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Short Communication

Trial to Evaluate Effects of Ambient Particulate Matter on Health: A Preliminary Study Using Two-Dimensional Gel Electrophoresis

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Abstract

Objectives: Particulate air pollution is a serious problem all over the world, and the development of a method to evaluate the health effects of ambient particles is necessary. In this study, cells cultured in vitro were exposed to particles sampled at the side of a main road, and their protein expression levels were examined.

Methods: Ambient particles were collected at the side of a main road using a high-volume air sampler. Some of the collected particles (crude particles) were treated with an organic solvent to remove chemical components, and the resulting residues were used as residual particles. Cells from the mouse alveolar epithelial cell line LA-4 were inoculated into tissue-culture dishes at 1.4×10^4/cm^2, exposed to each type of particle or artificial carbon particles (Printex 90) that were dispersed using an ultrasonic homogenizer by mixing in the medium twice at 24 and 48 hours, and incubated for up to 72 hours after the start of inoculation. After exposure, the number of cells and intracellular dehydrogenase activity were measured. Proteins extracted from the cells were subjected to two-dimensional gel electrophoresis with isoelectric focusing at pHs 4–7 using a 10% acrylamide gel, and their expression levels were analyzed after fluorescent staining.

Results: The intracellular dehydrogenase activity of the cells significantly decreased as a result of exposure to the residual (0.70-fold) and crude (0.84-fold) particles compared with that of the control, but it showed no change as a result of exposure to Printex 90. The protein expression levels in the cells exposed to the particles increased or decreased similarly, but different expression levels were also observed. There were differences in the effects observed between the cells exposed to the artificial carbon particles and those exposed to particles collected from ambient air.

Conclusion: This study indicates that protein expression levels in cells change in response to exposure to particles collected from ambient air. To evaluate the effects of particles on health, it is considered necessary to use particles collected from ambient air.

Key words: particulate matter, air pollution, health effect, biomarker, two-dimensional gel electrophoresis

Introduction

Currently, air pollution caused by particulate matter emitted from cars is a serious problem all over the world. The health effects of air pollutants have been examined by epidemiological studies and animal experiments. Ambient particulate matter contains solid carbon components, metals, and chemicals, such as polyaromatic hydrocarbons (PAHs), and its toxicity is considered to vary with particle-generation processes and its constituents. Therefore, the development of a method to evaluate the health effects of ambient particles is necessary.

To simplify the examination of the health effects of ambient particles, new biomarkers have recently been developed (1, 2). These biomarkers are known to respond to substances emitted as a result of biological damage and substances induced against various damage factors, such as stress proteins (3, 4). Methods for the global analysis of genes and proteins detected in samples include DNA microarray analysis (5–7) and two-dimensional gel electrophoresis (8–10). In one study, DNA microarray analysis showed that the expression of genes related to acute lung injury was induced by exposure to diesel exhaust particles (DEPs) and lipopolyssaccharide (LPS) (7), and in another study, proteomic analysis using two-dimensional gel

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electrophoresis showed that DEPs induced a hierarchical oxidative stress response in a macrophage cell line (10). In this study, to develop biomarkers to evaluate the effects of ambient particles by two-dimensional gel electrophoresis, cells cultured in vitro were exposed to particles sampled at the side of a main road that was heavily trafficked with diesel cars.

Methods

Sampling of ambient particles

Ambient particles were sampled using a high-volume air sampler (HV-1000F, SIBATA Ltd., Tokyo, Japan) placed on a roof of a 5-story building of Hyogo College of Medicine, adjacent to Route 43 and the Hanshin Expressway in Nishinomiya, Hyogo Prefecture, Japan. Particles were collected on glass fiber filters (GB-100R, ADVANTEC TOYO Ltd., Tokyo, Japan) for seven days at a rate of 1 m^3/min. Some of the collected particles (crude particles) were subjected to extraction using dichloromethane and a Soxhlet extraction apparatus, and the resulting residues were used as residual particles. Particles remaining on a filter with a pore size of 0.22 μm (0.22 μm PES Plus Membrane, IWAKI, Tokyo, Japan) and particles passing through a filter with a pore size of 2.5 μm (MILLIPORE 2.5 μm AN25, Millipore Co., Billerica, MA) in deionized distilled water were lyophilized, and resuspended in deionized distilled water. Artificial carbon particles (Printex 90, Degussa, Frankfurt, Germany) were treated using the same procedures, and used in experiments.

Cell culture

Cells from the mouse alveolar epithelial cell line LA-4 (Dainippon Sumitomo Pharm Co., Ltd., Tokyo Japan) were cultured in a nutrient mixture F-12 HAM (Sigma-Aldrich Inc., St. Louis, MO) containing 10% fetal bovine serum (FBS) (HyClone Laboratories, Inc., South Logan, UT).

Exposure of cells to particles

The cells were exposed to the particles on 24-well plates to measure the number of cells, 96-well plates to measure intracellular dehydrogenase activity, and 9-cm dishes for two-dimensional gel electrophoresis. The LA-4 cells were inoculated at 1.4×10^4/cm^2, exposed to each type of particle by changing the medium to one mixed with particles twice at 24 and 48 hours, and incubated for up to 72 hours after the start of inoculation. The crude and residual particles were sonicated using an ultrasonic homogenizer (Heat Systems, Plainview, NY) at a power of 20 W for 30 min, and dispersed on dishes at 1.1 μg/cm^2. In addition, Printex 90, which has carbon particles that are on average 14 nm in size and is used for a considerable amount of research on particles (11), was sonicated similarly. As a control, a medium mixed with deionized distilled water was used.

Determining number of cells

To evaluate the effects of exposure to the particles on cell growth, the number of cells was determined after exposure. The number of cells in the control well was defined as 1, and that counted in a certain area at the center of the well bottom in the exposure groups was expressed using a ratio.

Cytotoxicity of particles

To evaluate the cytotoxicity induced by exposure to the particles, intracellular dehydrogenase activity was measured using Cell Counting Kit-8 (Dojindo Lab., Kumamoto, Japan) and expressed as the enzymatic activity per cell.

Sample preparation

The cells exposed to the particles were rinsed twice in phosphate-buffered saline (PBS), scraped, collected by centrifugation at 300×*g*, and stored at −80°C. These frozen cells were lysed in 20 parts cell lysis buffer (9 M urea, 4% 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS), 60 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM ethylenediamine—*N,N,N',N'*-tetraacetic acid, disodium salt, dihydrate (EDTA), 40 mM Tris-HCl pH 6.8) at room temperature for 20 min and centrifuged at 18,000×*g*. The supernatant was used to extract proteins, and quantified by a BIO-RAD protein assay (BIO-RAD Laboratories, Inc., Hercules, CA).

Isoelectric focusing electrophoresis

The proteins extracted were separated on a gel (ImmobilineDryStrip, pHs 4–7, 11 cm, GE Healthcare Bio-Sciences Corp., Piscataway, NJ) using isoelectric focusing electrophoresis (Ettan IPGphor IEF system, GE Healthcare Bio-Sciences Corp.). The protein content was 150 mg, and the electrophoretic conditions were determined according to the recommended program (GE Healthcare Bio-Sciences Corp.).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The one-dimensional gel obtained by isoelectric focusing electrophoresis was equilibrated with Buffer A (6 M urea, 30% glycerol, 2% SDS, 1% DTT, 50 mM Tris-HCl, pH 6.8) for 15 min and then with Buffer B (6 M urea, 30% glycerol, 2% SDS, 2.5% iodoacetamide, 0.002% bromophenol blue, 50 mM Tris-HCl, pH 6.8) for 15 min. Next, SDS-PAGE was performed using a separating gel (10% acrylamide/bis [29.2:0.8 acrylamide:bisacrylamide], 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.05% ammonium persulfate (APS), and 0.05% *N,N,N',N'*-tetramethylenediamine (TEMED)) and a stacking gel (5% acrylamide/bis [29.2:0.8 acrylamide:bisacrylamide], 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.125% TEMED, and 0.075% APS) with a thickness of 1 mm. The electrophoretic buffer used contained 25 mM Tris, 192 mM glycine, and 0.1% SDS.

Analysis of protein spots by two-dimensional gel electrophoresis

Protein spots obtained by two-dimensional gel electrophoresis were treated with Flamingo Fluorescent Gel Stain (BIO-RAD Laboratories, Inc.), and the electrophoretic images were scanned using an image analyzer (Typhoon 8600S, GE Healthcare Bio-Sciences Corp.). After 3–4 images were obtained under the same conditions, protein expression was analyzed using image analysis software (Progenesis 220, PerkinElmer Life And Analytical Sciences, Inc., Wellesley, MA). The value obtained by subtracting the background value from the density and area of the spot was defined as the spot intensity, and the ratio of the intensity of each spot to the total

intensity of the spots contained in a single gel was considered as the protein content. The mean intensity of the same protein spots after matching was determined in each group.

Evaluation of protein expression

Changes in the protein content were evaluated using the ratio of each spot in the gel groups. Spot intensities that increased by more than 1.5-fold or decreased by less than 0.67-fold of that of the control were regarded as changes in protein expression levels.

Results

Measurement of number of cells and cytotoxicity of particles

The number of cells on the dish bottom was slightly smaller in the exposure groups than in the control group, but the difference was not significant (Table 1). The intracellular dehydrogenase activities were significantly lower in the residual-particle-exposure group (0.70-fold, P=0.001) and in the crude-

Table 1 Changes in number of cells and intracellular dehydrogenase activity induced by exposure to particles

	Number of cells	Intracellular dehydrogenase activity
Control	1.00±0.15	1.00±0.10
Printex 90	0.82±0.19	0.97±0.14
Residual particles	0.90±0.29	0.70±0.10*
Crude particles	0.92±0.23	0.84±0.11**

Values are means±standard deviations, relative to control.
* P=0.001, ** P=0.037 compared with control.

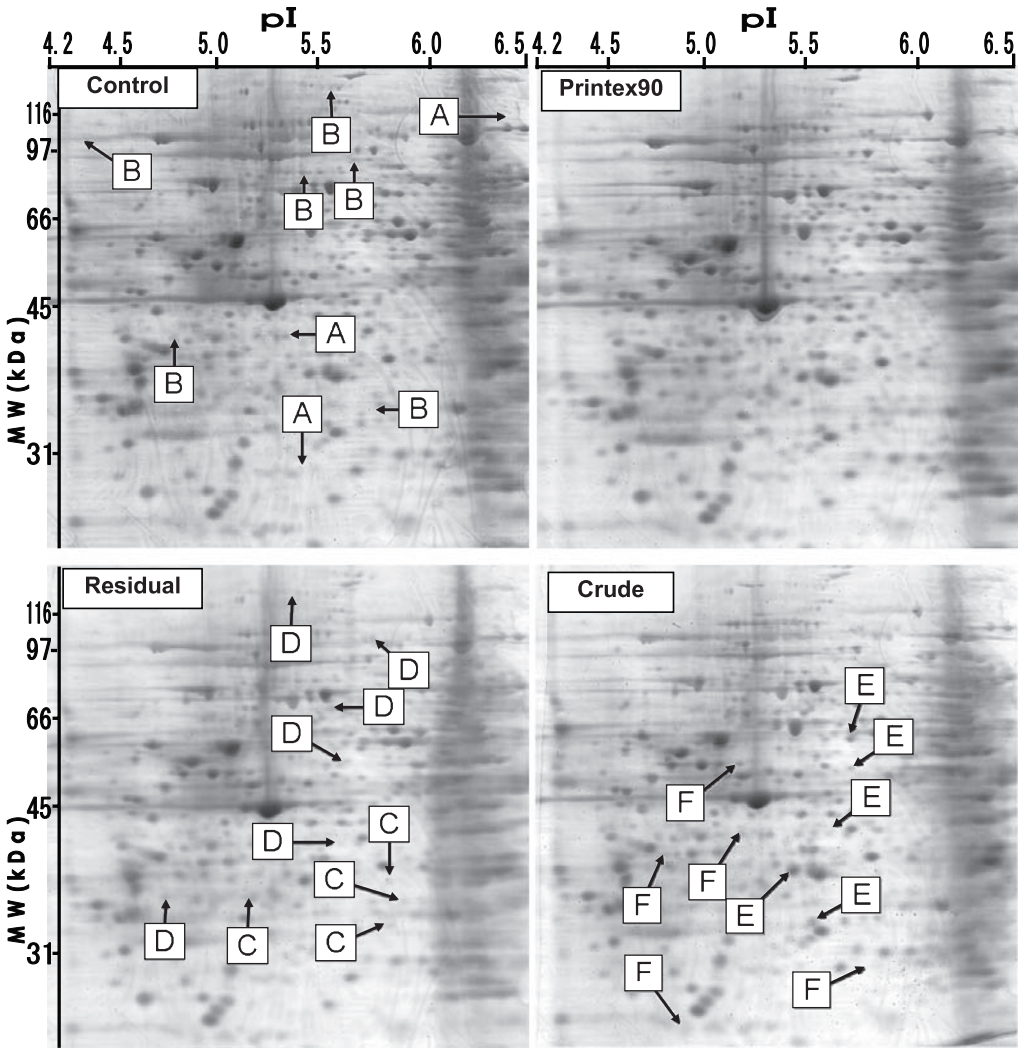

Fig. 1 Changes in protein expression levels detected by two-dimensional gel electrophoresis. Group A: Protein expression levels increased by more than 1.5-fold in cells exposed to Printex 90, residual particles, or crude particles compared with those in control cells. Group B: Protein expression levels decreased by less than 0.67-fold in cells exposed to Printex 90, residual particles, or crude particles, compared with those in control cells. Group C: Protein expression levels increased by more than 1.5-fold in cells exposed to residual particles compared with those in cells exposed to Printex 90. Group D: Protein expression levels decreased by less than 0.67-fold in cells exposed to residual particles compared with those in cells exposed to Printex 90. Group E: Protein expression levels increased by more than 1.5-fold in cells exposed to crude particles compared with those in cells exposed to residual particles. Group F: Protein expression levels decreased by less than 0.67-fold in cells exposed to crude particles compared with those in cells exposed to residual particles.

particle-exposure group (0.84-fold, P=0.037) than in the control group. However, the difference between the residual-particle-exposure and the crude-particle-exposure groups was not significant. There was no significant difference in intracellular dehydrogenase activity between the Printex 90-exposure and control groups (Table 1).

Two-dimensional gel electrophoresis

The protein expression levels in the cells exposed to Printex 90, and the residual and crude particles were higher than those in the control group in three spots (group A) and lower in six spots (group B) (Fig. 1). The protein expression levels in the residual-particle-exposure group were higher than those in the Printex 90-exposure group in four spots (group C) and lower in six spots (group D). In the crude-particle-exposure group, the protein expression levels were higher than those in the residual-particle-exposure group in five spots (group E) and lower in five spots (group F).

Discussion

The direct injection of various particles contained in urban air into animal bronchi causes changes in the chemotactic responses of alveolar macrophages, phagocytotic disorders, and cytoskeletal dysfunction (12–14). However, for simple and rapid screening assays, it is also necessary to develop in vitro tests to evaluate the health effects of environmental pollutants (1).

In this study, we examined the cytotoxicity of particles collected from ambient air and found that there was no significant difference in the cell growth rate between the particulate-matter-exposure and control group, however, the intracellular dehydrogenase activity was lower in the former than in the latter. In contrast, the intracellular dehydrogenase activity showed no change as a result of exposure to Printex 90 particles. The expression of proteins in the cells exposed to the artificial carbon particles differed from that exposed to the particles collected from ambient air. The decrease in intracellular dehydrogenase activity suggests that cellular dysfunction is induced by ambient particles. If the proteins expressed in response to such dysfunction are identified, they could serve as new biomarkers for the effects ambient particles on health.

To examine the in vitro effects of air pollutants, the levels of known biochemical markers such as interleukin-8, endothelin-1, and heat shock protein 70, which are induced by exposure to TiO_2, SiO_2, and urban particles, were measured (3, 4). A global analysis of the cell response to extracts from DEPs was performed by DNA microarray analysis (5, 6) and two-dimensional gel electrophoresis (9, 10). DNA microarray analysis is an appropriate method of evaluating gene expression. On the other hand, the analysis of proteins using two-dimensional gel electrophoresis has the advantage that it can obtain more accurate results by being enhanced by subsequent steps such as TOF mass spectrometry.

Most previous studies were performed using specific particles, and only a few studies noted differences between particulate matter samples collected from various regions. In animal experiments, inflammatory responses caused by exposure to particles varied in relation to the type of particle (15). In one study, there were differences in the amounts of PAHs and their related chemicals that act as electron acceptors, presumably resulting in oxidative stress due to the generation of reactive oxygen species (16). Epidemiological studies (17, 18) have indicated that different components were detected in particulate matter samples collected from ambient air in two regions where the incidences of allergic and respiratory diseases differed (19).

In this study, we evaluated the expression of proteins in the cells exposed to residual and crude particles. Different patterns of soluble proteins in the cells exposed to each type of particle were observed by two-dimensional gel electrophoresis, suggesting that the biological effects of the particles varied depending on their components. Although the expression level is expected to vary with the type of protein, we extensively examined the expression of proteins, including the mild changes in this study, since it is a preliminary study. Differences in the Th1/Th2 response (20) and levels of expression of cytokines and chemokines (21) were observed between the extracted chemicals and residuals in DEPs. Furthermore, it was also reported that endotoxin or fungus that adhered to particles enhanced the effects of DEPs or asian sand dust (7, 22). The effects of endotoxin, which may be attached to crude particles, should be further considered.

The effects of ambient particles might not be sufficiently evaluated in studies using artificial particles and DEPs collected under limited conditions. Therefore, the use of particles collected from ambient air is desirable to evaluate the realistic effects of particulate air pollution. In this study, we used the LA-4 cell line because an established human alveolar epithelial cell line is commercially unavailable, and human lung primary cultures are restricted in terms of subcultures. In a future study, spots showing changes in protein expression should be identified by TOF mass spectrometry. The identification of such proteins will help establish new parameters for a simple evaluation of the effects of particles in ambient air.

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