Cytotoxicity of Airborne Particulates Sampled Roadside in Rodent and Human Lung Fibroblasts

Takako Yamaguchi* and Hiroyasu Yamazaki

Faculty of Pharmaceutical Sciences, Kobe-Gakuin University, 518 Arise, Ikawadani-cho, Nishi-ku, Kobe 651–2180, Japan

(Received January 5, 2001; Accepted February 1, 2001)

The number of diesel-powered vehicles has been increasing in Japan in recent years. And chronic exposure to relatively low levels of diesel exhaust may be a risk factor for respiratory diseases. It has been demonstrated that the airborne particulates sampled at a roadside contain exhaust from diesel engines. The present study has been undertaken to evaluate the cytotoxicity of airborne particulates in human and Chinese hamster lung fibroblast cell lines (WI-38 and CHL/IU). Conversion of mitochondrial MTS, leakage of lactate dehydrogenase (LDH), and the effect on cell proliferation curves were monitored. A dose–related effect of airborne particulate extracts was observed in MTS and LDH leakage assay with both cell lines. Crude extract was most cytotoxic in the MTS assay and had a significant effect on cell proliferation. The response of extracts and fractions of these extracts on MTS assay were similar to the effect on cell proliferation in both cell systems. An increase in LDH leakage was detected in all extracts and fractions except crude extract. In comparison, CHL/IU proved to be more sensitive than WI-38 in MTS assay, but most extracts and fractions were more toxic to WI-38 in LDH leakage assay. These results suggest that even a small amount of substances in airborne particulates was toxic to both cell systems.

Key words —— airborne particulates, cytotoxicity, lung fibroblast, air pollution, respiratory toxicology

INTRODUCTION

Huge quantities of chemicals are released into the atmosphere, and many of these are the products of combustion and pyrolysis. Pollutants released from industry,[1] diesel and gasoline engines,[2,3] incineration,[4] home heating[5] and other activities, consist of complex chemical components, among them, potential and proven mutagens and carcinogens.[6,7] These complex chemical components might interact. Air pollution is a great concern to public health,[8] and is significantly related to the prevalence of certain respiratory diseases. Recent epidemiological studies confirm that airborne particulates are an important health risk factor,[9,10] especially, diesel exhaust particles. Hazardous effects of such particles include annoyance reaction, acute irritation of eyes and respiratory tracts, chronic inflammatory disease, such as asthma and allergic nasal catarrh, and even cancer. The number of diesel-powered cars has been increasing steadily in Japan in recent years, because the cost of light oil is cheaper than that of conventional gasoline. Diesel vehicles emit some 2 to 20 times more nitrogen oxides and some 30 to 100 times more particles than gasoline-powered cars. Chronic exposure to relatively low levels of diesel exhaust may be a risk factor in human cancer.[7] Epidemiological studies on workers occupationally exposed to diesel exhaust indicate an increased relative risk of developing lung cancer.[11–13]

Several *in vitro* models have been developed for the study of respiratory toxicology, carcinogenesis and mutagenesis utilizing rodent and human tracheal epithelial cells in culture.[14–17] Airborne particulate extract showed a substantial impairment of phagocytosis in human macrophages isolated from peripheral blood.[18] The respiratory tract is the major site of exposure to airborne particulates, retained in the nose, the tracheobronchial tree and the pulmonary alveoli.[19]

We report here the results of the cytotoxicity of airborne particulates collected roadside in heavy traffic using rodent and human lung fibroblasts.

*To whom correspondence should be addressed: Faculty of Pharmaceutical Science, Kobe-Gakuin University, 518 Arise, Ikawadani-cho, Nishi-ku, Kobe 651–2180, Japan. Tel.: +81-78-974-4479; Fax: +81-78-974-5689; E-mail: yamaguti@pharm.kobegakuin.ac.jp

MATERIALS AND METHODS

Collection and Extraction of Airborne Particulates —— Airborne particulates were collected on

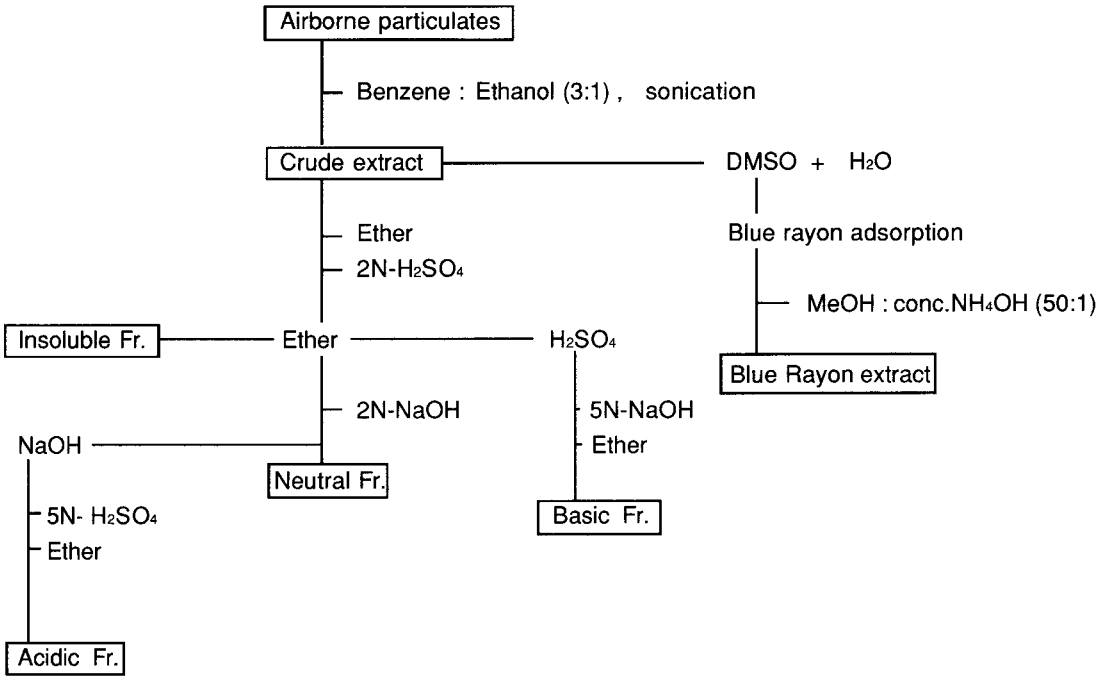

Fig. 1. Fractionation of the Extracts from Airborne Particulates

glass fiber filters with a high-volume air sampler (HVC-500, Shibata Scientific Technology Ltd., Tokyo, Japan) in the period April 1996 to March 1997. The average flow rate of air was set at 700 l/min. The air sampler was placed 5 m away from a busy road in Kobe (Japan). The filters, which had been weighed before sampling, were removed from the sampler and allowed to equilibrate at room temperature (20–22°C) and humidity (20–30%) for 24 hr, reweighed to determine the net weight of particulate deposited, and then stored at –20°C.

The filters were sonicated in benzene-ethanol (3 : 1, v/v), and the solvents were evaporated to dryness to give a crude organic sample (crude extract). As indicated in Fig. 1, the crude extract was further fractionated by acid-base partitioning[20] into 3 fractions, namely acidic, basic and neutral fractions, respectively. Furthermore, the crude extract was treated with Blue-Rayon (Funakoshi Co., Ltd., Tokyo, Japan), which is a selective adsorbent for polycyclic planner compounds,[21] and was named Blue-Rayon extract.

All extracts were evaporated to dryness and dissolved in dimethyl sulfoxide (DMSO) for cytotoxicity assay. Solutions were filtered through a 0.22 μm porous membrane (DIMEX-13, Millipore, Japan). Air volumes corresponding to specific amounts of airborne particulates were measured and the dosage of particulates was expressed in m^3 of air.

Cell Cultures —— Chinese hamster lung fibroblasts of the cell line CHL/IU and normal human fibroblasts of the cell line WI-38 were obtained from Dainippon Pharmaceutical Co., Ltd., (Japan). CHL/IU and WI-38 were grown in a minimum essential medium with Earle's salt (MEM-E) supplemented with 10% inactivated calf serum (CS) and in MEM-E supplemented with 10% fetal bovine serum (FBS), respectively. Cultures were incubated at 37°C in 5% CO_2 in air. Growing cultures were suspended by trypsinization when the monolayer was 80% confluent. The cellular viability was estimated by the trypan blue dye exclusion test.

Cytotoxicity Evaluations —— MTS assay[22,23] was modified from MTT assay,[24,25] performed with a Celltiter 96™ A2queous Non-Radioactive Cell Proliferation assay kit (Promega, U.S.A.). Growing CHL/IU and WI-38 cultures were suspended by tripsinization and 2 or 3×10^3 cells in 100 μl of medium were seeded on a 96-well microtiter culture plate (Nunk, Denmark), respectively. One day after seeding, cultures were exposed in triplicate to various concentrations of airborne particulate extracts for 24 hr. Samples were dissolved in DMSO, which did not exceed 1.0% (v/v) of the final incubation medium. Control and positive control cultures were treated with DMSO and cycloheximide (0.1 μg/well). After 24 hr treatment, MTS assay was performed.

Cytotoxicity, monitored as an increase in lactate dehydrogenase (LDH) leakage, was evaluated with LDH Cytotoxic Test Wako (Wako Pure Chemical Industries, Ltd., Japan). Cultures were seeded on microtiter plates at a density of 5×10^3 cells/0.1 ml of medium per well. After 24 hr, the medium was removed and the cultures washed with phosphate-buffered saline [PBS(–)], then exposed to various concentrations of airborne particulate extracts and positive control (5% Tween 20) for 2 hr.

Effect on Cell Proliferation Curve —— Cytotoxicity was evaluated from the effects of the extracts and fractions on cell proliferation curves. Cultures were seeded on 35-mm plates at a density 2×10^3/plate. After 1 hr incubation, cultures were exposed to airborne particulate extracts (10 m^3 eq.) and incubated for 120 hr or 168 hr. Every 24 hr, the cell number per dish was counted to assess proliferation. Proliferation curves were plotted and compared with the control.

Statistical Evaluation —— Experiments were carried out at least three times using different cell preparations. Each cytotoxic determination was made in four wells. The statistical difference was determined by Student's *t*-test.

RESULTS AND DISCUSSION

Quantity and Cytotoxicity of Airborne Particles

The amount of airborne particulates varied from 53.7 to 195.8 (mean value, 128.9) $\mu g/m^3$ (Table 1).

Table 1. Quantity of Airborne Particulates

	$\mu g/m^3$
Mean ($n = 50$)	128.9
S.D.	36.3
Min.	53.7
Max.	195.8

The response of the CHL/IU and WI-38 cell lines following exposure to airborne particulate extracts is illustrated in Fig. 2 and 3. A dose–related effect was seen on MTS assay. Crude extract had the most potent cytotoxicity, followed by the neutral fraction, acidic fraction, basic fraction and Blue-Rayon extract in both cell lines. The cytotoxicity of the extracts and fractions is shown in Table 2. The IC_{50} was determined from dose–response curves. In the case of crude extract, the IC_{50} was 4.46 and 9.93/m^3 air eq. for CHL/IU and WI-38 cells, respectively. CHL/IU cells were more sensitive to the extracts and fractions than WI-38 cells in the MTS assay (Table 2). The results for LDH leakage assay are shown in Fig. 3 and Table 3. The LDH leakage could not be detected in crude extract, because it inhibits LDH activity. The acid fraction was considerably more cytotoxic than other fractions which produced a similar response in both cell lines (Fig. 3). The concentrations causing the release of 50% of total cellular LDH (EC_{50}) are shown in Table 3. The EC_{50}

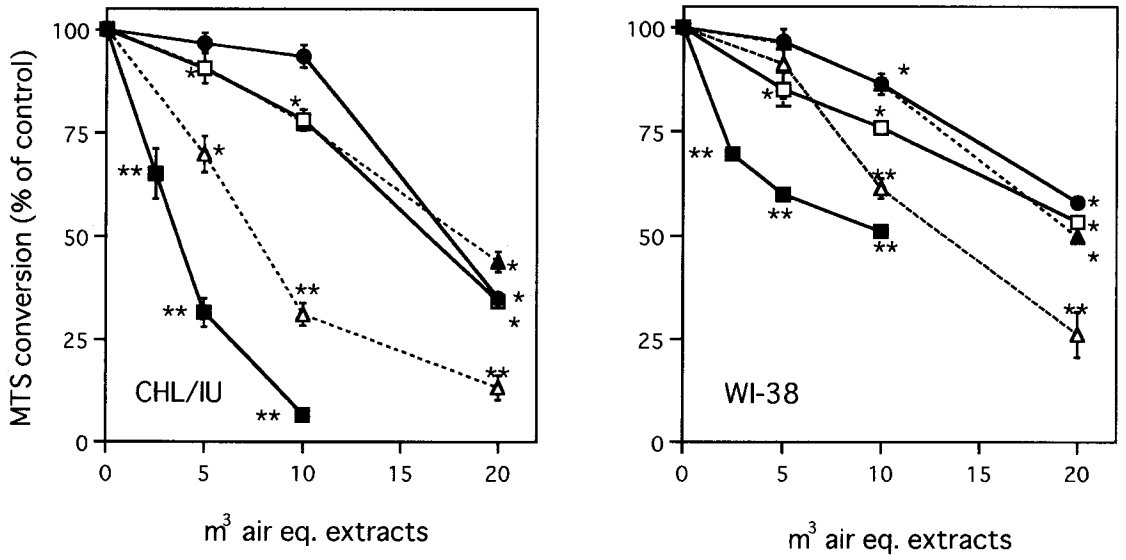

Fig. 2. Cytotoxicity of Airborne Particulate Extracts in MTS Assay with Chinese Hamster and Human Lung Fibroblasts
■ = crude; ▲ = acidic; ● = basic; △ = neutral; □ = Blue-Rayon. Significantly different from control levels: *$p < 0.05$; **$p < 0.01$.

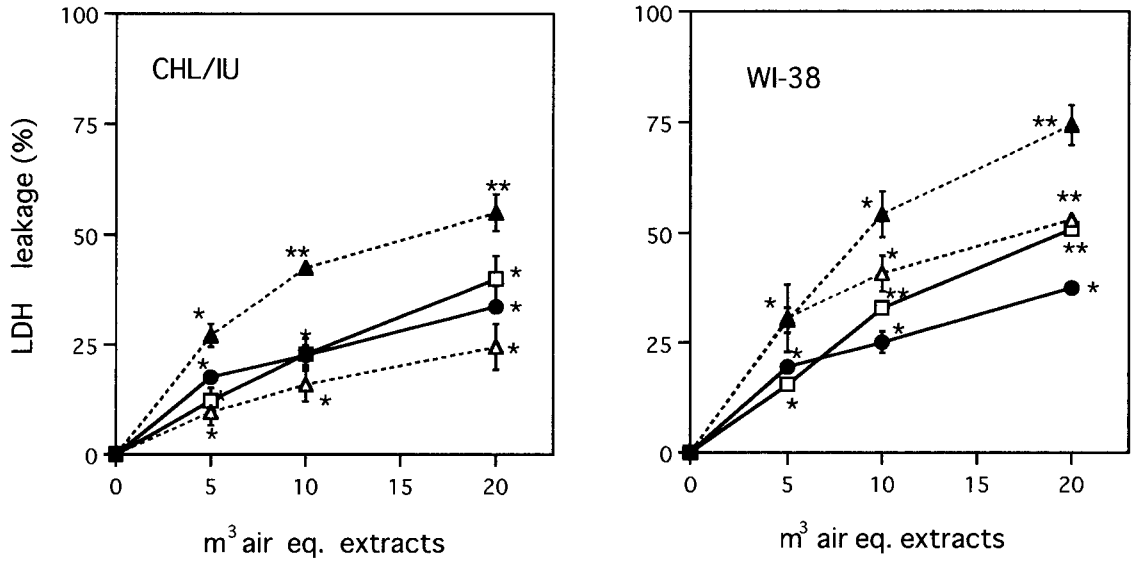

Fig. 3. Cytotoxicity of Airborne Particulate Extracts in LDH Assay with Chinese Hamster and Human Lung Fibroblasts
▲ = acidic; ● = basic; △ = neutral; □ = Blue-Rayon. Significantly different from control levels: $*p < 0.05$; $**p < 0.01$.

Table 2. Summary of the Toxicity of Airborne Particulate Extracts in MTS Assay with Lung Fibroblasts

Cell line	IC_{50} (m^3 air eq. extract)					
	Crude	Acidic	Basic	Neutral	Blue-rayon	Cycloheximide (μg/well)
CHL/IU	4.46	18.4	16.7	9.48	16.2	0.075
WI-38	9.93	20.6	24.1	17.4	21.2	0.125

Table 3. Summary of the Toxicity of Airborne Particulate Extracts in LDH Assay with Lung Fibroblasts

Cell line	EC_{50} (m^3 air eq. extract)			
	Acidic	Basic	Neutral	Blue-rayon
CHL/IU	16.3	27.1	38.4	21.8
WI-38	11.4	24.1	12.2	18.4

values of the acidic fraction were 16.3 and 11.4/m^3 air eq. with CHL/IU and WI-38, respectively. All extracts and fractions were more cytotoxic to WI-38 in LDH leakage assay. The effects on cell proliferation curves are shown in Fig. 4. Crude extract was the most toxic to both cell lines. Other extracts and fractions were moderately toxic in both cell lines.

Airborne particulates with mutagenic and carcinogenic activity pose a health risk.[17] A variety of aerosols are suspended in the atmosphere we breathe. The respiratory tract is the major site of exposure to airborne particulates.[19] It is also the most common site of cancer in humans. Therefore, tracheal and lung cells in culture are possible indicators of the cytotoxicity of airborne particulates.

In this study, the concentration of airborne particulates was in the range 53.7 to 195.8 μg/m^3 (mean value, 128.9 μg/m^3). The values are higher than those collected at a distance from the road (23.9–84.3 μg/m^3), probably because the samples were collected roadside in heavy traffic. Crude extract and its fractions induced in both cell lines, significant, dose–related inhibitory effects on cell proliferation in MTS assay. Crude extract caused potent inhibition; 5% of control cell proliferation was observed at a concentration of 10 m^3. It is known that the acidic and neutral fraction of the crude extract derived from airborne particulates contains fatty acids, phenols, aldehydes and polyaromatic compounds.[20,21] The cytotoxicity of the crude extract from airborne particulates is probably due to their interaction.

The results of LDH leakage assay showed that airborne particulates have a potent inhibitory effect on LDH activity. On the other hand, the fractionation of crude extract had some effect on LDH leakage of cytosol. It may be that the inhibitory effect of crude extract is due to the interaction of its compounds.

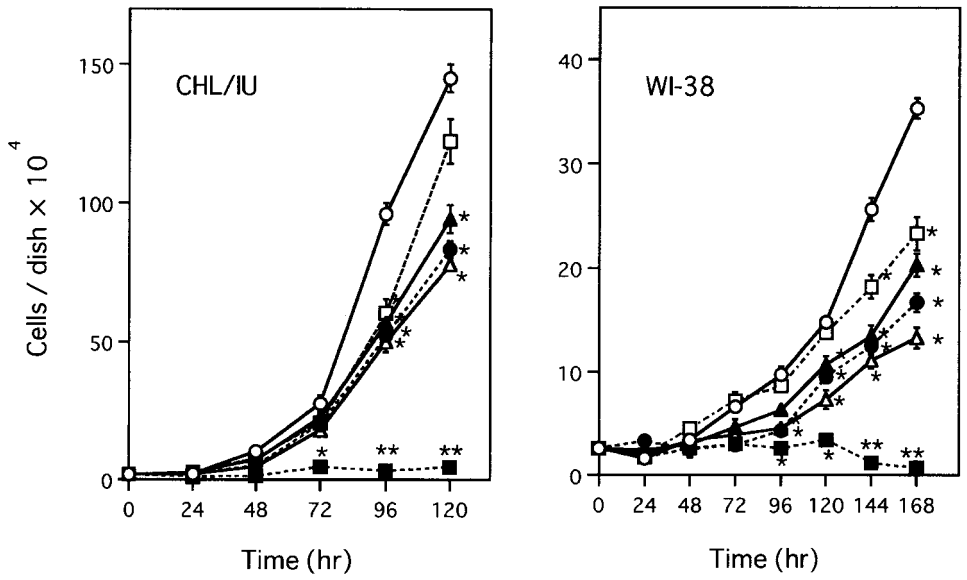

Fig. 4. Effect of Airborne Particulate Extracts on Cell Proliferation
○ = control; ■ = crude; ▲ = acidic; ● = basic; △ = neutral; □ = Blue-Rayon. Significantly different from control levels: $*p < 0.05$; $**p < 0.01$.

Inhibition of cell proliferation was observed in all extracts and fractions, especially in the crude extract, but the cells treated with the fractions of crude extract proliferated to some degree with time.

MTS assay is being widely exploited to investigate the mechanisms of both cell activation and cell damage. The assay is based upon the mitochondrial bioreduction of a tetrazozium salt and can detect reversible functional cell damage. Cell proliferation assay detects reversible cell damage and also irreversible cell modification. On the other hand, LDH assay detects irreversible cell modification.

Our studies of airborne particulates of roadside atmosphere have shown that crude extract was the most cytotoxic in MTS assay and cell proliferation. Therefore, these results reveal that the extract of airborne particulates have an effect on cell function. It has been definitely shown by the result of LDH assay that crude extract has a potent inhibitory effect on LDH activity, and fractionated samples, especially an acidic fraction, exerted irreversible cell modification. The different reactions observed in both cell lines were probably due to a difference in the cellular sensibility to samples.

The compounds in airborne particulates responsible for the cytotoxicity are unknown, but those extracts are composed of hundreds of chemicals[26] and their interaction may cause antagonistic, synergistic, additive or even toxic effects on the assay system. Chemical fractionation of extracts showed that the neutral fraction accounted for most of the cytotoxic activity. The dose–related cytotoxicity of airborne particulate extracts in human and rodent lung fibroblast cells reveals a risk for humans. To evaluate the health risk we must take into consideration a daily respiratory ventilation of 12–14 m^3 air. Further studies will be needed to assess the risk of the permanent inhaling of cytotoxic compounds.

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