Overexpression of survivin in primary ATL cells and sodium arsenite induces apoptosis by down-regulating survivin expression in ATL cell lines

Xiao-Fang Che1, Chun-Lei Zheng1, Satsuki Owatari2, Masato Mutoh4, Takenari Gotanda1, Hei-Cheul Jeung1, Tatsuhiko Furukawa1, Ryuji Ikeda1, Masatatsu Yamamoto1, Misako Haraguchi1, Naomichi Arima3, Shin-ichi Akiyama1,*

1Department of Molecular Oncology, Graduate School of Medical & Dental Sciences, 2Department of Hematology and Immunology, Kagoshima University Hospital, 3Center for Chronic Viral Diseases, Division of Host Response, Kagoshima University, 8-35-1, Sakuragaoka, Kagoshima, 890-8520, Japan.

4Pharmaceutical Research Laboratories, Toray Industries, Inc. 1, 111 Tebiro, Kamakura-city, Kanagawa 248-8555, Japan.

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* To whom request for reprints should be addressed, at Department of Molecular Oncology, Graduate School of Medical & Dental Sciences, Kagoshima University, 8-35-1, Sakuragaoka, Kagoshima 890-8520, Japan. Tel: 81-99-275-5490; FAX: 81-99-265-9687; E-mail: akiyamas@m3.kufm.kagoshima-u.ac.jp
Abstract

Patients with acute or lymphoma type adult T-cell leukemia (ATL) have a poor outcome because of the intrinsic drug resistance to chemotherapy. Protection from apoptosis is a common feature involved in multidrug-resistance of ATL. IAP (inhibitor of apoptosis) family proteins inhibit apoptosis induced by a variety of stimuli. In this study, we investigated the expression of IAP family proteins (survivin, cIAP1, cIAP2, XIAP) in the primary leukemic cells from patients with ATL. We found that survivin was overexpressed in ATL, especially in acute type ATL. Sodium arsenite was shown to down-regulate the expression of survivin at both the protein and RNA levels in a time- and dose-dependent manner, thus inhibiting cell growth, inducing apoptosis and enhancing the caspase 3 activity in ATL cells. NF-κB enhances the transcriptional activity of survivin. Sodium arsenite suppressed the constitutive NF-κB activation by preventing the IκB-α degradation and the nuclear translocation of NF-κB. These findings suggest that survivin is an important anti-apoptotic molecule which confers drug resistance on ATL cells. Sodium arsenite was shown to down regulate the expression of survivin through the NF-κB pathway thus inhibiting cell growth and promoting apoptosis of ATL cells.
Introduction

Adult T-cell leukemia (ATL) is an aggressive malignancy of CD4+ T cells associated with human T-cell leukemia virus type I (HTLV-1) infection.1 There is evidence suggesting that HTLV-1 viral Tax protein activates the expression of a number of cellular genes, such as growth factors, growth factor receptors, and oncogenes through the induction of transcription factors, such as NF-κB,2 cAMP response element binding protein (CREB)/AP-1 transcription factor (ATF)3 and serum response factor (SRF).4 However, the presence of tax gene expression has not yet been clearly established in freshly isolated ATL cells; moreover, defective viruses, which cannot produce Tax, have been observed in ATL cells, thus suggesting that the tax gene is necessary for the initial stages of leukemogenesis, but it is not essential for the late stage of leukemia.5 As suggested by the multistep model of tumorigenesis,6 mutations of various genes are considered to contribute to leukemogenesis.

ATL is divided into 4 clinical subtypes, acute, lymphoma, chronic and smoldering.7 Chronic and smoldering-type ATLs have a mild clinical course and do not require treatment with intensive chemotherapy. Acute and lymphoma-type ATLs require intensive chemotherapy, and the median survival period is less than one year because of resistance to chemotherapy. Several mechanisms are involved in the multidrug-resistance of ATL, including the overexpression of P-glycoprotein (P-gp),8 multidrug resistance protein (MRP)19 and lung resistance-related protein (LRP).10 Accumulating evidence suggests that the activation of NF-κB is a critical process in the inhibition of apoptosis and resistance to chemotherapy.11

Survivin, cIAP1, cIAP2 and XIAP belong to the IAP (inhibitor of apoptosis) family which is defined by one or more repeats of a highly conserved 70 amino acid domain termed the baculovirus IAP repeat (BIR) located at the amino-terminus. IAP family proteins directly bind and inhibit certain caspases and also inhibit apoptosis induced by a variety of stimuli.12 Previous studies have revealed that several IAPs, such as survivin, NAIP, and XIAP, were overexpressed in AML.13 Survivin was overexpressed in chronic lymphocytic leukemia (CLL) and ATL.14,15 The IAPs are probably involved in the drug
resistance in leukemia. Survivin was also demonstrated to be an unfavorable prognostic factor in leukemia, oral squamous cell carcinoma and bladder cancer.\textsuperscript{16-19} Among the known IAPs, survivin was prominently and consistently expressed in ATL and the expression level of the \textit{survivin} mRNA has correlated with a shorter survival of the patients.\textsuperscript{15} However, the expression of other IAP members such as IAP1, IAP2 and XIAP in ATL is still uncertain. Ishitsuka and his colleagues showed arsenic trioxide (As\textsubscript{2}O\textsubscript{3}) to have a therapeutic potential for the treatment of ATL.\textsuperscript{20} They suggested that As\textsubscript{2}O\textsubscript{3} induced apoptosis of ATL cells by the destruction of the bcl-2 protein and the enhancement of the Bak protein production.\textsuperscript{21}

In the present study, we investigated the expression levels of the members of the IAP family in primary ATL cells. We thus found survivin to be overexpressed in ATL while sodium arsenite inhibited cell growth and apoptosis through the down-regulation of survivin.
Patients and Methods

Patients

Between July 1999 and July 2003, we studied 38 ATL patients (Table 1) consisting of 18 males and 20 females with a median age of 62 years (range, 21-87 years). According to previously reported diagnostic criteria, 23 of these patients were acute, 12 were chronic, and 3 were smoldering type ATL. The performance status (PS) was based on the 5-grade scale of the World Health Organization. Belonging to PS 0, 1, 2, 3, and 4 is 17, 8, 6, 3, and 4 patients, respectively. During this study, we treated acute ATL patients with combination chemotherapy regimens, such as a response-oriented multidrug protocol; a cyclophosphamide, DOX, vincristine, and prednisone protocol; or an LSG15 protocol. None of the chronic and smoldering ATL patients required treatment with intensive chemotherapy. Approval was obtained from the Kagoshima University institutional review board for this study. Informed consent was provided according to the Declaration of Helsinki.

Reagents and antibodies

RPMI 1640 was purchased from Nissui Seiyaku (Tokyo, Japan). Fetal calf serum (FCS) was from Equitech-Bio, Inc. (TX, USA). Sodium arsenite (NaAsO$_2$, As$\text{III}$) and Ac-DEVD-MCA (Ac-Asp-Glu-Val-Asp-MCA) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MO).

Antibodies against NF-κB, polyclonal antibody p65 and monoclonal antibody p50, monoclonal antibodies Bcl-2, PARP, IκB-α were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody against α-tubulin was from Oncogene (Boston, MA). A polyclonal antibody against survivin, MP001, was prepared in our laboratory. MRPm6, a monoclonal antibody against MRP1 was purchased from Progen Biotechnick (Heidelberg, Germany).
Cell lines and cell cultures

The human ATL cell line MT2 is derived from normal human leucocytes transformed by the leukemic T cells of an ATL patient, S1T is HTLV-1-infected CD4⁺ T cells derived from ATL patient, which have no Tax expression. The acute T-cell lymphoma cell line Jurkat was used as a control. The cell lines were grown in RPMI 1640 containing 10% FCS, 2 mM glutamine and 100 U/ml of penicillin at 37°C in a 5% CO₂ humidified atmosphere.

cDNA synthesis

Peripheral blood mononuclear cells were separated by Ficoll-Conray (Immuno-Biological, Gunma, Japan) density gradient centrifugation and stored at -80°C until use for RNA or protein extraction. The total RNA from peripheral blood mononuclear cells and the cultured cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). One microgram of RNA was reverse transcribed using a first-strand cDNA synthesis kit (ReverTra Ace α, TOYOBO, Osaka, Japan).

RT-PCR

One microliter of the resulting first strand cDNA was used for each PCR reaction. The human survivin primers were as follows: forward, 5’-GAT TTG AAT CGC GGG ACC GTG-3’; and reverse, 5’-TCA AGA CAG AAC AGG AGC ACA GTG-3’. The primers of β-actin for internal control were as follows: forward, 5’-CAG CTT CGG AAC AAG AGA CC-3’; and reverse, 5’-GTC CGA TGA TTC CTG CTG AT-3’.

The PCR amplification mixture was adjusted to a final volume of 20 μl. Twenty-five cycles were performed at 94°C for 30 sec for denaturation, 58°C for 30 sec for annealing, and 72°C for 1 min for extension. The PCR products were separated on 1% agarose gel and then were stained with ethidium bromide.

Real-time reverse-transcription PCR quantification
One microliter of the resulting first strand cDNA was assayed by real-time reverse PCR (PRISM 7900HT, Applied Biosystems, Foster City, CA) according to a technical brochure of the company. The sets of primers and TaqMan probes were designed with a primer design software Primer Express version 2.0 (Applied Biosystems). The primers and TaqMan probes were as follows: The sequence of the forward primer for *survivin* mRNA was 5’-TTC AAG AAC TGG CCC TTC TTG-3’ and that of the reverse primer was 5’-TGG CTC CCA GCC TTC CA-3’; the TaqMan probe was FAM-CCT GCA CCC CGG AGC GGA T-TAMRA. For *XIAP* mRNA, the forward primer was 5’-GCC TTA GAC AGG CCA TCT GAG A-3’ and the reverse primer was 5’-TTC CTC GGG TAT ATG GTG TCT GAT-3’; the TaqMan probe was FAM-TGC AGA CTA TCT TTT GAG AAC TGG GCA GGT-TAMRA. For *cIAP1* mRNA, the forward primer was 5’-CAG ACA CAT GCA GCT CGA ATG-3’ and the reverse primer was 5’-AAG CCA CCA TCA CAA CAA AAG-3’; the TaqMan probe was FAM-TGT TCC AGT TCA GCC TGA GCA GCT TG-TAMRA. The forward primer for *GAPDH* mRNA was 5’-GAA GGT GAA GGT CGG AGT-3’ and the reverse primer was 5’-GAA GAT GGT GAT GGG ATT TC-3’; the TaqMan probe was FAM-CAA GCT TCC CGT TCT CAG CC-TAMRA. The conditions for the one-step RT-PCR were as follows: 2 min at 50°C and 10 min at 95°C and then 40 cycles of amplification for AmpliTaq Gold Activation at 95°C for 15 sec, annealing and extension at 60°C for 1 min. Human GAPDH was used for normalization. Quantification of the target gene expression was done using the comparative cycle threshold method according to the instructions of the manufacturer. All experiments were performed in triplicate for each data point. Each quantitation was performed with the standard curve method.

**Protein extraction and Western blotting**

Treatment with various concentrations of sodium arsenite, cells were harvested and lysed with RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na$_3$VO$_4$, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF]. The lysates were passed through a 21 G needle to break up the cell aggregates, and cleared by centrifugation at 14,000 × g for 15 min at 4°C and the supernatant (total cell lysate) was immediately used or stored at -80°C until use.
For the detection of NF-κB, the cells were washed with ice-cold PBS and lysed in 400 µl lysis buffer [buffer A, containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 0.6% Nonidet P-40]. The lysates were centrifuged at 250 × g for 10 min. The supernatant was collected as the cytosolic fraction. The pellets containing the nuclei were washed in buffer A without Nonidet P-40 and then were resuspended in 50 µl nuclear lysis buffer [buffer C, containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 2 mM EDTA, 1 mM DTT, and 1 mM PMSF], incubated for 30 min at 4°C, and centrifuged at 20,000 × g for 20 min. The supernatants were either used as nuclear fractions immediately or stored at -80°C until use. The protein concentration was determined by a DC Bio-Rad assay according to the manufacturer's protocol (Bio Rad Laboratories, Hercules, CA).

Lysates containing 100 µg of protein were subjected to 12.5% or 9.4% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then were transferred to Immobilon-P membrane (Millipore). The membrane was incubated with the primary antibody (dilution of 1:1,000) overnight at 4°C and then with a peroxidase-linked secondary antibody (dilution of 1:2,000) for 1 h at room temperature and proteins were visualized by enhanced chemiluminescence.

For detection of MRP1, 100 µg crude membranes prepared from Jurkat and S1T cells and 10 µg membrane vesicles prepared from KB/MRP which was used as a positive control were subjected to 7.5% SDS-PAGE and an m6 monoclonal antibody against MRP1 was used for MRP1 detection. The density of the bands for MRP1 was quantified using CCD camera (BIO-RAD Laboratories-Segrate, Milan, Italy).

Cell survival by the MTT assay

MT2 (2×10^4/ml), S1T and Jurkat cells (1×10^4/ml) were incubated either with or without various concentrations of sodium arsenite for the indicated time in 96-well plates. Ten µl of MTT (5 mg/ml) was added to each well and then the plates were incubated for an additional 4 h. The MTT formazan precipitate was dissolved in 100 µl of isopropanol containing 0.04 N HCl. The plates were shaken for 5 min and read immediately at 570 nm using a model 550 Micro Plate Reader (Bio-Rad, Richmond, CA,
USA).

For chemosensitivity *in vitro*, MT2 and S1T and Jurkat cells (1×10⁴/ml) were incubated in culture medium with various concentrations of indicated drugs at a final volume of 100 μl. After 3 days, 10 μl of MTT was added to each well and then the plates were incubated for an additional 4 h. OD570 was measured as above.

**Apoptosis analysis by FACS**

MT2, S1T and Jurkat cells (1×10⁶) were treated with various concentrations of sodium arsenite for various periods. The cells were harvested, washed once with PBS, suspended in 50 μl PBS and mixed with 50 μl of Coulter DNA-prep LRP (COULTER, Miami, FA) and then 1 ml of Coulter DNA-prep Stain was added. The mixtures were then incubated for 15 min at room temperature. The sub-G₁ fraction was determined using a Coulter FACSCAN (Beckman Dicson) as previously described.²²

**Caspase 3 activity assay**

Enzymatic reactions were carried out in the mixture containing 50 μg cell lysate treated by various concentrations of sodium arsenite, ICE-Like enzyme assay buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS), and 10 mM DTT. The reaction mixtures were incubated at 30°C for 30 min. Next, the 50 μM synthetic fluorogenic substrate, Ac-DEVD-MCA (Ac-Asp-Glu-Val-Asp-MCA), was added and incubated at 30°C for another 60 min. The activity of caspase 3 was measured at an excitation wavelength of 360 nm and an emission wavelength of 460 nm using a FP750 microplate fluorescence reader (Jasco, Tokyo, Japan).

**Statistical analysis**

All statistical analyses were performed by the Statview 5.0 software package for Windows. For ATL patients, differences in the numerical data between the two groups were evaluated using the Mann–Whitney *U*-test. A *p*-value of less than 0.05 was considered to be statistically significant.
For the MTT assay, the FACS analysis and the caspase 3 activity, the quantitative data were expressed as the mean ± SD. Statistical comparisons were performed using one-way ANOVA or the unpaired Student’s t-test. Differences were regarded as significant when the probability values were <0.01.
Results

Expression of mRNA for IAP proteins determined by real-time quantitative PCR or RT-PCR

Drug resistance of ATL is one of the main obstacles to successful chemotherapy. Kamihira et al. reported that survivin was prominently and consistently expressed in ATL, thus correlating with the ATL prognosis. However, the role of other IAP members such as IAP1, IAP2 and XIAP in the resistance of ATL to chemotherapy is uncertain. To quantitate the mRNA expression levels of the IAP family in ATL cells, real-time quantitative RT-PCR was performed. As shown in Fig. 1A, the expression of survivin was significantly higher \((p<0.01)\) in acute ATL, chronic and smoldering ATL than that in healthy controls. The expression of survivin in acute ATL was higher than that in chronic and smoldering ATL \((p<0.05)\). The expression of survivin in the performance status (PS) 3-4 ATL was higher than that in PS 0-2 ATL \((p<0.05)\). The mRNA level of \(cIAP1\), \(XIAP\) (Fig. 1B, C) did not differ substantially among the subtypes of ATL and the healthy controls, and between PS 0-2 and PS 3-4 ATls. The expression of \(cIAP2\) was also detected by RT-PCR, but the expression level in ATL did not increase significantly more than that in the control (data not shown). The patients’ age, sex, WBC number of ATL were not correlated with the expression of IAP proteins (data not shown). These results show that only survivin was overexpressed in ATL, thereby showing a correlation with the PS of the patients.

Protein expression of survivin in ATL

We next examined the expression levels of survivin in ATL cells from 17 patients by Western blotting (Fig. 2A), and compared with the survivin mRNA levels in ATL cells of each patient. As shown in Fig. 2B, the expression level of survivin mRNA was significantly correlated with that of the protein \((r=0.516, p=0.033)\). These results suggest that survivin alone is overexpressed among the members of the IAP family in ATL, especially in acute ATL, the survivin expression level was correlated with the ATL
progression (Fig. 1A).

**Down-regulation of survivin by sodium arsenite in ATL cells**

Arsenic trioxide (As$_2$O$_3$) was shown to have a therapeutic effect against acute promyelocytic leukemia (APL) and other types of haematological malignancy by decreasing the expression of BCL-2 and inducing apoptosis. It was recently reported that As$_2$O$_3$ combined with interferon α (IFN-α) could also induce apoptosis in ATL cells. We thus investigated whether sodium arsenite can regulate the expression of survivin in ATL cells. MT2 (Tax-positive) and S1T (Tax-negative), and a T-cell lymphoma cell line, Jurkat (as a control) cells were treated with 0, 2 and 5 μM sodium arsenite for 1, 2 and 3 days and the expression of survivin and BCL-2 was detected by Western blotting. α-Tubulin was used as a control for protein loading. The expression of survivin in MT2 and S1T cells dramatically decreased from 2 days after treatment with 2 and 5 μM sodium arsenite (Fig. 3A). The relative density of survivin compared with the untreated controls is shown in Fig. 3B. However, no clear down-regulation of survivin in Jurkat cells was detected (Fig. 3A, B). Sodium arsenite did not down-regulate BCL-2 expression in all three cell lines used in this study. The RNA level of survivin was also decreased by sodium arsenite treatment in S1T cells, but not in Jurkat cells (Fig. 3C). These data suggest that survivin is a target of sodium arsenite in ATL cells. Sodium arsenite could both dose- and time-dependently down-regulate the survivin expression at the RNA and protein levels in ATL cells.

**Effects of sodium arsenite on cell proliferation**

We next examined whether the growth inhibition of ATL cells by sodium arsenite correlates with the suppression of survivin expression. MT2, S1T and Jurkat cells were treated with 0, 0.5, 1, 2, 5 and 10 μM sodium arsenite for 5 - 7 days and cell proliferation was measured by an MTT assay. As shown in Fig. 4, the growth of MT2 and S1T cells was inhibited at a dose of 0.5 μM sodium arsenite (Fig. 4A, B). However, no significant growth suppression was observed at a dose of 2 μM sodium arsenite in Jurkat cells (Fig. 4C).
The dose-response curves of sodium arsenite for S1T and MT2 cells were compared with that for Jurkat cells. As shown in Fig. 4D, MT2 and S1T cells were more sensitive to sodium arsenite than Jurkat cells. IC₅₀ values (the concentrations of sodium arsenite that inhibit 50% of cell growth) for MT2, S1T and Jurkat cells were 2.17 ± 0.33, 1.99 ± 0.03 and 10.54 ± 0.17 μM, respectively. These results suggest that ATL cell lines, MT2 and S1T, are sensitive to sodium arsenite even at low concentrations, and the suppression of survivin expression is, at least in part, involved in the growth inhibition of the ATL cells.

Effects of sodium arsenite on apoptosis

To investigate whether the inhibition of cell proliferation was due to enhanced apoptosis, the proportion of sub-G₁ fraction was investigated in the presence of 0, 1, 2, 5 and 10 μM sodium arsenite for 1, 2, 3 days. Sodium arsenite at 1 μM did not increase the proportion of sub-G₁ fraction, but the proportion of sub-G₁ fraction both dose- and time-dependently increased at 2, 5, 10 μM in MT2 and S1T cells (Fig. 5A, B). However, the increase in the proportion of the sub-G₁ fraction of Jurkat cells treated with the same concentrations of sodium arsenite was less than that of the ATL cells (Fig. 5C). Sub-G₁ fraction in MT2 and S1T cells treated with various concentrations of sodium arsenite for 3 days was significantly higher than that in Jurkat cells (p < 0.01) (Fig. 5D). This result suggests that sodium arsenite inhibits the growth of ATL cells by inducing apoptosis.

Effects of sodium arsenite on caspases activation in ATL cells

Survivin was shown to bind directly with caspase 3 to inhibit the caspase activity in human cells exposed to apoptotic stimuli. We investigated whether caspases 3 are activated in ATL cells by treatment with sodium arsenite. As shown in Fig. 6, the caspases 3 activity increased in MT2 (Fig. 6A) and S1T (Fig. 6B) cells treated with 2 and 5 μM of sodium arsenite. The increased cleavage of PARP was also detected in MT2 and S1T cells treated with sodium arsenite (Fig. 3A). These results indicate that the down-regulation of survivin by sodium arsenite thus causes caspase 3-dependent cell death.
Decreased expression of survivin through the suppression of NF-κB activity by sodium arsenite

NF-κB is known to be constitutively activated in ATL. Either a Tax-dependent or Tax-independent mechanism of activation of the NF-κB pathway is crucial for the proliferation of malignant cells, protection from apoptosis and drug resistance in ATL. Tax regulates the expression of survivin through NF-κB pathway, and the combination of As$_2$O$_3$ and IFN-α decreases the activation of NF-κB in ATL cells. We therefore investigated the levels of nuclear p50 and p65 in ATL cells treated with sodium arsenite. As shown in Fig. 7A, sodium arsenite increased the level of IκB-α in MT2 cells. p50 and p65 in the nucleus of MT2 cells was decreased by sodium arsenite in a dose- and time-dependent manner. Cytosolic and nuclear survivin decreased in accordance with the decrease of nuclear p50 and p65. Although no significant decrease of p50 was seen in the nuclei of S1T cells, p65 in the nuclei dose-dependently decreased by sodium arsenite (Fig. 7B). These results suggest that sodium arsenite suppress the expression of survivin by preventing IκB-α degradation and the translocation of NF-κB into the nuclei.

Expression of MRP1 and the resistance to sodium arsenite in Jurkat cells

To elucidate the molecular basis for the difference in sensitivity to sodium arsenite between Jurkat and S1T cells, we examined the expression levels of MRP1 in these cells. MRP1 is involved in arsenite resistance and supposed to transport arsenite conjugated with glutathione. As shown in Fig. 8A, MRP1 was expressed in both Jurkat and S1T cells, but the expression level of MRP1 in Jurkat cells was about 3-fold higher than that in S1T cells. The higher molecular weight of MRP1 in S1T cells might be caused by a variation in glycosylation of MRP1 in the cells.

As shown in Fig. 8B, Jurkat cells were about 4-fold more resistant to sodium arsenite than S1T cells (IC$_{50}$ values of S1T and Jurkat cells for sodium arsenite were 1.99 ± 0.03 and 9.73 ± 0.71 μM, respectively). An inhibitor of MRP1, AG-A alone had no cytotoxic effect at 30 μM and decreased the IC$_{50}$ value of Jurkat cells for sodium arsenite to 4.03 ± 0.04 μM. A specific inhibitor of MRP1, MK571 at 30 μM completely abolished the difference in sensitivity to sodium arsenite between the two cells lines.
These findings suggest that the difference in sensitivity to sodium arsenite between Jurkat and S1T cells is attributed to the different expression levels of MRP1 in these cells.

**Discussion**

Patients with acute or lymphoma type ATL have a poor outcome with a median survival of about 6 months for acute type and 10 months for lymphoma and the expected 4-year survival is only about 5%. Combination chemotherapy regimens, especially those for the treatment of aggressive non-Hodgkin lymphoma or acute lymphoblastic leukemia, have little effect on ATL. The poor outcome might be due to multi-drug resistance in ATL cells. An overexpression of P-gp, MRP-1, LRP tends to be common features of the malignant cells and they confer intrinsic and acquired drug resistance on the cells. Another important mechanism of the resistance to apoptosis-inducing anticancer agents is the failure to activate apoptosis. Members of the IAP family are also overexpressed in many tumors and leukemia, and they also inhibit apoptosis. In order to find out the specific target for ATL therapy, we investigated the expression levels of the members of the IAP family, survivin, IAP1, IAP2, XIAP, in ATL cells from patients using real-time PCR or RT-PCR. Among them, survivin alone was overexpressed in ATL, especially in acute type ATL. The expression level of survivin in poor performance status (PS) was higher than that in good PS.

Survivin is broadly expressed in embryonic and fetal organs, but it becomes undetectable in most terminally differentiated normal tissues. Survivin was overexpressed in the majority of human tumor types. In gene-profiling studies, survivin was identified as the fourth ‘transcriptome’ expressed in the most common human cancers, but not in normal tissues. Retrospective trial studies suggest that the expression level of survivin contributes to the clinical outcome of tumors, including an abbreviated overall survival, increased rates of recurrences, resistance to therapy, and reduced apoptotic index. Recent studies using real-time PCR showed that high expression levels of *survivin* mRNA were a risk factor for prognosis of ATL in the clinical setting. These results are consistent with our results in this study.
There are some reports regarding the relationship between survivin expression and sensitivity to anticancer agents. Forced overexpression of survivin increased the resistance to paclitaxel in prostate cancer cell lines. The inhibition of survivin sensitizes prostate cancer cells to paclitaxel-induced apoptosis through caspase-dependant mechanism \textit{in vitro} and \textit{in vivo}.\textsuperscript{27} Down-regulation of survivin expression with ribozyme or siRNA increased sensitivity to topotecan and adriamycin in JR8 melanoma cell line and HL-60/ADR cells.\textsuperscript{28, 29} We have knocked down survivin in KB-3-1 cells using siRNA and found that the cells with the decreased level of survivin were more sensitive to doxorubicin, etoposide and sodium arsenite than the control KB-3-1 cells (data not shown). Survivin is an attractive therapeutic target in cancer for its differential expression in tumors versus normal tissues, and for its role in maintaining cancer-cell viability.\textsuperscript{25} A survivin-based therapy would be effective in removing the general cell-viability machinery exploited by cancer cells and it is also expected to have less side-effects since survivin is not detected in most normal tissues. Targeting survivin with antisense oligonucleotides,\textsuperscript{26} ribozymes\textsuperscript{28} or expression of dominant-negative mutants\textsuperscript{30} resulted in caspase-dependent cell death and suppression of tumor growth \textit{in vivo}. However, it remains difficult to apply these approaches to ATL therapy so far. In this study, we found, for the first time, that trivalent arsenite, sodium arsenite could down-regulate the expression of survivin at RNA and protein level in ATL cells. Sodium arsenite may provide a new avenue to suppressing survivin which is an attractive target for treatment of ATL patients.

\textit{As}_2\textit{O}_3\textsubscript{3} is very effective in the treatment of acute promyelocytic leukemia (APL), which carry the t(15;17) translocation involving the RAR-\textit{\alpha} and PML genes.\textsuperscript{31} \textit{As}_2\textit{O}_3\textsubscript{3} could also induce apoptosis in breast cancer, oesophageal carcinoma, multiple myeloma and ATL.\textsuperscript{32-35} It has recently been shown that \textit{As}_2\textit{O}_3\textsubscript{3} synergizes with IFN-\textit{\alpha} to induce cell-cycle arrest and apoptosis in cells infected with HTLV-1 and in the adult T-cell leukemia and lymphoma cells.\textsuperscript{36} Our study showed that sodium arsenite alone could suppress the growth and induce apoptosis in ATL cells. Sodium arsenite suppressed the growth of ATL cells at low concentrations and induced apoptosis of ATL cells at the relatively high concentration (2 \textmu M), at which concentration, the expression of survivin was down-regulated.

Survivin plays an important role in the suppression of mitochondria-dependent apoptosis by either
directly or indirectly inhibiting the activity of caspases. Complexes between survivin and caspase 9,37
caspase 3 or caspase 738 have been demonstrated. Survivin has also been shown to be associated with
Smac/DIABLO,39 a proapoptotic protein that is released from mitochondria and it prevents the inhibitory
effect of IAPs on caspase activation. Moreover, apoptosis induced by dominant-negative survivin
mutants have the characteristics of mitochondria-dependent apoptosis with cytochrome c release and
caspase-3 activation.40 These findings suggest that survivin protects apoptosis by interacting with
Smac/DIABLO and caspase 9. In the present study, caspase 3 was activated by sodium arsenite. It is
probable that sodium arsenite induced mitochondria-dependent apoptosis by decreasing the expression
level of survivin in ATL cells.

NF-kB is known to be constitutively activated in ATL. The Tax-dependent or Tax-independent
activation of the NF-kB pathway is crucial for proliferation, protection from apoptosis, and drug
resistance in adult T-cell leukemia and lymphoma. The most common p50-RelA (p65) dimer,
“specifically” known as NF-kB, is more abundant and controls the expression of more genes than any
other heterodimers or homodimers. NF-kB exists as an inactive cytoplasmic complex, predominantly
made up of p50-p65, bound to IkB-α, an inhibitory protein of the NF-kB. Recently, El-Sabban et al.
showed that IFN-α/As2O3 treatment significantly, and As2O3 alone slightly decreased the expression of
the viral transactivator protein Tax and repressed the activation of NF-kB pathway.41 As2O3 induced
apoptosis of HL-60 cells by repressing the constitutive activation of NF-kB.42 Moreover, Tax induced
survivin expression through NF-kB pathway.43 p50 and RelB were bound to NF-kB binding site in
survivin promoter between -354 and -345.43 Tax transactivated the survivin promoter through this
NF-kB binding site. Our results showed that the level of p65 and p50 in nuclei was decreased, while the
level of cytoplasmic IkB-α was increased by treatment with sodium arsenite in Tax-expressing MT2
cells. This was consistent with the studies of El-Sabban and Kawakami.41, 43 The NF-kB activity in the
cells that were not infected with HTLV-1 was also repressed by As2O3.42 In accordance with this report,
we found that sodium arsenite down-regulated the expression of survivin through the inhibition of
NF-kB pathway in S1T cells that did not express Tax. Sodium arsenite might suppress the NF-kB activity
by repressing the p65 translocation to nuclei, but not through Tax.

Sodium arsenite down-regulated the expression of survivin, but not of Bcl-2. Since there are a number of genes besides survivin and Bcl-2 that are up-regulated by NF-κB and involved in facilitating tumor cell survival, further study is needed to elucidate whether the down-regulation of these genes is also involved in apoptosis and decreased cell growth of ATL cells caused by sodium arsenite.

In this study, the concentration of sodium arsenite required to inhibit the growth of ATL cells in vitro was >0.5 μM and the concentration required to induce apoptosis was >2 μM. These concentrations appear to be beneficial and safe for clinical use in ATL patients. The plasma arsenic rapidly reached a mean maximum serum level of 6.85 μmol/L (range, 5.54 to 7.30 μmol/L) at 4 h after intravenous injection of 10 mg As₂O₃. A recent phase II trial in seven patients with a relapsed or refractory ATL has shown that the combination of IFN-α and As₂O₃ is a hopeful therapy for ATL. These preliminary results highlight that the treatment of ATL with As₂O₃ and IFN-α is feasible and has a clear antileukemic effect, even in patients with refractory disease. Further study is needed to clarify whether the combination of arsenite and IFN-α can down-regulate survivin more effectively, and whether arsenite can enhance the sensitivity of ATL cells to conventional anti-cancer agents.

T-cell leukemia Jurkat cells, which were used as a control, were more resistant to sodium arsenite than ATL cells. MRP1 is a member of the ATP binding cassette (ABC) superfamily of transport proteins and is involved in arsenite resistance. We have previously demonstrated the clinical significance of MRP1 expression and that high MRP1 expression correlated with short survival in patients with acute-type and lymphoma-type ATL. The expression level of MRP1 in Jurkat cells was about 3-fold higher than that in S1T cells and MK571, a specific inhibitor of MRP1, abolished the difference in sensitivity to sodium arsenite between the two cell lines. Since MRPI transported inorganic arsenic as a tri-GSH conjugate, we examined the GSH level in the cells and found that the level of GSH in Jurkat cells was similar to that in S1T cells (data not shown). These findings suggested that MRP1, at least in part, might be responsible for the decreased sensitivity to sodium arsenite of Jurkat cells.

In this study, we proved for the first time that arsenite could down-regulate survivin by repressing
NF-κB activation in ATL cells irrespective of the Tax expression. Our findings provide a rational basis for the new therapy for ATL using arsenite.

**Acknowledgements**

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(36) Bazarbachi A, El-Sabban ME, Nasr R, Quignon F, Awaraji C, Kersual J, Dianoux L, Zermati Y,


Figure Legends

**Fig. 1.** IAP family mRNA expression. A quantitative RT-PCR analysis for survivin (A), cIAP1 (B) and XIAP (C) is shown in acute ATL, chronic and smoldering ATL patients and healthy control(left), low PS and high PS (right). Each mRNA expression level was normalized on the basis of the GAPDH mRNA expression and expressed relative to the mRNA level in a healthy control. Boxes correspond to the interquartile range. Lines in the boxes represent median values. The vertical lines represent the 10th and 90th percentiles, and the circles represent the outliers. Differences were analyzed by Mann–Whitney’s U-test.

**Fig. 2.** Protein expression of survivin in ATL. Whole cell lysates (50 μg protein) from ATL patients were separated by 12.5% SDS-PAGE and then were transferred to a PVDF membrane. The transferred proteins were reacted with antibody against survivin (A) as described in the “Materials and Methods”. Concentrations of survivin protein and mRNA are expressed relative to the survivin protein and mRNA levels in control normal cells, which were assigned values of 1. (B) A comparison between the survivin mRNA level (Y-axis) and the protein expression level (X-axis) in 17 ATL patients. The survivin mRNA level correlated with the protein level (r=0.516, p=0.033)

**Fig. 3.** The effects of sodium arsenite on the survivin expression in ATL cells. (A) MT2, S1T, Jurkat cells were incubated in the presence of 2 or 5 μM arsenic trioxide for 24, 48 or 72 h. Whole cell lysates (100 μg protein) were prepared and separated by 12.5% SDS-PAGE and transferred to a PVDF membrane. The transferred proteins were reacted with the antibody against survivin, Bcl-2 or PARP as described in the “Materials and Methods”. As an internal control, α-tubulin expression was detected. (B) The quantification of the survivin levels in MT2, S1T and Jurkat cells. The relative density of the bands
for survivin obtained by a densitometric analysis and α-tubulin was used to 
normalize the respective intensities. (C) S1T and Jurkat cells were treated 
with sodium arsenite at the indicated concentration and time. Total RNA 
was then subjected to RT-PCR using primers specific for the amplification 
of survivin. β-Actin expression was examined as an internal control to 
ensure the RNA integrity and proper amplification.

**Fig. 4. Growth inhibition of MT2, S1T and Jurkat cells by sodium arsenite.** Proliferation of MT2 (A) S1T (B) and Jurkat (C) cells in the absence or presence of the indicated concentrations of sodium arsenite was assessed by MTT assay. (D) The sodium arsenite toxicity in MT2, S1T and Jurkat cells was determined by an MTT assay. The points represent the means of triplicate determinations, while the bars show SD.

**Fig. 5. Effects of sodium arsenite on the proportion of sub-G₁ fraction of MT2, S1T and Jurkat cells.** MT2, S1T and Jurkat cells were treated with 0, 1, 2, 5, 10 μM sodium arsenite for 1, 2, 3 days. The cells were then stained by propidium iodide (PI) and analyzed by flow cytometry. The proportion of sub-G₁ fraction of MT2 (A) and S1T (B) was higher than that of Jurkat (C) cells under the indicated concentrations of sodium arsenite and the indicated time. Each column and bar represents the mean ± SD of three independent experiments. (D) The sub-G₁ fraction of MT2, S1T and Jurkat cells treated with sodium arsenite for 3 days was compared. Each column and bar represents the mean ± SD of three independent experiments. *p < 0.01.

**Fig. 6. The activities of caspase-3 in MT2 and S1T cells in the absence or presence of sodium arsenite.** The caspase-3 activity was measured in the cell lysates using the specific substrate Ac-DEVD-MCA. The data are expressed in arbitrary units. Each value represents the mean of three independent experiments. Bars, SD. *p < 0.01.
**Fig. 7.** The effect of sodium arsenite on the levels of IκB-α, NF-κB and survivin in nuclear and cytosolic fraction from MT2 and S1T cells. (A) MT2 cells were treated with sodium arsenite at the indicated concentrations, nuclear and cytosolic fractions were prepared at the indicated time. Fifty micrograms of nuclear protein was subjected to Western blotting using the antibody against p50, p65 or survivin. Cytoplasmic fraction (100 μg of protein) was subjected to Western blotting using the specific antibody against IκB-α or survivin. α-Tubulin expression was used as a loading control. NE, nuclear extract; CE, cytoplasmic extract; *, non-specific band. (B) p50 and p65 in the nucleus and cytoplasmic survivin in S1T cells treated with sodium arsenite in the indicated concentration and time was measured by Western blot as above.

**Fig. 8.** Expression of MRP1 and the resistance to sodium arsenite in Jurkat cells. (A) Crude membranes (100 μg protein) were prepared and separated by 7.5% SDS-PAGE and transferred to a PVDF membrane. The transferred proteins were reacted with an antibody against MRP1 as described in the “Materials and Methods”. Ten microgram KB/MRP membrane vesicles were used as a positive control. (B) Jurkat and S1T cells were incubated with the indicated concentrations of drugs for 72h, and cell viability was determined by the MTT assay. The points represent the means of triplicate determinations, and the bars show SD.
### Table 1  Patient Characteristics

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**Fig. 1**

(A) Relative Survivin mRNA Expression

- Acute: n=21
- Chronic+: n=15
- Normal: n=17

- **p<0.001**
- *p=0.036**
- **p<0.001**

(B) Relative cIAP1 mRNA Expression

- Acute: n=23
- Chronic+: n=15
- Normal: n=17

- *p=0.496*
- *p=0.073*

(C) Relative XIAP mRNA Expression

- Acute: n=23
- Chronic+: n=15
- Normal: n=17

- *p=0.816*
- *p=0.282*
- *p=0.136*
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**Survivin**

**Fig. 2**

\[ Y = 59.352 + 20.116X \]

\[ r = 0.516 \]

\[ p = 0.033 \]
Fig. 3
Fig. 4
Fig. 5
Fig. 6

A

MT2

B

S1T

Fig. 6
Fig. 7
Fig. 8

A

B

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Overexpression of survivin in primary ATL cells and sodium arsenite induces apoptosis by down-regulating survivin expression in ATL cell lines

Xiao-Fang Che, Chun-Lei Zheng, Satsuki Owatari, Masato Mutoh, Takenari Gotanda, Hei-Cheul Jeung, Tatsuhiko Furukawa, Ryuji Ikeda, Masatatsu Yamamoto, Misako Haraguchi, Naomichi Arima and Shin-ichi Akiyama