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Arsenic trioxide induces down-regulation of gp46 via protein oxidation: Proteomics analysis of oxidative modified proteins in As_2O_3-treated HTLV-1-infected cells

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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) retrovirus. Epidemiology studies strongly indicate that an increase in HTLV-1 virus load is an important factor during the onset of ATL. Therefore, inhibition of the growth/transmission of HTLV-1 infected cells is a promising strategy in preventing the disease. In our previous study, we revealed that arsenic trioxide (As_2O_3), a drug used to treat acute promyelocytic leukemia (APL), exerts an inhibitory effect on syncytium formation between HTLV-1 infected cells and HeLa cells *via* suppression of HTLV-1 envelope protein gp46 expression at low concentrations. In this study, we analyze the mechanism of action of As_2O_3 using a proteomics approach. Our results suggest that down-regulation of gp46 might be related to As_2O_3-induced oxidation of the 71-kDa heat shock cognate protein (HSC70) and the 78-kDa glucose-regulated protein (BiP/GRP78). We postulate that As_2O_3 exerts an inhibitory effect on HTLV-1 virus transmission *via* down-regulation of gp46-production, which might be caused by oxidative modification of various proteins such as chaperones.

1. Introduction

The human T-cell leukemia virus type 1 (HTLV-1) belongs to the delta-retrovirus family and is known as the etiologic agent of adult T-cell leukemia (ATL) (Hinuma et al. 1981; Takatsuki et al. 1984). ATL is caused by malignant lympho-proliferation of mature activated T-cells, mostly CD4 positive T cells, characterized by the appearance of tumor cells having a constricted nucleus called as a flower cell (Poiesz et al. 1980; Hinuma et al. 1981; Takatsuki 2005; Matsuoka and Jeang 2007). ATL develops after a long period of latency following HTLV-1 infection and transmission (Hinuma et al. 1981; Takatsuki et al. 1984). HTLV-1 infection and transmission results in the immortalization of the infected CD4 positive T cells; and this immortalization establishes a life-long persistent infection in a host (Miyoshi et al. 1981; Yamamoto et al. 1982). HTLV-1 carriers currently number 10-20 million people worldwide and are especially common in southwestern Japan, Africa, the Caribbean Islands and South America (Proietti et al. 2005). Around 5% of HTLV-1 carriers could potentially develop ATL.

Infectivity of free HTLV-1 tends to be uncommon. Indeed, HTLV-1 appears to be transmitted most efficiently by cell to cell contact and cell to cell fusion (also termed syncytium formation) (Donegan et al. 1994). HTLV-1 can induce syncytium formation between infected cells and certain uninfected cell types (Hoshino et al. 1983; Nagy et al. 1983). HTLV-1 infection is dependent upon viral envelope glycoprotein-catalyzed fusion of the viral and cellular membranes (Land and Braakman 2001). The HTLV-1 envelope glycoprotein, which plays important role as the virus attachment protein, is synthesized as a 61-kDa precursor that is cleaved to generate the cell-surface (gp46) and transmembrane (gp21) proteins (Hattori et al. 1984; Schneider et al. 1984). This syncytium formation can be blocked by antibodies against the gp46 protein and gp46-derived peptides (Tanaka et al. 1991; Brighty and Jassal 2001).

As_2O_3 has several effects on living cells, including: an increase in intercellular oxidant levels and the generation of reactive oxygen species (ROS) (Barchowsky et al. 1996, 1999; Pelicano et al. 2003); stimulation of apoptosis (Bazarbachi et al. 1999; El-Sabban et al. 2000; Mahieux et al. 2001) in several types of cells including ATL cells and HTLV-1 infected cells (Iwama et al. 2001; McCafferty-Grad et al. 2003). Moreover, As_2O_3 induces pleiotropic effects including oxidative stress and, at a low dose, partial differentiation (Zhu et al. 2002a,b). In our previous study, we showed that As_2O_3-treatment inhibits syncytium formation related to HTLV-1 transmission *via* suppression of gp46 expression (Nabeshi et al. 2009). Thus, As_2O_3 is a promising therapeutic agent for ATL. We reasoned that the inhibitory

Table: Identification of carbonylated proteins induced by treatment of MT-2 cells with As_2O_3

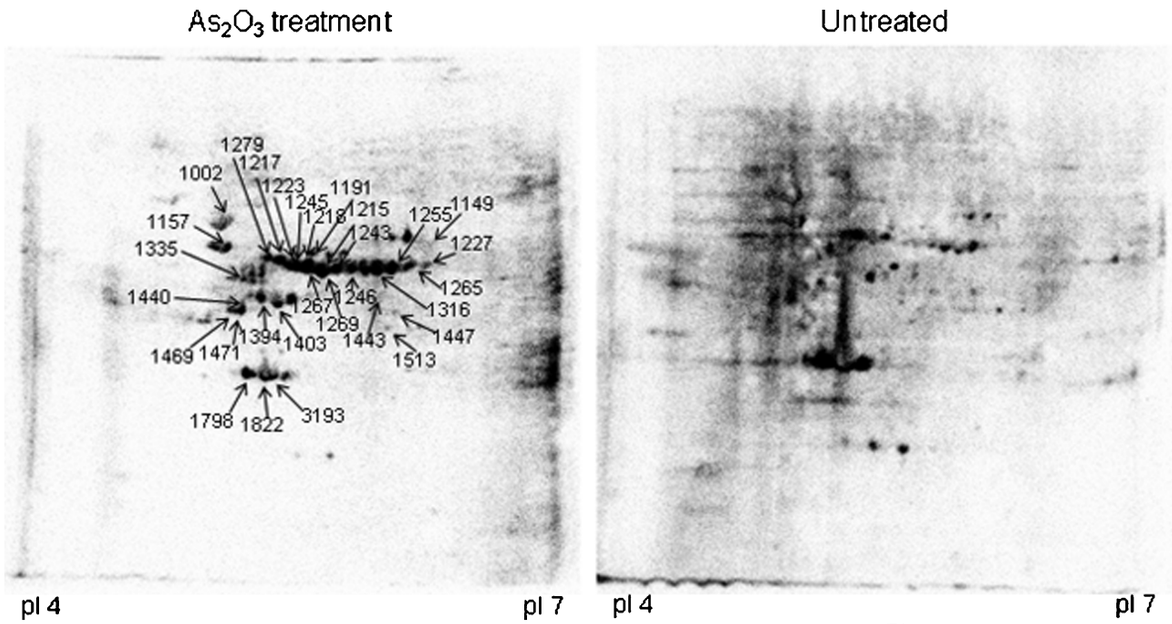
No.	Protein name	Accession No.	MW (Da)	pI	2D-DIGE
1002	Heat shock 90 kDa protein 1, beta	gi|20149594	83212	4.97	Up[1)]
1149	GARS protein (glycyl-tRNA synthetase classII)	gi|14043544	83087	6.61	NC[2)]
1157	Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	gi|16507237	72288	5.07	NC
1191	Heat shock 70 kDa protein 8 isoform 1 (HSC70)	gi|5729877	70854	5.37	NC
1215	Heat shock 70 kDa protein 1A variant	gi|62089222	77448	5.97	Up
1217	Lamin B1	gi|5031877	66368	5.11	NC
1218	Heat shock 70 kDa protein 1A	gi|62089222	77448	5.97	Up
1223	L-plastin	gi|4504965	70245	5.20	Up
1227	Unnamed protein product (HSP70 protein)	gi|35222	70810	5.67	Up
1243	Heat shock 70 kDa protein 1A variant	gi|62089222	77448	5.97	Up
1245	L-plastin isoform. 2	gi|114651555	63839	5.41	NC
1246	serum albumin	gi|28592	69321	6.05	Up
1255	Heat shock 70 kDa protein 6(HSP70 B')	gi|34419635	70984	5.81	Up
1265	dihydropyrimidinase-like 2 variant	gi|62087970	68142	5.85	Up
1267	lymphocyte cytosolic protein 1 (L-plustin)	gi|8217500	70244	5.29	Up
1269	HSP A 1A(HSP70)	gi|17511780	70009	5.48	Up
1279	Lamin B1	gi|5031877	66368	5.11	Down[3)]
1316	t-complex polypeptide 1	gi|36796	60356	6.03	Up
1335	serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit alpha isoform	gi|231443	65182	4.96	Up
1394	heat shock protein 60	gi|77702086	61174	5.70	Up
1403	HSP60	gi|77702086	61174	5.70	Up
1440	Tubulin-alpha 6	gi14389309	49863	4.96	Up
1443	protein disulfied isomerase ER6o precursor	gi|2135267	56716	6.23	NC
1447	protein disulfied isomerase ER6o precursor	gi|2135267	56716	6.23	NC
1469	Tubulin beta	gi|18088719	49640	4.75	NC
1471	Vimentin	gi|57471646	49623	5.19	NC
1513	Heterogeneous nuclear ribonucleoprotein H1	gi|5031753	49498	5.89	NC
1798	Actin, beta	gi|14250401	40978	5.56	NC
1822	Actin, beta	gi|14250401	40978	5.56	NC
3193	Actin beta	gi|14250401	40978	5.56	NC

[1)] Up: Up-regulated by As_2O_3-treatment (>1.5-fold); [2)] NC: No change between untreated and As_2O_3-treated cells; [3)] Down: Down-regulated by As_2O_3-treatment (<1.5-fold)

effect of gp46 expression and syncytium formation by treatment with As_2O_3 might be primarily caused by ROS-induced oxidative damage against cellular proteins. Oxidative stress by ROS causes the oxidation of intracellular genes and proteins, leading to carbonyl modifications of proteins. Carbonylation is a marker of protein oxidation, and can lead to degradation *via* the proteasome and loss of specific protein functions (Davies 1987; Oliver et al. 1987; Stadtman and Oliver 1991). However, the detection of oxidized protein does not explain how this might affect HTLV-1 mediated cell fusion and ultimately lead to syncytium formation. Here, we use an array of proteomics tools to perform a global analysis of changes in intracellular proteins of HTLV-1 infected cell lines in order to analyze the mechanism by which As_2O_3-treatment inhibits syncytium formation from the perspective of protein oxidation.

2. Investigations and results

In order to search for the target proteins of oxidation that are related to inhibition of syncytium formation, we performed global analysis of carbonylated proteins, a marker of protein oxidation, using a combined method of 2,4-dinitrophenylhydrazine (DNPH)-labeled protein carbonyls detection method and 2D-PAGE separation method (2D-Oxyblot). We detected carbonylated proteins from MT-2 cells treated with As_2O_3 (Fig. 1A) or untreated control cells (Fig.1B). Subsequent analysis identified carbonylated proteins that were up-regulated after As_2O_3-treatment. Approximately 1800 spots corresponding to carbonylated proteins were detected, of which 79 were increased by at least 1.5-fold in As_2O_3-treated cells compared with untreated control cells. The relevant spots were identified using matrix-assisted laser desorption ionization time-of-flight/time-of-flight tandem mass spectrometer (MALDI-TOF/TOF) and the results summarized in Table. Using this approach 20 different proteins were identified in MT-2 cells. Next, in order to confirm the changes of expression of As_2O_3-induced carbonylated proteins, we performed 2D-DIGE analysis with untreated and As_2O_3-treated MT-2 cells (Fig. 2). In total, approximately 3200 spots were detected. Comparison of the two cell extracts by image analysis showed that the intensity of about 980 spots were increased (>1.5-fold) and about 300 spots decreased (<1.5-fold) upon exposure to As_2O_3. The changes in the levels of As_2O_3-induced carbonyl protein spots are summarized in Table. Comparison of the result of 2D Oxyblots with the protein expression profile from 2D-DIGE analysis revealed that many, but not all, individual protein spots exhibit anti-protein carbonyl immunoreactivity. Furthermore, increases in anti-protein carbonyl immunoreactivity sometimes, but not always, paralleled increasing protein expression levels after treatment with As_2O_3 (Table). Among them, glycyl-tRNA synthetase class II, GRP78/BiP, HSC70, lamin B1, L-plastin isoform 2, protein disulfide isomerase ER60 precursor (PDI), tubulin beta, vimentin, heterogeneous nuclear ribonucleoprotein H1 and actin beta, showed unchanged expression levels but increased carbonylation, indicating these proteins were subject to oxidation. Next, among the As_2O_3-induced oxidation proteins, we focused on the increase in oxidation of HSC70. HSC70 is known to bind to HTLV-1 envelope protein (gp46 and gp62) corresponding to amino acids 197 to 216 (Sagara et al. 1998). Consequently, we analyzed whether As_2O_3-treatment affects the interaction of HSC70 with HTLV-1 envelope protein using an immunoprecip-



(A) (B)

Fig. 1: 2D-Oxyblot analysis of protein carbonylation induced by As_2O_3-treatment. Cell lysates from As_2O_3-treated MT-2 cells (A) were derivatized with DNP. Following 2D gel electrophoresis and transfer to PVDF, the derivatized proteins were detected using a DNP-specific antibody. Untreated MT-2 cells (B) were used as control. The arrows show As_2O_3-induced carbonylated proteins identified by MALDI-TOF/MS. The numbers correspond to the spots listed in Table

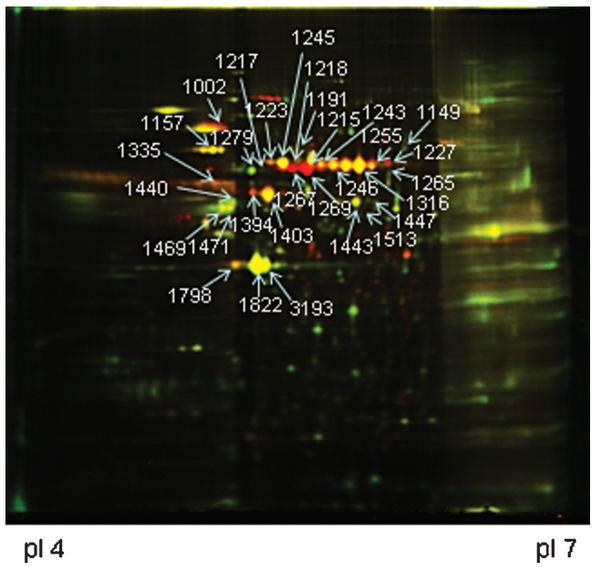


Fig. 2: 2D-DIGE analysis of protein expression alteration induced by As_2O_3-treatment. MT-2 cells were treated with As_2O_3 (final concentration 10 μ() for 48 h. Untreated control cells were prepared at the same time. Proteins were labeled with Cy3 (untreated), Cy5 (As_2O_3-treated), and Cy2 (mixture of untreated and As_2O_3-treated as internal reference (data not shown); see "Experimental") and separated on pH 4-7 IPG strips in the first dimension. This procedure was followed by separation on 12.5% SDS-polyacrylamide gels in the second dimension. Gels were subsequently scanned with a Typhoon Trio+ fluorescent scanner. Red and green spots represent proteins that were up- and down-regulated following As_2O_3-treatment, respectively. Yellow spots represent proteins that were equally abundant before and after As_2O_3-treatment. The arrows point to the 30 spots (*A*) identified as As_2O_3-induced carbonylated proteins in MT-2 cells. The numbers correspond to the spots listed in Table

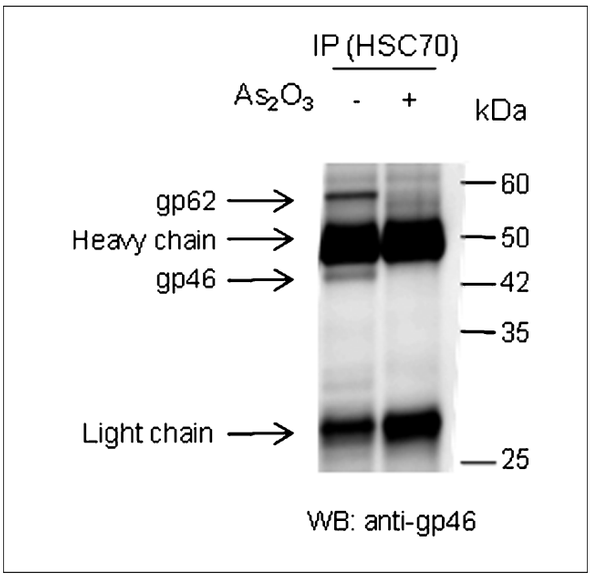
itation technique. Our results show that the binding of HTLV-1 envelope protein gp62 to HSC70 is significantly reduced following treatment with As_2O_3 (Fig. 3A). We used rat anti-HSC70 antibody for immunoprecipitation and mouse anti-gp46 antibody, which also recognizes gp62. Thus, the cross reactivity of the second antibody caused the heavy and light chain of anti-HSC70 antibody to appear in Fig. 3A. We verified that HSC70 protein recovered by immunoprecipitation was equal in each lane using biotinylated anti-HSC70 antibody (Fig. 3B). These results suggested that oxidative stress induced by As_2O_3-treatment might cause a loss of function of proteins.

3. Discussion

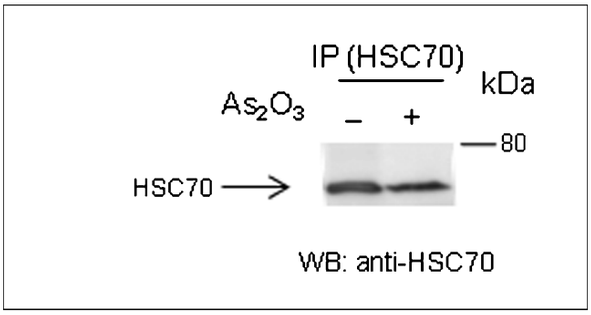
In our previous study, we revealed that As_2O_3-treatment induced down-regulation of gp46 expression. HTLV-1 envelope protein displayed on the infected cell surface is essential for syncytium formation (Nagy et al. 1983). Gp46 plays a very important role in HTLV-1 transmission, which is responsible for receptor recognition and interaction with HTLV-1 receptor candidate proteins, including ubiquitous glucose transporter GLUT1, HSC70 and heparan sulfate. These complexes facilitate cell-to-cell fusion events related to HTLV-1 transmission (Sagara et al. 1998, 2001; Manel et al. 2003; Coskun and Sutton 2005). Syncytium formation can be blocked by antibodies against the gp46 protein and gp46-derived peptides (Tanaka et al. 1991; Brighty and Jassal 2001). Taken together, these results suggest a decrease of gp46 is the main mechanism of inhibition of syncytium formation by As_2O_3.

In this study, we revealed for the first time that oxidative modification of HSC70 was increased upon treatment with As_2O_3. Moreover, in As_2O_3-treated cell lines the binding affinity of HTLV-1 envelope protein for HSC70 was greatly diminished. It is known that HSC70 is a major cytosolic component and belongs to the heat shock 70 kDa protein family and possess protein folding, unfolding and peptide binding capacity (Haug et al. 2007). HSC70 binds a number of proteins and mediates their correct folding and unfolding (Bercovich et al. 1997). Our findings indicate that As_2O_3-induced oxidative modification of HSC70 might induce a loss of chaperone function.

Furthermore, we showed that As_2O_3-treatment induced specific oxidative modifications of BiP/GRP78 as well as HSC70.



(A)



(B)

Fig. 3: As_2O_3-treatment induces inhibition of the interaction between HSC70 and gp46. As_2O_3-treated MT-2 cell lysate were immunoprecipitated with an anti-HSC70 monoclonal antibody using protein G beads. Precipitates were analyzed by Western blots using an anti-gp46 antibody (A) and an anti-HSC70 antibody (B)

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) (Hattori et al. 1984; Schneider et al. 1984). gp62 is modified by glycosylation events, proteolytically cleaved and inserted into the host cell membrane during virus particle assembly and release (Pique et al. 1992; Paine et al. 1994). It has been reported that inhibition of glycosylation induces a lack of both gp62 cleavage and syncytium formation (Pique et al. 1992). Interestingly, several researchers suggested that the maturation of glycoproteins, such as HIV-1 envelope glycoprotein gp160, is regulated by molecular chaperones including BiP/GRP78 in the endoplasmic reticulum (Earl et al. 1991; Otteken et al. 1996; Land and Braakman 2001). Our results suggest that As_2O_3-treatment induces oxidative modification of BiP/GRP78 (Fig. 3). We propose that As_2O_3 mediated down-regulation of gp46 may be caused by oxidative modification of BiP/GRP78 causing inhibition of glycosylation.

Intriguingly, microtubule and actin filaments were drastically oxidized by As_2O_3-treatment. We are focused on clarifying the relationship between oxidative modification of these cytoskeletal proteins and syncytium formation. Recently, Igakura et al. reported that cell-cell contact rapidly induces reorganization of the microtubule organizing center (MTOC) and actin cytoskeleton around the cell-cell junction. Moreover, the same group showed that HTLV-I core (Gag protein) complexes and the HTLV-I genome accumulates at the cell-cell junction, which are then transferred to the uninfected cell (Igakura et al. 2003; Piguet and Sattentau 2004). This interface is called a virological synapse (VS), similar to the immunological synapse formed between T-cells or natural killer cells and antigen presenting cells (Grakoui et al. 1999; Davis 2002). Microtubule and actin filaments play an important role in transporting proteins and structural proteins inside the cell during MTOC reorganization. Thus, microtubule and actin filaments are also considered to be crucial in HTLV-1 transmission *via* VS. Furthermore, it was reported that microtubule formation and actin filament formation is inhibited by oxidative modification of tubulin and actin (Tuma et al. 1991; DalleDonne et al. 1995). We propose that As_2O_3-induced oxidative modification of tubulin beta and actin beta might lead to inhibition of transportation and accumulation of virus proteins at the cell-cell junction.

In summary, we firstly demonstrated that As_2O_3 inhibits HTLV-I mediated syncytium formation. We believe the inhibition of syncytium formation is probably mediated by oxidative modification of HSC70 and BiP/GRP78, which interferes with gp46 maturation. Although more detailed studies are necessary to elucidate the relationship between HTLV-1 transmission and the oxidation specific increase of actin beta, tubulin-beta and heat shock proteins, these proteins might be involved in the As_2O_3-mediated inhibitory effect on syncytium formation. Furthermore, with the correct dosing regimen, we believe that As_2O_3 could be a promising preventive treatment for ATL in the future. For example, (1) As_2O_3 has been approved as the medicine for acute promyelocytic leukemia (APL), (2) half-life of As_2O_3 is about 100 hours and As_2O_3 is metabolized to dimethylarsinic acid and excreted in the urine, (3) As_2O_3 is included in orally-available traditional Chinese remedy called Ai-Lin I, which triggered the original discovery of As_2O_3 effect for APL (Sears 1988; Hu et al. 1999). Thus, we believe that this study provides useful information for the target discovery of anti-ATL drugs and will assist in the development of novel anti-ATL preventive drugs.

4. Experimental

4.1. 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% CO_2 at 37 °C.

4.2. Identification of protein oxidation (2D-Oxyblot)

10^7 cells of MT-2 were seeded on 10 cm tissue culture dishes (ASAHI GLASS CO. LTD., Chiba, Japan) and then treated with As_2O_3 (final concentration 10 μM) for 48 h. As_2O_3-treated cells and untreated control cells were solubilized by adding 500 μl of 2D sample buffer (7 M urea, 2 M thiourea, 30 mM Tris-HCl, and 4% (w/v) CHAPS, pH 8.5). The 2D-DIGE technique involves extraction of proteins from the whole cell, with the exception of some plasma membrane proteins. To identify carbonyl groups that were introduced into amino acid side chains after oxidative modification of proteins, 2D-oxyblot analysis was performed. In this method, the derivative produced by reaction with 2,4-dinitrophenylhydrazine (DNPH, Chemicon International Inc., Temecula, CA) were immunodetected by an antibody specific to the attached DNP moiety of proteins using the Oxyblot Oxidation Detection kit (Chemicon International Inc.). Briefly, the electrophoresis was performed as described earlier, and the proteins in the gels were transferred to a PVDF membrane using a semidry transfer system (GE Healthcare, Tokyo, Japan). Membranes were blocked in a buffer (50 mM Tris-HCl (pH 7.5), 154 mM NaCl, 0.1% Tween-20) containing 1% BSA for 1 h, and then incubated with a rabbit anti-DNP antibody (1:150; Chemicon International Inc.) for 1 h at room temperature. Incubation with secondary antibody was performed using horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:300; Chemicon International Inc.) for 1 h at room temperature.

Immunoreactivity was visualized by enhanced chemiluminescence using ECL plus (GE Healthcare). After scanning the chemiluminescence image, membranes were restained with Deep Purple Total Protein Stain (GE Healthcare). The Deep Purple staining protocol involved washing membranes in deionized water for 5 min on a rocking platform shaker, followed by a wash in 200 mM Na_2CO_3 for 5 min. The membranes were then placed in 100 ml of fresh deionized water together with 500 µl of Deep purple and stained in the dark on a rocking platform for 15 min. Next, the membranes were destained with 7.5% v/v acetic acid for 5 min and 100% methanol for 3 min.

4.3. Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE)

Cell lysates were prepared by the same protocol as 2D-oxyblot. 50 µg of each sample were labeled for DIGE analysis using CyDye DIGE fluors according to the manufacturer's guidelines (GE Healthcare). Briefly, samples were labeled using Cy5 or Cy3 N-hydroxysuccinamide ester DIGE dyes freshly dissolved in anhydrous dimethylformamide (Sigma-Aldrich, St. Louis, MO) by mixing 50 µg proteins with 1 µl of 400 µM CyDye. Equal volumes of samples from As_2O_3-treated and -untreated cells, containing 25 µg protein each, were mixed and labeled with Cy2 as an internal control. In each case, the labeling reaction was allowed to proceed on ice in the dark for 30 min. The reaction was terminated by the addition of 10 nmol lysine and subsequent incubation on ice in the dark for 10 min.

The three labeled samples were mixed together and run on the same gel. For the first dimension, a mixture of Cy2-, Cy3- and Cy5-labeled samples were combined. Rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 40 mM dithiothreitol (DTT), 0.5% (v/v), IPG Buffer (pH 3-11 NL: GE-Healthcare) and 0.002% (w/v) bromophenol blue) was added to give a total volume of 250 µl. The sample was then loaded onto a 13 cm Immobiline DryStrip gel (pH 4-7 non-linear: GE-Healthcare) by passive rehydration for a minimum of 10 h. Following rehydration, isoelectric focusing was performed using IPGphor by applying 500 V for 4 h, 1,000 V for 1 h, and 8,000 V for 5.5 h until a total of 64,000 Vh had been achieved. After isoelectric focusing, strips were equilibrated in SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 0.002% (w/v) bromophenol blue) containing 1% (w/v) DTT for 15 min at room temperature followed by an incubation in SDS equilibration buffer containing 2.5% (w/v) iodoacetamide for 15 min at room temperature. After equilibration, the strips were applied to a 10% (w/v) SDS-PAGE gel and run at 25 mA/gel on the SE-600 system (GE Healthcare) until the blue dye front reached the bottom of the gel.

4.4. Analysis of gel images

A Typhoon TRIO+ variable mode imager (GE Healthcare) was used to analyze gels and membranes to compare protein and carbonyl content between control and As_2O_3-treated samples. Images were then analyzed using DeCyder Differential In Gel Analysis V4.0 software (GE Healthcare) to identify whether the intensity of each spot changed after As_2O_3-treatment.

4.5. In-gel tryptic digestion

A preparative gel was prepared and 500 µg each of unlabeled (untreated) and As_2O_3-treated proteins were loaded per gel. Then, electrophoresis the gel was stained for total protein using Deep Purple Total Protein Stain (GE Healthcare). Selected protein spots were cut from the gel using an automated 2D spot picker (Ettan Spot Picker; GE Healthcare) and digested with trypsin as described below. The gel pieces were then destained with 50% acetonitrile/50 mM NH_4HCO_3 for 2 × 20 min, dehydrated with 75% acetonitrile for 20 min, and dried using a contrifugal concentrator (Tomy, Tokyo, Japan). Next, 5 µl of 20 µl/ml trypsin (Promega Corp., Madison, WI) solution was added to each gel piece and then incubated for 16 h at 37 °C. We used three solutions to extract the resulting peptide mixtures from the gel pieces. First, 50 µl of 50% (v/v) acetonitrile in 1% (v/v) aqueous trifluoroacetic acid (TFA) was added to the gel pieces, which were then sonicated for 5 min. Next, we collected the solution and added 80% (v/v) acetonitrile in 0.2% TFA. Finally, 100% acetonitrile was added for the last extraction. The peptides were dried and then resuspended in 10 µl of 0.1% TFA before being cleaned using ZipTip^{TM} µC_{18} pipette tips (Millipore Corporation, Temecula, CA). The tips were wetted with three washes in 50% acetonitrile and equilibrated with three washes in 0.1% TFA, then the peptides were aspirated 10 times to ensure binding to the column. The column and peptides were washed three times in 0.1% TFA before being eluted in 1 µl of 80% acetonitrile/ 0.2% TFA.

4.6. Mass spectrometry and database search

The tryptic digests (0.6 µl) were mixed with 0.6 µl α-cyano-4-hydroxy-trans-cinnamic acid (HCCA) saturated in a 0.1% trifluoroacetic acid and acetonitrile solution (1:1 vol/vol). The mixture was deposited onto each well of the 96-well target plate. Samples were then analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS; autoflexII, Bruker Daltonics, Bremen, Germany) in the Reflectron mode. The mass axis was adjusted with a calibration peptide (Bruker Daltonics) peaks (M/z 1047.19, 1296.68, or 2465.19) as lock masses. Bioinformatic databases were searched to identify the proteins based on the tryptic fragment sizes. The Mascot search engine (http://www.matrixscience.com) was initially used to query the entire theoretical tryptic peptide database in the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/, a public domain database provided by the National Institutes of Health, Bethesda, MD) as well as SwissProt (http://www.expasy.org/, a public domain database provided by the Swiss Institute of Bioinformatics, Geneva, Switzerland). The search query assumed that (i) the peptides were monoisotopic (ii) they might be oxidized at methionine residues, and (iii) all cysteines are modified by iodoacetamide.

4.7. Immunoprecipitation (IP) assay

500 µg of MT-2 cell lysates in IP lysis buffer (25 mM Tris-HCl (pH7.4), 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) were mixed with 7 µg precipitating antibody (anti-HSC70 monoclonal antibody; Stressgen Biotechnologies, Victoria, BC, Canada) and incubated overnight at 4 °C on a rotator. Protein G plus agarose (Sigma-Aldrich; 50 µl) was added next and the mixtures incubated at 4 °C on a rotator for 1 h. The immunoprecipitates were collected by centrifugation and washed three times with lysis buffer. The samples were resolved by SDS-PAGE, transferred to a PVDF membrane, and HTLV-1 envelope protein (gp62 and gp46) visualized by Western blotting using anti-HTLV-1 gp46 antibody. After detection, the antibody was stripped away using the Re-Blot Plus Western Blot Recycling Kit (Chemicon International Inc.). The membrane was incubated in 1 × Mild Antibody Stripping Solution with gentle mixing for 15 min at room temperature. The membrane was washed briefly (2 × 5 min) in blocking buffer and then soaked in blocking buffer for 1 h. For detection of HSC70 protein using the stripped membrane, the Western blot primary and secondary antibodies were anti-HSC70 Biotin Conjugate (Stressgen Biotechnologies; 1:2000 dilution) and HRP-Streptavidine Conjugate (1:5000 dilution), respectively.

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