Potential of a Novel Safety Evaluation of Nanomaterials Using a Proteomic Approach

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We studied changes in protein expressions using human monoblastic leukemia cells (U937) exposed to carbon black (CB) to explore the possibility of a new safety evaluation method using a proteomic approach. The cells exposed to CB with a particle size of approximately 85 nm did not show cell growth inhibition by 96 hr. However, when we performed two-dimensional electrophoresis with U937 cell lysates four times and analyzed the gel images, we found that the CB-exposed cells had changed significantly in 14 protein spots, with expression amounts being different by more than two-fold those at 96 hr. Only one protein was up-regulated while the others were down-regulated. Ten of the 14 spots were identified using peptide mass fingerprinting: transportin 1, thioredoxin domain containing 5, annexin A2, otubain 1, 14-3-3 protein γ, *etc*. The functions of these proteins are associated with metabolism, responses to stress, signal transduction, and cell differentiation. According to our research, CB undoubtedly causes biological responses, but those responses are not detectable by conventional methods. Regarding safety risk, the proteomic approach could possibly detect biological responses more sensitively than conventional *in vitro* evaluation methods.

Key words —— safety evaluation, proteomics, nanomaterial, carbon black, *in vitro*, protein expression

INTRODUCTION

Nanomaterials are expected to be very useful technological components in this century. In particular, nano-sized carbon materials have seen significant developments; they are being mass produced and are already widely utilized in diverse fields at the commercial level.[1, 2)] With the rapid spread of commercial use, the effects of such nano-sized materials on the human body have come to receive widespread attention in recent years, along with environmental pollutants and the problem of asbestos. Although safety evaluations of the carbon-based nanomaterials have been performed with earnest, they have not yet produced a conclusion.

Some biological responses by nanomaterials, especially carbon-based nanomaterials, have been reported. In *in vitro* experiments, nanomaterials cause such biological responses as inflammatory reaction, oxidization stress, cytokine production, and apoptosis.[3–9)] In *in vivo* experiments, some biological responses (inflammation, fibrosis, granulomas, mutagenesis, *etc.*) have been observed.[10–13)]

A problem of conventional safety evaluation methods, except carcinogenicity tests, is that they do not clarify whether these biological responses lead eventually to serious disease. Conventional safety evaluation methods, such morphological observations and cytotoxicity, only evaluate the results of biological responses in the acute phase. Cytokine inductions are immunoreactions in a healthy body that has taken in foreign substances, and this temporary change is not necessarily a risk. We need an approach based on a completely new idea that takes into consideration the risk of disease development.

In recent years, vigorous efforts have been put into the search for biomarkers of a variety of diseases using high-throughput microarray and mass spectrometry technologies.[14–16)] The concept of these technologies is to look at the changes in a variety of mRNA and proteins in one swoop without

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paying attention to specific molecules in the sample. Although many biomarkers of disease have been identified thus far in diseased tissues and cultured cells, these markers are not necessarily producing good results as diagnostic markers. This is because the amount of these markers is minimal even in the focal point of disease, since they are proteins that reflect the early phase of disease. Furthermore, they are not detectable even if leaked into the blood. However, from the safety evaluation point of view, which is our objective, the results from disease-related proteins obtained from diseased tissue or cultured cells can be used effectively as is.

If disease were to develop due to nanomaterials, then the changes should start in the exposed cell. We undertook a new safety evaluation method using the proteomic techniques of two-dimensional electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS) instead of the old conventional safety evaluation methods used to detect the changes in cells. Protein changes reflect biological responses more accurately than the results of a DNA/RNA microarray. We suggest the potential of a new safety evaluation method of nanomaterials using biological responses and taking into consideration whether proteins that are related to disease onsets, including those in the biomarker database, are included.

MATERIALS AND METHODS

Materials —— Carbon black (Diablack R; CB) of approximately 85 nm in size was supplied by Mitsubishi Chemicals (Tokyo, Japan). It was sterilized with ethylene oxide gas overnight, suspended with 10% gelatin, and sonicated in a water-bath sonicator for 2 hr. Before use, the suspended material was heated at 40°C and mixed vigorously.

Cell Culture and CB Exposure —— Human monoblastic leukemia (U937) cell lines were purchased from RIKEN (Ibaraki, Japan) and grown in an RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO_2 atmosphere. The cells were seeded at 5×10^4 cells/ml and split approximately twice per week to maintain them. Fifty μl of CB (10 mg/ml) were added to a culture medium of 5 ml having 2.5×10^5 cells. The same volume of 10% gelatin was added to the control.[17] The cells were counted using trypan blue solution at 24-hr intervals.

Two-dimensional Electrophoresis and Image Analysis —— The U937 cells cultured with CB for 4 days were centrifuged, washed 3 times with 250 mM saccharose in 10 mM Tris-HCl (pH 7.0) and solubilized in a lysis solution containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 5% β-mercaptoethanol (β-ME), and 0.5% IPG buffer (pH 3–10; Bio-Rad, Hercules, CA, U.S.A.). The cells were ground using a mini-grinder for 1 min on ice and sonicated for 10 min at 10°C in a water-bath sonicator. The samples were centrifuged at 16000 rpm for 15 min at 10°C. The supernatant was transferred to a new microtube and kept at −80°C until use. The protein concentration was determined using a protein assay solution (Bio-Rad).

IPG strips (11 cm, nonlinear pH 3–10; Bio-Rad) rehydrated overnight at room temperature were loaded with 200 μg of proteins. Isoelectric focusing was performed by a program of progressively increasing voltage for a total of 30000 Volt-hr with cooling at 15°C. The focused IPG strips were then equilibrated for 30 min with slow shaking in an equilibration solution containing 2% sodium dodecyl sulfate (SDS), 16.7 mM Tris-HCl (pH 6.8), 10% glycerol, and 5% β-ME. The equilibrated IPG strips were laid on the second dimension gels (12% SDS-polyacrylamide gels—13.8 cm × 12 cm × 1 mm) and a current, 20 mA/gel, applied for 6 hr. Each gel was then fixed in a solution of 50% methanol and 10% acetic acid for a minimum of 30 min and stained for a minimum of 60 min with Quick-CBB (Wako, Osaka, Japan). Gels destained with distilled water overnight were sandwiched between cellophane soaked in 5% methanol and 5% glycerol and scanned with a transmission scanner (CanoScan 9950F; Canon, Tokyo, Japan) at a 16-bit depth and a resolution of 300 dpi. The scanned gel images were analyzed with PDQuest Advanced software (Bio-Rad, version 8.0) to quantify the protein spots. Four sets ($n = 4$) of independent 2-dimensional gel electrophoresis (2-DE) gel analyses were conducted and averaged. We selected protein spots with more than a twofold expression change and significance compared with the spots of the control.

Protein Identification by MALDI-TOF MS —— In-gel digestion of 2-DE gel spots was performed as described before.[18] Briefly, protein spots excised from 2-DE gel were destained with 100 mM ammonium in 50% acetonitrile. The gel pieces were dried and digested with sequencing grade modified trypsin (Promega, Madison, WI, U.S.A.).

The peptide solution was recovered and residual peptide was extracted by shaking with 5% trifluoroacetic acid (TFA) in 50% acetonitrile. The combined solution was concentrated using a lyophilizer. The tryptic peptides dissolved in 0.2% TFA were mixed with a matrix solution (α-cyano-4-hydroxycinnamic Acid 10 mg/ml in 50% acetonitrile/0.1% TFA) and applied to a target plate. MS spectra were obtained using MALDI-TOF MS (Voyager Elite, Applied BioSystems, Foster City, CA, U.S.A.). The MS spectra were analyzed in the positive-ion mode. The peptide mass fingerprint (PMF) search was performed through MASCOT (http://www.matrixscience.com) using the NCBInr database. The PMF search parameters were: animal species, Homo sapiens; enzyme specificity, trypsin (maximum missed cleavage = 1); modification, propionamide (cysteine), *N*-acetyl (protein), oxidation (methionine), and pyro-glutamate (N-terminal glutamate and glutamine); and mass tolerance, 0.5 Da. Protein identification was based on the combination of probability-based MOWSE scores for PMF. We declared protein identities based on the criteria that the probability-based PMF MOWSE score exceeds 65 ($p < 0.05$). The identified proteins were categorized according to three ontological aspects (cell component, cell process, and physiological function) using the Generic Gene Ontology (GO) Term Finder (http://go.princeton.edu/cgi-bin/GOTermFinder) and GO Term Mapper (http://go.princeton.edu/cgi-bin/GOTermMapper).[19]

Statistical Analysis —— Unpaired two-tailed Student's *t* tests were used for the statistical analyses of the differences in cell count data and raw quantitative data for each protein spot on the 2-DE gels. *p* values of 0.05 or less were considered to indicate significance.

RESULTS

Cell Proliferation

Although U937 cells have the capacity to differentiate into macrophage-like cells when treated with a differentiation-inducing agent (*e.g.*, phorbol 12-myristate 13-acetate), CB exposure did not induce differentiation in this study. Figure 1 shows the cell proliferation in the control group and in the CB exposure group. No difference in the proliferation of the U937 cells was observed, even on day 4, and no CB-induced influence was observed on the cell-growth nature.

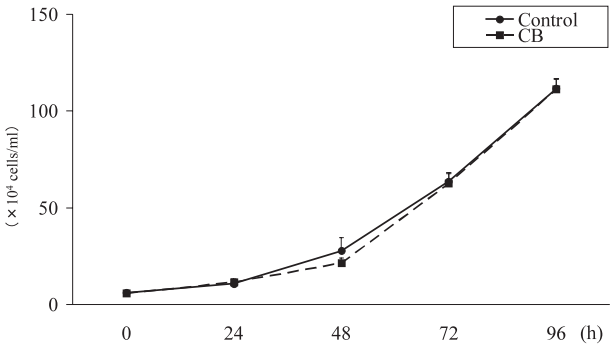

Fig. 1. Proliferation of U937 Cells Exposed to CB Compared with the Control

The growth curve was generated by seeding cells at 0.5×10^5 cells and counting cells using a hemocytometer under light microscopy by the trypan blue exclusion method at intervals of 24-hr. The representative data of four separate experiments are shown.

Cells stained by trypan blue were not observed in either group, nor were there any morphological changes (data not shown).

Two-dimensional Electrophoresis and Protein Identification

Two-dimensional electrophoresis was used to investigate the altered expression of proteins in U937 cells exposed to CB for four days. Analysis of the images obtained from running electrophoresis four times for each group revealed a total of more than 700 protein spots on the gels. Fourteen protein spots with a two-fold or greater change in expression level and quantitatively significant differences were detected by comparing matched spots on gels in the control and exposed groups (Fig. 2). Among these, only one spot showed an increased expression level in the exposed group compared to the control, where as the other 13 spots showed a decreased expression level.

The 14 protein spots were all cut from the gels, and protein identification was performed using MALDI-TOF MS. Ten proteins were identified, as shown in Table 1. Furthermore, a search of the functions of these proteins found that seven participate in metabolism, three participate in development, and one protein each participates in anti-apoptosis, signal transduction/cell communication, and cytoskeleton organization (Table 2). Thus, some proteins are multifunctional.

DISCUSSION

This study investigated the potential of a new

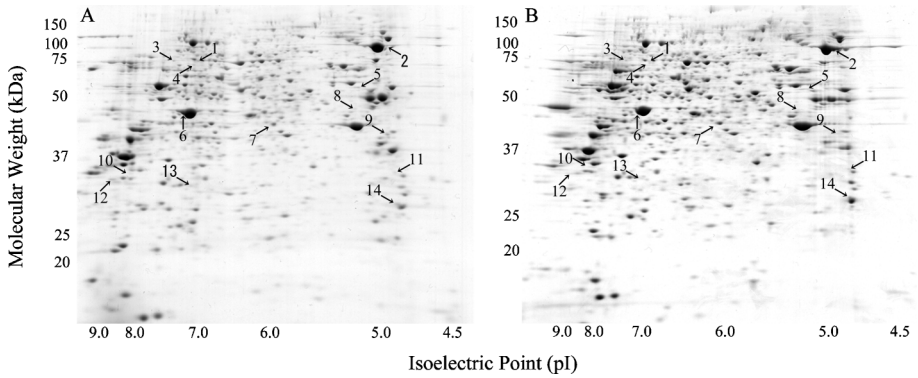

Fig. 2. Two-DE Gel Images of U937 Cell Proteins. Cell Lysate (300 μg) were Separated by 2-DE

A: Control gel. B: CB gel. The protein spots indicated with arrows have more than a twofold expression change and significance compared with the spots of the control.

Table 1. List of Proteins Identified by Peptide Mass Fingerprint. Spot Numbers Correspond to Those Shown in Fig. 2B. Spot Nos. 1, 3, 4, and 7 were Not Identified

Spot No.	Protein Name	Theoretical MW	Theoretical pI	Score*	Sequence Coverage (%)	Ratio	Accession No.
2	transportin 1	103091	4.81	102	13	3.80	Q92973
5	splicing factor 3a subunit 3	59238	5.27	76	14	0.46	Q12874
6	phosphogluconate dehydrogenase	53745	6.80	71	12	0.47	P52209
8	thioredoxin domain containing 5	44636	5.77	68	12	0.20	Q8NBS9
9	spermine synthase	24942	5.16	71	19	0.34	P52788
10	annexin A2	38864	7.57	114	28	0.34	P07355
11	otubain 1	31549	4.85	81	26	0.23	Q96FW1
12	DnaJ homolog subfamily C member 8	29823	9.04	89	28	0.42	O75937
13	actin related protein 2/3 complex subunit 2	34454	6.84	71	20	0.49	O15144
14	14-3-3 protein γ	28498	4.80	150	36	0.39	P61981

*Probability-based MOWSE score. Scores higher than 65 indicate the level of statistical significance at $p < 0.05$.

Table 2. The Main Functions of Identified Proteins

Metabolism
actin related protein 2/3 complex subunit 2, phosphogluconate dehydrogenase, spermine synthase, splicing factor 3a subunit 3, transportin 1, otubain 1, DnaJ homolog subfamily C member 8
Development
14-3-3 protein γ, thioredoxin domain containing 5, annexin A2
Anti-apoptosis
thioredoxin domain containing 5
Signal transduction/Cell communication
14-3-3 protein γ
Cytoskeleton organization
actin related protein 2/3 complex subunit 2

The protein functions were based on the Gene Ontology Annotation database.

safety assessment of nanomaterials using proteomics. CB, which is an easily obtained nanomaterial for which a relatively large number of *in vivo* and *in vitro* safety assessments have been conducted, was used in this study for simplify the model.

CB is reported to have induced an inflammatory response in *in vivo* experiments.[20–22] CB is reported to influence cytokine and chemokine production and neurotransmission,[23–25] and to have other effects. For *in vitro* experiments, changes in the expression level of cytokine and cytotoxity have been reported.[22,26,27] On the other hand, there are some reports in which significant changes were not observed;[28,29] even in this experiment, cell proliferation and apoptosis/necrosis were not observed. Based on such results, the current safety evaluation of CB says that it may induce some biological responses such as inflammation but that there is little association with serious disease.

In this experiment, CB did not show any biological responses, such as cell growth, apoptosis and necrosis. In this point of view, since many of biological responses were reported by used CB particle 50 nm or less, we thought the particle size is important factor to biological response. Moreover, we thought the possibility that the established

leukemia cell line had a less affected by cytotoxic substance, because it's abnormally and strong cell proliferation. Therefore, further study will be necessary to investigate the size dependent-, exposing time dependent-, and dose dependent-effect of CB in our experimental condition.

A proteomics approach was used to examine protein expression in cultured cells in which CB exposure was not observed to have inhibited cell proliferation or resulted in cytotoxicity. But, significant alterations were detected at 14 spots, and so protein identification was conducted using MALDI-TOF MS, resulting in the identification of 10 proteins. Although most of the identified proteins (7 out of 10) are associated with metabolism, proteins that participate in development and cell death were also contained in the altered spots. Intercellular changes were confirmed at a level undetectable through the confirmation of proliferation changes or cell death using trypan blue stain. Thus, the obtained results could predict the biological responses indicated in previous reports. Moreover, nanomaterials are also reported to influence the cytoskeleton,[30] and change in one protein related to the cytoskeleton was detectable. However, changes in tumor marker proteins or other disease marker proteins were not observed in the proteins identified in this study. At the least, this study predicts a low risk of specific disease development from the initial changes in the cells exposed to the CB used in this study. Witzmann and Monteiro-Riviere have reported the effect of multi-walled carbon nanotubes using proteomics on the human keratinocyte.[31] Although the altered expression of many proteins is shown by 48 hr after exposure, the state that cell viability declined is observed, and many protein changes related to cell death is detected. However, we thought that the sub acute- or chronic safety assessment is important because of thinking the problem of asbestos. For aiming at sub acute- or chronic safety assessments, it is considered that it should be examined on condition without the acute cytotoxicity as much as possible.

It must be kept in mind that the associations between individual proteins and diseases are still under examination and have not yet been made into a comprehensive database. A database of proteins that are affected by nanomaterial exposure should be made without waiting for the completion of a database of disease-related proteins. Even at present it is possible to obtain a certain degree of information about disease associations by searching the functions of individual proteins.

The safety evaluation method that we are considering is a completely new evaluation method that can be used not only for nanomaterials but for the safety assessment of novel compounds by combining biological responses and databases of disease-related proteins. The results of this study produced basic information on this potential.

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