The advent of nanomaterials has provided incredible opportunities for biomedical applications such as therapeutic and diagnostic tools, in addition to applications in engineering, electronics and optics.1–3 Bio-medical applications under development include targeted drug delivery systems for the brain and tumor tissues and intravascular nanosensor and nanorobotic devices for imaging and diagnosis. However, the potential adverse effects of nanomaterials on human health remains to be established.4–6

Potential exposure pathways for nanomaterials have been proposed in humans.5,7 Although inhalation might be a major route of exposure, ingestion and dermal exposure are also possible during the nanomaterial manufacture. After inhalation, nanomaterials are deposited in the respiratory tract, and their small size allows cellular uptake and transcytosis into the blood and lymphatic system. Our experiments have shown that both carbon black (CB) and water-soluble fullerene (C60(OH)24) exhibit cytotoxicity in human umbilical vein endothelial cells (HUVEC), such as decreased cell density and cell growth. Furthermore, CB and C60(OH)24 upregulated the expression of inflammation-related genes and ubiquitin-proteasome-related genes in HUVEC. These results suggest that exposure to CB and/or C60(OH)24 might be a risk for atherothrombotic diseases.8,9

In addition to endothelium, macrophages and platelets are important players for atherogenesis. Macrophages under the endothelium may take up denatured-low-density lipoprotein (LDL) (oxidized-LDL; Ox-LDL and acetylated-LDL; Ac-LDL) and become foam cells. The lipid-laden macrophages play a key role in inducing plaque rupture by secreting proteases, such as a matrix metalloprotease-9 (MMP-9) that destroys the extracellular matrix. Finally, rupture of advanced atherosclerotic plaques can lead to platelet aggregation, which results in atherothrombotic events.

In the present study, we aimed to clarify the effects of chronic exposure to nanomaterials, such as CB and C60(OH)24, on macrophage phenotypes and the aggregating effects of CB and C60(OH)24 on platelets.

Methods

Materials

CB (Association of Powder Process Industry and Engineering, Japan) and C60(OH)24 (Tokyo Progress System, Tokyo, Japan) were prepared as described previously. Fluoresbrite carboxylate microspheres (diameter, 6 μm) were obtained from Polysciences Inc (PA, USA).

Cell Culture

Mouse macrophages cell lines (RAW264.7) were obtained from Dainippon Sumitomo Phrma (Osaka, Japan) and were cultured in RPMI1640 with 10% (v/v) fetal bovine serum (Hyclone, Utah, USA). Cells were cultured in RPMI1640 with 2% (v/v) fetal bovine serum plus sonicated CB (0–100 μg/ml) or C60(OH)24. Cells were passaged every 3–4 days.
Lactate Dehydrogenase (LDH) Assay

LDH activity was analyzed with a CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison, USA) according to the manufacturer's protocols. RAW264.7 cells at 50–60% confluence were treated with CB or C60(OH)24 alone for 24 h, or 13 or 50 days. In addition, cells at 50–60% confluence were treated with CB or C60(OH)24 for 50 days or 8 days, respectively, and Ox-LDL was then added (Biomedical Technologies, MA, USA) (0–100μg/ml) for a further 2 days. LDH activity in the culture medium was measured based on absorbance at 490 nm using a microplate reader (ARVO; PerkinElmer, Japan). Cytotoxicity was expressed relative to basal LDH release in untreated controls. Cells at 50–60% confluence were treated with C60(OH)24 for 8 days or 6–18 μm beads for 3 days, and Ox-LDL or Dil-Ac-LDL (Biomedical Technologies) was then added for 24 h. Cells were examined under a microscope (Zeiss Axiosvert 25, Göttingen, Germany).

Proliferation Assay

Cell proliferation assay was carried out using a Cell Counting-8 kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocols. Cells were cultured in 12-well plates, and were treated with Ox-LDL (100μg/ml) for 5 days to induce foam cell-like formation, after which C60(OH)24 (1–100ng/ml) was added to the cells for a further 2 days. Cell growth was measured based on absorbance at 450 nm as detected by the ARVO micro-plate reader.

Treated and untreated cells were fixed with 4% paraformaldehyde for 15 min at room temperature. After washing, cells were stained with filtered Oil Red O solution (60% isopropanol) or Giemsa solution for 0.5 h–2 h at room temperature. After washing, the cells were examined under a microscope (Olympus BX-51, Tokyo, Japan). The Dil-Ac-LDL incorporated macrophages were captured directly from an RGB camera attached to the microscope (Zeiss Axiosvert 25, Göttingen, Germany) and displayed on an Adobe Photoshop CS2 to quantify fluoro-intensity in macrophages.

Western Blotting of LOX-1 and SR-AI

Samples were obtained from RAW264.7 cells treated with lysis buffer (20 mmol/L Tris-HCl, pH 7.4, with 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 2.5 mmol/L lysis buffer (20 mmol/L Tris-HCl, pH 7.4, with 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 2.5 mmol/L NaCl, 1 mmol/L Na3VO4, 1 μg/ml leupeptin, 0.1% protease inhibitor cocktail (Nacarai Tesque, Kyoto, Japan), and 1% Triton X-100). Equal amounts of proteins (10μg/ml) were subjected to reducing sodium dodecyl sulfate–polyacrylamide gel (10%) electrophoresis electrophoresis after boiling with 1 mmol/L dithiothreithol and were transferred to nitrocellulose membranes (Pall Corporation, Ann Arbor, MI, USA). After blocking with 5% (w/v) skim-milk in T-TBS (10 mmol/L Tris-HCl, pH 7.5 with 10 mmol/L sodium chloride and 0.1% Tween 20) buffer, the membrane was incubated with primary antibody (R&D Biosystems, MN, USA) (LOX-1: 1:100 dilution, scavenger receptor-type AI (SR-AI): 2μg/ml) at 4°C overnight. After washing, the membrane was incubated with secondary antibody (anti-mouse horseradish peroxidase-conjugated antibody and anti-rat horseradish peroxidase-conjugated antibody, respectively: 1:5,000–10,000 dilution) at room temperature for 1 h. LOX-1, SR-AI and β-actin protein were visualized using the ECL system (Amersham Biosciences, Buckinghamshire, UK).

Pro-MMP-9 Secretion Assay

Culture medium was collected from cells treated with C60(OH)24 alone for 10 days or with C60(OH)24 for 8 days followed by 2 days of co-treatment with Ox-LDL (100μg/ml). Pro-MMP-9 in culture medium was analyzed using a mouse pro-MMP-9 sandwich enzyme-linked immunosorbent assay kit (B&D systems, MN, USA) according to the manufacturer's protocols. Pro-MMP-9 in culture medium was detected by absorbance at 450 nm, and concentration was calculated using a standard curve.

Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) for Tissue Factor mRNA Expression

Expression of tissue factor mRNA in cells treated with C60(OH)24 alone for 10 days or with C60(OH)24 for 8 days followed by 2 days with Ox-LDL (100μg/ml) and were quantified by RT-PCR. Tissue factor primer sets were as follows: tissue factor; forward 5'-CGGTTGCAGGGATTCCAGAG-3' and reverse 5'-CTCCGTTGGACAGAGGAC-3', glucose-3-phosphate dehydrogenase; forward 5'-ACCACATGCTGCCATCA-3' and reverse 5'-TCCACACCTGTGTCCAT-GA-3'. The PCR products were electrophoresed on 2% agarose gels, and were stained with ethidium bromide, visualized using ultraviolet and recorded. Expression levels of tissue factor were adjusted against glucose-3-phosphate dehydrogenase expression levels.

Platelet Aggregation Assay

Whole blood was collected from the ear artery of male Japanese white rabbits (n=3; body weight, 3–5 kg) into tubes containing 3.8% (w/v) tri-sodium citrate, and were kept at room temperature for 5–10 min. After pre-treatment with or without CB (5μg/ml) or C60(OH)24 (5μg/ml) at room temperature for 5 min, ticlopydine hydrochloride (0–4 mmol/L) was added to whole blood, which was then left to stand at room temperature for a further 15 min. Adenosine diphosphate (ADP) (0–80 nmol/L) induced aggregation was analyzed using a whole blood filtration pressure aggregometer (WBA analyzer, Yokohama, Japan) Reaction tubes containing 200μl aliquots of whole blood were placed an incubation chamber at 37°C for 2 min, followed by addition of 22.2μl of ADP. The pressure rate was standardized using a grading curve of 4 different ADP concentrations (0, 1, 2, 4, 8 mmol/L or 0, 10, 20, 40, 80 mmol/L, respectively) on the x-axis and pressure rate on the y-axis. The concentration of ADP causing an increase in pressure rate was calculated and was applied as the platelet aggregatory threshold index. Experiments were performed at least 3 times.

Statistical Analysis

Data are shown as means±SD. Statistical evaluation was performed by ANOVA. Values of p<0.05 were considered statistically significant.

Results

Effects of CB on RAW264.7

The effects of chronic exposure to CB were examined by treating RAW264.7 cells with CB (0–100μg/ml) for 24 h, 13 days and 50 days. We observed marked CB (100μg/ml) uptake in RAW264.7 cells at days 13 and 50 (Figs 1B, d and C, d). However, cytotoxic morphological changes, such as cytosolic phagosome formation, cell disorientation and decreased cell density, were not observed (Figs 1A, B and C, a–d). No significant cytotoxic injury (based on LDH
activity) was seen in RAW264.7 cells at any time point (24 h, 13 days and 50 days) (Figs 1A–C).

**CB Together With Ox-LDL Induces Injury in RAW264.7**

To determine the effects of Ox-LDL (100 μg/ml) on chronic exposure to CB (0–100 μg/ml) in RAW264.7 cells, we performed Oil Red O staining. Cells were cultured in CB-containing medium for 48 days and were then treated with Ox-LDL for 2 days. Dose-dependent Oil Red O staining was seen with CB treatment (Fig 2A). LDH activity after Ox-LDL treatment was also dependent on CB concentration (peak LDH activity was observed at 10 μg/ml CB) (Fig 2B). These data suggest that CB itself may not be cytotoxic to RAW264.7 cells, but when cells are co-treated with CB and Ox-LDL, LDH secretion was elevated in a CB dose-dependent manner, and Ox-LDL uptake was also increased.

**C60(OH)24 Induces Cytotoxic Morphological Changes in RAW264.7**

RAW264.7 cells were treated with C60(OH)24 for 24 h to 10 days, C60(OH)24 (20 ng/ml), which is the predicted natu-
ral environmental concentration induced cytotoxic morphological changes in RAW264.7, including phagosome-like formation in the cytosol and decreased cell density (Fig 3A). Phagosome formation was confirmed by Giemsa staining (Fig 3B, arrowheads).

**C60(OH)24 Together With Ox-LDL Induces Injury in RAW264.7**

To determine the effects of Ox-LDL (100 μg/ml) on C60(OH)24 (0–100 ng/ml)-induced cell injury, we performed Oil Red O staining. Cells were cultured with C60(OH)24 for 8 days, and were then co-treated with Ox-LDL for 2 days. Enhanced Oil Red O staining was seen in a dose-dependent manner with C60(OH)24 and Ox-LDL co-treatment (Figs 4A, d–f), but Oil Red O staining cells was not seen when treated with C60(OH)24 alone (Figs 4A, a–c). Increases in LDH activity were dependent on C60(OH)24 concentration (LDH maximum activity was observed at 100 ng/ml C60(OH)24) in the presence of Ox-LDL (Fig 4B). RAW264.7 cells were also treated with Ox-LDL (100 μg/ml) for 5 days followed by C60(OH)24 (20, 100 ng/ml) for a further 48 h. Cells were stained by Oil Red O in a C60(OH)24 dose-dependent manner (Fig 4C). In addition, cell growth was dose-dependently suppressed by C60(OH)24 in the pre-treatment with Ox-LDL (Fig 4D). CB alone had no cytotoxic effects, however, C60(OH)24 alone had significant cytotoxicities appeared in macrophages. We focused on the characterization of C60(OH)24 cytotoxicities in further experiments.

**C60(OH)24 Induces LOX-1 Expression in RAW264.7**

CB and C60(OH)24 induced endocytotic uptake of Ox-LDL in RAW264.7 cells, which were strongly stained with Oil Red O. This indicates that expression of Ox-LDL receptors, such as LOX-1, is elevated in RAW264.7 cells. To identify LOX-1 protein expression in RAW264.7, we performed immunoblotting using whole cell extracts. LOX-1 expression was induced by C60(OH)24 in a dose-dependent manner.

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**Fig 3.** Water-soluble fullerene (C60(OH)24) induced phagosome formation in RAW264.7 cells. (A) Cells were treated with C60(OH)24 (a, 0 ng/ml; b, 20 ng/ml) for 24 h or 10 days (c, 0 ng/ml; d, 20 ng/ml), and cell morphology was then visualized (×200 magnification). (B) Cells were treated with C60(OH)24 (0, 20 ng/ml) for 10 days, and the cells were then fixed with 4% (v/v) paraformaldehyde neutralized buffered solution, and stained with Giemsa. Cells were then examined by microscope (×1,000 magnification). Arrowheads indicate phagosomes.

**Fig 4.** Oxidized low-density lipoprotein (Ox-LDL)-induced cytotoxic injury in water-soluble fullerene (C60(OH)24)-treated RAW 264.7 cells. (A) RAW264.7 cells were cultured with C60(OH)24 (0 ng/ml, 20 ng/ml, 100 ng/ml) for 8 days and were co-treated with Ox-LDL (100 μg/ml) for a further 48 h. Cells were then fixed with 4% paraformaldehyde neutralized buffered solution and were then stained with Oil Red O (a–f). Cells were visualized by microscopy (×400 magnification). (B) Cells were cultured with C60(OH)24 (0, 20 or 100 ng/ml) for 8 days, and were then co-cultured with (+) or without (–) Ox-LDL (100 μg/ml) for a further 48 h. Culture medium was collected and lactate dehydrogenase (LDH) activity was measured. *p<0.05 vs controls. (C) RAW264.7 cells were cultured with Ox-LDL (100 μg/ml) for 5 days, and were then co-cultured with C60(OH)24 (a, 0 ng/ml; b, 20 ng/ml; c, 100 ng/ml) for a further 48 h. Cells were then fixed and stained with Oil Red O. (D) Cell growth was inhibited by co-treatment with C60(OH)24 and Ox-LDL. Cell growth was analyzed using cell counting kit-8. Results are relative to controls (n=4). *p<0.05 vs controls.
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manner, and was further stimulated in the presence of Ox-LDL (Fig 5A). In contrast, SR-AI expression was not significantly induced by co-treatment with C60(OH)24 and Ox-LDL or Ac-LDL in RAW264.7 cells (Fig 5B). In addition, 6-μm fluoro beads induced endocytotic uptake of Ac-LDL in RAW264.7 cells (Fig 5D). LOX-1 expression was not induced by 6-μm beads, but was stimulated by co-treatment with Ox-LDL or Ac-LDL (Figs 5C,E). LOX-1 protein expression was more strongly stimulated by Ox-LDL than Ac-LDL (Figs 5B,E).
Fig 6. Water-soluble fullerene (C$_{60}$(OH)$_{24}$)-induced pro-matrix metalloprotease-9 (MMP-9) secretion and tissue factor (TF) mRNA expression. Cells were cultured with C$_{60}$(OH)$_{24}$ (0, 20 or 100 ng/ml) for 8 days and were then co-cultured with (+) or without (−) oxidized low-density lipoprotein (Ox-LDL) (100 μg/ml) for a further 48 h, after which culture medium was collected for pro-MMP-9 enzyme-linked immunosorbent assay (n=4) (A), and total RNA was extracted from the cells for TF reverse transcription-polymerase chain reaction experiments (n=3) (B). *p<0.02 vs controls (0 ng/ml), **p<0.05 vs controls (0 ng/ml).

Fig 7. Platelet aggregation, as analyzed by screen filtration pressure method in carbon black (CB) or water-soluble fullerene (C$_{60}$(OH)$_{24}$)-treated whole blood. (A) Concentration response curve of platelet aggregation to adenosine diphosphate (ADP) (•; 0, 10, 20, 40, 80 mmol/L), CB (□; 0, 50, 100, 200 μg/ml) and C$_{60}$(OH)$_{24}$ (△; 0, 5, 10, 25, 50 μg/ml). (B) High-concentration ADP (●; 0, 10, 20, 40, 80 mmol/L), CB (♦; 50 μg/ml, □; 100 μg/ml), C$_{60}$(OH)$_{24}$ (△; 25 μg/ml, △; 50 μg/ml), and (C) low-concentration ADP (○; 0, 1, 2, 4, 8 mmol/L), ADP-induced aggregation was facilitated by C$_{60}$(OH)$_{24}$ (△; 2.5 μg/ml, △; 10 μg/ml) in a dose-dependent manner, but CB (♦; 50 μg/ml, □; 100 μg/ml) had no effect. (D) High-concentration ADP (0, 10, 20, 40, 80 mmol/L), C$_{60}$(OH)$_{24}$ (△; 2.5 μg/ml, △; 10 μg/ml) upregulated platelet aggregation, despite inhibition by ticlopidine hydrochloride (○; 2 mmol/L).
C60(OH)24 Induces Pro-MMP-9 Secretion and Tissue Factor mRNA Expression in RAW264.7

To assess whether pro-MMP-9 might be secreted from in RAW264.7 cells, cells were treated with C60(OH)24 (0–100 ng/ml) for 8 days and Ox-LDL (100 μg/ml) was added for 2 further days of culture. Sandwich ELISA against pro-MMP-9 was then performed. The amounts of pro-MMP-9 secreted into culture medium were significantly increased in the presence of C60(OH)24, and under co-treatment conditions with Ox-LDL and C60(OH)24, although the amount of secreted pro-MMP-9 was 25% lower under the co-treatment conditions (Fig6A). Tissue factor mRNA expression was approximately 2-fold higher with C60(OH)24, and was approximately 6-fold higher after C60(OH)24 stimulation (Fig6B). These results show that C60(OH)24 alone facilitates pro-MMP-9 secretion and tissue factor mRNA expression via an unknown pathway in RAW264.7 cells.

C60(OH)24 and CB Effects on Platelet Aggregation

The effects of CB or C60(OH)24 on platelet function were examined by evaluating ADP-induced whole blood aggregation using the filtration pressure method. C60(OH)24 does not stimulate platelet aggregation in vitro.27 We also found that CB and C60(OH)24 alone do not induce platelet aggregation (Fig7A). However, we hypothesized that CB or C60(OH)24 might affect ADP-dependent platelet aggregation. When whole blood was pretreated with C60(OH)24, ADP-induced aggregation threshold index values were elevated in a dose-dependent manner (Figs7B,C). Thus, C60(OH)24 facilitates ADP-induced aggregation, and this function was dependent on the ADP receptor (Fig7D), as collagen- and thrombin-induced platelet aggregation threshold index values did not significantly change when whole blood was pre-treated with CB or C60(OH)24 (data not shown). These results show that C60(OH)24 specifically facilitates ADP-induced platelet aggregation.

Discussion

Epidemiologic and animal studies have suggested that exposure to nanoparticles plays a role in cardiovascular diseases such as atherosclerosis and myocardial infarction.13–20 For example, traffic-derived nanoparticles are suspected to be a risk for cardiovascular diseases.21

Our studies have recently shown that CB and C60(OH)24 exert cytotoxic effects on HUVEC, presumably via phagocytic cell death dependent on further ubiquitination of cytosolic proteins.22 Endothelial cell injury, inflammation, and impairment of membrane integrity are closely related to the initiation of atherosclerosis and ischemic heart disease.22,23 Nanomaterial cytotoxicity in cells varies with chemical characteristics and surface properties of the molecule, including hydrophobicity, hydrophilicity and surface area per molecule.18,19 The purpose of the current study was to clarify the effects of chronic exposure to low-dose nanomaterials, such as CB or C60(OH)24, particularly with regard to the cardiovascular system in vitro and in vivo. The present study indicates that CB alone has no significant cytotoxic actions in macrophages; however, cytotoxicity was markedly enhanced by co-treatment with Ox-LDL. In contrast, C60(OH)24 alone has significant cytotoxic actions and cytotoxicity was markedly enhanced by co-treatment with Ox-LDL (Figs2B,4B).

We analyzed the phagocytotic functions of RAW264.7 cells toward Ox-LDL or Ac-LDL after pre-treatment with 6-μm fluoro beads or 20 ng/ml C60(OH)24 in cells. Treatment with 6-μm fluoro beads stimulated Ac-LDL incorporation to a greater degree than pre-treatment with C60(OH)24 in RAW264.7 cells (Fig5D). We proposed that microparticles, as well as nanoparticles, are able to stimulate phagocytic system: however, microparticles more strongly stimulated the phagocytic function of RAW264.7 cells. Further stimulated phagocytic function caused cell death in RAW264.7 cells treated with 6-μm fluoro beads for 4 days (data not shown). Interestingly, C60(OH)24 alone might induce LOX-1 protein expression; C60(OH)24 activated stress-related kinases, such as p38 MAPK, thus contribute to phagosome maturation in macrophages24,25 and leading to activation of nuclear factor (NF)-κB, which is a major transcriptional factor for LOX-1. LOX-1 gene expression is dynamically modulated by tumor necrosis factor-α, transform growth factor-β, angiotensin II, endothelin-1 and peroxisome proliferator-activated receptor-a via NF-κB activation27–25 and stimulate cell injury or suppress cell growth. Nanomaterials might affect the activity of Ox-LDL receptors, such as CD36, SR-A and LOX-1, on the macrophage plasma membrane, thus triggering the process of phagocytosis and Ox-LDL uptake. We also found that C60(OH)24 induced secretion of pro-MMP-9. Macrophage-mediated proteolysis participates in the rupture of atherosclerotic plaques and MMP-9 might be involved in this process. Recent studies have indicated that the proteolytic activity of MMP-9 is sufficient to induce the rupture of advanced atherosclerotic lesions in apoE-/- mice.26,31 These results suggest that nanomaterials might contribute to the rupture of advanced atherosclerosis by stimulating MMP-9 secretion from macrophage-derived foam cells.

CB and C60(OH)24 did not directly cause activation and aggregation of platelets in vitro. This observation was also reported by Radomski et al in experiments on nanomaterials and platelet aggregation.27 Although C60(OH)24 alone did not activate platelet aggregation, when platelets were pre-treated with C60(OH)24, ADP-induced aggregation was 10–20% higher and this increase was C60(OH)24 dose-dependent (Figs7B,C). Furthermore, ADP-induced platelet aggregation was inhibited by more than 80% by ticlopidine hydrochloride (2–4 mmol/L), an ADP receptor (P2Y12) antagonist22 but after platelets were pre-treated with C60(OH)24, inhibition by ticlopidine hydrochloride (2 mmol/L) was suppressed. This indicates that C60(OH)24 increases the affinity of ADP for its receptor, possibly via a C60(OH)24-dependent conformational change or via C60(OH)24-mediated inhibition of ticlopidine hydrochloride binding to P2Y12. Interestingly, C60(OH)24 did not affect collagen- or thrombin-induced platelet aggregation (data not shown). C60(OH)24 also failed to affect acetylsalicylic acid (aspirin)-mediated inhibition of collagen-induced aggregation, and RGDS peptide-mediated inhibition of thrombin-induced aggregation (data not shown). These results suggest that C60(OH)24 specifically stimulates ADP-induced platelet aggregation via an ADP receptor such as P2Y12, and that C60(OH)24 might contribute to thrombosis.

We used the ng/ml or μg/ml order concentration of CB and C60(OH)24 in experiments of macrophages. This concentration is similar to the maximal concentration of particulate matter <2.5 μm (PM2.5) in Chongqing, a city in China, ~700 μg/m³ (daily average), and thus an individual might inhale 10,000 μg of particulate matter in a 24 h period. This is equivalent to approximately 1 μg/ml, considering that the extracellular fluid volume of a 60kg individual is...
However, the effective concentration of CB and C_{60(OH)}_{24} was much higher than predicted physiological 12 L. However, the effective concentration of CB and 444 4 ex vivo are required. experiments regarding the influence of nanomaterials on 5 the cardiovascular system in vivo are required.

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