

Carbon fullerenes (C60s) can induce inflammatory responses in the lung of mice

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ABSTRACT

Fullerenes (C60s) occur in the environment due to natural and anthropogenic sources such as volcanic eruptions, forest fires, and the combustion of carbon-based materials. Recently, production and application of engineered C60s have also rapidly increased in diverse industrial fields and biomedicine due to C60' unique physico-chemical properties, so toxicity assessment on environmental and human health is being evaluated as a valuable work. However, data related to the toxicity of C60s have not been abundant up to now. In this study, we studied the immunotoxic mechanism and change of gene expression caused by the instillation of C60s. As a result, C60s induced an increase in sub G1 and G1 arrest in BAL cells, an increase in pro-inflammatory cytokines such as IL-1, TNF- α , and IL-6, and an increase of Th1 cytokines such as IL-12 and IFN- γ in BAL fluid. In addition, IgE reached the maximum at 1 day after treatment in both BAL fluid and the blood, and decreased in a time-dependent manner. Gene expression of the MHC class II (H2-Eb1) molecule was stronger than that of the MHC class I (H2-T23), and an increase in T cell distribution was also observed during the experiment period. Furthermore, cell infiltration and expression of tissue damage related genes in lung tissue were constantly observed during the experiment period. Based on this, C60s may induce inflammatory responses in the lung of mice.

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Introduction

Fullerene was discovered in 1985 by Robert Curl and is a carbon allotrope that is composed entirely of carbon. The most abundant form is buckminsterfullerene (C60) with 60 carbon atoms arranged in a spherical structure (Kroto et al., 1985). C60s occur in the environment due to natural and anthropogenic sources such as volcanic eruptions, forest fires, and the combustion of carbon-based materials (e.g., coal, soot) as MWCNT (multiwall carbon nanotube). Recently, production and application of engineered C60s have also rapidly increased in diverse industrial fields and biomedicine due to C60' unique physico-chemical properties, so toxicity assessment on environmental and human health is being evaluated as more valuable work. In this respect, it is fortunate that full-scale exposure to the public has not been started yet, so the current study results can be directly fed back to the product development plan. Manufacturers can produce safer products without risks of waiting for toxicology studies to be finished after the products are widely marketed (Tsuda et al., 2008).

Currently, there are contradictory reports whether C60s act as a radical scavenger or as a generator. Krusic et al. characterized

fullerenes as “radical sponges” in 1991, and many researchers supported the hypothesis by publishing other results. However, others have reported that C60s induced damage in tissues and cells through lipid peroxidation and oxidative stress in vivo and in vitro (Krusic et al., 1991; Misirkic et al., 2009; Sayes et al., 2005; Oberdorster, 2004; Hu et al., 2007; Zhu et al., 2006, 2008; Usenko et al., 2008).

In addition, particles stimulate the immune system in the body. C60s induced MHC (major histocompatibility complex)-mediated immunotoxicity under the systemic inhalation exposure condition and a significant increase of protein in BAL (Bronchoalveolar Lavage) fluid due to a nose-only exposure system (Baker et al., 2008; Fujita et al., 2009). In this study, we investigated the immunotoxic mechanism of C60s in mice intratracheally instilled with this material. Furthermore, to give useful information concerning the hazards of nanoparticles to the public, characterization related to physico-chemical properties of nanoparticles such as morphology, size, and surface chemistry had to be performed before the toxicity test. C60s have a low solubility of about 1.3×10^{-11} $\mu\text{g/L}$ in water, and form water-stable aggregates with diameters up to 500 nm (Spohn et al., 2009). Toxicologists get water-soluble suspension of C60s by utilizing various organic solvents, including THF, benzene, toluene, and DMSO to overcome this problem. Prior studies suggest that this solvent can alter the physico-chemical properties and the toxicity of C60s. Therefore, we measured the size, conductance, mobility, and surface charge of C60s,

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which was modified by toluene as the solvent for PBS-soluble suspension, and confirmed the shape, aggregation, and agglomeration state using TEM prior to the toxicity test; we also utilized the solvent as control vehicle.

Materials and methods

Animals. ICR male mice (25 ± 1 g) were purchased from Orient-Bio Animal Company (Seongnam, Gyeonggi-do, Korea) and were allowed to adapt to the animal room conditions prior to initiation of the study. The environmental conditions were a temperature of 23 ± 1 °C, relative humidity of $55 \pm 5\%$, and a 12 h light/dark cycle. All the animals used in this study were cared for in accordance with the principles outlined in the “Guide for the Care and Use of Laboratory Animals” issued by the Animal Care and Use Committee of NVRQS (National Veterinary Research and Quarantine Service). The animals were treated to observe dose-dependent ($n = 5$) and time-dependent ($n = 11$) change, respectively.

Preparation of test material. C60s were purchased from Tokyo Kasei Kogyo Co., Ltd. C60-water suspension was prepared using the method proposed by Andrievsky (Andrievsky et al., 1995). C60s 0.1 g was added to 50 mL toluene. It was added to ion-free distilled water 300 mL. This layered mixture was then sonicated with heating until all of the toluene evaporated. The brown suspension was then filtered sequentially through a 200 nm pore membrane filter, and then C60s in water-phase were obtained. Finally, the water-soluble suspension was diluted to 1:1 by $2 \times$ PBS (0.15 M, pH 7.2). Particle size measurement was performed using a submicron particle sizer (NICOMPTM, CA, USA), and images of the nanoparticles were acquired by transmission electron microscopy (TEM; JEM1010, JEOL, Japan). Then, the surface charge was measured using a zeta potential analyzer (Brookhaven Instruments Corp., NY, USA).

Intratracheal instillation and sample preparation. C60s were delivered with a 24-gauge catheter at the described dose (dose-dependent change: 0.5 mg/kg, 1 mg/kg and 2 mg/kg, time-dependent change: 2 mg/kg) by intratracheal instillation under light tiletamine anesthesia (day 0). The animals were terminated at days 1, 7, 14, and 28 after treatment. The control group was treated by a solvent that was manufactured by the same method. At the selected time intervals after instillation, 1 mL of blood was collected per mouse from the retro-orbital venous plexus into heparinized capillary tubes. One hundred microliters of whole blood was used for cell phenotype and the rest was centrifuged at 3000 rpm for 20 min for serum preparation. About 350 μ L of serum was obtained from each mouse pooled for cytokine assay. BAL fluid collection was performed by cannulating the trachea and lavaging the lungs with 1 mL of cold sterile (Ca^{2+} plus Mg^{2+})-free PBS (0.15 M, pH 7.2). Approximately 500–600 μ L BAL fluid was harvested per mouse, and this was centrifuged at 3000 rpm for 10 min, and supernatant of 400 μ L per mouse was pooled for cytokine assay, and fluid of 50 μ L containing pellet was pooled for cell cycle.

To isolate the splenocytes, the spleen was aseptically removed from an ICR mouse, and suspended by passage through a sterile plastic strainer in Dulbecco's modified Eagle's medium (DMEM) with 2% fetal bovine serum (FBS). After centrifugation at 1500 rpm for 3 min, the supernatant was discarded, and the pellet was briefly vortexed in 500 μ L distilled water and then resuspended in DMEM with 2% FBS. The cells were filtered through a nylon mesh to yield the final splenocyte preparation (Byun et al., 2006; Vendrame et al., 2006; Manosroi et al., 2005; Cho et al., 2007).

Cell cycle analysis. BAL cells were stained using propidium iodide and RNase. The cell cycle was analyzed by measuring their DNA content with the FACSCalibur system and CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA).

Measurement of cytokines. The concentrations of each cytokine in the supernatant of the BAL fluid and serum were determined using commercially available ELISA kits (eBioscience, San Diego, CA, USA). Briefly, each well of a microplate was coated with 100 μ L of capture antibody, and incubated overnight at 4 °C. After washing and blocking with assay diluent, and BAL fluid, serum, or standard antibody was added to individual wells, then the plates were maintained for 2 h at room temperature. The plates were washed, and biotin-conjugated detecting antibody was added to each well, and incubated at room temperature for 1 h. The plates were washed again and further incubated with avidin-HRP for 30 min before detection with TMB solution. Finally, reactions were stopped by adding 1 M H_3PO_4 , and the absorbance at 450 nm was measured with an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). The amount of cytokine was calculated from the linear portion of the generated standard curve (Byun et al., 2006).

Immunophenotyping. All monoclonal antibodies were purchased from eBioscience (San Diego, CA, USA). T cells (CD3, 1:50), B cells (CD19, 1:50), NK cells (DX5, 1:100), CD4+ T cells (CD4+, 1:160), and CD8+ T cells (CD8+, 1:50) were identified using directly conjugated anti-mouse antibodies. Briefly, splenocytes of $3\text{--}5 \times 10^3$ cells/mL were blocked with Fc-block (eBioscience, San Diego, CA, USA) to reduce non-specific antibody binding. Cells were then incubated in the dark with 10 μ L of the appropriate fluorochrome-conjugated antibody for 20 min at 4 °C. Cells were then washed with 500 μ L FACS (Fluorescence Activated Cell Sorter) buffer. The blood was lysed for 5 min with FACS lysis buffer (BD Bioscience, Franklin Lakes, NJ, USA) at room temperature and then re-washed with FACS buffer. Finally, each sample was fixed with 1% paraformaldehyde until further analysis. Flow cytometry analysis was performed on the FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA). Control samples were matched for each fluorochrome. Data were analyzed using CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA) (Silva et al., 2005; Vendrame et al., 2006).

Measurement of IgE. The concentration of IgE in serum and the supernatant of BAL fluid was determined using commercially available IgE ELISA kits (Komabiotek, Seoul, Korea). Briefly, each well of a microplate was coated with coating antibody and incubated overnight at 4 °C. After the wells were washed and blocked with blocking solution for 1 h at room temperature, BAL fluid, serum and IgE standards were added to individual wells, then left for 1 h at room temperature. The plates were washed, detection antibody was added to each well, and the plates were incubated at room temperature for 1 h. After washing, the plates were further incubated with color development solution. Finally, the reaction was terminated by the

Table 1
Primer sequences used in this study.

Primer	GB NO.	Sequence
H2-Eb1	NM_010382	(L) : gccatgaaacatgtctctgc (R) : gaagtgcagacaaaaggca
H2-T23	NM_010398	(L) : aggttcctcacagaccagg (R) : tccacgtagccgacaaatgat
MMP-2	NM_008610	(L) : tgtttcttcgacgggaatgag (R) : ggatggcattccaggagtct
MMP-3	NM_010809	(L) : gttctgggctatcagagggc (R) : ggcagcatcatcttcttca
MMP-11	NM_008606	(L) : cccatgccttcttccctaag (R) : gctgtgggtgtgttagccc
Slpi	NM_011414	(L) : catggctcctgcctgaaacag (R) : ctcttccacagtcagggt
Timp 1	NM_011593	(L) : tatgcccaagaatccagaa (R) : cctgatccgtccacaacag
SOD 1	NM_011434	(L) : cgtccatcagtaggggaca (R) : aatggacacattggccacac

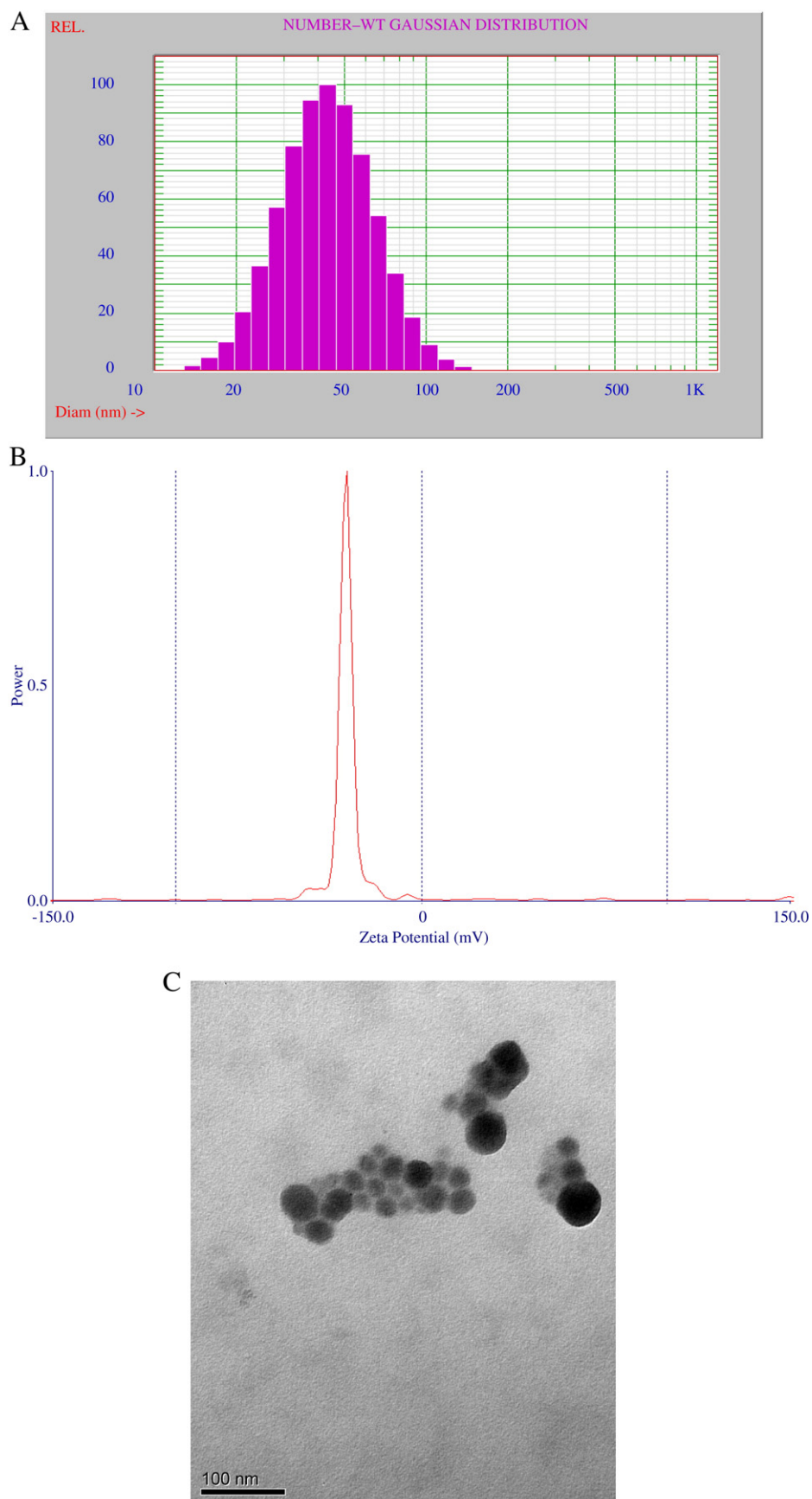


Fig. 1. The physico-chemical properties of C60s. (A) The size distribution, (B) surface charge, (C) TEM image.

addition of 2 M H₂SO₄, and the absorbance was measured at 450 nm with an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). The amounts of secreted IgE were calculated from the linear portion of the prepared standard curve.

Gene expression of tissue. Gene expression analysis using RT-PCR was also evaluated. To prepare the total RNA from lung tissue, the RNeasy Total RNA Isolation System (Qiagen, Madison, WI, USA) was used according to the manufacturer's instructions. Amplified cDNA products were separated on 1.5% agarose gel by electrophoresis. The primer sequence showed on Table 1.

Histopathology. Histopathology analysis was performed and read by a board-certified pathologist in Biototech Cheongwon-gun, Chungbuk, Korea), one of the GLP institutes of Korea. Briefly, lungs from mice of the control group and the treated group were fixed with 10% neutral buffered formalin and processed using routine histological techniques. After paraffin embedding, 3 µm sections were cut and stained with hematoxylin and eosin (H&E) for histopathologic evaluation.

Statistical analysis. The results obtained from the chemically-treated groups were compared to those of the control group. The values were compared using Dunnett's *t*-test after one-way ANOVA analysis, and levels of significance were represented compared to the control group.

Results

Stability of C60s in PBS

Before instillation, we investigated the stability of C60s in PBS. Particle size was 46.7 ± 18.6 nm (Fig. 1A), and surface charge was -30.24 mV, conductance was $22 \mu\text{S}$, and average mobility was -2.36 ($\mu\text{s})/(\text{V}/\text{cm})$ (Fig. 1B). TEM micrographs confirmed that the C60s were spherical in shape and were mildly agglomerated in PBS (Fig. 1C).

Cell cycle of BAL cell

Distribution of the G1 stage increased to 95.04%, 96.89%, 97.2%, and 97.29% at days 1, 7, 14, and 28 after treatment with 2 mg/kg C60s, respectively, whereas that of the control group was 79.64%. And, the distribution of sub G1 regard to cell apoptosis was significantly increased to 24.83% and 25.41% at days 1 and 7 after treatment, respectively, but returned to the same level with the control group at day 28 after treatment (Fig. 2).

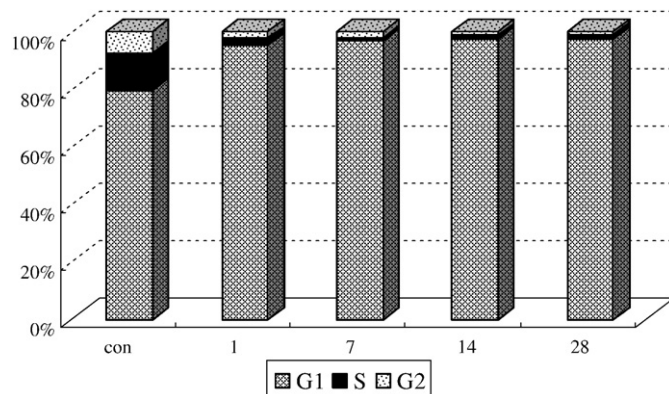


Fig. 2. Change of cell cycle following instillation of C60s. Mice were treated with 2 mg/kg of C60s, and BAL cells were harvested and pooled at days 1, 7, 14 and 28, respectively ($n = 11$). The cell cycle was analyzed by measuring the DNA content with PI staining and RNase digestion in the FACSCalibur system.

Cytokines in the BAL

To identify inflammatory responses induced by C60s instillation, the concentrations of pro-inflammatory cytokines (IL-1, TNF- α , and IL-6), Th0 cytokine (IL-2), Th1-type cytokines (IL-12 and IFN- γ), Th2-type cytokines (IL-10) and antioxidant cytokine (TGF β) were measured in the BAL fluid (Fig. 3). Secretion of pro-inflammatory cytokines (IL-1, TNF- α and IL-6), Th1-type cytokines (IL-12, IFN- γ) and Th2-type cytokines (IL-10) was increased in a dose-dependent manner at day 1 after treatment (Fig. 3A). Pro-inflammatory cytokines (IL-1, TNF- α , and IL-6) reached the maximum at day 1 after a single instillation with 2 mg/kg C60s and decreased in a time-dependent manner. Th0 cytokine, IL-2, reached the maximum at day 14 after a single instillation. IL-12 reached the maximum at day 1 after treatment

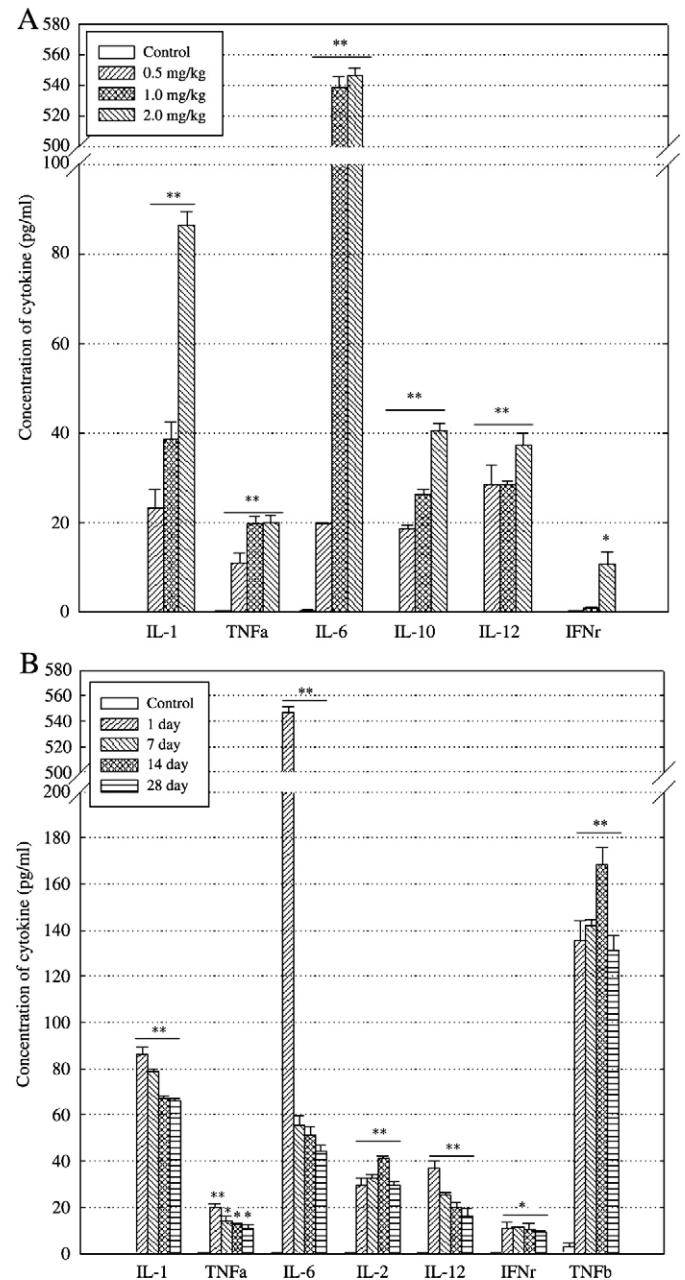


Fig. 3. Changes in cytokine levels in BAL fluid after a single instillation of C60s. BAL fluid was harvested and pooled (400 µL per mouse) at days 1, 7, 14 and 28, respectively ($n = 11$), after instillation of C60 (2 mg/kg), and cytokine concentrations were determined using ELISA kits. * $P < 0.05$, ** $P < 0.01$. (A) Dose-dependent change ($n = 5$). (B) Time-dependent change ($n = 11$).

and decreased in a time-dependent manner, whereas IFN- γ remained at a similar level until day 28. Furthermore, TGF- β constantly remained at a high level during the experiment period (Fig. 3B).

Cytokines in the blood

As shown in Fig. 4, the secretion of TNF- α , IL-6, IL-12, and IFN- γ increased in a dose-dependent manner in the blood by C60s treatment, but IL-1 and IL-10 were not detected. In addition, when instilled with 2 mg/kg of C60s, TNF- α , IL-6, and IL-12 reached the maximum at day 1 after treatment, and decreased in a time-dependent manner as in the BAL. IFN- γ reached the maximum at day 14 after treatment. However, IL-1, IL-2, and TGF- β were not detected at any of the designed time points.

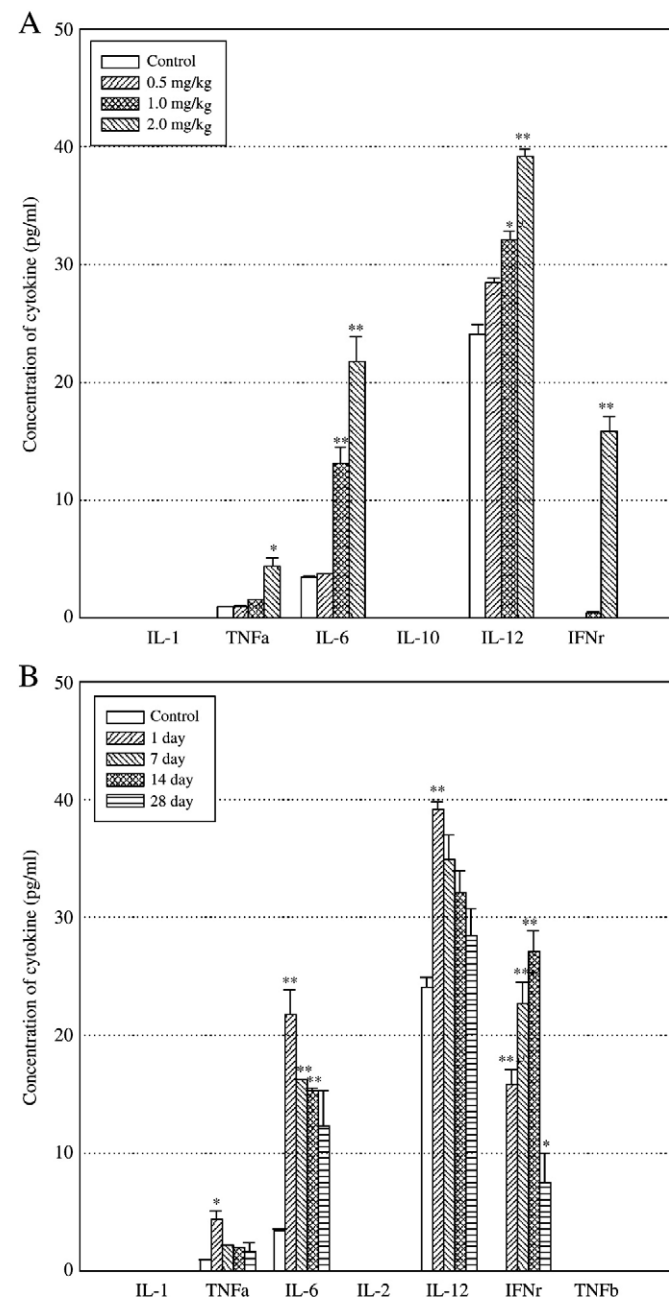


Fig. 4. Changes in cytokine levels in the blood after a single instillation of C60s. Serum was harvested and pooled (350 μ L per mouse) at days 1, 7, 14 and 28, respectively ($n=11$), after instillation of C60 (2 mg/kg), and cytokine concentrations were determined using ELISA kits. * $P<0.05$, ** $P<0.01$. (A) Dose-dependent change ($n=5$). (B) Time-dependent change ($n=11$).

Secretion of IgE

The time-dependent change of IgE following instillation of 2 mg/kg of C60s was also examined. As shown in Fig. 5, IgE reached the maximum at 1 day after treatment in both the BAL and the blood, and decreased in a time-dependent manner. In case of the blood, IgE was detected at the level of 18.58 ± 1.50 ng/mL, 13.35 ± 1.54 ng/mL, 7.71 ± 0.76 ng/mL, and 7.34 ± 0.10 ng/mL at days 1, 7, 14 and 28 after instillation, respectively, and the level of the control group was 5.84 ± 0.70 ng/mL.

Lymphocyte phenotype

A single instillation of a C60s dose of 2 mg/kg significantly increased the proportion of T cells in both blood and spleen. The proportion of T cells in blood lymphocytes was 33.47% in the control group and was 42.82%, 52.53%, and 41.68% at days 1, 14, and 28 after C60s instillation, respectively (Figs. 6A–D). In the spleen, the proportion of T cells composed of 23.35% of the lymphocytes in the control was increased to 31.41%, 29.37%, and 26.82% at the day 1, 14, and 28 groups after C60s instillation (data not shown). The ratio of CD4+/CD8+ T cells also was increased at 1 day after instillation, from 3.47 to 4.53 in blood (Figs. 6E, F) and from 3.75 to 3.85 in spleen (data not shown), which means a significant increase in CD4+ distribution.

Histopathology of lung tissue

Fig. 7 and Table 2 show the histopathological changes in the lung tissue following instillation of C60s. C60s induced cell infiltration in lung tissue during the experiment period.

Gene expression by C60s instillation

As shown in Fig. 8, gene expression of MHC class I (H2-T23) and MHC class II (H2-Eb1) molecules, which is related to the presence of antigens, was increased by the instillation of 2 mg/kg C60s during the experiment period. The expression of genes related to tissue damage, including MMP (matrix metalloproteinase) –2, –3 and –11, Sipi (secretory leukocyte peptidase inhibitor) and Timp (tissue inhibitor of

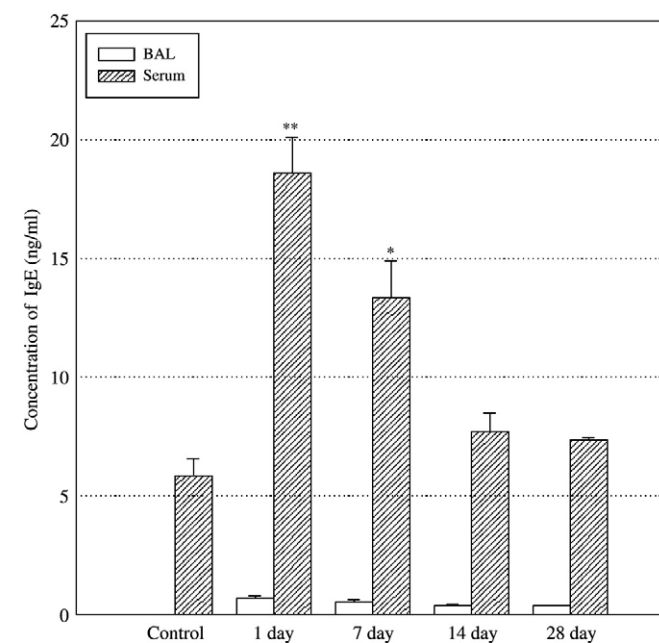


Fig. 5. Levels of IgE in BAL fluid and in blood after a single instillation of C60s. BAL fluid and serum were harvested and pooled at days 1, 7, 14 and 28, respectively ($n=11$), after instillation of 2 mg/kg C60s ($n=11$), and IgE concentration in each samples was determined using commercially available kits. * $P<0.05$, ** $P<0.01$.

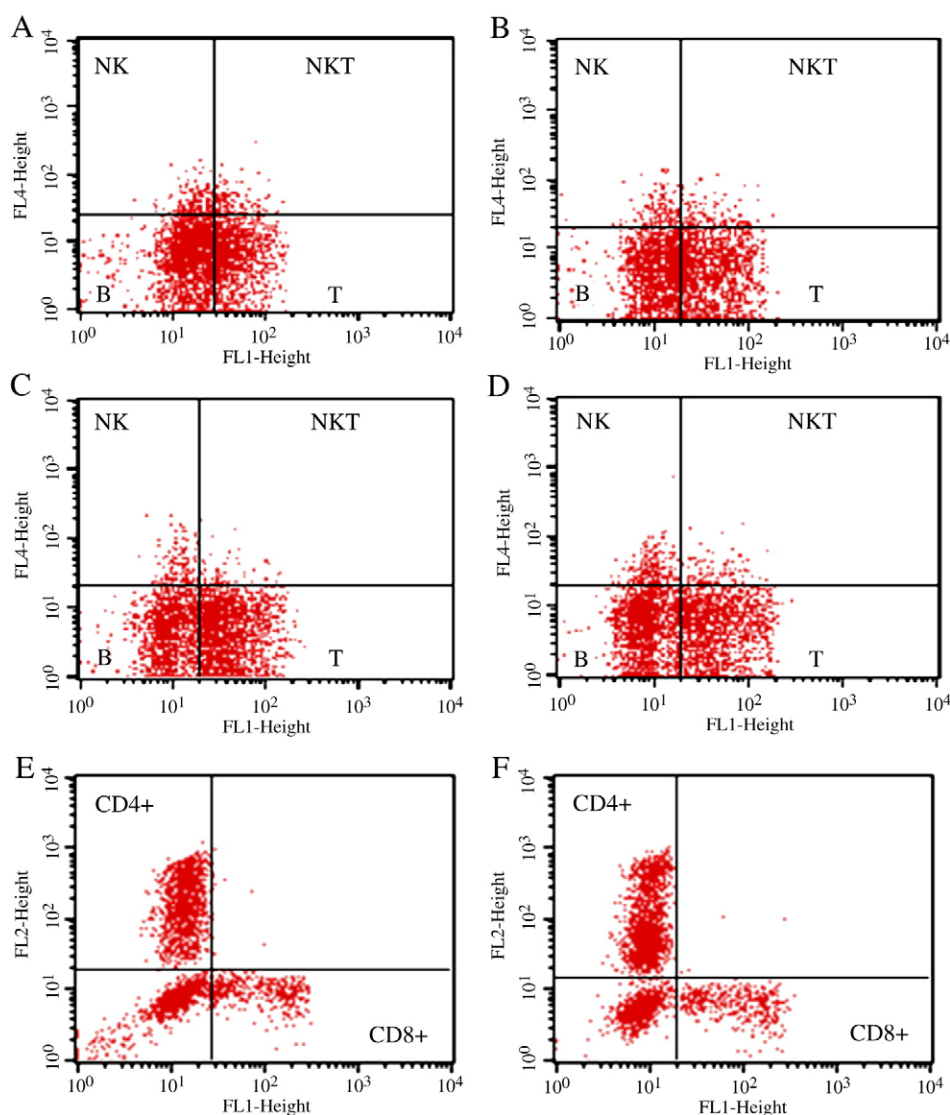


Fig. 6. Lymphocyte phenotypes in blood by instillation of C60s (2 mg/kg). Experiment was repeated 3 times using sample pooled by $n=3$, 3 and 4, and representative data are shown. Control samples were matched for each fluorochrome. (A) Lymphocyte of control, (B) lymphocyte of day 1, (C) lymphocyte of day 14, (D) lymphocyte of day 28, (E) T subtype of control, (F) T subtype of day 28.

metalloproteinase) 1 also increased. Furthermore, gene expression of SOD (super oxide dismutase), which is related to oxidative stress, increased during the experiment period.

Discussion

The carbon atoms in C60 together join to form a hexagonal structure resembling a soccer ball. Now, it is known that C60s have electron-donating or -accepting unique properties, but it is not clear yet whether C60s act as an anti- or pro-oxidant in biological systems (Zhu et al., 2006; Ryan et al., 2007). And, the organic solvent utilized to prepare water-soluble suspension can modify the properties of C60s and the modified C60s can alter the toxicity of pristine C60s. Sayes et al. investigated the pulmonary toxicity effects after instillation with nano-C60s dispersed by THF (tetrahydrofuran) in rats. At the experiment, C60s had a diameter of 160 ± 50 nm, the surface charge was -30 mV, and both nano-C60s and C60(OH)₂₄ showed little or no difference in lung toxicity effects when compared to controls (Sayes et al., 2007).

In this study, we used toluene as the dispersing agent and prepared the PBS soluble-C60s (diameter; 46.7 ± 18.6 nm, surface charge; -30.24 mV). Toluene is a volatile solvent, and able to dissolve

paints, silicon sealants, rubber, and adhesives. It can also be used as a fullerene indicator, able to dissolve C60s to 3 mg/mL, a higher dose than in THF (0.006 mg/mL) (Bezmel'nitsyn et al., 1998). A significant increased apoptosis was observed in the BAL cells of day 14 after the instillation of C60s, and G1 arrest was also observed in BAL cells of day 28. These are in agreement with previous results that C60s induced cytotoxicity in human umbilical vein endothelial cells, Chinese hamster ovary cells, and Madin-Darby canine kidney cells (Han and Karim, 2008; Yamawaka and Iwai, 2006).

Cytokines play an important role in the generation of inflammation by particles. Baker et al. compared inhalation toxicity and lung toxicokinetics of C60s nanoparticles and microparticles using a nose-only exposure system. They reported that C60s induced a significant increase in protein concentration, greater body burdens, and longer lung half-lives, but a significant increase in cytokines was not observed in either size. However, in our study, secretion of cytokines, including IL-1, TNF- α , IL-6, IL-10, IL-12, and IFN- γ , was significantly increased in the BAL fluid of day 1 after instillation, and these cytokines remained elevated level during the rest of experiment period.

Furthermore, inflammatory responses triggered by MHC were presented by antigen presenting cells which phagocyte xenobiotics.

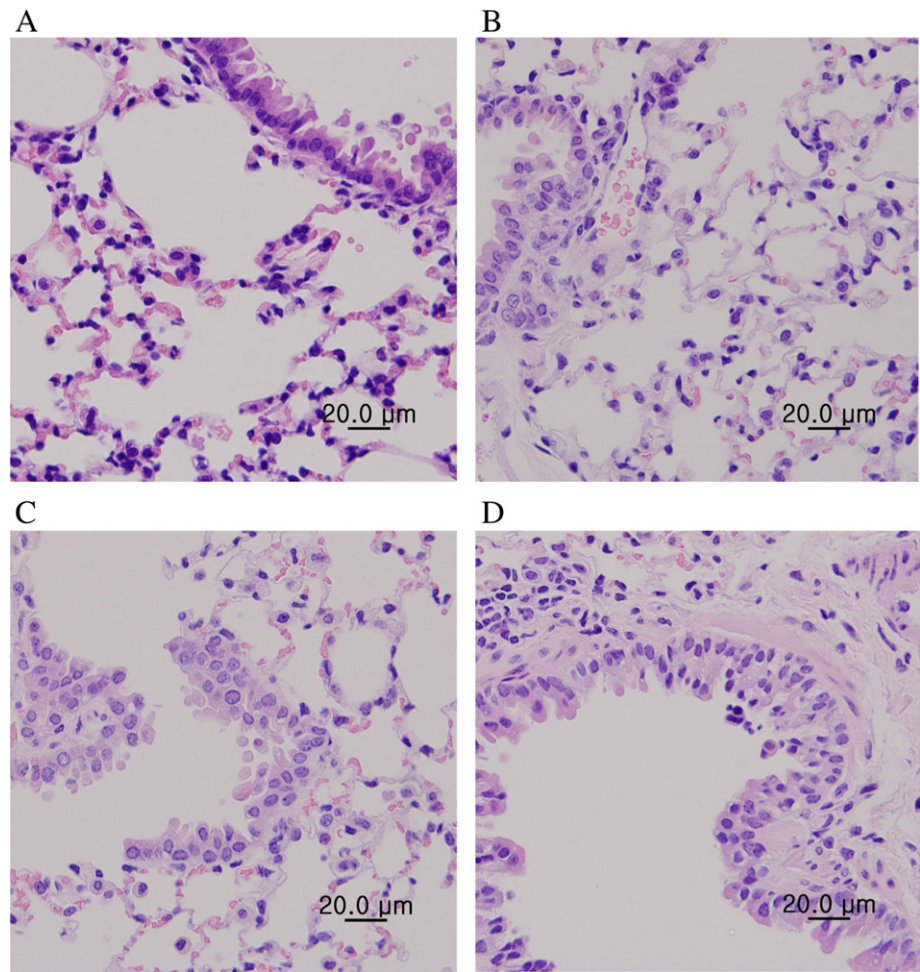


Fig. 7. Histopathological change of lung tissues after a single intratracheal instillation of C60s (2 mg/kg). Lung sections were stained with hematoxylin and eosin stains (×200). (A) Solvent control, (B) day 1, (C) day 14, (D) day 28.

Generally, MHC class I and II stimulate CD8+ T cell (TH2) and CD4+ T cell (TH1), respectively. However, under many circumstances in vivo, there is a mixed TH1 and TH2 response, and the overall effect is determined by which responses dominate. In this study, H2-Eb1, which is an MHC class II antigen presenting related gene, was more dominant than H2-T23, which is an MHC class I antigen presenting related gene. CD4+ T cells were more dominant than CD8+ T cells in the cell phenotype. These results are different from those of Fujita et al. (2009), in which inhaled C60s are presented by MHC class I. We guess that this may be different by experiment conditions such as exposure method, exposure time, exposure dose and physico-chemical properties of C60s used in the experiment.

Table 2
Time-dependent histopathological change.

	Grade	control	Day 1	Day 7	Day 14	Day 28
Number of animals (total)		3	3	3	3	3
Microgranulomatous change, around terminal bronchioles	+			1		
Cell infiltration, mononuclear, macrophage, alveolar space, alveolar wall	±	0	2	3	3	3
Cell infiltration, mononuclear, macrophage, neutrophil, alveolar space, alveolar wall	±	0	1	0	0	0

Grade: ±, minimal; +, mild.

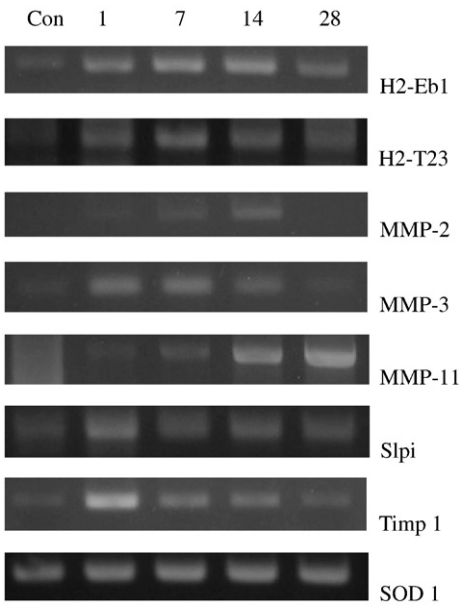


Fig. 8. Change of gene expression following instillation of C60s. RNA was extracted from the lungs of mice that had been treated with 2 mg/kg of C60s according to the shown time schedule, and amplified by RT-PCR using the respective primers described in Table 1. Results were confirmed by several separate experiments, and representative images are shown.

In an intact organism, TGF- β was known to be involved in wound repair processes and in inflammatory reactions (Lawrence, 1996). In this study, TGF- β was sustained at an obviously high level in the BAL. Furthermore, the expression of tissue damage related MMPs, Timp and Sipi gene, and the expression of oxidative stress related SOD gene were increased until day 28 after a single instillation. Cell infiltration in lung tissue was also observed until day 28 (Nukiwa et al., 2008; Niu et al., 2008; Wan et al., 2008; Visse and Nagase, 2003). These means that C60s remaining in lung tissue can induce continuous tissue damage.

Based on this, C60 may induce inflammatory responses in mice by a single intratracheal instillation.

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