Arsenic trioxide induces apoptosis in human T-cell leukemia virus type 1– and type 2–infected cells by a caspase-3–dependent mechanism involving Bcl-2 cleavage

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Treatment of patients with adult T-cell leukemia–lymphoma (ATLL) using conventional chemotherapy has limited benefit because human T-cell leukemia virus type 1 (HTLV-1) cells are resistant to most apoptosis-inducing agents. This may in part be because HTLV-1 leukemic cells overexpress the multidrug resistance protein and the lung-resistance protein, resulting in the pumping of a wide spectrum of agents from the plasma membrane and preventing them from entering the cytoplasm. In addition, the down-regulation of Fas-ligand expression and rare cases of mutations in the Fas gene entering the cytoplasm could also impair the induction of apoptosis in HTLV-1– and -2–infected cells and in ATLL immortalized cells or in ex vivo ATLL samples. Fluorescence-activated cell sorter analysis, fluorescence microscopy, and measures of mitochondrial membrane potential (ΔΨm) demonstrated that arsenic trioxide alone was sufficient to induce programmed cell death in all HTLV-1 and -2 cells tested and in ATLL patient samples. IκB-α phosphorylation strongly decreased, and NF-κB translocation to the nucleus was abrogated. Expression of the antiapoptotic protein Bcl-XL, whose promoter is NF-κB dependent, was down-regulated. The collapse of ΔΨm and the release of cytochrome c to the cytosol resulted in the activation of caspase-3, as demonstrated by the cleavage of PARP. A specific caspase-3 inhibitor (Ac-DEVD-CHO) could reverse this phenotype. The antiapoptotic factor Bcl-2 was then cleaved, converting it to a Bax-like death effector. These results demonstrated that arsenic trioxide induces apoptosis in HTLV-1– and -2–infected cells through activation of the caspase pathway. (Blood. 2001; 98:3762-3769)

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia–lymphoma (ATLL). Treatment of patients with ATLL using conventional chemotherapy has limited benefit given that HTLV-1 cells are resistant to most apoptosis-inducing agents. This may in part be because HTLV-1 leukemic cells overexpress the multidrug resistance protein and the lung-resistance protein, resulting in the pumping of a wide spectrum of agents from the plasma membrane and preventing them from entering the cytoplasm. In addition, the down-regulation of Fas-ligand expression and rare cases of mutations in the Fas gene sequence have been reported and could also impair the induction of apoptosis through this pathway. In vivo, but not in vitro, the combination of zidovudine (AZT) with interferon-α is a partially effective treatment that induces cell death in HTLV-1–infected cells. In vitro, retinoic acid or arsenic trioxide (As_{2}O_{3}), in combination with IFN-α, can also induce cell death in HTLV-1–transformed cells through mechanisms that remain unclear but that may involve the down-regulation of NF-κB in the latter case.

Programmed cell death, or apoptosis, consists of a highly regulated series of events allowing the organism to control cell number and tissue size. It occurs in 3 phases—initiation, commitment to cell death, and execution. During apoptosis, a complex set of proteins is activated. Among them, caspases play a key role and act as initiators (for review, see 15). These cysteine proteases are normally present as pro-enzymes and are proteolytically cleaved to active heterodimers. They have an active-site cysteine and mediate apoptosis by proteolysis of specific substrates. Substrate specificity is determined by the 4-residue amino-terminal to the cleavage site. Caspase-dependent cleavage can either activate or inactivate the target protein. Close to 100 caspase substrates have been reported. Poly (ADP-ribose) polymerase (PARP), a regulator of DNA repair, and Bcl-2 are known substrates of caspase-3. Mitochondria have been recently recognized to play a major role in the control of apoptosis or programmed cell death. Members of the Bcl-2 family that interact with the permeability transition pore complex regulate permeabilization of mitochondrial membranes, a decisive feature of early cell death.

Bcl-2 is a member of an expanding family of related proteins. Some of them are proapoptotic (Bax, Bak, Bid, Bcl-XL) and some are antiapoptotic (Bcl-2, Bcl-XL) (for review, see 14,18). It has been shown that the presence of Bcl-2 blocked the activation of caspase-3. However—and adding another level of complexity—Bcl-2, which inhibits cell death, can also be converted to a Bax-like death effector through its cleavage at Asp-34 by caspase-3. Interestingly, Bcl-2 and Bcl-XL are overexpressed in HTLV-1 cells. In the latter case, this phenomenon is linked with the constitutive activation of the NF-κB pathway by Tax. 23 Tax is a 42-kd protein whose expression is sufficient to induce
murine cell transformation or human T cell immortalization. Tax has pleiotropic effects: not only does Tax transactivate the viral promotor, it is also able to activate or repress the expression or functions of a wide array of genes. Many of them are regulators of the cell cycle (p21, p53, MAD-1, p16INK4A) or of apoptosis (Bcl-2, Bcl-XL, and caspases). Tax is also competent for inhibiting DNA repair through the suppression of the nucleotide-excision repair and the base excision pathways. The pro- or antiapoptotic role of Tax is still a matter of debate. Recent data suggest a proapoptotic function for this protein, even though it inhibits the caspase cascade.

Tax-expressing cells have a striking feature—constitutive NF-κB activation. This promotes the expression of antiapoptotic factors and tumor formation in transgenic mice. Interestingly, arsenic treatment inhibits NF-κB activation in different non-HTLV cell lines through interactions with the IκB complex. Because NF-κB activation has been strongly linked to Tax-induced immortalization, we investigated whether the expression or function of several apoptosis-regulating proteins, whose expression or function is regulated through the NF-κB pathway, was modified by arsenic trioxide–IFN-α treatment in HTLV-1– and -2–infected cells.

In this report, we demonstrate that arsenic trioxide treatment induced apoptosis in all HTLV-1 and -2 cell lines tested, whether they were interleukin-2 (IL-2) dependent or independent, and in cells obtained from patients with HTLV-1 ATL. Drug treatment induced the disruption of the mitochondrial ΔΨm and the reversion of NF-κB activation. Apoptosis was correlated with caspase-3 activation and caspase-3–dependent cleavage of PARP and of Bcl-2. Cleavage of Bcl-2 promoted the release of cytochrome c from the mitochondria. Addition of a specific caspase-3 inhibitor prevented arsenic trioxide–induced apoptosis. Finally, Bcl-XL protein, whose promoter is NF-κB dependent, was also down-regulated. These results demonstrated that though multiple apoptosis pathways are inhibited in HTLV-1– and -2–infected cells, these effects were reversible. This may open new avenues for treating patients with HTLV ATL.

Materials and methods

Drugs

IFN-α (Roche Molecular Biochemicals, Basel, Switzerland) or arsenic trioxide (Sigma, l’Isle d’Abeau, France) was used at 100 units/mL and 1 μM, respectively, as previously described. The cell-permeable reversible caspase-3 inhibitor Ac-DEVD-CHO (Bachem, V oisins-le-Bretonneux, France) was dissolved in dimethyl sulfoxide (Sigma) and was used at 50 μM and 100 μM.

Cell lines and ATLL patient cells

HUT-102, MT-2, C8166, and C91/PL are HTLV-1–transformed cell lines, and C19 is an HTLV-2–transformed cell line. CEM, MOLT-4, and Jurkat are leukemic cells not infected by HTLV viruses. All these cell lines were grown in Roswell Park Memorial Institute medium (RPMI-1640; Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum, L-Glu, and penicillin-streptomycin. Boul and Bes (HTLV-1 IL-2–dependent cell lines isolated from a patient with TSP/HAM and a patient with ATL, respectively) and PH878 and PH868 (short-term cultured cells from 2 patients with ATL) were grown in the same medium supplemented with 20% fetal calf serum and 10% recombinant IL-2 (Roche Molecular Biochemicals). PH868 is a patient with chronic ATL (WBC showed 2.6 × 10^9 lymphocytes/L), and PH878 is a patient with acute ATL (WBC showed 10.7 × 10^9 lymphocytes/L).

FACS analysis and 4’,6’-diamidino-2-phenylindole dihydrochloride staining

After 48 to 72 hours of treatment, cells were harvested and washed in phosphate-buffered saline (PBS) without Ca^2+ /Mg^2+ (Life Technologies). They were then stained using the Vybrant Apoptosis kit (Molecular Probes, Eugene, OR). Briefly, annexin V conjugated to fluorescein allowed the identification of apoptotic cells, whereas propidium iodide (PI) allowed the identification of dead cells. Apoptotic cells were annexin V positive and PI negative. For 4’,6’-diamidino-2-phenylindole dihydrochloride (DAPI) staining experiments, the chambers were coated with poly-L-lysine overnight. Cells were then added to the slide, fixed with 7% paraformaldehyde, washed in PBS without Ca^2+ /Mg^2+, and incubated with DAPI (4’,6’-diamine-2-phenylindole dihydrochloride) (Sigma) at 0.1 μg/mL. Cells were then mounted using Vectashield (Vector Laboratories, Burlingame, CA).

Mitochondrial membrane potential (∆Ψm) changes

To investigate the changes in mitochondrial membrane potential that occur during apoptosis, the Apoalert Mitochondrial Membrane Sensor kit (Clontech, Palo Alto, CA) was used. Briefly, after 48 to 72 hours of treatment, cells were incubated with Mitosensor reagent for 20 minutes at 37°C. After centrifugation, they were resuspended in incubation buffer and analyzed by fluorescence-activated cell sorter scanner (FACS). Mitochondria containing Mitosensor (normal mitochondria) aggregates were detectable in the PI channel, and Mitosensor monomers (mitochondria with altered membrane permeability) were detectable in the fluorescein isothiocyanate channel.

Cell proliferation and viability assay

To measure cellular proliferation or viability, a cell proliferation–viability kit (XTT; Roche Molecular Biochemicals) was used. In this assay, tetrazolium salt XTT is cleaved to form an orange formazan dye by metabolic active cells. This dye is directly quantified using an enzyme-linked immunosorbent assay reader at 942 nm.

Whole-cell extracts and Western blot analysis

Cells were lysed in TNM buffer (50 mM Tris HCl, pH 7.4, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM Na₃VO₄, 1 mM dithiothreitol) in the presence of protease inhibitors (Complete, Boehringer Mannheim, Germany) for 20 minutes on ice. The lysate was then centrifuged for 10 minutes at 4°C, and the supernatant was frozen at −80°C.

Electrophoretic mobility shift assay

Nuclear and cytoplasmic extracts were made as previously described. The 8 mC/EBP oligonucleotide was used as an NF-κB–specific probe, as previously described.

Mitochondria purification

Cells were washed in PBS without Ca^2+ /Mg^2+, the pellet was resuspended in lysis buffer (0.15 mM MgCl₂, 10 mM KCl, 10 mM Tris-HCl, pH 7.6, and proteases inhibitors) and kept for 30 minutes on ice. Membranes were then disrupted with a dounce homogenizer, and RLM buffer was added (0.2 mM saccharose, 10 mM Tris-HCl, pH 7.4, ethylene glycol tetraacetic acid (EGTA)-Tris 0.1 mM final). Lysates were then centrifuged for 3 minutes, at 900g at 4°C. Supernatants were then centrifuged at 7000g for 10 minutes at 4°C. Pellets were resuspended in RLM buffer and centrifuged again at 4°C for 10 minutes at 7000g for 10 minutes at 4°C. Finally, the pellet was resuspended in protein extraction buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate) containing proteases...
inhibitors. Lysates were cleared by centrifugation in a microcentrifuge at 14,000 rpm for 15 minutes and were used for Western blot analysis, as described above.

**Antibodies**

Anti-Bcl-2 (sc-509), Bcl-2 ΔC21 (sc-783), Bax (sc-526), anti-caspase-3 (sc-7148), phospho-IκB-α (sc-8404), and β-tubulin (sc-9104) were purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-cytochrome c (65971A) and anti-PARP (65196E) were purchased from PharMingen (San Diego, CA). Anti Bcl-XL/XS (06-851) was purchased from Upstate Biotechnology (Lake Placid, NY), and anti p53 DO-1 (Ab-6) was purchased from Oncogene Research (Cambridge, MA). Anti-Tax Tab172 was previously described. Anti-gag p24 (9281) was purchased from Promega (Madison, WI), and anti p53 DO-1 (Ab-6) was purchased from Oncogene Research (Cambridge, MA). Anti-cytochrome c antibody (65971A) (PharMingen) for 1 hour at room temperature. Cells were washed in PBS/2% BSA and incubated with anti-cytochrome c antibody (65971A) (PharMingen) for 1 hour at room temperature. The coverglass was finally washed, mounted with Vectashield (Vector Laboratories), and examined using laser confocal microscope argon-krypton (Leica).

**Confocal microscopy**

Cells were centrifuged (Shandon, Pittsburgh, PA) on Superfrost/Plus glass slides (Menzel-Glaser, Braunschweig, Germany) and fixed with 4% paraformaldehyde. They were then permeabilized (PBS/0.1% Triton), blocked (2% PBS–bovine serum albumin (BSA)), and incubated with anti-cytochrome c antibody (65971A) (PharMingen) for 1 hour at room temperature. Cells were washed in PBS/2% BSA and incubated with anti-cytochrome c antibody (65971A) (PharMingen) for 1 hour at room temperature. The coverglass was finally washed, mounted with Vectashield (Vector Laboratories), and examined using laser confocal microscope argon-krypton (Leica).

**Results**

**Arsenic trioxide alone or in combination with IFN-α induces apoptosis of HTLV-1– and HTLV-2–immortalized or –transformed cells**

Arsenic trioxide (As2O3) and IFN-α were recently demonstrated to induce apoptosis in 2 HTLV-1–transformed cells. To investigate the mechanism of action of arsenic trioxide, associated or not associated with IFN-α, we treated with these chemicals a series of HTLV-1–immortalized (IL-2–dependent) cells (Bes, Boul), HTLV-1–transformed (IL-2–independent) cells (C8166, HUT-102, MT-2), HTLV-2–transformed (C19) cells, or noninfected control cells (Jurkat and MOLT-4). After 60-hour treatment, the cells were analyzed by FACS using double staining (DAPI/annexin V). Apoptotic cells were scored as annexin V+/PI− versus dead cells, which were PI+. Figure 1A shows a typical FACS result obtained with MT-2 cells. An apoptotic population was detected when As2O3 was added to the cell culture. Similar FACS results were obtained with C8166 HTLV-1–infected cells (data not shown). Control Jurkat and MOLT-4 cells were not affected by As2O3 treatment (data not shown and Figure 2). DAPI staining of MT-2 cells (Figure 1B) demonstrated the expected nuclear condensation after arsenic trioxide or arsenic trioxide–IFN-α treatment, whereas control treatment or IFN-α alone had no or little effect.

Changes in ΔΨm are a critical step in cells undergoing apoptosis, regardless of the death signal. Therefore, ΔΨm was also measured in MT-2 cells (Figure 1C). Arsenic trioxide or As2O3 + IFN-α–treated cells that entered apoptosis showed an altered mitochondrial membrane permeability that led to ΔΨm collapse. By contrast, MT-2 cells treated with buffer control or with IFN-α did not show any change in ΔΨm (Figure 1C; compare panels 3 and 4 with panels 1 and 2).

To investigate whether all HTLV-1– or HTLV-2–infected cell lines or cells from patients were also sensitive to As2O3 with or without IFN-α, we conducted a series of XTT assays that allowed us to measure cellular proliferation and viability after drug treatment (Figure 2). Confirming the FACS results, HTLV-1–immortalized (IL-2–dependent) cell lines obtained from Boul and Bes patients and HTLV-1–transformed HUT-102, C8166, C91/PL (data not shown), HTLV-2–transformed C19 or short-term cultured cells from 2 patients with ATLL (PH878 and PH868) displayed a significant reduction in cellular proliferation and viability after As2O3 or As2O3 + IFN-α treatment. By contrast, control treatment
or IFN-α treatment had no effect (Figure 2A-C). As control cells, CEM, T2, MOLT-4, and Jurkat were tested. CEM, MOLT-4, and Jurkat proliferation was not affected by any treatment. Strikingly, however, As2O3 and As2O3 + IFN-α induced viability and growth alteration in T2 (CEM-derived) cells. This suggested that As2O3 did not specifically induce apoptosis in HTLV-1– or HTLV-2–infected cells but did affect a cellular pathway that might have been altered in HTLV-1 and -2 cells and in the T2 cell line (Figure 2D).

NF-κB activity down-regulated after arsenic trioxide or arsenic trioxide–IFN-α treatment

Because arsenic was reported to inhibit NF-κB activity in various cell lines,40,41 we investigated whether, as suggested by others,13 this was the case in HTLV-1 cell lines. Retardation gels using an NF-κB–specific probe and IκB-α phospho-specific WB confirmed that As2O3, whether used alone (data not shown) or in combination with IFN-α, was able to inhibit the constitutive IκB-α phosphorylation ordinarily present in HTLV-1 cell lines (Figure 3A; compare lanes 2 and 1). This led to a decrease in NF-κB translocation to the nucleus and consequently diminished binding to an NF-κB–specific probe (Figure 3C; compare lanes 2 and 3). In contrast, control treatment (Figure 3A, lane 1) or IFN-α alone (data not shown) did not induce any change. Supershift experiments and competition with a cold probe demonstrated the binding specificity (data not shown).

Switch from Bcl-XL to Bcl-XS after As2O3 or As2O3 + IFN-α treatment

NF-κB factors affect the transcription of some Bcl-2 family members such as Bcl-XL.21,22 They also alter p53 transcriptional activity.44,45 Moreover, p53 drives Bax expression. Because we showed that As2O3 affected NF-κB activity, the level of expression of Bax, Bcl-XL, Bcl-XS, and p53 was investigated after drug treatment using whole-cell extracts from C8166 (Figure 4A-C) or from MT-2 and HUT-102 (data not shown). Overall levels of Bax (Figure 4A) and p53 (Figure 4C) remained unchanged, even after the addition of As2O3 + IFN-α. Similar results were obtained for p21 (data not shown). Alternative splicing of the Bcl-X gene gives rise to 2 proteins with antagonistic functions: Bcl-XL (antiapoptotic) or Bcl-XS (proapoptotic).46 HTLV-1–infected cells display high levels of Bcl-XL.22 Strikingly, we noted a strong decrease in Bcl-XL expression after As2O3 or As2O3 + IFN-α treatment (Figure 4B).
Of note, increased expression of the alternatively spliced, proapoptotic protein Bcl-Xs was seen in all the IFN-\(\alpha\)-treated C8166 samples. Such an increase in Bcl-Xs expression was not observed in the other HTLV-1–cell lines tested, suggesting that this is not a common phenomenon in HTLV-1 cells treated with IFN-\(\alpha\) (data not shown). The altered expression levels of Bcl family members might, therefore, have been a factor that influenced the shift toward apoptosis in C8166 cells.

\[\text{b-Tubulin Western blot (Figure 4D) confirmed equal loading of protein extracts.}\]

Involvement of caspase-3 in arsenic trioxide–induced apoptosis

\(\Delta\Psi_m\) Collapse results in the activation of caspases. Caspase-3 is a critical downstream protease in the caspase cascade, and PARP is one substrate of the caspase-3 protease activity associated with apoptosis. Interestingly, the activation of caspase-3 was detected after As\(_2\)O\(_3\) (data not shown) or As\(_2\)O\(_3\) + IFN-\(\alpha\) treatment, as demonstrated by cleavage of the pro-enzyme (Figure 5A; compare lane 2 [arsenic treated] with lane 1 [control]). This demonstrates that this cysteine protease was activated by the addition of As\(_2\)O\(_3\). We then looked for potential PARP cleavage in As\(_2\)O\(_3\)-treated MT-2 cells. Indeed, the 85-kd fragment representing the cleaved form of PARP was detected after treatment of MT-2 HTLV-1 cells by As\(_2\)O\(_3\) alone or in combination with IFN-\(\alpha\) (Figure 5B; compare lanes 3 and 4 with lanes 1 and 2). This demonstrates the involvement of caspase-3 in this process.

Because Bcl-2 is also cleaved by caspase-3 and results in a proapoptotic form, we performed MT-2 and HUT-102 subcellular fractionation and mitochondrial enrichment and then Western blot analysis to search for such Bcl-2 posttranslational modification. The proapoptotic, 23-kd Bcl-2 form was detected in As\(_2\)O\(_3\)-treated MT-2 cells (Figure 5C; compare lanes 3 and 4

\[\text{Figure 3. Inhibition of NF-\(\kappa\)B activation by As\(_2\)O\(_3\) in HTLV-1–infected cells. (A) Sixty micrograms cytoplasmic protein was resolved on 10\% to 20\% Tris-glycine, transferred to PVDF membranes, and probed with an antibody specific for the phosphorylated form of I\(\kappa\)B\(\alpha\). Results are representative of 2 independent experiments. (B) Comparable protein loading was verified using an antibody specific for the housekeeping gene product \(\beta\)-tubulin. Results are representative of 2 independent experiments. (C) Electrophoretic mobility shift assay using a previously published NF-\(\kappa\)B–specific probe\(^{23}\) mixed with no extract (lane 1), mixed with 5 \(\mu\)g nuclear extract obtained from MT-2 treated with control buffer (lane 2), or treated with As\(_2\)O\(_3\) + IFN-\(\alpha\) and run on a 6\% DNA retardation gel (lane 3). Specificity of the NF-\(\kappa\)B binding was checked by competition with a 50-fold excess of cold probe (data not shown).}\]

\[\text{Figure 4. Bcl\(X_L/X_S\) expression, but not Bax or p53 expression, is modified by As\(_2\)O\(_3\) in HTLV-1–transformed and –immortalized cell lines. Sixty micrograms total protein from C8166 cells was resolved on 10\% to 20\% Tris-glycine, transferred to PVDF membranes, and probed with specific antibodies for (A) Bax, (B) Bcl\(X_L/X_S\), (C) and p53. Results are representative of at least 2 independent experiments using different HTLV-1–infected cells. Comparable protein loading was verified using an antibody specific for the housekeeping gene product \(\beta\)-tubulin (D). Lane 1, control treatment; lane 2, IFN-\(\alpha\); lane 3, As\(_2\)O\(_3\); lane 4, As\(_2\)O\(_3\) + IFN-\(\alpha\).}\]

\[\text{Figure 5. As\(_2\)O\(_3\) induces caspase-3 activation and Bcl-2 cleavage. Sixty micrograms total proteins from MT-2 cells were resolved on 4\% to 20\% Tris-glycine gels transferred to PVDF membranes and probed with specific antibodies for (A) caspase-3 and (B) PARP. Comparable protein loading was verified using an antibody specific for the housekeeping gene product \(\beta\)-tubulin (data not shown). (C) Sixty micrograms mitochondrial-enriched fraction proteins from MT-2 or HUT-102 cells was resolved on 4\% to 20\% Tris-glycine transferred to PVDF membranes and probed with specific antibodies for Bcl-2. (A-C) Results are representative of at least 2 independent experiments using different HTLV-1–infected cells (B-C, panel 1). Lane 1, control treatment; lane 2, IFN-\(\alpha\); lane 3, As\(_2\)O\(_3\); lane 4, As\(_2\)O\(_3\) + IFN-\(\alpha\). (A, C, panels 2 and 3) Lane 1, control treatment; lane 2, As\(_2\)O\(_3\) + IFN-\(\alpha\).}\]
with lanes 1 and 2 in panel 1 and lane 2 with lane 1 in panel 2) and in HUT-102 cells (Figure 5C; compare lane 2 [arsenic trioxide + IFN-α] with lane 1 [control] in panel 3). This confirmed that As₂O₃-induced apoptosis in HTLV-infected cells was caspase-3 dependent.

Ultimately, HUT-102 cells were also treated with Ac-DEVD-CHO, a specific caspase-3 inhibitor, in the presence of As₂O₃ and IFN-α. As seen on Figure 6, incubating HTLV-1-infected cells with DEVD-CHO during As₂O₃ treatment completely prevented death. Ac-DEVD-CHO treatment of HUT-102 in the absence of As₂O₃ had no effect on cell growth (data not shown). This result clearly demonstrated the role of the caspase-3 in As₂O₃-induced apoptosis.

**Figure 6. Ac-DEVD-CHO, a caspase-3–specific inhibitor, can prevent As₂O₃-induced apoptosis.** HUT-102 cells were preincubated with Ac-DEVD-CHO at 100 μM for 2 hours, then treated with control buffer or with As₂O₃ + IFN-α for 60 hours in the presence of Ac-DEVD-CHO. XTT assay was then performed, as described in “Materials and methods.” Graph represents the mean of 2 separate experiments performed in triplicate. Lane 1, control treatment; lane 2, As₂O₃ + IFN-α; lane 3, As₂O₃ + IFN-α + Ac-DEVD-CHO.

Bcl-2 cleavage induced the release of cytochrome c from the mitochondria to the cytosol in baby hamster kidney cells. We performed a series of confocal microscopy experiments to look for cytochrome c release. Staining of cytochrome c was punctate and in a mitochondrial distribution around a well-defined nucleus in cells treated with buffer control (Figure 7A).
or with IFN-α (data not shown). By contrast, after As₂O₃ treatment, cytochrome c staining was no longer clearly defined. (Figure 7B).

### Discussion

Programmed cell death, or apoptosis, is a genetic program that allows the control of cellular homeostasis. Disruption of apoptosis can contribute to a number of diseases, including cancer. It is now well established that anticancer agents induce apoptosis and that disruption of apoptotic programs can reduce treatment sensitivity. In vitro and in vivo HTLV-1 cells are resistant to most apoptosis-inducing agents; this might, in part, be because HTLV-1 cells overexpress PgpP. Inorganic arsenic trioxide (As₂O₃) was recently reported to induce complete remission in a high proportion of patients with acute promyelocytic leukemia (APL). In the APL cell line NB4, apoptosis might involve the down-regulation of Bcl-2 expression and the modulation of PML-RARα expression or localization. However, in other APL cells, the involvement of PML-RARα is a matter of controversy. Because HTLV-1 causes ATLL, investigators have been prompted to determine whether this chemical is also active in vitro on cell lines infected with HTLV-1. Indeed, As₂O₃ in combination with IFN-α kills HTLV-1–transformed cells possibly through Tax down-regulation, leading to NF-κB repression. The caspase cascade is activated in HTLV-1–infected cells. Here we demonstrate that this inhibition is reversible and participates in arsenic-induced programmed cell death. We show that concomitant with NF-κB inactivation, caspase cascade is reactivated, leading to PARP and to Bcl-2 cleavage and to the release of cytochrome c. Finally, the reversion of As₂O₃ effects can be achieved by a specific caspase-3 inhibitor, DEVD-CHO. These results are in disagreement with those of El-Sabban et al. The DEVD-CHO concentration we used (100 μM vs 50 μM used by El-Sabban et al.) or the fact that we used DEVD-CHO instead of z-VAD might explain the differences observed.

Bcl-2 cleavage by caspase-3 can also lead to apoptosis. Indeed, this was the case when HTLV-1 cells were treated with As₂O₃. Therefore, our results illustrate that though Bcl-2 is postulated to inhibit apoptosis, arsenic trioxide–treated HTLV-1 cells. We do note that in examining the level of Bax and p24 KGF, 2 well-characterized p53-responsive genes, we did not see an increase in HTLV-infected cells treated with arsenic trioxide. This suggests that perhaps the apoptosis induced by arsenic trioxide, though caspase dependent, was p53 independent.

In addition, we think that As₂O₃–induced NF-κB down-regulation in HTLV-1 cells did not result from Tax down-regulation itself but rather from a direct effect of the chemical component on the IκB complex, as reported by other investigators in different cellular systems. Therefore, p53 inhibition may still be activated. In fact, conjoined with a Tax decrease, we reproducibly detected by Western blot analysis an increase in p24 gag protein expression after As₂O₃ treatment (Figure 8A-B). This could indicate that, after stress, there is a shift toward viral production. Indeed, such an effect has been reported in HTLV-1 cells treated with sodium arsenite and in HHV-8–infected primary effusion lymphoma cells treated with arsenic trioxide (G. Torelli, written communication, 2000). These results would also explain why the addition of As₂O₃ is effective on freshly isolated ATLL cells, which show little, if any, Tax expression but do show NF-κB activity. This hypothesis is now under study, but it has also been raised by other investigators who treated ATLL cells with all-trans retinoic acid. These results also suggest that using As₂O₃ in vivo to treat HTLV-1– or HTLV-2–infected patients might have pernicious effects. The drug treatment would be anticipated to increase viral production and, therefore, to expand the number of newly infected cells. However, given that in vitro AZT protects cells from infection with HTLV-1, one could suggest that treatment based on the combination of As₂O₃ and AZT would be expected to induce cell death in HTLV-1–infected cells and prevent new infection.

In conclusion, these results confirm that although multiple apoptotic pathways are inhibited in HTLV-1–infected cells, these effects are reversible. This may open new avenues for treating HTLV-1–infected patients.

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References


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