Moesin Is a Biomarker for the Assessment of Genotoxic Carcinogens in Mouse Lymphoma

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1,2-Dibromoethane and glycidol are well known genotoxic carcinogens, which have been widely used in industry. To identify a specific biomarker for these carcinogens in cells, the cellular proteome of L5178Y mouse lymphoma cells treated with these compounds was analyzed by 2-dimensional gel electrophoresis (2-DE) and MALDI-TOF mass spectrometry (MS). Of 50 protein spots showing a greater than 1.5-fold increase or decrease in intensity compared to control cells on a 2-D gel, we focused on the candidate biomarker moesin. Western analysis using monoclonal rabbit anti-moesin confirmed the identity of the protein and its increased level of expression upon exposure to the carcinogenic compounds. Moesin expression also increased in cells treated with six additional genotoxic carcinogens, verifying that moesin could serve as a biomarker to monitor phenotypic change upon exposure to genotoxic carcinogens in L5178Y mouse lymphoma cells.

INTRODUCTION

Carcinogens are substances that are capable of causing cancer in humans or animals and are generally classified into two groups, genotoxic carcinogens and nongenotoxic carcinogens (van Delft et al., 2004). Especially, genotoxic carcinogens are compounds that have potential to induce cancer by altering the genetic material directly (Hayashi, 1992). Compounds of this class damage chromosomes or DNA in cells and are evaluated based on their effects on mutagenesis, chromosome aberrations, DNA strand breakage, and DNA repair. Since genotoxic compounds are known to be potent mutagens or carcinogens, methods to monitor this class of compounds during the process of drug development are necessary because molecules could have the potential for causing toxicity or undesirable side effects. The Ames test and the micronucleus formation assay have been commonly used for the assessment of genotoxicity and carcinogenicity. Recently, the method linked with proteins, the real players for biological activity, has attracted as a new and complementary means to provide an efficient monitoring of the chemicals in a number of applications, including drug development and environmental toxicology.

Toxicoproteomics is an emerging field which aims to identify critical proteins and pathways in biological systems that are affected by and respond to adverse chemicals and environmental exposures using global protein expression technologies (Bandara and Kennedy, 2002). Proteome profiling permits the screening and selection of candidate biomarkers based on the differential expression of the proteins of interest. Selected proteins can also be validated by using molecular and cell biological tools. Biomarkers discovered by proteomics could be applied for the assessment of toxicity and safety of compounds, immunohistochemistry, and reducing animal test, a common method of safety assessment.

In this study, we used L5178Y mouse lymphoma cells and two well-known genotoxic carcinogens, 1,2-dibromoethane and glycidol, to identify a candidate biomarker for screening genotoxic carcinogens. L5178Y mouse lymphoma cells are commonly used in chemical testing because of the high sensitivity to DNA damage due to loss of cell cycle control (Clark et al., 1998). 1,2-Dibromoethane is widely used as a soil fumigant, a gasoline additive, and an industrial solvent (Fishbain and Vilasuso, 1980). 1,2-Dibromoethane is known to cause liver and kidney toxicity and it has been categorized as a carcinogen (Humphreys et al., 1999). In addition, 1,2-dibromoethane is a genotoxic compound, as verified by the Ames test (Kim et al., 2006a). 1,2-Dibromoethane affects gene mutation, recessive lethal mutation, and mitotic recombination. Glycidol is another genotoxic carcinogen and has been used as a stabilizer in the manufacture of vinyl polymers; it is an intermediate in pharmaceutical production and is also used as a diluting agent (Yamamoto et al., 2001). The carcinogenicity of glycidol has been evaluated by animal test (Lijinsky and Kovatch, 1992) and its genotoxicity has been confirmed by the Ames test (Kim et al., 2006b).

We investigated the consequence of exposure of L5178Y mouse lymphoma cells to these two established genotoxic carcinogens using 2-DE and MALDI-TOF MS and identified moesin as a candidate biomarker for genotoxic carcinogens. The biomarker protein was further validated by western analysis and by examination of its expression level in cells treated with additional genotoxic carcinogens. Collectively, we report for the first time that moesin could serve as a candidate bio-

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marker to monitor exposure to genotoxic carcinogens in L5178Y mouse lymphoma cells.

MATERIALS AND METHODS

Chemicals and reagents

1,2-Dibromoethane, glycidol, methylcarbamate, *O*-nitrotoluene, and TCDD were purchased from Accustandard Inc. (USA). Diethylstilbestrol was purchased from Fluka (Switzerland). 1,4-Dioxane, tetrachloroethylene, and nitromethane were purchased from Sigma-Aldrich (Italy). Urethane, chlorambucil, dibenz (a,h)anthracene, methyl methanesulfonate, and *N*-nitroso-*N*-methylurea were obtained from Sigma (USA). Chloroprene was purchased from Fisher Scientific (USA). Nitrobenzene was purchased from Aldrich (USA). RPMI 1640, antibiotics, and horse serum were purchased from Invitrogen (USA). PVDF membranes and the ECL kit were purchased from Millipore (USA) and GE Healthcare (UK), respectively. Rabbit anti-moesin monoclonal antibodies were obtained from Epitomics (USA), mouse anti- β -actin monoclonal antibodies were from Abcam (UK). HRP-conjugated sheep anti-mouse and donkey anti-rabbit were purchased from GE Healthcare (UK).

Cell culture and sample preparation

L5178Y mouse lymphoma cells were grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated horse serum and 1% antibiotics at 37°C in a humidified atmosphere of 5% CO₂/95% air. Cells were seeded in T75 flasks under the conditions described above and incubated for 2 h. After 2 h incubation, followed by addition of test compounds and an additional 2 h incubation. Treated cells were harvested and test compounds were removed by centrifugation at 3,000 rpm in RPMI. Cells were reseeded in T75 flasks, incubated for 22 h, and harvested for sample preparation.

2-DE

2-DE was conducted as described previously (Cho et al., 2005). Briefly, 1.5 volumes of sample buffer (40 mM Tris, pH 8.8, containing 7 M urea, 2 M thiourea, 100 mM DTE, 4.55% CHAPS, and protease inhibitor cocktails) was added to cells. Samples were applied to an immobilized pH 3-10 nonlinear gradient strip. Isoelectric focusing was carried out in the first dimension at 80,000 Vh and molecular weight determination was carried out in the second dimension in 9-17% linear gradient polyacrylamide gels at a constant current of 40 mA/gel for approximately 5 h. Following electrophoresis gels were fixed in 40% methanol/5% phosphoric acid for 1 h, stained with Coomassie Brilliant Blue G-250 for 12 h, and destained in distilled water. Gels were scanned with a GS-710 imaging densitometer (Bio-Rad) and converted to electronic files. Images were analyzed with the Image Master Platinum 5 program (GE Healthcare).

Identification of proteins by MS

For 2-D gel mapping of the compound-treated proteome, spots were identified by peptide mass fingerprinting (Lu et al., 2009). Protein spots excised from 2-D gels were destained, reduced, alkylated, and digested with trypsin. Trypsin-digested peptides were desalted using a porous resin and purified. Trypsin digestion and desalting were carried out as previously described (Cho et al., 2005). Peptides were prepared for MALDI-TOF MS by mixing with matrix (alpha-cyano-4-hydroxy cinnamic acid, CHCA), 2% formic acid in 70% acetonitrile, and droplets were allowed to dry on the MALDI plate (Opti-TOF™ 384 well Insert, Applied Biosystems). MALDI-TOF MS was performed on a 4800 MALDI-TOF/TOF™ Analyzer (Applied Biosystems) and

the mass spectra were obtained in the reflectron mode with an accelerating voltage of 20 kV and sum from either 500 laser pulses and calibrated using the 4700 calibration mixture (Applied Biosystems). Data Explorer 4.4 (PerSeptive Biosystems) was used for data acquisition and extraction of the monoisotopic masses. NCBI human protein database (<http://www.ncbi.nlm.nih.gov>) searching was performed with the MASCOT search engine (<http://www.matrixscience.com>).

Western analysis

L5178Y mouse lymphoma cells were seeded at 1×10^6 cells/ml in 6-well plates and treated with test compounds for 2 h. Proteins present in the cell lysates were separated by 10% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 3% skim milk in 1X Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature followed by overnight incubation at 4°C with the primary antibodies rabbit monoclonal anti-moesin and mouse monoclonal anti- β -actin. On the following day membranes were washed with TBS-T and incubated for 1 h with either HRP-conjugated anti-mouse or anti-rabbit secondary antibody. Immunoreactivity was assessed by ECL.

Statistical analysis

Results are expressed as the mean $\pm$ standard deviation. Student's *t*-test was used to determine statistical significance between control and test groups. $P < 0.05$ was considered statistically significant.

RESULTS

Proteomic analysis of L5178Y mouse lymphoma cells treated with 1,2-dibromoethane and glycidol

L5178Y mouse lymphoma cells were treated with 1,2-dibromoethane and glycidol at concentrations of 100 and 400 μ g/ml, respectively, followed by extraction of proteins for two dimensional separation. The effective concentrations of test compounds were determined by cytotoxicity test (Go and Sheen, 2008). Briefly, three doses of test compounds were treated to L5178Y mouse lymphoma cells for 2 h. The 80% of cell viability was considered as a standard and the cells were incubated in normal media. After 22 h incubation, doses which showed more than 60% of cell viability were defined as the effective concentrations of each compound. Proteins were separated by 2-DE and image analysis of each gel was carried out. Fifty spots showing greater than 1.5-fold change in intensity following treatment with genotoxic carcinogens compare to solvent control were selected (Fig. 1). The 50 proteins were identified by MALDI-TOF MS and listed in Table 1. Unnamed, hypothetical, and highly abundant proteins like immunoglobulins or keratin were excluded in the Table.

Among the identified proteins, moesin was chosen for validation of the result of proteomics analysis, because moesin expression level could be correlated with genotoxic carcinogenic potential. Moesin is a member of the ERM (ezrin/radixin/moesin) family of proteins. ERM family proteins serve as linkers between plasma membranes and the cytoskeleton. According to previous studies moesin is expressed in about 31% of all cancer types. Moesin expression is indirectly related to thyroid cancer and oral squamous carcinoma as well as being a marker of breast cancer (Charafe-Jauffret et al., 2007; Krawetz et al., 2006; Seabrooke and Stewart, 2008).

Fig. 1. The 2-D gel of proteins isolated from L5178Y mouse lymphoma cells treated with genotoxic carcinogens. (A) Sample gel image of solvent control-treated proteome profile. Proteins were separated on the basis of pI (x-axis) and molecular weight (y-axis). Spots were visualized by Coomassie blue staining. (B) Enlarged images of up-regulated protein spots. (C) Enlarged images of down-regulated protein spots. Selected spots were defined as altered and identified by MALDI-TOF MS analysis.

Table 1. Proteins identified by 2-DE and MALDI-TOF MS analysis

	Spot no.	Protein name	NCBI accession number	Fold difference (Compound/control)	MW (Da)/pI	No. of peptides matched	Sequence coverage (%)
Up-regulated	397	Moesin	gi 6754750	2.3	67725/6.22	19	31
	509	CDK5RAP3	gi 148684104	2.0	56125/4.77	14	31
	588	Pyruvate kinase	gi 31981562	4.0	57808/7.18	19	45
	826	PIP kinase	gi 115529473	5.6	231933/6.19	29	18
	915	Ornithine aminotransferase	gi 8393866	6.0	48324/6.19	13	34
	1982	Macrophage scavenger receptor 1	gi 85861254	2.0	38749/6.05	13	41
Down-regulated	168	Nucleolin	gi 148708274	0.2	53478/4.95	12	30
	193	Tumor rejection antigen gp96	gi 6755863	0.4	92418/4.74	31	39
	1822	Rps16 protein	gi 52078405	0.0	17542/10.21	11	62
	2068	Cofilin	gi 6680924	0.0	18548/8.22	5	45
	2097	Histone 1	gi 28316756	0.0	14048/11.21	7	65

Moesin expression increases following treatment of two genotoxic carcinogens

To determine the expression level of moesin, L5178Y mouse lymphoma cells were treated with solvent control (DMSO), 100 μ g/ml 1,2-dibromoethane, or 400 μ g/ml glycidol as described in "Materials and Methods", and cell lysates were subjected to western analysis. Moesin expression level was increased to 170% and 160% with treatment of 1,2-dibromoethane and glycidol, respectively, compared to solvent control (Fig. 2). In contrast, it was only 120% when methylcarbamate treated. Methylcarbamate is a nongenotoxic carcinogen and served as a negative control (Kwon et al., 2007). Since the result of proteome analysis was confirmed by western analysis with a greater than 150% increase in moesin expression, we selected moesin for further investigation.

Moesin can serve as a specific biomarker for genotoxic carcinogens

To investigate the potential of moesin as a specific biomarker for genotoxic carcinogens, we examined the expression level of moesin in L5178Y mouse lymphoma cells exposed to other known genotoxic and nongenotoxic carcinogens. Six genotoxic carcinogens; diethylstilbestrol, urethane, chlorambucil, dibenz(a,h)anthracene, methyl methanesulfonate, and N-nitroso-N-methylurea, were chosen (Gladek and Liehr, 1989; Hübner et al., 1997; Miloch et al., 1993; Palmer et al., 1984; Tinwell et al., 1998; Zarbl et al., 1985). In addition, six nongenotoxic carcinogens; O-nitrotoluene, 1,4-dioxane, tetrachloroethylene, 2,3,7,8-tetrachlorodibenzo-p-dioxine (TCDD), chloroprene, nitrobenzene, and nitromethane were selected (Biswas et al., 2008; Hetch, 2002; Hsu et al., 2007; Hurst, 2007; Paulu et al., 1999; Sills et al., 2004; Stickney et al., 2003). L5178Y mouse lymphoma cells were exposed to genotoxic and nongenotoxic carcinogens at the biologically effective concentrations (Tables 2 and 3) determined by cytotoxicity test (Go and Sheen, 2008). Moesin expression level was detected by western analysis (Figs. 3A and 3B) and the specificity was calculated. Moesin expression level following treatment of test compounds was measured using Image J program and expressed as a percentage of the expression level observed following treatment with solvent alone. The specificity is the ratio of the number of compounds showing a greater than 150% increase to the total number of compounds. Of eight genotoxic carcinogens, six compounds, 1,2-dibromoethane, glycidol, diethylstilbestrol, urethane, dibenz(a,h)anthracene, and N-nitroso-N-methylurea,

Fig. 2. Validation of increased expression of moesin by western analysis in response to genotoxic carcinogens treatments. Con: solvent control (DMSO), 1 to 3; 1,2-dibromoethane, glycidol (genotoxic carcinogen), and methylcarbamate (nongenotoxic carcinogen), respectively. L5178Y mouse lymphoma cells were treated with each compound for 2 h. β -actin was used as a loading control. Moesin expression level was increased to 170% and 160% with treatment of 1,2-dibromoethane and glycidol, respectively, compared to solvent control. In contrast, methylcarbamate did only 120% increase.

showed greater than 150% (± 0.48 , $n = 3$) increase in moesin expression level, indicating that moesin has a 75.0% specificity for the genotoxic carcinogens tested in this study. Chlorambucil or methyl methanesulfonate-treated samples were showed just 130% increase in moesin expression. In contrast, only one of the eight nongenotoxic carcinogens, TCDD, caused greater than 150% (± 0.10 , $n = 3$) increase in expression; the specificity of moesin for nongenotoxic carcinogens is just 12.5%.

In addition, the specificity of moesin as a biomarker for genotoxic carcinogens was assessed in NIH3T3 mouse fibroblasts. Since L5178Y mouse lymphoma cells are tumor cells, test in normal cell line was required for the cell line specificity. Experimental design for NIH3T3 mouse fibroblasts was as same as for L5178Y mouse lymphoma cells. Western analysis was conducted for detection of moesin expression level (Figs. 3C and 3D) and the specificity was calculated. Of eight genotoxic carcinogens, five compounds, glycidol, chlorambucil, dibenz(a,h)anthracene, methyl methanesulfonate, and N-nitroso-N-methylurea, showed greater than 150% (± 0.32 , $n = 2$) increase in moesin expression level, indicating that the specificity for genotoxic carcinogens is 6.25%. 1,2-Dibromoethane, diethyl-

Table 2. Chemical structure and concentrations of genotoxic carcinogens

Genotoxic carcinogen	Chemical structure	Concentration ($\mu\text{g/ml}$)
1,2-Dibromoethane		100
Glycidol		400
Diethylstilbestrol		15
Urethane		5,000
Chlorambucil		75
Dibenz(a,h)anthracene		80
Methyl methanesulfonate		125
N-nitroso-N-methylurea		625

stilbestrol, or urethane-treated samples were showed just slight increase in moesin expression. Of tested nongenotoxic carcinogens, only nitrobenzene caused greater than 150% (± 0.22 , $n = 2$) increase in moesin expression; the specificity of moesin for nongenotoxic carcinogens is just 12.5%. Although the results of NIH3T3 mouse fibroblasts and L5178Y mouse lymphoma cells were not exactly matched, the specificity of each case was highly correlated. These results suggest that moesin has the potential as a specific biomarker for detection of genotoxic carcinogenicity of the given compounds.

DISCUSSION

The primary goal of this study was the identification of a specific molecular or protein biomarker for genotoxic carcinogens. The assessment of toxicity is important for new compounds in drug development and diagnosis (Bandara and Kennedy, 2002). We

used proteomic technologies to identify moesin as a specific biomarker for genotoxic carcinogens.

In this study, L5178Y mouse lymphoma cells were used and the cells are commonly used for toxicity test of chemicals because of high sensitivity to DNA damage by the mutation of thymidine kinase gene. Cells were treated with two genotoxic carcinogens and the cellular proteomes were then analyzed by 2-DE. The proteomes of compound-treated samples were separated in two dimensions and image analysis of each gel was conducted. Fifty protein spots that showed a greater than 1.5-fold variation in expression in the carcinogen-treated samples compared to samples treated with solvent alone were chosen from the detected spots (Fig. 1). The selected proteins were identified by MALDI-TOF MS analysis (Table 1).

We conducted a literature search to determine if a relationship had been observed between any of the identified proteins and cancers. Moesin, one of the proteins upregulated by treat-

Table 3. Chemical structures and concentrations of nongenotoxic carcinogens

Nongenotoxic carcinogen	Chemical structure	Concentration ($\mu\text{g/ml}$)
Methylcarbamate		5,000
O-nitrotoluene		200
1,4-Dioxane		4,000
Tetrachloroethylene		100
TCDD		10
Chloroprene		20
Nitrobenzene		125
Nitromethane		5,000

ment with genotoxic carcinogens is a known marker of basal breast carcinomas (Charafe-Jauffret et al., 2007) and CDK 5RAP3 is a marker of lung adenocarcinomas (Stav et al., 2007). Histone 1 showed a pattern of downregulation by 2-DE following carcinogen treatment and is known to be expressed at a lower level in human breast cancer cells than in normal cells (Vani et al., 2006). The results of proteomic profiling of genotoxic carcinogen-treated L5178Y mouse lymphoma cells allowed us to select several promising proteins for further investigation of potential biomarkers. To verify the data derived from proteomics analysis of these proteins we selected moesin and characterized its expression pattern.

To confirm the expression level of moesin, cells were treated with effective concentrations of solvent control (DMSO), 1,2-dibromoethane, glycidol, and methylcarbamate and western analysis was conducted. Genotoxic carcinogen-treated samples showed a pattern of sharply increased moesin expression

whereas cells treated with nongenotoxic carcinogen had a pattern of only slightly increased expression (Fig. 2). Since the pattern of moesin upregulation in genotoxic carcinogen-treated samples were confirmed, moesin was selected for further investigation.

To further explore a potential role for moesin as a specific biomarker for genotoxic carcinogens, we investigated the expression level of moesin in L5178Y mouse lymphoma cells in response to eight genotoxic and eight nongenotoxic carcinogens at their effective concentrations (Tables 2 and 3). As the result, moesin has the specificity of 75.0% for the genotoxic carcinogens and 12.5% for nongenotoxic carcinogens (Figs. 3A and 3B). In addition, the genotoxic carcinogenic potential of test compounds with their effective concentrations was also detected on NIH3T3 mouse fibroblasts. In NIH3T3, moesin showed 62.5% specificity for genotoxic carcinogens, and 12.5% specificity for nongenotoxic carcinogens (Figs. 3C and 3D). To clarify

Fig. 3. Specific expression of moesin by genotoxic carcinogen treatments. Cells were treated with compounds for 2 h. The effective concentration of each compound is shown in Tables 2 and 3. (A, B) Treatment of test compounds to L5178Y mouse lymphoma cells. (C, D) Treatment of test compounds to NIH3T3 mouse fibroblasts. (A, C) Treatment of genotoxic carcinogens. Con: solvent control (DMSO), 1 to 8: 1,2-dibromoethane, glycidol, diethylstilbestrol, urethane, chlorambucil, dibenz(a,h)anthracene, methyl methanesulfonate, and N-nitroso-N-methylurea, respectively. Cells were treated with each compound for 2 h at the concentrations shown in Table 2. (B, D) Treatment of nongenotoxic carcinogens. Con: solvent control (DMSO), 1 to 8: methylcarbamate, *O*-nitrotoluene, 1,4-dioxane, tetrachloroethylene, TCDD, chloroprene, nitrobenzene, and nitromethane, respectively.

dose-response, test compounds were treated to L5178Y mouse lymphoma cells with one tenth concentrations. As the result, the effect of moesin as a genotoxic carcinogenic biomarker was not shown in lower concentrations (data not shown). These findings suggest that moesin might serve as a specific biomarker for detection of genotoxicity and carcinogenicity of the given compounds.

The data generated in this study are in agreement with the results of previous studies indicating that moesin is a biomarker for carcinogens. Here, we used 2-DE to examine changes in the expression patterns of proteins in cells treated with genotoxic carcinogens compared to cells treated with solvent alone. Data derived using this approach permitted the identification of moesin as a specific biomarker for genotoxic carcinogens. As a specific biomarker for genotoxic carcinogens, moesin could be applied to a tissue biomarker by immunohistochemistry and used for safety/toxicity assessment of chemicals instead of animal testing that eventually contributes for an effective assessment of genotoxic carcinogens in molecular level.

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