Production in the BM Microenvironment. We have reported previously that adhesion of MM cells to BMSCs increases the secretion of IL-6 (38) and VEGF (5, 7) in BMSCs, mediating proliferation and survival of MM cells, as well as angiogenesis in the BM milieu. Binding of MM.1S cells to BMSCs induced significant increases in IL-6 (Fig. 7*A*) and VEGF (Fig. 7*B*) secretion compared with BMSCs alone. The cytokine secretion by MM cells alone was negligible. As_2O_3 (1–10 μM) reduced the secretion of both cytokines in cultures of MM cells adherent to BMSCs.

As_2O_3 Inhibits TNF-α-induced Adhesion of MM Cells to BMSCs. We have reported previously that TNF-α increases the expression of adhesion molecules on both MM cells and BMSCs via activation of NF-κB, resulting in increased MM cell to BMSC binding (23). As shown in Fig. 8*A,* As_2O_3 did not alter constitutive binding of MM cells to BMSCs. However, As_2O_3 blocked the increased binding of MM.1S cells to BMSCs triggered by TNF-α. To delineate the mechanism of this effect of As_2O_3, we first demonstrated that As_2O_3 blocked TNF-α-induced up-regulation of ICAM-1 on BMSCs (Fig. 8*B*). Given that TNF-α-induced ICAM-1 expression is conferred via NF-κB (23), we next examined the effect of As_2O_3 on TNF-α-induced NF-κB nuclear translocation in BMSCs. Specifically BMSCs, with or without As_2O_3 pretreatment, were stimulated with TNF-α (5 ng/ml) for 0, 10, and 20 min, and EMSA was performed with nuclear extracts. As can be seen in Fig. 8*C,* TNF-α-induced nuclear translocation of NF-κB in BMSCs was completely blocked by As_2O_3.

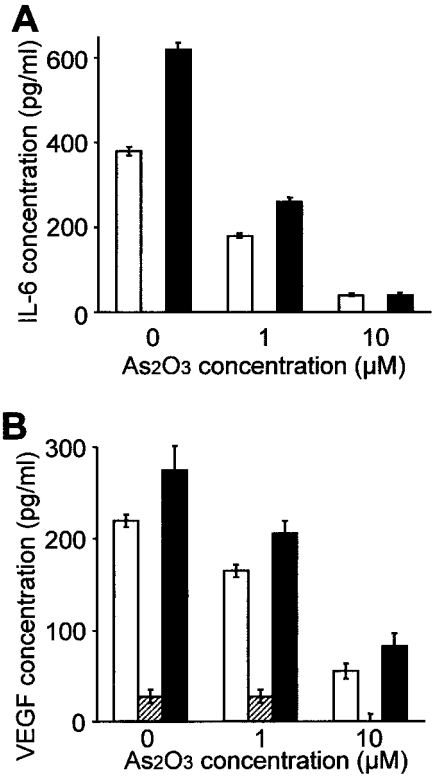

Fig. 7. As_2O_3 inhibits cytokine production in the BM microenvironment. BMSCs (□) and MM.1S cells (▨) were cultured alone or together (■) in the presence or absence of As_2O_3 (0–10 μM). IL-6 (*A*) and VEGF (*B*) in 24-h culture supernatants were measured by ELISA. Values represent the mean ± SD of triplicate cultures.

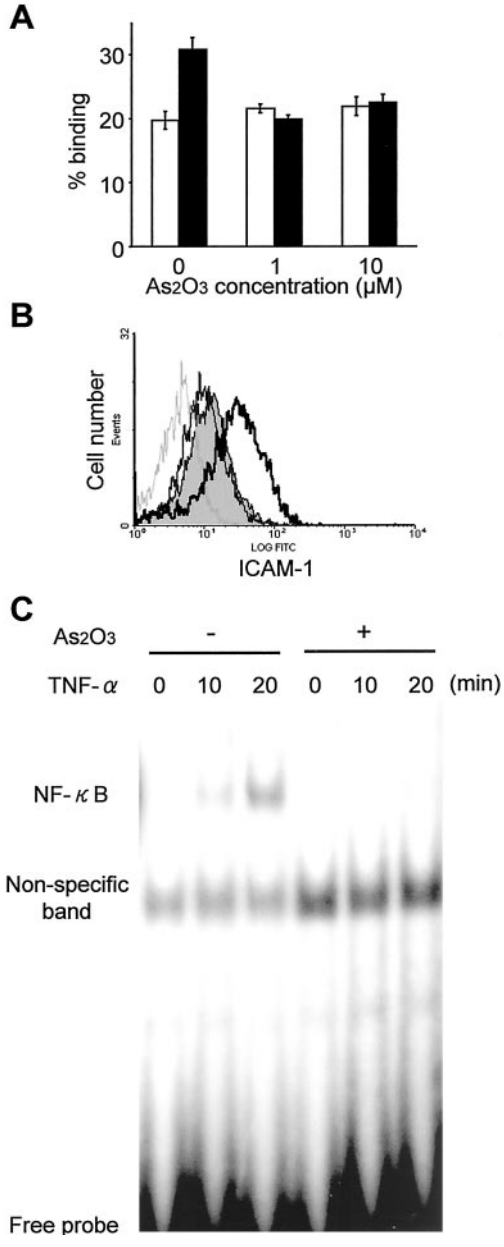

Fig. 8. As_2O_3 inhibits TNF-α-induced adhesion of MM cells to BMSCs. *A,* BMSCs were incubated with As_2O_3 (0, 1, or 10 μM) for 12 h, followed by 0 (□) or 5 (■) ng/ml TNF-α. After replacing culture media, ^{51}Cr-labeled MM.1S cells were added, and the percentage of adherent cells was evaluated at 1 h. *B,* BMSCs were treated with As_2O_3 (10 μM) and TNF-α (5 ng/ml) and then harvested with 0.5 mM EDTA. Expression of ICAM-1 was assessed by flow cytometry: isotype control, *gray line*; nontreated BMSCs, *thin black line*; TNF-α-treated BMSCs, *thick black line*; and BMSCs treated with both TNF-α and As_2O_3, *gray-shaded area*. *C,* nuclear extracts were prepared from BMSCs in the presence or absence of TNF-α (5 ng/ml) for the indicated times, after pretreatment of BMSCs with As_2O_3 (0 or 25 μM) for 1 h. Radiolabeled oligonucleotide specific for NF-κB and 6 μg of nuclear extract were incubated and electrophoresed in 4% polyacrylamide gel.

Discussion

In the present study, we show that As_2O_3 has direct effects not only on MM cells, including drug-resistant MM cells, but also on their growth in the BM microenvironment. As_2O_3 induces apoptosis of MM cells via caspase-9 and overcomes the protective effect of IL-6 in the BM milieu by inhibiting JAK-STAT survival signaling in tumor cells. Furthermore, As_2O_3 reduces TNF-α-induced adhesion to BMSCs and the resultant induced secretion of cytokines (IL-6 and VEGF) that promote MM cell growth, survival, and migration. Importantly, As_2O_3 inhibits the growth even of MM cells adherent to BMSCs at clinically achievable concentrations. These studies provide the rationale for clinical trials of As_2O_3 in MM.

We first demonstrate that As_2O_3 acts directly on MM cells, inhibiting the proliferation of both MM cell lines and patients' MM cells. As_2O_3 induced apoptosis in MM cells, as evidenced by an increase in the annexin V-positive and PI-negative apoptotic cell population. These results confirm and extend previous investigations (16, 17). Furthermore, our study shows that As_2O_3 activates caspase-9, but not caspase-8, followed by cleavage of procaspase-3, in both MM cell lines and MM patients' cells. Two major pathways of activation of caspase-3 are reported, *i.e.*, via caspase-8 and/or via caspase-9 (31, 32). Both have been implicated in As_2O_3-triggered apoptosis: caspase-9 in head and neck cancer (39) and caspase-8 in APL (40). Other reports suggested that As_2O_3-induced apoptosis is associated with accumulation of hydrogen peroxide (41), which is enhanced by ascorbic acid (42) or collapse of mitochondrial transmembrane potential (43), followed by release of cytochrome *c* and

consequent activation of caspase-9. Our recent demonstration that Dex induces caspase-9 activation in MM cells (19), coupled with this report implicating caspase-9 in As_2O_3-associated apoptosis, provides further support for the additive anti-MM activities *in vitro* of Dex and As_2O_3 and the potential clinical utility of combination therapy.

As_2O_3 inhibited the proliferation even of doxorubicin, melphalan, or Dex-resistant MM cell lines. These data suggest that As_2O_3 can overcome conventional drug resistance. As_2O_3 has previously been reported to overcome drug resistance by inducing hyperacetylation of histones H3 and H4 in APL (14) or by inhibiting activation of NF-κB when used with IFN-α in human T-cell lymphotropic virus type I-associated adult T-cell leukemia/lymphoma (44). In MM cells, constitutive STAT3 activation and up-regulation of Bcl-x_L confer resistance to apoptosis and a multidrug-resistant phenotype (33, 45). Moreover, IL-6-induced up-regulation of Mcl-1 is mediated via JAK-STAT signaling, thereby promoting MM cell survival (34, 46). In our study, As_2O_3 reduced IL-6-induced phosphorylation of STAT3 and expression of Mcl-1, but not Bcl-x_L. Mcl-1 is a prosurvival member of the Bcl-2 family (47) that confers resistance to apoptosis induced by several cytotoxic agents *in vitro* (48), and it is elevated at the time of recurrence in acute leukemia (49). In this context, the suppression of Mcl-1 expression in MM cells may contribute, at least in part, to overcoming conventional drug resistance.

IL-6 can completely rescue MM cells from Dex-induced apoptosis (28, 35), but the present study showed no similar protective effect of IL-6 against As_2O_3, as has recently been reported (16). Our previous studies show that IL-6 protects against Dex-induced apoptosis by activation of the SH2 domain-containing protein tyrosine phosphatase (28) and phosphatidylinositol 3-kinase/AKT signaling (50). To delineate the mechanisms whereby As_2O_3 blocks protection conferred by IL-6, we investigated the effect of As_2O_3 on IL-6 signaling pathways in MM cells. Specifically, IL-6 activates Ras/Raf/mitogen-activated protein/ERK kinase/MAPK (24), JAK/STAT (20, 25), and phosphatidylinositol 3-kinase/AKT (50, 51) signaling pathways in MM cells. Our results show that As_2O_3 inhibits the IL-6-induced phosphorylation of STAT3, thereby inhibiting its DNA binding (52, 53), and also blocks early activation of JAK1 and JAK2 in the JAK/STAT pathway, known to mediate cell survival (20, 33). In contrast, our data show little, if any, suppressive effect of As_2O_3 on MAPK and AKT signaling in MM cells induced by IL-6. Our recent studies indicating that IL-6 confers protection against Dex-induced apoptosis via AKT signaling (50), coupled with the present study showing selective inhibition of As_2O_3 on IL-6-induced STAT3 (but not MAPK or AKT) pathways, suggest distinct mechanisms of As_2O_3 *versus* Dex killing in MM cells. These preclinical studies provide the rationale for protocols using combinations of As_2O_3 and Dex to enhance clinical anti-MM activity.

Many novel biologically based drugs, including thalidomide and its immunomodulatory derivatives as well as proteasome inhibitors, act on both MM cells and the BM microenvironment to alter growth and survival signals (4, 9), and our study suggests that As_2O_3 has similar effects. First, adhesion of MM cells to fibronectin and BMSCs both localizes MM cells in BM and promotes tumor growth, survival, and drug resistance (20, 21). Although As_2O_3 does not inhibit the constitutive binding of MM cells to BMSCs, we show that As_2O_3 blocks the increase in MM to BMSC adhesion induced by TNF-α, as we have previously shown using proteasome inhibitor PS-341 (9). This is related, at least in part, to blockade of NF-κB-dependent up-regulation of ICAM-1 expression on BMSCs (23). Because adhesion confers resistance to drug-induced apoptosis (22), this inhibition of tumor cell binding may restore drug sensitivity. Second, our previous studies have shown that adhesion of MM cells to BMSCs triggers NF-κB-dependent up-regulation of IL-6 and VEGF secretion, with related MM cell growth and migration (7, 37, 38). In this study, we showed that As_2O_3, like thalidomide (5) and proteasome inhibitor PS-341 (9), abrogates the production of both cytokines triggered by MM cell to BMSC binding. Third, we show here that proliferation even of adherent MM cells is inhibited by As_2O_3. Fourth, we have recently shown that VEGF mediates migration of MM cells and BM neovascularization (6, 7), suggesting that As_2O_3 may also block these processes. A prior report (54) has shown that As_2O_3 also decreases VEGF production by the leukemic cell line HEL, with related inhibition of capillary tube formation. Fifth, both IL-6 (55) and VEGF (56) inhibit the antigen-presenting function of DCs by blocking the differentiation of monocytes to DCs and by inhibiting DC maturation, respectively. Therefore, As_2O_3 may improve the immune-compromised status in MM by overcoming these effects on DCs. Finally, it has also been reported that As_2O_3, as we have observed with thalidomide (8), augments lymphokine activated killer-mediated cytotoxicity (57), suggesting that As_2O_3 also has the potential to enhance both specific and nonspecific immunological reactivity against MM cells.

Recently, preliminary results of Phase I/II clinical trials of As_2O_3 alone or coupled with ascorbic acid in patients with refractory or recurrent MM have been reported (58, 59). In a study with As_2O_3 alone, 7 of 10 evaluable patients had minor decreases in M-protein or stable disease; grade 3 leukopenia, anemia, abdominal pain and diarrhea, fever, and fatigue were observed (58). Another study coupled As_2O_3 with ascorbic acid and reported that six of six patients achieved minor responses or stabilization of disease, with similar toxicities (59). As shown in our results (Fig. 4), combination with Dex enhances the effects of As_2O_3 *in vitro*; higher response rates and less toxicity are expected using this combination, and a clinical trial is currently on going. Additional large-scale clinical studies are needed to evaluate the clinical efficacy of As_2O_3.

In summary, our data demonstrate multiple direct and indirect effects of As_2O_3 on MM cells *in vitro* and provide the framework for use of As_2O_3, either alone or coupled with Dex, to overcome drug resistance and improve outcome for patients with MM.

References

1. Attal, M., Harousseau, J. L., Stoppa, A. M., Sotto, J. J., Fuzibet, J. G., Rossi, J. F., Casassus, P., Maisonneuve, H., Facon, T., Ifrah, N., Payen, C., and Bataille, R. A prospective, randomized trial of autologous bone

marrow transplantation and chemotherapy in multiple myeloma. Intergroupe Francais du Myelome. N. Engl. J. Med., *335:* 91–97, 1996.

2. Barlogie, B., Jagannath, S., Desikan, K. R., Mattox, S., Vesole, D., Siegel, D., Tricot, G., Munshi, N., Fassas, A., Singhal, S., Mehta, J., Anaissie, E., Dhodapkar, D., Naucke, S., Cromer, J., Sawyer, J., Epstein, J., Spoon, D., Ayers, D., Cheson, B., and Crowley, J. Total therapy with tandem transplants for newly diagnosed multiple myeloma. Blood, *93:* 55–65, 1999.

3. Singhal, S., Mehta, J., Desikan, R., Ayers, D., Roberson, P., Eddlemon, P., Munshi, N., Anaissie, E., Wilson, C., Dhodapkar, M., Zeddis, J., and Barlogie, B. Antitumor activity of thalidomide in refractory multiple myeloma. N. Engl. J. Med., *341:* 1565–1571, 1999.

4. Hideshima, T., Chauhan, D., Shima, Y., Raje, N., Davies, F. E., Tai, Y. T., Treon, S. P., Lin, B., Schlossman, R. L., Richardson, P., Muller, G., Stirling, D. I., and Anderson, K. C. Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy. Blood, *96:* 2943–2950, 2000.

5. Gupta, D., Treon, S. P., Shima, Y., Hideshima, T., Podar, K., Tai, Y. T., Lin, B., Lentzsch, S., Davies, F. E., Chauhan, D., Schlossman, R. L., Richardson, P., Ralph, P., Wu, L., Payvandi, F., Muller, G., Stirling, D. I., and Anderson, K. C. Adherence of multiple myeloma cells to bone marrow stromal cells upregulates vascular endothelial growth factor secretion: therapeutic applications. Leukemia (Baltimore), *15:* 1950–1961, 2001.

6. D'Amato, R. J., Loughnan, M. S., Flynn, E., and Folkman, J. Thalidomide is an inhibitor of angiogenesis. Proc. Natl. Acad. Sci. USA, *91:* 4082–4085, 1994.

7. Podar, K., Tai, Y. T., Davies, F. E., Lentzsch, S., Sattler, M., Hideshima, T., Lin, B. K., Gupta, D., Shima, Y., Chauhan, D., Mitsiades, C., Raje, N., Richardson, P., and Anderson, K. C. Vascular endothelial growth factor triggers signaling cascades mediating multiple myeloma cell growth and migration. Blood, *98:* 428–435, 2001.

8. Davies, F. E., Raje, N., Hideshima, T., Lentzsch, S., Young, G., Tai, Y. T., Lin, B., Podar, K., Gupta, D., Chauhan, D., Treon, S. P., Richardson, P. G., Schlossman, R. L., Morgan, G. J., Muller, G. W., Stirling, D. I., and Anderson, K. C. Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. Blood, *98:* 210–216, 2001.

9. Hideshima, T., Richardson, P., Chauhan, D., Palombella, V. J., Elliott, P. J., Adams, J., and Anderson, K. C. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. Cancer Res., *61:* 3071–3076, 2001.

10. Shen, Z. X., Chen, G. Q., Ni, J. H., Li, X. S., Xiong, S. M., Qiu, Q. Y., Zhu, J., Tang, W., Sun, G. L., Yang, K. Q., Chen, Y., Zhou, L., Fang, Z. W., Wang, Y. T., Ma, J., Zhang, P., Zhang, T. D., Chen, S. J., Chen, Z., and Wang, Z. Y. Use of arsenic trioxide (As_2O_3) in the treatment of acute promyelocytic leukemia (APL). II. Clinical efficacy and pharmacokinetics in relapsed patients. Blood, *89:* 3354–3360, 1997.

11. Chen, G. Q., Shi, X. G., Tang, W., Xiong, S. M., Zhu, J., Cai, X., Han, Z. G., Ni, J. H., Shi, G. Y., Jia, P. M., Liu, M. M., He, K. L., Niu, C., Ma, J., Zhang, P., Zhang, T. D., Paul, P., Naoe, T., Kitamura, K., Miller, W., Waxman, S., Wang, Z. Y., de The, H., Chen, S. J., and Chen, Z. Use of arsenic trioxide (As_2O_3) in the treatment of acute promyelocytic leukemia (APL). I. As_2O_3 exerts dose-dependent dual effects on APL cells. Blood, *89:* 3345–3353, 1997.

12. Wang, Z. G., Rivi, R., Delva, L., Konig, A., Scheinberg, D. A., Gambacorti-Passerini, C., Gabrilove, J. L., Warrell, R. P., Jr., and Pandolfi, P. P. Arsenic trioxide and melarsoprol induce programmed cell death in myeloid leukemia cell lines and function in a PML and PML-RAR independent manner. Blood, *92:* 1497–1504, 1998.

13. Zhu, X. H., Shen, Y. L., Jing, Y. K., Cai, X., Jia, P. M., Huang, Y., Tang, W., Shi, G. Y., Sun, Y. P., Dai, J., Wang, Z. Y., Chen, S. J., Zhang, T. D., Waxman, S., Chen, Z., and Chen, G. Q. Apoptosis and growth inhibition in malignant lymphocytes after treatment with arsenic trioxide at clinically achievable concentrations. J. Natl. Cancer Inst. (Bethesda), *91:* 772–778, 1999.

14. Perkins, C., Kim, C. N., Fang, G., and Bhalla, K. N. Arsenic induces apoptosis of multidrug-resistant human myeloid leukemia cells that express Bcr-Abl or overexpress MDR, MRP, Bcl-2, or Bcl-x_L. Blood, *95:* 1014–1022, 2000.

15. Konig, A., Wrazel, L., Warrell, R. P., Jr., Rivi, R., Pandolfi, P. P., Jakubowski, A., and Gabrilove, J. L. Comparative activity of melarsoprol and arsenic trioxide in chronic B-cell leukemia lines. Blood, *90:* 562–570, 1997.

16. Rousselot, P., Labaume, S., Marolleau, J. P., Larghero, J., Noguera, M. H., Brouet, J. C., and Fermand, J. P. Arsenic trioxide and melarsoprol induce apoptosis in plasma cell lines and in plasma cells from myeloma patients. Cancer Res., *59:* 1041–1048, 1999.

17. Park, W. H., Seol, J. G., Kim, E. S., Hyun, J. M., Jung, C. W., Lee, C. C., Kim, B. K., and Lee, Y. Y. Arsenic trioxide-mediated growth inhibition in MC/CAR myeloma cells via cell cycle arrest in association with induction of cyclin-dependent kinase inhibitor, p21, and apoptosis. Cancer Res., *60:* 3065–3071, 2000.

18. Chauhan, D., Pandey, P., Ogata, A., Teoh, G., Krett, N., Halgren, R., Rosen, S., Kufe, D., Kharbanda, S., and Anderson, K. Cytochrome *c*-dependent and -independent induction of apoptosis in multiple myeloma cells. J. Biol. Chem., *272:* 29995–29997, 1997.

19. Chauhan, D., Hideshima, T., Rosen, S., Reed, J. C., Kharbanda, S., and Anderson, K. C. Apaf-1/cytochrome *c*-independent and Smac-dependent induction of apoptosis in multiple myeloma (MM) cells. J. Biol. Chem., *276:* 24453–24456, 2001.

20. Hallek, M., Bergsagel, P. L., and Anderson, K. C. Multiple myeloma: increasing evidence for a multistep transformation process. Blood, *91:* 3–21, 1998.

21. Tricot, G. New insights into role of microenvironment in multiple myeloma. Lancet, *355:* 248–250, 2000.

22. Damiano, J. S., Cress, A. E., Hazlehurst, L. A., Shtil, A. A., and Dalton, W. S. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. Blood, *93:* 1658–1667, 1999.

23. Hideshima, T., Chauhan, D., Schlossman, R., Richardson, P., and Anderson, K. C. The role of tumor necrosis factor α in the pathophysiology of human multiple myeloma: therapeutic applications. Oncogene, *20:* 4519–4527, 2001.

24. Ogata, A., Chauhan, D., Teoh, G., Treon, S. P., Urashima, M., Schlossman, R. L., and Anderson, K. C. Interleukin-6 triggers cell growth via the Ras-dependent mitogen-activated protein kinase cascade. J. Immunol., *159:* 2212–2221, 1997.

25. Hideshima, T., Chauhan, D., Teoh, G., Raje, N., Treon, S. P., Tai, Y. T., Shima, Y., and Anderson, K. C. Characterization of signaling cascades triggered by human interleukin-6 *versus* Kaposi's sarcoma-associated herpes virus-encoded viral interleukin 6. Clin. Cancer Res., *6:* 1180–1189, 2000.

26. Hardin, J., MacLeod, S., Grigorieva, I., Chang, R., Barlogie, B., Xiao, H., and Epstein, J. Interleukin-6 prevents dexamethasone-induced myeloma cell death. Blood, *84:* 3063–3070, 1994.

27. Lichtenstein, A., Tu, Y., Fady, C., Vescio, R., and Berenson, J. Interleukin-6 inhibits apoptosis of malignant plasma cells. Cell. Immunol., *162:* 248–255, 1995.

28. Chauhan, D., Pandey, P., Hideshima, T., Treon, S., Raje, N., Davies, F. E., Shima, Y., Tai, Y. T., Rosen, S., Avraham, S., Kharbanda, S., and Anderson, K. C. SHP2 mediates the protective effect of interleukin-6 against dexamethasone-induced apoptosis in multiple myeloma cells. J. Biol. Chem., *275:* 27845–27850, 2000.

29. Tai, Y. T., Teoh, G., Shima, Y., Chauhan, D., Treon, S. P., Raje, N., Hideshima, T., Davies, F. E., and Anderson, K. C. Isolation and characterization of human multiple myeloma cell enriched populations. J. Immunol. Methods, *235:* 11–19, 2000.

30. Maeda, H., Hori, S., Nishitoh, H., Ichijo, H., Ogawa, O., Kakehi, Y., and Kakizuka, A. Tumor growth inhibition by arsenic trioxide (As_2O_3) in the orthotopic metastasis model of androgen-independent prostate cancer. Cancer Res., *61:* 5432–5440, 2001.

31. Thornberry, N. A., and Lazebnik, Y. Caspases: enemies within. Science (Wash. DC), *281:* 1312–1316, 1998.

32. Wickremasinghe, R. G., and Hoffbrand, A. V. Biochemical and genetic control of apoptosis: relevance to normal hematopoiesis and hematological malignancies. Blood, *93:* 3587–3600, 1999.

33. Catlett-Falcone, R., Landowski, T. H., Oshiro, M. M., Turkson, J., Levitzki, A., Savino, R., Ciliberto, G., Moscinski, L., Fernandez-Luna, J. L., Nunez, G., Dalton, W. S., and Jove, R. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity, *10:* 105–115, 1999.

34. Puthier, D., Bataille, R., and Amiot, M. IL-6 up-regulates Mcl-1 in human myeloma cells through JAK/STAT rather than ras/MAP kinase pathway. Eur. J. Immunol., *29:* 3945–3950, 1999.

35. Chauhan, D., Pandey, P., Ogata, A., Teoh, G., Treon, S., Urashima, M., Kharbanda, S., and Anderson, K. C. Dexamethasone induces apoptosis of multiple myeloma cells in a JNK/SAP kinase independent mechanism. Oncogene, *15:* 837–843, 1997.

36. Ward, A. C., Touw, I., and Yoshimura, A. The Jak-Stat pathway in normal and perturbed hematopoiesis. Blood, *95:* 19–29, 2000.

37. Uchiyama, H., Barut, B. A., Mohrbacher, A. F., Chauhan, D., and Anderson, K. C. Adhesion of human myeloma-derived cell lines to bone marrow stromal cells stimulates interleukin-6 secretion. Blood, *82:* 3712–3720, 1993.

38. Chauhan, D., Uchiyama, H., Akbarali, Y., Urashima, M., Yamamoto, K., Libermann, T. A., and Anderson, K. C. Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-κB. Blood, *87:* 1104–1112, 1996.

39. Seol, J. G., Park, W. H., Kim, E. S., Jung, C. W., Hyun, J. M., Lee, Y. Y., and Kim, B. K. Potential role of caspase-3 and -9 in arsenic trioxide-mediated apoptosis in PCl-1 head and neck cancer cells. Int. J. Oncol., *18:* 249–255, 2001.

40. Kitamura, K., Minami, Y., Yamamoto, K., Akao, Y., Kiyoi, H., Saito, H., and Naoe, T. Involvement of CD95-independent caspase 8 activation in arsenic trioxide-induced apoptosis. Leukemia (Baltimore), *14:* 1743–1750, 2000.

41. Jing, Y., Dai, J., Chalmers-Redman, R. M., Tatton, W. G., and Waxman, S. Arsenic trioxide selectively induces acute promyelocytic leukemia cell apoptosis via a hydrogen peroxide-dependent pathway. Blood, *94:* 2102–2111, 1999.

42. Dai, J., Weinberg, R. S., Waxman, S., and Jing, Y. Malignant cells can be sensitized to undergo growth inhibition and apoptosis by arsenic trioxide through modulation of the glutathione redox system. Blood, *93:* 268–277, 1999.

43. Cai, X., Shen, Y. L., Zhu, Q., Jia, P. M., Yu, Y., Zhou, L., Huang, Y., Zhang, J. W., Xiong, S. M., Chen, S. J., Wang, Z. Y., Chen, Z., and Chen, G. Q. Arsenic trioxide-induced apoptosis and differentiation are associated respectively with mitochondrial transmembrane potential collapse and retinoic acid signaling pathways in acute promyelocytic leukemia. Leukemia (Baltimore), *14:* 262–270, 2000.

44. El-Sabban, M. E., Nasr, R., Dbaibo, G., Hermine, O., Abboushi, N., Quignon, F., Ameisen, J. C., Bex, F., de The, H., and Bazarbachi, A. Arsenic-interferon-α-triggered apoptosis in HTLV-I transformed cells is associated with tax down-regulation and reversal of NF-κB activation. Blood, *96:* 2849–2855, 2000.

45. Tu, Y., Renner, S., Xu, F., Fleishman, A., Taylor, J., Weisz, J., Vescio, R., Rettig, M., Berenson, J., Krajewski, S., Reed, J. C., and Lichtenstein, A. BCL-X expression in multiple myeloma: possible indicator of chemoresistance. Cancer Res., *58:* 256–262, 1998.

46. Puthier, D., Derenne, S., Barille, S., Moreau, P., Harousseau, J. L., Bataille, R., and Amiot, M. Mcl-1 and Bcl-xL are co-regulated by IL-6 in human myeloma cells. Br. J. Haematol., *107:* 392–395, 1999.

47. Adams, J. M., and Cory, S. The Bcl-2 protein family: arbiters of cell survival. Science (Wash. DC), *281:* 1322–1326, 1998.

48. Zhou, P., Qian, L., Kozopas, K. M., and Craig, R. W. Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood, *89:* 630–643, 1997.

49. Kaufmann, S. H., Karp, J. E., Svingen, P. A., Krajewski, S., Burke, P. J., Gore, S. D., and Reed, J. C. Elevated expression of the apoptotic regulator Mcl-1 at the time of leukemic relapse. Blood, *91:* 991–1000, 1998.

50. Hideshima, T., Nakamura, N., Chauhan, D., and Anderson, K. C. Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma. Oncogene, *20:* 5991–6000, 2001.

51. Tu, Y., Gardner, A., and Lichtenstein, A. The phosphatidylinositol 3-kinase/AKT kinase pathway in multiple myeloma plasma cells: roles in cytokine-dependent survival and proliferative responses. Cancer Res., *60:* 6763–6770, 2000.

52. Ihle, J. N. STATs: signal transducers and activators of transcription. Cell, *84:* 331–334, 1996.

53. Darnell, J. E., Jr. STATs and gene regulation. Science (Wash. DC), *277:* 1630–1635, 1997.

54. Roboz, G. J., Dias, S., Lam, G., Lane, W. J., Soignet, S. L., Warrell, R. P., Jr., and Rafii, S. Arsenic trioxide induces dose- and time-dependent apoptosis of endothelium and may exert an antileukemic effect via inhibition of angiogenesis. Blood, *96:* 1525–1530, 2000.

55. Chomarat, P., Banchereau, J., Davoust, J., and Palucka, A. K. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. Nat. Immunol., *1:* 510–514, 2000.

56. Gabrilovich, D. I., Chen, H. L., Girgis, K. R., Cunningham, H. T., Meny, G. M., Nadaf, S., Kavanaugh, D., and Carbone, D. P. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. Nat. Med, *2:* 1096–1103, 1996.

57. Deaglio, S., Canella, D., Baj, G., Arnulfo, A., Waxman, S., and Malavasi, F. Evidence of an immunologic mechanism behind the therapeutical effects of arsenic trioxide (As_2O_3) on myeloma cells. Leuk. Res., *25:* 227–235, 2001.

58. Hussein, M. A., Mason, J., Ravandi, F., and Rifkin, R. M. A Phase II clinical study of arsenic trioxide (ATO) in patients (Pts) with relapsed or refractory multiple myeloma (MM); a preliminary report. Blood, *98:* 378a, 2001.

59. Bahlis, N. J., Jordan-McMurry, I., Grad, J. M., Reis, I., Neel, J., Kharfan-Dabaja, M. A., Eckman, J., Goodman, M., Fernadez, H. F., Boise, L. H., and Lee, K. P. Phase I results from a Phase I/II study of arsenic trioxide (As_2O_3) and ascorbic acid (AA) in relapsed and chemorefractory multiple myeloma. Blood, *98:* 375a, 2001.

Molecular Cancer Therapeutics

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