Arsenic Trioxide Inhibits Human T Cell-Lymphotropic Virus-1-Induced Syncytiums by Down-Regulating gp46

Hiromi NABESHI,^{a,b} Tomoaki YOSHIKAWA,^{a,b} Haruhiko KAMADA,^{a,c} Hiroko SHIBATA,^{a,b} Toshiaki SUGITA,^{a,b} Yasuhiro ABE,^a Kazuya NAGANO,^{a,b} Tetsuya NOMURA,^{a,b} Kyoko MINOWA,^{a,d} Takuya YAMASHITA,^{a,b} Norio ITOH,^{a,b} Yasuo YOSHIOKA,^{a,c} Shin-ichi TSUNODA,^{*,a,c} and Yasuo TSUTSUMI^{a,b,c}

^aLaboratory of Pharmaceutical Proteomics, National Institute of Biomedical Innovation; 7–6–8 Saito-Asagi, Ibaraki, Osaka 567–0085, Japan; ^bGraduate School of Pharmaceutical Sciences, Osaka University; ^cThe Center for Advanced Medical Engineering and Informatics, Osaka University; 1–6 Yamadaoka, Suita, Osaka 565–0871, Japan; and ^dDepartment of Biopharmaceutics, Kyoto Pharmaceutical University; 5 Misasagi-Nakauchicho, Yamashina-ku, Kyoto 607–8414, Japan. Received January 16, 2009; accepted May 8, 2009; published online May 11, 2009

Adult T-cell leukemia (ATL) is a severe chemotherapy-resistant malignancy associated with prolonged infection by the human T cell-lymphotropic virus 1 (HTLV-1). One approach to prevent the onset of ATL is to inhibit the growth/transmission of HTLV-1 infected cells using arsenic trioxide (As₂O₃). However, there are no reports on the transmission inhibitory effect of As₂O₃. In this study, we reveal that As₂O₃ exerts an inhibitory effect on syncytium formation between HTLV-1 infected MT-2 and HeLa cells. In addition, Western blot analysis revealed that the HTLV-1 derived envelope protein gp46 was down regulated by As₂O₃ treatment, suggesting that As₂O₃ may inhibit HTLV-1 virus transmission via down-regulation of gp46. These results suggest that As₂O₃ may be a promising drug to treat refractory HTLV-1-related diseases.

Key words adult T-cell leukemia; human T cell-lymphotropic virus 1; arsenic trioxide; syncytium

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia (ATL).^{1,2)} ATL is characterized by malignant lympho-proliferation of mature activated T cells, predominantly CD4-positive T cells. This proliferation develops after a long period of latency following HTLV-1 infection. It is currently estimated that there are about twenty million HTLV-1 carriers worldwide. Of these, around 5% are at risk for ATL onset, and it has been reported that the incidence of ATL has been increasing. Epidemiological studies have indicated strongly that an increased HTLV-1 virus load is an important factor in ATL onset, although the detailed mechanisms of ATL onset are unknown. Therefore, an ideal anti-ATL agent would have both a growth inhibitory effect on ATL cells and an inhibitory effect on transmission of HTLV-1-infected cells. As₂O₃ show promising results in the treatment of patients with ATL in combination with interferon (IFN)- α .^{3–8)} Although As₂O₃-induced apoptosis is thought to play a major role in its observed therapeutic growth inhibitory effects in treating ATL,^{9,10)} the effects of As₂O₃ on HTLV-1 infection/transmission have not been reported.

HTLV-1 can be distinguished from all other retroviruses on the basis of the infection process: it is transmitted almost exclusively *via* cell-to-cell contact and cell-to-cell fusion (also termed syncytium formation), and the free viral particle is very poorly infectious.¹¹⁾ The HTLV-1 envelope glycoprotein is synthesized as a 61-kDa precursor that is cleaved into cell-surface (gp46) and transmembrane (gp21) proteins^{12,13)} and the gp46 product is thought to play an important role as the virus attachment protein. HTLV-1 can induce syncytium formation between infected cells and certain uninfected cell types^{14,15)} but this process can be blocked by antibodies against the gp46 protein or against gp46-derived peptides.^{16,17)} Thus, gp46 is a key protein for HTLV-1 transmission *via* syncytium formation, which is a critical process for HTLV-1 transmission *via* cell-to-cell contact.

In the present study, we reveal that HTLV-1-induced syncytium formation can be blocked by As₂O₃ treatment. Western blot analysis indicated that gp46 was down-regulated by As₂O₃ treatment. Our results suggest that As₂O₃ can prevent the HTLV-1 transmission by reducing levels of gp46, in addition to its therapeutic effect on ATL by promoting apoptosis. These results suggested that As₂O₃ may open new avenues for treating HTLV-1-related diseases, including ATL.

MATERIALS AND METHODS

Cell Culture The HTLV-1-infected cell line MT-2 (a kind gift from Hayashibara Biochemical Laboratories, Inc.) was grown in RPMI-1640 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% heat-inactivated fetal calf serum and 1% Antibiotic–Antimycotic Mix stock solution (Nacalai Tesque, Osaka, Japan) in a humidified atmosphere of 95% air/5% CO₂, at 37 °C. A HeLa cell line was maintained in minimum essential medium (MEM- α ; Wako) supplemented with 10% heat-inactivated fetal calf serum and 1% Antibiotic–Antimycotic Mix stock solution.

Detection of Syncytium Formation HeLa cells were plated at 8.0×10^4 cells per well onto 12-well plates (Corning, NY, U.S.A.) and incubated for 36 h. MT-2 cells were pre-incubated for 12 h with or without As₂O₃, following irradiation at 12000 rads. As₂O₃-treated and irradiated HTLV-1-infected cells were then added to HeLa cells seeded into a 12-well plate at a concentration of 8.0×10^4 viable cells per well. After 24 h co-culture, MT-2 cells were removed by washing with pre-warmed culture medium, and 200 μ l of Hoechst 33342 (Dojindo, Kumamoto, Japan; 20 μ g/ml) and 40 μ l of CFSE (Dojindo; 100 μ g/ml) were added to stain the nuclei and cytoplasm, respectively. After fixing with 4% paraformaldehyde, cells were analyzed using an ImageXpress^{MICRO} High Content Screening System (Molecular Devices, Sunnyvale, CA, U.S.A.) and a fluorescence micro-

* To whom correspondence should be addressed. e-mail: tsunoda@nibio.go.jp

scope. Syncytia containing >5 nuclei per cell were counted in triplicate wells.

Western Blots to Detect gp46 MT-2 was solubilised in IP lysis buffer (25 mM Tris-HCl pH 7.4, 100 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5% (w/v) Triton X-100, 0.1% (v/v) protease inhibitor cocktail (Sigma, St. Louis, MO, U.S.A.)). 2.5 μ g of cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The proteins were visualized by immunoblot analysis; primary and secondary antibodies were anti-HTLV-1 gp46 antibody (ZeptoMetrix, Buffalo, NY, U.S.A.; 1:5000 dilution) and peroxidase-conjugated anti-mouse IgG (H+L-chain) (MBL; 1:10000 dilution), respectively. As a loading control, beta actin was detected using anti-beta actin antibody (GeneTex, San Antonio, TX, U.S.A.; 1:80000 dilution).

RESULTS AND DISCUSSION

The infectivity of free HTLV-1 tends to be very poor, and the virus appears to be transmitted most efficiently by the cell-to-cell route. HTLV-1 infection is dependent upon the viral envelope glycoprotein-catalyzed fusion of the viral and cellular membranes.¹⁸ Like many other retroviruses, HTLV-1 can induce syncytium formation between infected cells and certain uninfected cell types. Thus, to determine whether As₂O₃-treatment inhibits HTLV-1 transmission, we first carried out a syncytium formation assay using As₂O₃-treated MT-2 cells and uninfected HeLa cells. As shown in Figs. 1A and B, a significant number of syncytia were observed when untreated MT-2 cells were co-cultured with HeLa cells. In contrast, this syncytium formation was significantly de-

creased when MT-2 cells were pre-treated with 10 μ M As₂O₃. The number of syncytia per field was 100 in As₂O₃-untreated MT-2 cells, but only 10 syncytia were observed in 10 μ M As₂O₃-treated cultures. We also assessed the viability of 10 μ M As₂O₃-treated MT-2 cells by WST-8 assay, and results indicated that there were no significant cytotoxicities (data not shown). Collectively, these results suggested that As₂O₃ had an inhibitory effect on syncytium formation mediated by HTLV-1 infected cell lines without inducing cytotoxicity.

It has been reported that the HTLV-1 envelope protein gp46 interacts with GLUT1, HSC70 and heparin sulfate. These interactions play an important role in HTLV-1 transmission *via* cell-to-cell contact between HTLV-1 infected cells.^{19–24} Therefore, we next performed Western blots using an anti-gp46 antibody to determine whether the expression level of gp46, a key protein involved in HTLV-1 transmission, was changed by As₂O₃ treatment. The gp46 expression level was significantly decreased in the MT-2 cell lines by As₂O₃ treatment, indicating that As₂O₃ treatment suppressed gp46 production. The HTLV-1 envelope protein is synthesized as a precursor protein (gp62), which is subsequently cleaved to a surface protein (gp46) and a transmembrane protein (gp21).^{12,13} Gp62 is modified by glycosylation events, proteolytically cleaved and inserted into the host cell membrane during virus particle assembly and release.^{25,26} It has been reported that inhibition of glycosylation, which is regulated by molecular chaperones, prevents both gp62 cleavage and syncytium formation.^{25,27–29} Because As₂O₃ treatment suppresses gp46 but not gp62 (Fig. 2), we speculated that it might inactivate the chaperone machinery. To clarify these mechanisms, we are now carrying out a proteomic analysis of As₂O₃-treated MT-2 cells. Previous reports^{25,27–29} indicated that as one of the factors of down-regulation of gp46 by As₂O₃ treatment was caused by oxidation of protein chaperones causing their dysfunction, leading to suppression of gp46 production. These findings contradict the observations of Andrews *et al.*, who reported that Na arsenite shock results in an increase in syncytium formation and gp62 expression. As₂O₃ reportedly exerts dose-dependent dual effects on APL. Therefore, the 8-fold difference in the arsenic ion concentration between their methods and our methods might influence the cellular responses.

In summary, we have demonstrated that As₂O₃ can inhibit HTLV-1 mediated syncytium formation, and that this is prob-

Fig. 2. As₂O₃ Treatment Down-Regulates gp46 Expression

Expression of gp46 and gp62 in untreated MT-2 cells and in MT-2 cells treated with As₂O₃ was detected by Western blot. β -Actin was used as a loading control.

Fig. 1. Syncytium Formation Assay

Irradiated HeLa cells were co-cultured with MT-2 cells (A, B), which were pre-incubated with or without As₂O₃. Nuclei and cytoplasm were stained with Hoechst 33342 and CFSE, respectively. (A) The effect of As₂O₃ on syncytium formation was observed under a fluorescence microscope ($\times 100$). (B) The number of syncytia containing >5 nuclei is the average of total syncytia per 147 mm² measured in three separate wells. The data represent means and S.D.s of triplicate wells.

ably due to suppression of gp46 production. Although more detailed studies are necessary to elucidate the causes of the observed effect on HTLV-1 transmission, it appears that dysfunction of chaperone proteins might contribute to the inhibitory effect of As₂O₃ treatment on syncytium formation. Thus, we indicated that As₂O₃ could inhibit HTLV-1 transmission as well as induce apoptosis to ATL cells. Because As₂O₃ have carcinogenesis, it is necessary for safety use of As₂O₃ to study the syncytium inhibition effect at low concentration. But, we believe that this study provides pertinent information related to the targeted discovery of drugs to treat ATL.

Acknowledgements This study was supported in part by some Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and Japan Society for the Promotion of Science (JSPS). And this study was also supported in part by some Health Labour Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan, in part by Health Sciences Research Grants for Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation. Finally, we would like to thank to the support form JSPS Research Fellowships for Young Scientists (No. 202324).

REFERENCES

- Hinuma Y., Nagata K., Hanaoka M., Nakai M., Matsumoto T., Kinoshita K. I., Shirakawa S., Miyoshi I., *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 6476—6480 (1981).
- Takatsuki K., Yamaguchi K., Kawano F., Hattori T., Nishimura H., Tsuda H., Sanada I., *Princess Takamatsu Symp.*, **15**, 51—57 (1984).
- Hermine O., Dombret H., Poupon J., Arnulf B., Lefrere F., Rousselot P., Damaj G., Delarue R., Femand J. P., Brouet J. C., Degos L., Varet B., de The H., Bazarbachi A., *Hematol. J.*, **5**, 130—134 (2004).
- Nasr R., Rosenwald A., El-Sabban M. E., Arnulf B., Zalloua P., Lepelletier Y., Bex F., Hermine O., Staudt L., de The H., Bazarbachi A., *Blood*, **101**, 4576—4582 (2003).
- Mahieux R., Pise-Masison C., Gessain A., Brady J. N., Olivier R., Perret E., Misteli T., Nicot C., *Blood*, **98**, 3762—3769 (2001).
- El-Sabban M. E., Nasr R., Dbaiibo G., Hermine O., Abboushi N., Quignon F., Ameisen J. C., Bex F., de The H., Bazarbachi A., *Blood*, **96**, 2849—2855 (2000).
- Bazarbachi A., El-Sabban M. E., Nasr R., Quignon F., Awaraji C., Kersual J., Dianoux L., Zermati Y., Haidar J. H., Hermine O., de The H., *Blood*, **93**, 278—283 (1999).
- Ishitsuka K., Hanada S., Suzuki S., Utsunomiya A., Chyuman Y., Takeuchi S., Takeshita T., Shimotakahara S., Uozumi K., Makino T., Arima T., *Br. J. Haematol.*, **103**, 721—728 (1998).
- Zhu J., Chen Z., Lallemand-Breitenbach V., de The H., *Nat. Rev. Cancer*, **2**, 705—713 (2002).
- Zhu Q., Zhang J. W., Zhu H. Q., Shen Y. L., Flexor M., Jia P. M., Yu Y., Cai X., Waxman S., Lanotte M., Chen S. J., Chen Z., Tong J. H., *Blood*, **99**, 1014—1022 (2002).
- Donegan E., Lee H., Operskalski E. A., Shaw G. M., Kleinman S. H., Busch M. P., Stevens C. E., Schiff E. R., Nowicki M. J., Hollingsworth C. G., *Transfusion*, **34**, 478—483 (1994).
- Hattori S., Kiyokawa T., Imagawa K., Shimizu F., Hashimura E., Seiki M., Yoshida M., *Virology*, **136**, 338—347 (1984).
- Schneider J., Yamamoto N., Hinuma Y., Hunsman G., *Virology*, **132**, 1—11 (1984).
- Hoshino H., Shimoyama M., Miwa M., Sugimura T., *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 7337—7341 (1983).
- Nagy K., Clapham P., Cheingsong-Popov R., Weiss R. A., *Int. J. Cancer*, **32**, 321—328 (1983).
- Brighty D., W. Jassal S. R., *J. Virol.*, **75**, 10472—10478 (2001).
- Tanaka Y., Zeng L., Shiraki H., Shida H., Tozawa H., *J. Immunol.*, **147**, 354—360 (1991).
- Jassal S. R., Lairmore M. D., Leigh-Brown A. J., Brighty D. W., *Virus Res.*, **78**, 17—34 (2001).
- Manel N., Kim F. J., Kinet S., Taylor N., Sitbon M., Battini J. L., *Cell*, **115**, 449—459 (2003).
- Coskun A. K., Sutton R. E., *J. Virol.*, **79**, 4150—4158 (2005).
- Fang D., Haraguchi Y., Jinno A., Soda Y., Shimizu N., Hoshino H., *Biochem. Biophys. Res. Commun.*, **261**, 357—363 (1999).
- Sagara Y., Ishida C., Inoue Y., Shiraki H., Maeda Y., *J. Virol.*, **72**, 535—541 (1998).
- Sagara Y., Inoue Y., Kojima E., Ishida C., Shiraki H., Maeda Y., *AIDS Res. Hum. Retroviruses*, **17**, 125—135 (2001).
- Pinon J. D., Klasse P. J., Jassal S. R., Welson S., Weber J., Brighty D. W., Sattentau Q. J., *J. Virol.*, **77**, 9922—9930 (2003).
- Paine E., Gu R., Ratner L., *Virology*, **199**, 331—338 (1994).
- Pique C., Pham D., Tursz T., Dokhelar M. C., *J. Virol.*, **66**, 906—913 (1992).
- Earl P. L., Moss B., Doms R. W., *J. Virol.*, **65**, 2047—2055 (1991).
- Otteken A., Earl P. L., Moss B., *J. Virol.*, **70**, 3407—3415 (1996).
- Land A., Braakman I., *Biochimie*, **83**, 783—790 (2001).
- Andrews J. M., Oglesbee M. J., Trevino A. V., Guyot D. J., Newbound G. C., Lairmore M. D., *Virology*, **208**, 816—820 (1995).
- Miller W. H. Jr., Schipper H. M., Lee J. S., Singer J., Waxman S., *Cancer Res.*, **62**, 3893—3903 (2002).