Negative Effect of Photocopier Toner on Alveolar Macrophages Determined by *In Vitro* Magnetometric Evaluation

Yasushi FURUKAWA¹, Yoshiharu AIZAWA¹*, Mitsushi OKADA¹, Mitsuyasu WATANABE¹, Masato NIITSUYA¹ and Makoto KOTANI²

¹Department of Preventive Medicine and Public Health, Kitasato University School of Medicine, Kanagawa 228-8555, Japan
²Department of Electronics, Faculty of Engineering, Tokyo Denki University, Tokyo 101-0054, Japan

Received November 17, 2000 and acceptted December 25, 2001

Abstract: Photocopier toner has been implicated in the etiology of some pulmonary diseases. We examined here the *in vitro* toxicity of toner particles to alveolar macrophages. Cell magnetometry revealed that relaxation was not delayed in macrophages exposed to toner, which represents a rapid decrease in the remaining magnetism emitted by phagocytosed magnetite. However, relaxation was delayed in macrophages exposed to silica (positive controls). The release of intracellular LDH enzyme activity to the extracellular space was negligible in cells exposed to toner compared with negative and positive controls. Morphological examinations by light and electron microscopy revealed no abnormal findings in the exposed cells. A histochemical study using TUNEL staining and the electrophoretic profile of DNA obtained from cells exposed to toner and to silica were negative for apoptosis. The results of the present and other investigations into animal exposure indicate that photocopier toner is toxicologically inert. However, although the present study examined only effects *in vitro*, exposure to toner should be minimized because lung overloading in animals has been reported.

Key words: Photocopier toner, Magnetometry, Alveolar macrophage, Enzyme release, Apoptosis, Carbon black, Silica

Introduction

Photocopier toner is a chemically synthesized powder that forms images on paper reproduced by copy machines. About 30,000 tons of toner are produced annually in Japan. Because copiers are now fixtures of small offices, the possibility of exposing office and maintenance workers to toner has increased. A few patients have developed pulmonary disease due to such exposure. Gallardo¹ described a woman who presented with siderosilicosis due to photocopier toner dust after 6 years of employment at a photocopy shop. X-ray energy dispersive microanalysis of intracellular particles in the lung, mediastinal lymph nodes and toner dust revealed Cu and Si. Toner was therefore implicated as a causative agent.

The diameter of photocopier toner particles is currently about 7–10 µm, although it is likely to be reduced in attempts to improve image quality. Such a decrease will result in an increased amount of inhaled toner, which will lead to increased deposition in the alveolar region. We therefore examined the toxicity of photocopier toner to alveolar macrophages *in vitro* by cell magnetometry, intracellular
enzyme release, electron microscopy and by histochemical means to detect apoptosis.

Magnetometric measurements were developed to evaluate the clearance of magnetite from the lungs of smokers\(^3,4\). Studies employing this technique show a rapid decrease in remnant magnetic fields, which have been generated by the external magnetization of magnetic iron oxide particles in alveolar macrophages. This phenomenon is known as relaxation, and it is principally attributed to the result of the cytoskeleton-driven rotation of phagocytosed magnetic iron particles in the alveolar macrophages\(^5,6\). Using the rate of relaxation as an indicator of the cytoskeletal function of cells, we identified several chemicals that are cytotoxic\(^7–10\).

Materials and Methods

Reagents

Iron oxide (Fe\(_3\)O\(_4\)) particles with a mean geometric diameter of 0.26 \(\mu\)m (Toda Kogyo Company, Hiroshima) constituted the index of magnetometry.

The photocopier toner examined in this study was enriched with inhalable particles compared with the commercial product. This toner was produced by heating a mixture of 5% carbon black and 87% styrene/butyl-acrylate polymer and 8% polyethylene wax. The average geometric diameter of the particles was 3.0 \(\mu\)m. Silica was the positive control and provided by Dr. K. Honma, former Departmental Chairman of the National Institutes of Industrial Health. The average geometric diameter of silica particles was 2.16 \(\mu\)m.

The materials were suspended in phosphate-buffered saline (PBS, Takara Shuzo Co., Ltd., Otsu) (pH 7.4) by ultrasonic oscillation.

Collection of alveolar macrophages

Male F344/NS1c rats weighing approximately 180 g were anesthetized with an intraperitoneal injection of pentobarbital sodium (1.5–2.5 ml/kg; Nembutal Injection, Abbott Laboratories, Chicago, IL, USA). After a median abdominal section, the abdominal aorta was cut and the lungs were collapsed. A silicone catheter (Intravenous Catheter 7 French for Cut-Down, Atom Medical Co., Tokyo) was then inserted into the exposed trachea. Thereafter, 4.0 ml of pH 7.4 cold PBS containing 0.1% ethylenediamine-tetraacetic acid (EDTA) was administered and aspirated through the catheter ten times, and then the lavage fluid was collected. Approximately 6–8 \(\times\) 10\(^6\) cells were obtained from each animal.

Exposure to chemicals and cell culture

Eagle’s minimum essential medium (MEM, Nissui Pharmaceutical Co., Tokyo; 1.0 ml) supplemented with 10% fetal bovine serum (FBS) and containing 1 \(\times\) 10\(^6\) cells was poured into each well (Nuncloon 4 Well Multidishes, Japan Inter Med Co., Tokyo) containing a cell disk (R1, Sumitomo Bakelite Co., Ltd., Tokyo) at the bottom. The cells were divided into 3 groups according to the product to which they were exposed. All groups included Fe\(_3\)O\(_4\) at a final concentration of 50 \(\mu\)g/ml. The final concentration of toner was 20, 40 or 60 \(\mu\)g/ml (toner group), that of silica particles was 60 \(\mu\)g/ml (silica group) and the volume of PBS was 50 \(\mu\)l (control group). This dose was sufficient to be engulfed by almost all cells according to microscopic examination.

Cytomagnetometry

Cell disks were removed from the wells after 18 hours in a 5%-CO\(_2\) incubator. Glass tubes containing medium and a cell disk on the bottom were magnetized in 70 mT for 10 milliseconds. The tubes were installed on a stage that was turned at 10 rpm above the probe of a fluxgate magnetometer and maintained at 37\(^\circ\)C by a heater with a thermostat.

Intracellular enzyme release

We evaluated cellular enzyme release by measuring lactate dehydrogenase (LDH). Serum-free medium (1.0 ml; Macrophage-SFM, liquid, Life Technologies, Inc., Rockville, MD, USA) containing 1 \(\times\) 10\(^6\) alveolar macrophages was poured into each well with a cell disk at the bottom and incubated for 18 hours with test chemicals in a 5%-CO\(_2\) incubator at 37\(^\circ\)C. The medium in the wells was centrifuged at 1800 rpm for 5 minutes, then 50 \(\mu\)l of supernatant was assayed using LDH-UV test kits (Wako Pure Chemical Industries, Ltd., Osaka) as described (Wroblewski and LaDue 1955)\(^11\) using a U-3000 type autospectrometer (Hitachi Ltd., Tokyo). Triton-X 100 was added to the control group to measure the total LDH activation index derived from both the intracellular and the extracellular matrices. The LDH release rate (%) was calculated using the following equation:

\[
\text{LDH release rate (\%)} = \left( \frac{\text{LDH activation index from cells exposed to silica or photocopier toner}}{\text{total LDH activation index}} \right) \times 100
\]

Morphological examination

Macrophages that adhered to glass treated with polycations were washed with 0.1 M cacodylate buffer (pH 7.4) and fixed with 1% of glutaraldehyde at 4\(^\circ\)C for 3 hours. The buffer was removed and the macrophages were postfixed with 1% osmium tetroxide at 4\(^\circ\)C for 3 hours. Ultrathin sections were stained with 3.0% uranyl acetate and Reynolds’...
lead citrate. Specimens were spattered with iron or dehydrated and embedded in resin for observation by SEM (S-4500FE, HITACHI Ltd.) and TEM (H-600, HITACHI Ltd.), respectively.

DNA ladder method

The DNA ladder method proceeded in two steps, DNA extraction and agarose gel-electrophoresis using an apoptosis ladder detection kit (Wako Pure Chemical Industries Ltd.) according to the manufacturer’s instructions. However, some steps were altered as follows. After DNA extraction with 2-propanol, cold 0.3M-CH3COONa 200 µl and cold 100%-ethanol 500 µl were added to the sediment and the sample was placed at –80°C for 15 minutes.

TUNEL method

After adding the chemicals as described, macrophages were cultured overnight (18 hours) in wells (Nunc LAB-TEK 8 Chamber Slide, Nunc Inc., Naperville, IL, USA) containing toner, silica or buffer. The medium was removed and the cells were fixed using 5% paraformaldehyde for over 90 minutes to detect in situ apoptosis using a kit (Apop Tag Plus-Peroxidase, Oncor Co., Gaithersburg, MD, USA) with some modifications (Satoh and Tsuchiya 1999) as follows. Cultured cells were initially digested with proteinase K (Wako Pure Chemical Industries Ltd.). The secondary antibody was anti-digoxigenin-alkaline phosphatase (Boehringer Mannheim Co., Tokyo), which omitted the quenching step. The cells were stained with 5-bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride (DAKO JAPAN Co., Kyoto) and washed with Tris buffered saline. Positive controls were 4 µm thick sections of 10% buffered formalin-fixed and human tonsils embedded in paraffin wax.

Statistical analysis

Differences between relaxation curves were analyzed using the two way analysis of variance (ANOVA). LDH release and apoptosis induction rates were examined by Dunnett’s multiple comparison procedure.

Results

Cytomagnetometry

Relaxation curves obtained from cells exposed to toner and silica are shown in Fig. 1. Relaxation of the silica group was delayed, whereas that of the toner groups at all tested doses did not differ from that of the control group. The plot shows a declining relaxation curve that is the plotted remnant magnetic field normalized to 100% immediately after stopping magnetization. Two way ANOVA indicated a significant difference among groups (p<0.001) and Dunnett’s multiple comparison test showed a significant difference between the silica and toner groups (p<0.001) as well as between the silica and control groups.

Intracellular enzyme release

The data are summarized in Fig. 2. The release of LDH was low grade but significantly higher in alveolar
macrophages exposed to silica than to toner or the control group (p<0.05).

Morphology
Observation by SEM showed an almost identical appearance except for several cytoplasmic projections towards particles in the toner and silica groups compared with controls. (Fig. 3a, b and c).
Observation by TEM revealed peripheral nuclear condensation in some cells exposed to silica. However, most cells exposed to silica and toner remained as intact as the control. (Fig. 3d, e and f).

DNA ladder
The electrophoretogram shown in Fig. 4 indicates no fragmentation of nucleosome units (about 180 bp) in the DNA profile of alveolar macrophages exposed to toner and silica particles compared with control cells.

TUNEL method
The results of apoptotic staining are summarized and a photograph are shown in Figs. 5 and 6. The ratio of apoptotic cells did not increase in the toner group. In contrast, the average positive rate of staining in the silica group was less than 10% with significant difference compared with the control and toner groups.

Discussion
The aim of the present study was to evaluate the toxicity of photocopier toner to rat alveolar macrophages. The literature states that some pulmonary diseases might be associated with exposure to photocopier toner1, 2), with support for such an association resting on the pathological finding that higher concentrations of specific chemicals found in lung cells are also found in toner. However, this does not constitute experimental proof of an association. The described patients had a combination of iron and silica or of copper and silica in their lung cells, as did the corresponding toner dust. According to the manufacturer’s information, the photocopier toner examined in the present study was composed of carbon black and a styrene and butyl-acrylate polymer and polyethylene wax. Magnetometric and morphological measurements, intracellular enzyme release and an examination of apoptosis indicates that this toner is not cytotoxic to rat alveolar macrophages. Magnetometry showed that the relaxation curves of the toner and control groups were identical, whereas relaxation was
delayed in the group exposed to silica.

Cytomagnetometry is a modified method of pneumomagnetometry\(^3\). The rapid decrease in the size of the magnetic field is called relaxation. This phenomenon is thought to be due to the random rotation of magnetized particles engulfed by alveolar macrophages, since harvested phagocytes exposed to magnetite in vitro behave in the same manner\(^13\).

Cytomagnetometry can evaluate the toxicity of chemicals to phagocytes in vitro, because the random rotation of magnetized particles is thought to be driven by the cytoskeleton connected with phagosomes\(^6\). Although details of the mechanism remain to be elucidated, we presume that delayed relaxation is due to cytoskeletal dysfunction induced by exposure to chemicals. Exposing the lungs and alveolar macrophages to gallium arsenide and silica respectively in vitro and in vivo delays relaxation\(^7, 9\). In vitro exposure to chrysotile fiber\(^6\), silicon carbide whiskers\(^9\), titanium oxide and arsenide chloride also delays relaxation. On the other hand, relaxation is not delayed by exposure to calcium carbonate either in vivo or in vitro\(^14, 15\).

The present study found that doses of up to 60 µg/ml of photocopier toner did not influence macrophage relaxation, whereas exposure to 60 µg/ml of silica delayed relaxation. Exposure to 60 µg/ml of chemicals influences relaxation according to our experience with other substances and this dose was sufficient to be engulfed by almost all cells according to microscopic examination.

The release of LDH from the cytoplasm of alveolar macrophages into the external fluid indicates alteration of the cell membrane, manifesting as chemically induced necrosis. The present study found that the LDH release rate of the silica group was greater than that of the control group. The toner group released negligible amounts of LDH levels that were almost identical to those of the control. We measured LDH in serum-free medium, because FBS contains LDH.

Morphological examinations revealed no remarkable changes in the toner group. Light microscopy revealed no degeneration of alveolar macrophages in the toner or silica groups. Electron microscopy did not reveal any structural degeneration of the cell membrane or intracytoplasmic organelles in the toner groups, whereas a mild nuclear change suggested apoptosis in some of the silica group.

Neither DNA electrophoresis nor TUNEL staining showed changes suggestive of apoptosis in cells exposed to toner, whereas the apoptotic incidence was low but significantly increased in the silica group.

Asbestos\(^{16}\), silica\(^{17, 18}\), TNF-α\(^{19, 20}\), endotoxin\(^{21}\) and benzo[a]pyrene adsorbed onto carbon black (CB+Bap)\(^{22}\) induce apoptotic changes in alveolar macrophages. The phagocytosis of diesel exhaust particles and silica by alveolar macrophages induces apoptosis by generating reactive oxygen radicals and by interacting with scavenger receptors, respectively\(^{18, 23}\). CB+Bap evoked programmed cell death or apoptosis in cultured macrophages was assessed by genomic DNA-laddering assays\(^{22}\). To examine the acute effects of carbon black exposure, 16 healthy individuals were exposed for 5-hours in an environmental chamber to Xerox toner (containing carbon black) at total dust levels of 2, 10

---

**Fig. 4. Electrophoretic profile of DNA extracted from alveolar macrophages.**

Lane a: indicators of known sizes (kbp). Lane b: Control. Lane c: Macrophages exposed to 60 µg/ml of photocopier toner for 18 hours. Lane d: Macrophages exposed to 60 µg/ml of silica for 18 hours.
and 25 mg/m$^3$, the latter two of which were almost three and six times the threshold limit value, respectively, for carbon black$^{24}$. The results of pulmonary function tests and respiratory symptoms were unremarkable, indicating that the acute effects of carbon black exposure are the same as those of any other insoluble particulates$^{25}$.

Tsunoda$^{26}$ reported that 41 workers exposed to toner dust and ozone showed a higher rate of profusion (0/1 or over) than non-exposed workers with no significant difference in ventilatory function tests.

Fibrosis was not demonstrated in rats given intratracheal instillations of carbon black$^{27}$. Mice, hamsters, guinea pigs and primates have been exposed to channel black and furnace black at 84.7 and 56.5 mg/m$^3$, respectively, for 7 hr/day, five days/week for up to an entire life-span$^{28}$. Radiographic examinations have revealed carbon deposition in the lungs of the mice and primates with little or no pulmonary fibrosis. Bellman et al.$^{29}$ reported that the alveolar clearance of two types of radioisotopically labeled particles (iron oxide and polystyrene) is substantially impaired at high levels of exposure to toner and moderately slow at medium levels. The excessive quantity of toner retained and the substantially retarded clearance in the high-exposure group indicate “lung overloading”. In the same experiment, the estimated fibrogenic potency of TiO$_2$, toner and SiO$_2$ was 1:5:418$^{30}$.

Cocarla et al.$^{31}$ observed 29 instances of pneumoconiosis in 143 carbon black workers with a mean exposure time of 19 years. Gardiner et al.$^{32}$ analyzed 1742 employees in 15 carbon production plants to identify symptoms of chronic bronchitis associated with increasing indices of current exposure. Lung function decreased in relation to increasing dust exposure and 25% of the chest radiographs revealed small opacities of category 0/1 or greater.

Polystyrene is an ingredient of photocopier toner that is considered to be physiologically inert. Polystyrene dust causes no known adverse effects other than irritation to the upper respiratory tract$^{33}$. Polystyrene particles did not affect relaxation and served as a negative control in a previous magnetometric study$^{34}$. Butyl-acrylate is another element of the photocopier toner used in the present study. It is a colorless, combustible, highly reactive compound that is a skin, eye and respiratory tract irritant. However, it does not seem to dissociate from toner$^{35}$.

The results of the present study indicate that toner dust is
toxicologically harmless. Filters are usually attached to the exhaust of copy machines to reduce the discharged dust concentration to 0.01 mg/m³, and this level should not adversely affect health. However, since even inert dust may induce generic responses of the respiratory system to saturation of its detoxification capacity such as “lung overloading”, worker exposure to toner should be minimized.

Acknowledgments

The authors are grateful to Ms. Y. Sugiura, Ms. Y. Komatsu, Ms. Y. Inoue, Mr. S. Miyazawa and Ms. N. Nemoto for technical assistance. This study was partly supported by the Academic Frontier Project of the Ministry of Education, Science, Sports and Culture.

References