Observation of phagocytosis of fullerene nanowhiskers by PMA-treated THP-1 cells

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Abstract. Phorbol 12-myristate 13-acetate (PMA)-treated THP-1 cells (macrophage-like cells) were exposed to the C60 fullerene nanowhiskers (C60NWs) with an average length of about 6.0 µm and an average diameter of about 660 nm and observed with an inverted optical phase-contrast microscope for 48 h. The C60NWs were well and stably dispersed onto the dishes of culture medium during the observation. The number of cells that internalised C60NWs gradually increased after the exposure to C60NWs. But no alteration of cellular morphology was observed compared to the control group without exposure to C60NWs during this period in this pilot study.

1. Introduction
Nanomaterials possess enormous potentials to wide applications in various fields owing to their distinctive unique properties. But potential risks caused by exposure to nanomaterials have not been cleared. With the release of industrial products of nanomaterials into environment, public concerns have raised their potential side effects. Carbon nanotubes (CNTs), one of the most promising nanomaterials, may be hazardous to health and environment owing to their needle-like morphology and strong mechanical properties like asbestos. Recently, an asbestos-like pathogenic behavior associated with CNTs indicated a structure-activity relationship based on length, to which asbestos and other pathogenic fibers conform [1].

Fullerene nanowhiskers (FNWs) are composed of the fullerene molecules that are usually bonded via van der Waals forces [2]. FNWs are expected to have various application fields such as low-dimensional semiconductors, field emission tips, nanoprobe for microdevices, fiber-reinforced nanocomposites, composite elements for lubrication, and so on. But FNWs also have the needle-like morphology like asbestos. Hence, it is of great importance to evaluate the biological impacts of FNWs for their sound application in advance.

Macrophages are one of the immune system cells and defend the host against the foreign substances in a non-specific manner during the initial phase of infection. Macrophages recognize, internalise and digest them. THP-1 is a human acute monocytic leukemia cell line and THP-1 cells have been isolated by Tsuchiya et al [3]. It is well known that THP-1 cells are induced to differentiate into macrophage-like cells by the addition of PMA [4].

In this paper, the interaction of C60NWs with macrophage-like cells is investigated as a pilot study for evaluating the biological impacts of C60NWs by use of an inverted optical phase-contrast microscope.

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2. Materials and methods

2.1. Preparation and characterization of C_{60}NWs

C_{60}NWs were prepared by the liquid-liquid interfacial precipitation method using a C_{60}-saturated toluene solution and isopropyl alcohol (Fig. 1) [2]. 20 mL of isopropyl alcohol was gently added to 20 mL of C_{60}-saturated toluene solution in a glass bottle at room temperature. After manual mixing, the solution was kept at 15°C for 15 minutes and 60 mL of isopropyl alcohol was poured into the solution to stop the crystal growth. The C_{60}NWs were separated by filtration using a GFP filter (0.8 \mu m, Kiriyama glass, Japan) from the solvents. The characterization was carried out by measuring the length and diameter of C_{60}NWs using an optical microscope (ECLIPSE ME600, Nikon, Japan) and a scanning electron microscope (JSM-6700, JEOL, Japan). C_{60} (99.5%) was purchased from MTR (OH). Toluene (99.5%) and 2-propanol (99.7%) were purchased from Wako Pure Chemical Industries (Japan).

2.2. Cell culture

THP-1 cells were purchased from American Type Culture Collection (ATCC, VA). THP-1 cells were cultured in RPMI 1640 medium (Invitrogen, CA) supplemented with 10% heat inactivated fetal bovine serum (JRH Biosciences, KS), 100 units/mL penicillin and 100 \mu g/mL streptomycin (Nacalai Tesque, Japan) (culture solution) at 37°C in an atmosphere of 5% CO₂ and saturated humidity. Cells were subcultured every three or four days, where the number of cells in culture were maintained by centrifugation (at 1000 rpm for 3 min) and subsequent resuspension at 2 x 10^5 viable cells/mL.

2.3. Differentiation of THP-1 cells into macrophage-like cells

2 x 10^5 cells were incubated in 2 mL of culture solution on a 35 mm polystyrene culture dish (Greiner Bio-One, Germany). PMA (Wako pure chemicals, Japan) was dissolved in dimethylsulfoxide at a concentration of 1 mM and diluted by a culture solution to be 50, 500 and 5000 nM. And 40 \mu L of each PMA solution was added to the cellular medium to be the final concentrations of 1, 10 and 100 nM. The cells were induced to differentiate into macrophage-like cells for 1, 3, 6, 24, 48 and 72 h at 37°C in an atmosphere of 5% CO₂ and saturated humidity. To estimate the degree of differentiation, the number of suspended living cells (undifferentiated cells) was measured using 0.4% trypan blue stain (Invitrogen, CA) and the morphological changes of cells were observed by an inverted optical phase-contrast microscope (DM IL-HC, Leica Microsystems, Germany).

2.4. C_{60}NWs' exposure

C_{60}NWs were dispersed in the culture solution at a concentration of 1 mg/mL and diluted by the culture solution to be 0.1 and 0.01 mg/mL. Macrophage-like cells were exposed to 20 \mu L of each concentration of C_{60}NWs suspension agitated by ultrasonication to be the final concentrations of 0.1, 1
and 10 µg/mL and incubated for 1, 3, 6, 24 and 48 h at 37°C in an atmosphere of 5% CO₂ and saturated humidity. Cells were observed with the inverted optical phase-contrast microscope to evaluate the effect of C₆₀NWs on the cell morphology.

3. Results and discussion

3.1. C₆₀NWs

3.1.1. Characterization of C₆₀NWs

C₆₀NWs were synthesized by the liquid-liquid interfacial precipitation method (Fig.2)[2]. The length of C₆₀NWs ranged from 1 to 17 µm approximately and the average length was about 6.0 µm (Fig.3). The diameter of C₆₀NWs ranged from 300 to 1340 nm and the average diameter was about 660 nm (Fig.4).

3.1.2. Dispersion of C₆₀NWs in culture

Before exposing cells to C₆₀NWs, the dispersion of C₆₀NWs in culture was examined visually by the inverted optical phase-contrast microscope for the same period. The suspensions of C₆₀NWs were poured into the cell-free medium by the same method as the exposure examination. The C₆₀NWs were well and stably dispersed onto the dishes of culture medium during observation (Fig.5).

3.2. Effect of PMA concentration and induction period on differentiation of THP-1 cells into macrophage-like cells

Optimal PMA concentration and induction period for the differentiation of THP-1 cells into macrophage-like cells were estimated under the condition of this study. In the case of 10 nM and 100 nM, cells adhered to the dish surface gradually (decrease of suspended cells) and about 90% of seeded cells adhered after 24 h of the PMA treatment (Fig.6). By contrast, the suspended cells grew by a factor of 5 in comparison with the seeded cells during 72 h in control culture (without PMA treatment). At the concentration of 1 nM, a few cells adhered the dish surface (data not shown), but the number of suspended cells increased about by a factor of 2 during 72 h. We observed that some of the cells showed elongation and pseudopodia formation after a few hours of the treatment by 10 nM and 100 nM (data not shown) of PMA, and most of the cells were observed to change their morphology by the PMA treatment of 24 h with the inverted optical phase-contrast microscope (Fig.7).

Because no significant difference was observed in the cellular morphology and the number of adherent cells between 10 nM and 100 nM of PMA treatment, the cells differentiated by 10 nM PMA treatment for 24 h were used for the exposure experiment of C₆₀NWs.

Figure 2. SEM image of C₆₀NWs.

Figure 3. Histogram of C₆₀NWs' length.

Figure 4. Histogram of C₆₀NWs' diameter.
3.3. Exposure experiments of C₆₀NWs

3.3.1. Phagocytosis of C₆₀NWs
Macrophages recognize, internalise and digest foreign materials. The uptake of them depends on their size and surface properties [5]. C₆₀ is also phagocytized by macrophages [6] and the uptake rate of C₆₀ is lower than that of graphite particles [7]. We observed that the macrophage-like cells gradually internalised C₆₀NWs after the exposure to C₆₀NWs with an inverted optical phase-contrast microscope (Fig.8). We will three-dimensionally locate the position of C₆₀NWs among the cells and study the phagocytosis of C₆₀NWs in more detail.

3.3.2. Effect of C₆₀NWs’ concentration and exposure period on the cell morphology.
No alteration of cellular morphology was observed in the macrophage-like cells compared to the control group without exposure to C₆₀NWs after the exposure of 48 h for any concentration of C₆₀NWs in the observations by the inverted optical phase-contrast microscope (Fig.9). However, we are going to investigate the endpoints such as cell viability, cytokines, LDH and active oxygen generation in the next follow-up study using positive controls.

We cannot explain the fiber paradigm that a hazardous fibre is thinner than 3 µm in diameter, longer than 20 µm and biopersistent in the lungs [1] in the present paper dealing with only short (< 20 µm) C₆₀NWs. But some previous studies have reported that C₆₀ (aggregate size was not described or larger than 1 µm) were nontoxic against mammalian cells [7, 8, 9] and dissolved inside lipid droplets in liver rats [10]. Owing to the weak van der Waals bonding forces acting between C₆₀ molecules, C₆₀NWs may decompose into individual C₆₀ molecules in living organisms. And on the basis of this assumption, C₆₀NWs may exhibit a biological effect like C₆₀. But these discussions are unclear now. We will carry out further researches on the digestion and dilution of C₆₀NWs using short and long C₆₀NWs.

Figure 5. C₆₀NWs’ dispersion in culture at a concentration of 10 µg/mL. (a) 1h and (b) 48 h after pouring the suspension of C₆₀NWs into the cell-free medium.

![Figure 5](image)

Figure 6. Suspended living cells after the PMA treatment. Circle, control (untreated); triangle, 1 nM PMA; diamond, 10 nM PMA; inverted triangle, 100 nM PMA. The results are expressed as the mean values for triplicate cultures with standard deviations.

![Figure 6](image)
Figure 7. Morphological changes of THP-1 cells after (a) 1 h, (b) 3 h, (c) 6 h and (d) 24 h of 10 nM PMA treatment.

Figure 8. Macrophage-like cells exposed to C₆₀NWs for (a) 1 h, (b) 3 h, (c) 6 h and (d) 24 h at a concentration of 10 μg/mL.
4. Conclusions
C$_{60}$NWs with an average length of about 6.0 µm and an average diameter of about 660 nm were well and stably dispersed onto the dishes of culture medium. Macrophage-like cells internalised the C$_{60}$NWs gradually, but no alteration of cellular morphology was observed in the macrophage-like cells for any concentration of C$_{60}$NWs (0.1, 1 and 10 µg/mL) compared to the control group without the exposure to C$_{60}$NWs for 48 h. We will complete this research for the biological impacts of C$_{60}$NWs in the next follow-up study using asbestos as a positive control and different sizes of C$_{60}$NWs and CNTs.

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References
[1] Poland C A, Duffin R, Kinloch I, Maynard A, Wallace W A H, Seaton A, Stone V, Brown S, Macnee W and Donaldson K 2008 Nature Nanotechnol. 3 423
[2] Miyazawa K, Kuwasaki Y, Obayashi A and Kuwabara M 2002 J. Mater. Res. 17 83
[3] Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T and Tada K 1980 Int. J. Cancer 26 171
[4] Tsuchiya S, Kobayashi Y, Goto Y, Okumura H, Nakae S, Konno T and Tada K 1982 Cancer Research 42 1530
[5] Tabata Y and Ikada Y 1988 Biomaterials 9 356
[6] Porter A E, Muller K, Skepper J, Midgley P and Welland M 2006 Acta Biomaterialia 2 409
[7] Fiorito S, Serafino A, Andreola F and Bernier P 2006 Carbon 44 1100
[8] Moussa F, Chretien P, Dubois P, Chuniard L, Dessante M, Trivin F, Sizaret P Y, Agafonov V, Ceolin R, Szwarc H, Greugny V, Fabre C and Rassat A 1995 Fullerene Sci. Technol. 3 333
[9] Baierl T, Drosselmeyer E, Seidel A and Hippeli S 1996 Exp. Toxic. Pathol. 48 508
[10] Gharbi N, Pressac M, Hadchouel M, Szwarc H, Wilson S R and Moussa F 2005 Nano Lett. 5 2578

Figure 9. Macrophage-like cells exposed to C$_{60}$NWs for 48 h at concentrations of (a) 0.1 µg/mL, (b) 1 µg/mL and (c) 10 µg/mL. (d) Control culture cultivated without C$_{60}$NWs.