Arsenic Trioxide Induces Apoptosis of Human Monocytes during Macrophagic Differentiation through Nuclear Factor-κB-Related Survival Pathway Down-Regulation

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Received July 19, 2005; accepted September 14, 2005

ABSTRACT

Arsenic trioxide (As₂O₃) is known to be toxic toward leukemia cells. In this study, we determined its effects on survival of human mononuclear cells during macrophagic differentiation, an important biological process involved in the immune response. As₂O₃ used at clinically relevant pharmacological concentrations induced marked apoptosis of human blood monocytes during differentiation with either granulocyte-macrophage colony-stimulating factor or macrophage colony-stimulating factor. Apoptosis of monocytes was associated with increased caspase activities and decreased DNA binding of p65 nuclear factor-κB (NF-κB); like As₂O₃, the selective NF-κB inhibitor (E)-3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile (Bay 11-7082) strongly reduced survival of differentiating monocytes. The role of NF-κB in arsenic toxicity was also studied in promonocytic U937 cells during phorbol 12-myristate 13-acetate-induced macrophagic differentiation. In these cells, As₂O₃ first reduced DNA binding of p65 NF-κB and subsequently induced apoptosis. In addition, overexpression of the p65 NF-κB subunit, following stable infection with a p65 retroviral expressing vector, increased survival of As₂O₃-treated U937 cells. As₂O₃ specifically decreased protein levels of X-linked inhibitor of apoptosis protein and FLICE-inhibitory protein, two NF-κB-regulated genes in both U937 cells and blood monocytes during their differentiations. Finally, As₂O₃ was found to inhibit macrophagic differentiation of monocytes when used at cytotoxic concentrations; however, overexpression of the p65 NF-κB subunit in U937 cells reduced its effects toward differentiation. In contrast to monocytes, well differentiated macrophages were resistant to low concentrations of As₂O₃. Altogether, our study demonstrates that clinically relevant concentrations of As₂O₃ induced marked apoptosis of mononuclear cells during in vitro macrophagic differentiation likely through inhibition of NF-κB-related survival pathways.

Arsenic trioxide (As₂O₃), an inorganic trivalent salt, is used at clinically relevant pharmacological concentrations induced marked apoptosis of human blood monocytes during differentiation with either granulocyte-macrophage colony-stimulating factor or macrophage colony-stimulating factor. In these cells, As₂O₃ first reduced DNA binding of p65 NF-κB and subsequently induced apoptosis. In addition, overexpression of the p65 NF-κB subunit, following stable infection with a p65 retroviral expressing vector, increased survival of As₂O₃-treated U937 cells. As₂O₃ specifically decreased protein levels of X-linked inhibitor of apoptosis protein and FLICE-inhibitory protein, two NF-κB-regulated genes in both U937 cells and blood monocytes during their differentiations. Finally, As₂O₃ was found to inhibit macrophagic differentiation of monocytes when used at cytotoxic concentrations; however, overexpression of the p65 NF-κB subunit in U937 cells reduced its effects toward differentiation. In contrast to monocytes, well differentiated macrophages were resistant to low concentrations of As₂O₃. Altogether, our study demonstrates that clinically relevant concentrations of As₂O₃ induced marked apoptosis of mononuclear cells during in vitro macrophagic differentiation likely through inhibition of NF-κB-related survival pathways.

Hematologic remissions are generally reported using As₂O₃ at the dose of 0.15 mg/kg per day for variable durations. Following i.v. infusions, blood arsenic levels reach to 5 to 7 μM and then fall approximately to 1 and 0.2 μM at 12 and 24 h after As₂O₃ administration, respectively (Shen et al., 1997). In vitro, low concentrations of As₂O₃ are markedly toxic toward acute promyelocytic leukemia and multiple myeloma cells and to other hematological tumoral cells such as non-Hodgkin’s lymphoma and chronic lymphocytic leukemia cells (Zhang et al., 1998). Cellular toxicity of As₂O₃ (0.5–2 μM) can first result from oxidative stress-induced mitochondrial damages and sub-

ABBREVIATIONS: As₂O₃, arsenic trioxide; NF-κB, nuclear factor-κB; XIAP, X-linked inhibitor of apoptosis protein; FLIP, FLICE-inhibitory protein; IκBα, inhibitor of κBα; GM-CSF, granulocyte-macrophage colony-stimulating factor; PMA, phorbol 12-myristate 13-acetate; SG, Sytox Green; A5, Annexin V; Bay 11-7082, (E)-3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile; M-CSF, macrophage colony-stimulating factor; FITC, fluorescein isothiocyanate; RT-PCR, reverse transcriptase-polymerase chain reaction; FLIPₕ, long-splice variant of FLIP; DEVD-AMC, Asp-Glu-Val-Asp-fluoromethylketone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole.
sequent caspase-dependent apoptosis of leukemia cells (Jing et al., 1999; Zhou et al., 2003). On the other hand, arsenic can also induce apoptosis through inhibition of the NF-κB pathway (Mahieux et al., 2001; Mathas et al., 2003; Nasr et al., 2003), which controls expression of various survival proteins, including the caspase inhibitors X-linked inhibitor of apoptosis protein (XIAP) and FLICE-inhibitory protein (FLIP) (Micheau et al., 2001; Zhang et al., 2003). In leukemia cells, arsenic is thought to decrease NF-κB activity by directly inhibiting IκBα kinase (Mathas et al., 2003), thus preventing NF-κB release and translocation to the nucleus.

Besides leukemia cells, As2O3 may also be toxic toward normal hematological cells. Indeed, this metallodioxid was reported to induce neutropenia in 8% and up to 80% of patients suffering from acute promyelocytic leukemia and multiple myeloma, respectively (Soignet et al., 2001; Munsch et al., 2002). In the case of multiple myeloma, severe cytopenia in As2O3-treated patients is likely related to myelosuppression due to extensive prior therapy; nonetheless, it appears that arsenic can be directly toxic, at least in vitro, to blood-circulating cells. Notably, it was recently reported that low concentrations of As2O3 in the range of clinically effective concentrations (1–5 μM), induce partial apoptosis of T lymphocytes by increasing oxidative stress and caspase activation (Gupta et al., 2003). In addition, sodium arsenite, another trivalent inorganic arsenic salt, was shown to reduce proliferation of normal T lymphocytes at low micromolar concentrations by delaying production and secretion of interleukin-2 (Galicia et al., 2003). Experimental studies have demonstrated that arsenide also markedly impairs functional integrity of monocytes/macrophages. In vivo, it alters macrophage functions such as adhesion or phagocytic activity (Sengupta and Bishayi, 2002) and reduces murine responses against experimental bacterial infection (Bishayi and Sengupta, 2003). In vitro, low concentrations of arsenite affect differentiation of human blood monocytes into mature macrophages, in part by reducing cell viability (Sakurai et al., 2005); molecular pathways mediating arsenic-induced monocytic cell death remain, however, to be determined. In this context, the present study was designed to analyze the effects of As2O3 on survival of human mononuclear cells, which constitutes a key biological process of macrophagic differentiation (Kiener et al., 1997; Perlman et al., 1999). Indeed, whereas human monocytes rapidly undergo Fas-mediated apoptosis in vitro, growth factor-induced macrophagic differentiation is associated with increased expression of NF-κB-related survival pathways (Perlman et al., 1999; Pennington et al., 2001; Zhang et al., 2003), which could be compromised by As2O3 as reported above.

We demonstrate in this work that clinically relevant concentrations of As2O3 induced marked apoptosis of human blood monocytes and promonocytic U937 cells during macrophagic differentiation induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) and phorbol 12-myristate 13-acetate (PMA), respectively. Apoptosis of monocytic cells likely resulted from decreased NF-κB activity and down-regulation of the NF-κB-regulated antiapoptotic proteins FLIP and XIAP.

Materials and Methods

Chemical Reagents and Antibodies

As2O3, sodium arsenite, cadmium chloride, and PMA were purchased from Sigma (Saint Quentin Fallavier, France). Hoechst 33342 and Sytox Green (SG) were from Molecular Probes (Interchim, Montluçon, France). Annexin V (A5) Alexa568 was purchased from Roche Diagnostic (Meylan, France). Bay 11-7082, an NF-κB inhibitor, was from Calbiochem (France Biochem, Meudon, France). GM-CSF (specific activity, 1.2 × 109 UI/mg) was obtained from Shering Plough (Levallois-Péret, France), and macrophage colony-stimulating factor (M-CSF) (specific activity, 1 × 109 UI/mg) was from Promocell (Heidelberg, Germany). Rabbit polyclonal antibodies against bcl-xL, caspase-3, IκBα, mcl-1, and p38 kinase were purchased from Santa Cruz Biotechnology (Tebu-bio S.A., Le Perray en Yvelines, France). Rabbit polyclonal anti-FIPL antibody was from Stressgen Biotechnologies (Victoria, BC, Canada), whereas mouse monoclonal anti-bcl-2 and anti-XIAP antibodies were obtained from BD Biosciences Pharmingen (San Diego, CA). Mouse monoclonal antibody against caspase-8 was purchased from Alexis Biochemicals (Paris, France). Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against CD14, CD71, CD11b, and CD11c was purchased from Immunotech (Marseille, France).

Cells and Treatments

Peripheral blood mononuclear cells were first isolated from bloody buffy coats of healthy donors through Ficoll gradient centrifugation. Human monocytes were then prepared by a 2-h adhesion step, which routinely obtained >90% of adherent CD14-positive cells as assessed by immunostaining. These monocytic cells were next cultured for 6 days in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 20 UI/ml penicillin, and 20 μg/ml streptomycin in the presence of 800 UI/ml GM-CSF or 50 UI/ml M-CSF to get macrophages as previously reported (Young et al., 1990; van Grevenyghel et al., 2003, 2004). The human promonocytic cell line U937 was grown in RPMI 1640 supplemented with 10% fetal bovine serum and was induced to differentiate into macrophage-like cells in the presence of 100 nM PMA during 4 days (Yan et al., 1997). Blood monocytes and U937 cells were induced to differentiate in the absence or presence of As2O3. In some experiments, blood monocytes were first differentiated with GM-CSF for 6 days and then treated with As2O3.

Measurement of Apoptosis and Necrosis

A5 and SG Staining Assays. To analyze alterations of the plasma membrane structure linked to apoptosis, exposition of phosphatidylserine to the extracellular environment was studied. We determined binding of A5, a calcium-dependent protein with high affinity for phosphatidylserine, using A5 conjugated to the fluorescent label Alexa568. Simultaneously, necrotic cells, which have lost their plasma membrane integrity, were detected with the green fluorescent DNA dye SG. Cells were induced to differentiate in the presence or absence of As2O3. Subsequently, cells were collected, washed, and incubated with dyes as previously described (Lemarie et al., 2004). Apoptotic (A5/SG−) and necrotic (A5/SG+ and A5+/ SG+) cells were quantified using a fluorescence Olympus BX60 microscope (Olympus, Tokyo, Japan) in comparison with living cells. At least 200 cells were counted for each cell suspension.

Hoechst 33342 Staining Assay. To look for changes in chromatin structure typical of apoptotic cells, condensed and fragmented nuclei were stained with the Hoechst 33342 fluorescent DNA dye as previously described (Lemarie et al., 2004). Cells with apoptotic nuclei, i.e., condensed or fragmented, were quantified as described above.

Western Blot Immunoassays

Cells were induced to differentiate into 100-mm dishes in the absence or presence of As2O3. Cells were then harvested, centri-
fuged, washed with phosphate-buffered saline, and lysed for 20 min on ice in radioimmunoprecipitation assay buffer supplemented with 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 0.5 μg/ml aprotinin, 0.5 mM dithiothreitol, and 1 mM orthovanadate. Cells were then centrifuged at 13,000 rpm for 15 min at 4°C. The resulting supernatants were collected and frozen at −80°C or used immediately. Protein concentration was quantified using the Bradford’s method (Bradford, 1976). Each sample (30 μg) or 20 μg of nuclear extract prepared as mentioned below was heated for 5 min at 100°C and then analyzed by 12.5% SDS-polyacrylamide gel electrophoresis and electroblotted overnight onto nitrocellulose membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). After blocking, membranes were hybridized with primary antibody overnight at 4°C and washed and incubated with appropriate horseradish peroxidase-conjugated secondary antibody. Immunolabeled proteins were visualized by chemiluminescence.

**Caspase Activity Assay**

Caspase activity was assessed as previously described (Huc et al., 2004; Lemarie et al., 2004). Crude cell lysate (50 μg) was incubated with 80 μM DEVD-AMC or IETD-AMC, two caspase substrates known to be essentially cleaved by caspase-3 or caspase-8, respectively, for 2 h at 37°C. Caspase-mediated cleavage of substrate-AMC was measured by spectrofluorometry (SpectraMax Gemini; Molecular Devices, Sunnyvale, CA) at the excitation/emission wavelength pair of 380:440 nm. Caspase activities were expressed as the ratio of relative activity of treated cells to that of untreated cells.

**Measurement of NF-κB DNA Binding**

Nuclear proteins were prepared using the Nuclear Extract Kit from Active Motif (Rixensart, Belgium). DNA binding of p65 NF-κB was analyzed using the enzyme-linked immunosorbent assay-based TransAM NF-κB kit (Active Motif) as previously described (Lemarie et al., 2004). In brief, nuclear cell extracts (10 μg) were incubated for 1 h in a 96-well plate to which oligonucleotide containing an NF-κB consensus binding site had been immobilized. After washing, the plate was incubated for 1 h with the rabbit anti-NF-κB p65 antibody (1:1000), which specifically detects an epitope accessible only when NF-κB p65 is activated and bound to its cognate oligonucleotide. The plate was then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. After washing, colorimetric readout was quantified by spectrophotometry at 450 nm. To monitor the specificity of this assay, wild-type and mutated consensus oligonucleotides were used as competitors for NF-κB binding.

**Stable Expression of p65 NF-κB in U937 Cells**

The retroviral vector pMSCV-Puro-p65 was obtained by subcloning the HindIII/HpaI fragment from pEGFP-p65 kindly provided by Dr. J. Schmid, Center for Biomolecular Medicine and Pharmacology, Medical University Vienna, Austria (Schmid et al., 2000) into a modified pMSCV-Puro (Clontech; Ozyme, Saint Quentin Yvelines, France) containing HindIII and HpaI in its multiple cloning site. Retroviral production and cell transduction were carried out as previously described ( Micheau et al., 2001).

**Total RNA Isolation and RT-PCR Assay**

Total RNAs were extracted from monocytes using the TRIzol method (Invitrogen, Carlsbad, CA), and RT-PCR analysis was then performed (Laupeze et al., 2002). The primers used for bcl-2, bcl-ξ, XIAP, mcl-1, and the long-splice variant of FLIP (FLIPL) have been previously described (Perlman et al., 1999; Cui et al., 2000; Yamaguchi et al., 2002). Glyceraldehyde-3-phosphate dehydrogenase detection was performed as a loading control. PCR products were separated on 1% agarose gel and stained with ethidium bromide.

**Flow Cytometric Immunolabeling Assays**

After treatment, floating and adherent cells were removed by a 15-min incubation at 37°C in phosphate-buffered saline supplemented with 100 μM ethylenediaminetetraacetic acid, collected, and centrifuged. Then phenotypic analysis of mononuclear cells was performed using flow cytometric direct immunofluorescence assays (Laupeze et al., 2002). Fluorescence related to immunolabeling was measured using a FACScalibur flow cytometer (BD Biosciences, San Jose, CA). Each measurement was conducted on 8000 events and analyzed on Cell Quest software (BD Biosciences).

**Endocytosis and Phagocytosis Assays**

Cells were incubated at 37°C with 1 mg/ml FITC-dextran (Sigma) for 60 min or with 15 μl of fluorescent latex microspheres (Polysciences, Warrington, PA) for 30 min for endocytosis or phagocytosis assays, respectively. Cellular uptake of FITC-dextran and phagocytosis of latex microspheres were then monitored by flow cytometry at 525 nm. Negative controls were performed in parallel by incubating cells with FITC-dextran or latex beads at 4°C instead of 37°C. Each measurement was conducted on 5000 events and analyzed on Cell Quest software.

**Statistical Analysis**

The results are presented as means ± S.E.M. Significant differences were evaluated with the multirange Dunnet’s t-test for experiments in which multiple comparisons were studied. Other differences were evaluated with the Student’s t test. Criterion of significance of the difference between means was p < 0.05.
Apoptosis was confirmed by chromatin and DNA condensation, as assessed by Hoechst 33342 staining, after a 3-day treatment (Fig. 2B). Accordingly, at this time point, Western blot analysis showed decreased levels of both procaspase-8 and procaspase-3 and apparition of the caspase-3 active cleaved fragment p17 (Fig. 2C). Involvement of caspases was confirmed by measurements of caspase-8 and -3 activities, using IETD-AMC and DEVD-AMC as specific substrates, respectively. Ratios of relative caspase-8 and -3 activities in cells treated for 6 days with 1 μM As₂O₃ compared with untreated cells were 3.64 ± 0.72 and 3.40 ± 1.25, respectively. Unfortunately, the use of caspase inhibitor to confirm involvement of these cysteine proteases in As₂O₃-induced apoptosis could not be addressed in our cell system. Indeed, as recently reported in M-CSF-treated monocytes (Sordet et al., 2002), we found that 30 μM z-VAD-fmk shifted differentiation of GM-CSF-treated monocytes to cell death: 9.03 ± 1.8 and 40.4 ± 6% of A5+/SG⁺ cells were measured in untreated and z-VAD-fmk-treated monocytes after 2 days, respectively.

Different studies report that survival of monocytic cells during growth factor-induced macrophagic differentiation requires NF-κB activity (Pennington et al., 2001; Zhang et al., 2003). We thus determined whether As₂O₃ could alter DNA binding activity of the transcriptionally active p65 NF-κB subunit, which is increased during macrophage differentiation (Conti et al., 1997; Ammon et al., 2000). Figure 3A demonstrates that 1 μM As₂O₃ significantly reduced DNA binding of p65 subunit to κB consensus sites by 20 and 50% in GM-CSF-treated monocytes after 3 and 6 days, respectively. In addition, like arsenic, the specific NF-κB inhibitor Bay 11-7082 significantly reduced p65 NF-κB DNA binding (Fig. 3B), prevented cell adhesion (data not shown), and induced potent apoptosis in GM-CSF-treated monocytes after a 3-day treatment (Fig. 3C). Besides NF-κB, we also examined potential roles of p38 kinase and c-Jun N-terminal kinase, two mitogen-activated protein kinases frequently involved in apoptosis induced by inorganic arsenic. Our results demonstrated that neither the p38 kinase inhibitor SB203580 (10 μM) nor the c-Jun N-terminal kinase inhibitor D-JNKI1 (1 μM) could prevent apoptosis of GM-CSF-treated monocytes exposed to As₂O₃ for 3 days (data not shown).

Arsenic Induced Apoptosis of U937 Cells during Macrophagic Differentiation through Inhibition of NF-κB Signals. To further study the role of NF-κB in arsenic toxicity toward monocytic cells, we used the promonocytic U937 cell line. Indeed, up-regulation of NF-κB during growth factor-induced macrophagic differentiation was associated with rapid cell adhesion and marked expression of the CD11c β2 integrin after 4 days (data not shown). As₂O₃ was not toxic toward differentiating U937 cells at 1 μM; in contrast, 4 μM As₂O₃ reduced cell adhesion and induced marked apoptosis of differentiating U937 cells (Fig. 4A). Figure 4B indicates that apoptosis was time-dependent and not detectable after 24-h treatment with As₂O₃.

PMA increased DNA binding activity of the p65 NF-κB subunit during U937 cell differentiation (Fig. 4C); this effect was associated with a decrease of IkBo protein levels (Fig. 4D). Addition of As₂O₃ during PMA-induced differentiation reduced p65 NF-κB DNA binding activity in a time-dependent manner (Fig. 4C). This effect was significant as early as 24 h and was associated with a decrease of nuclear levels of p65 NF-κB (Fig. 4C, insert) and an inhibition of IkBo degradation (Fig. 4D). It thus appears that decrease of NF-κB DNA
binding preceded apoptosis and was not a consequence of cell death. To determine whether NF-κB inhibition was a causal event mediating As₂O₃-induced apoptosis, a p65 NF-κB subunit expressing retroviral vector was prepared to stably express p65 in U937 cells; human primary monocytes are difficult to transfect and could not be used in this study. As shown in Fig. 4E, both p65 DNA binding activity and nuclear levels were significantly higher in p65-transduced U937 cells compared with control empty vector, treated or not with PMA. Interestingly, As₂O₃ toxicity was significantly reduced in p65-overexpressing U937 cells during differentiation with PMA for 48 h (Fig. 4E): percentages of A5/SG⁻ cells were 28.6 ± 3.7 and 13.6 ± 1.3 in control and p65-overexpressing U937 cells, respectively.

As₂O₃ and Bay 11-7082 Inhibited FLIP and XIAP Expression during Differentiation. NF-κB controls expression of various antiapoptotic proteins, notably FLIP and XIAP (Lin et al., 2001; Micheau et al., 2001; Zhang et al., 2003), two endogenous caspase inhibitors known to increase survival during macrophagic differentiation (Perlman et al., 1999; Lin et al., 2001; Zhang et al., 2003). We analyzed cellular expression of these proteins in response to As₂O₃ treatment. As shown in Fig. 5A, As₂O₃ prevented up-regulation of both FLIP and XIAP in U937 cells during differentiation with PMA without altering mcl-1 expression. Similarly, As₂O₃ did not significantly impair protein (Fig. 5B) or mRNA (Fig. 5C) levels of the bcl-2, bcl-xL, and mcl-1 antiapoptotic factors in human blood monocytes during differentiation with GM-CSF. In contrast, it markedly inhibited both mRNA and protein levels of FLIP and XIAP in these cells; Bay 11-7082 also markedly reduced mRNA levels of FLIP and XIAP but not those of bcl-2 and bcl-xL in differentiating monocytes (Fig. 5C).

As₂O₃ Inhibited Macrophagic Differentiation of Blood Monocytes and U937 Cells. Besides its effect on survival, As₂O₃ reduced, in a similar dose-dependent manner, expression of the transferrin receptor CD71, a well-known macrophagic differentiation marker (Fig. 6A) (van Grevenynghe et al., 2003). At 0.125 μM, a dose that did not alter monocyte survival, no effect was observed on CD71 expression. In addition, 1 μM As₂O₃ inhibited expressions of the integrins CD11b and CD11c, two other macrophagic markers (Pennington et al., 2001; van Grevenynghe et al., 2003) (Fig. 6B); it also reduced levels of both endocytosis and phagocytosis, two major functions of macrophages (Fig. 6C).

As observed with blood monocytes, cytotoxic concentrations...
of metalloid altered expression of CD11c in U937 cells during differentiation with PMA; Fig. 6D demonstrates that 4 \( \mu \)M As2O3 prevented up-regulation of CD11c expression, whereas 1 \( \mu \)M As2O3 had no effect. Interestingly, Fig. 6E shows that overexpression of the p65 NF-\( \kappa \)B subunit in U937 cells not only reduced As2O3-induced apoptosis but also prevented inhibition of CD11c expression.

**Mature Macrophages Are Resistant to As2O3-Induced Apoptosis.** Once differentiated, mature macrophages display increased NF-\( \kappa \)B activity and consequently potently resist to apoptosis (Pagliari et al., 2000). Figure 7, A and B, demonstrates that 1 \( \mu \)M As2O3 neither altered viability of human primary macrophages nor decreased levels of FLIP1, or XIAP proteins, respectively; however, a 4-fold higher concentration of As2O3 induced macrophage apoptosis and reduced protein levels of these caspase inhibitors (Fig. 7, A and C).

**Discussion**

Clinical and experimental studies have demonstrated that anticancerous effects of As2O3 are mainly due to apoptosis of leukemia cells. In the present study, we demonstrated that low concentrations of As2O3 (0.25–1 \( \mu \)M) also markedly decreased survival of human monocyctic cells during in vitro macrophagic differentiation.

Our results demonstrate that As2O3 induced time-dependent apoptosis of human blood monocytes and promonocytic U937 cells during differentiation with GM-CSF and PMA, respectively. Kinetics of death were slow and similar to those reported for human promyelocytic NB4 cells or human lymphoma cells treated with low concentrations of As2O3 (Chen et al., 1997; Mathas et al., 2003). Apoptosis of GM-CSF-treated monocytes was characterized by phosphatidylserine externalization, chromat condensation, DNA fragmentation, and caspase activation. Involvement of caspases in arsenic-treated monocytes was supported by 1) decreased protein levels of procaspase-8 and procaspase-3 and an increased protein level of the active caspase-3 fragment \( \mu \)17, and 2) increased activity of both caspase-8 and caspase-3. Caspase-8 is an initiator caspase mainly activated within the death-inducing signaling complex by death receptors of the tumor necrosis factor family. It can either directly activate the effector caspase-3 in the cytoplasm and trigger apoptosis or induce disruption of the outer mitochondrial membrane allowing cytochrome \( c \) release and activation of effector caspases, including caspase-3, via the apoptosome. Prevention of arsenic toxicity by caspase inhibition could not be tested; indeed, the pan-caspase inhibitor z-VAD-fmk was found, by itself, to shift GM-CSF-induced differentiation to cell death. It thus appears that the level of caspase activity is, in fact, a critical factor since its down-regulation or its excessive up-regulation, like in As2O3-treated monocytes, results in failure of macrophagic differentiation and cell death.

Treatment of monocyctic cells with As2O3 was associated with marked reduction of DNA binding activity of the transcriptionally active p65 NF-\( \kappa \)B subunit. Different arguments support the idea that NF-\( \kappa \)B inhibition can be a causal event mediating arsenic toxicity toward monocyctic cells. First, NF-\( \kappa \)B activity increases survival of monocyctic cells during macrophagic differentiation with M-CSF (Zhang et al., 2003), PMA (Pennington et al., 2001), and likely with GM-CSF since we showed that Bay 11-7082, a selective NF-\( \kappa \)B inhibitor, induced their apoptosis. Second, our results clearly demonstrate that inhibition of NF-\( \kappa \)B DNA binding preceded apo.
As$_2$O$_3$ Induced Monocyte Apoptosis by NF-kB Inhibition

NF-kB regulates expression of some antiapoptotic proteins, notably FLIP and XIAP, in different cell types (Lin et al., 2001; Micheau et al., 2001; Zhang et al., 2003). Our results suggest that NF-kB also controlled expression of these two genes during GM-CSF-induced macrophagic differentiation since Bay 11-7082 specifically reduces their mRNA levels; indeed, this inhibitor had no effect on bcl-2 and bel-xL gene expressions, which are not regulated by NF-kB pathways in mature macrophages (Pagliari et al., 2000). In addition, our results demonstrated that As$_2$O$_3$ selectively reduced mRNA and protein levels of FLIP$_L$ and XIAP in differentiating human primary monocytes with GM-CSF in the absence or presence of Bay 11-7082 (C) for 3 days. Expressions of antiapoptotic genes were then analyzed by Western blot (B) and RT-PCR (C). Experiments in A, B, and C were repeated at least three times with similar results. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Fig. 4. As$_2$O$_3$ induces apoptosis of the human promonocytic U937 cells during differentiation with PMA through NF-kB inhibition. Untreated and PMA-treated U937 cells were cultured in the absence or presence of As$_2$O$_3$ (A) for 4 days (d) at the indicated concentrations or (B) at 4 µM for the indicated time intervals. Then cells were costained with Annexin V-Alexa568 (A5) and SG to detect apoptotic (A5+/SG+) and necrotic cells (A5+/SG-), respectively, and viewed by fluorescence microscopy. Only apoptotic cells (A5+/SG+) are represented in B. Values are means ± S.E.M. of four independent experiments. *, p < 0.05, untreated cells versus As$_2$O$_3$-treated cells. C, untreated and PMA-treated cells were cultured in the absence or presence of 4 µM As$_2$O$_3$ for the indicated time intervals. Nuclear extracts of untreated and As$_2$O$_3$-treated cells were used to measure p65 NF-kB DNA-binding activity and p65 protein levels (insert), as described under Materials and Methods. Wild-type (wt) or mutated (mut) consensus oligonucleotides of p65 NF-kB were used as competitors of nuclear extracts prepared from untreated cells to verify specificity of the assay. Values are means ± S.E.M. of three independent experiments. D, Western blot of IκBα protein. Untreated and PMA-treated cells were cultured in the absence or presence of 4 µM As$_2$O$_3$ for 24 h. Whole-cell lysates were prepared and separated by a 12.5% SDS-polyacrylamide gel electrophoresis. Equal gel loading and transfer efficiency were checked by protein hybridization with an anti-p38 kinase antibody. Equal gel loading and transfer efficiency were checked by protein hybridization with an anti-p38 kinase antibody. These Western blots were repeated three times with similar results. E, U937 cells were stably transduced with empty pMSCV retroviral vector (U937 empty) or a pMSCV-p65 NF-kB construct (U937 p65). Cells were selected by use of puromycin, and populations expressing stably p65 were analyzed as follows. Untreated and PMA-treated cell populations were cultured in the absence or presence of 4 µM As$_2$O$_3$ for 2 days. a, nuclear extracts were used to measure DNA-binding activity of p65 NF-kB and p65 protein levels (insert); b, cells were costained with Annexin V-Alexa568 (A5) and SG to detect apoptotic (A5+/SG+) and necrotic cells (A5+/SG- and A5+/SG-), respectively. Only apoptotic cells (A5+/SG+) are represented in the graph. Cells were viewed by fluorescence microscopy. Values are means ± S.E.M. of three independent experiments. *, p < 0.05.

Fig. 5. As$_2$O$_3$ inhibits FLIP$_L$ and XIAP expression in monocycteic cells during macrophagic differentiation. A, untreated and PMA-treated U937 cells were cultured in the absence or presence of 4 µM As$_2$O$_3$ for 4 days, and expression of antiapoptotic proteins was then analyzed by Western blot. Equal gel loading and transfer efficiency were checked by protein hybridization with an anti-p38 kinase antibody. Human primary monocytes were cultured with GM-CSF in the absence or presence of 1 µM As$_2$O$_3$ (B and C) or 2.5 µM Bay 11-7082 (C) for 3 days. Expressions of antiapoptotic genes were then analyzed by Western blot (B) and RT-PCR (C). Experiments in A, B, and C were repeated at least three times with similar results. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
As$_2$O$_3$ inhibited macrophagic differentiation of monocytic cells. A–C, blood monocytes were cultured with GM-CSF or M-CSF in the absence or presence of As$_2$O$_3$ for 6 days (d). Parental monocytes and macrophages were stained with monoclonal antibodies directed against the macrophagic differentiation markers CD71 (A) and CD11b and CD11c (B). C, GM-CSF-differentiated monocytes were incubated with FITC-dextran (endocytosis) or fluorescent latex microbeads (phagocytosis) at 4°C (negative control) or 37°C. Marker expression and cellular uptakes of FITC-dextran or microbeads were then determined by flow cytometry. U937 cells (D) and U937 cells stably transduced with an empty pMSCV retroviral vector (U937 empty) or with a pMSCV-p65 NF-$\kappa$B construct (U937 p65) (E) were treated or not with PMA in the absence or presence of indicated concentrations of As$_2$O$_3$ for 4 days (D) or 2 days (E). Then cells were stained with monoclonal antibodies directed against CD11c and analyzed by flow cytometry. Representative histograms of at least three individual experiments are shown.

Fig. 6.
monocytes and U937 cells. Down-regulation of these NF-κB-regulated genes did not result from a general toxic effect of As$_2$O$_3$ on transcription, since like Bay 11-7082, it neither alter bcl-2 nor bcl-xL expressions. FLIPL has a strong structure homology to procaspase-8, but it lacks catalytic activity; it directly interacts with procaspase-8 in the death-inducing signaling complex, blocks its cleavage into active fragments, and finally prevents caspase-8-dependent apoptosis (Krueger et al., 2001). Down-regulation of FLIPL expression could thus explain, at least in part, the enhanced caspase-8 activity measured in monocytes exposed to As$_2$O$_3$. XIAP is also a potent suppressor of apoptosis, and its effects are mainly mediated by direct caspase inhibition. It tightly interacts with caspase-9 and -3 but not with caspase-8 (Salvesen and Duckett, 2002). Consequently, arsenic might impair function of both initiator and effector caspases in monocytic cells. In contrast to monocytes, mature macrophages were less sensitive to 1 μM As$_2$O$_3$, which is in agreement with their known resistance to apoptosis. At this concentration, metalloid decreased neither cell viability nor FLIPL protein levels but, unexpectedly, increased those of XIAP. A 4-fold higher concentration (4 μM), however, allowed both FLIPL and XIAP down-regulation and reduced viability of mature macrophages. Altogether, these results suggest that alteration of FLIPL and XIAP expression, likely due to NF-κB inhibition, is involved in As$_2$O$_3$ toxicity toward monocytes/macrophages.

Finally, our study shows that As$_2$O$_3$ markedly inhibited macrophagic differentiation of blood monocytes and U937 cells. Different observations strengthen the idea that such an inhibition was mainly related to As$_2$O$_3$-induced apoptosis; first, 0.125 and 1 μM As$_2$O$_3$ modified neither macrophagic marker expressions nor viability in differentiating monocytes and U937 cells, respectively, whereas higher concentrations similarly altered both parameters. Second, blocking of As$_2$O$_3$-induced apoptosis in p65-overexpressing U937 cells allowed CD11c up-regulation during differentiation of U937 cells exposed to As$_2$O$_3$. Differentiation of monocytes into macrophages constitutes a cellular process involved in numerous physiological functions. Indeed, once differentiated, macrophages play a pivotal role in immune defenses by producing cytokines, chemokines, growth factors, or eicosanoids. They also protect against microbial infection and play a role in the tumor cell killing most likely via their ability to present antigens to lymphocytes. In addition, they play an important role in inflammation and lipid metabolism, and bone narrow macrophages are notably involved in erythropoiesis. Inhibition of macrophagic differentiation by a clinically relevant concentration of As$_2$O$_3$ may therefore lead to deleterious adverse effects in As$_2$O$_3$-treated patients. On the other hand, macrophages participate to the physiopathology of several diseases, such as hemophagocytic syndrome or rheumatoid arthritis (Koch et al., 1994). In these circumstances, As$_2$O$_3$-induced inhibition of macrophagic differentiation may present a clinical interest. In conclusion, our study demonstrated that low clinically achievable concentrations of As$_2$O$_3$ prevented macrophagic differentiation of human mononuclear cells by altering NF-κB-regulated survival pathways.

References
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