Effects of C60 Fullerene on Cell Differentiation with EL-M3 and ES-R1-EGFP B2/EGFP Cell Lines

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Synopsis
The effects of nanomaterials on human reproduction and development remain unclear. Thus, their embryotoxicity should be examined to ensure the biological safety of the next generation. In the present study, the effects of C60 fullerene on cell differentiation were investigated using EL-M3 and ES-R1-EGFP B2/EGFP cell lines that require feeder cells, instead of the ES-D3 cells used for the EST method, an in vitro embryotoxicity test. As a result, the effects of C60 fullerene on cell differentiation increased in a concentration-dependent manner for both cell lines, demonstrating the absence of severe developmental toxicity. The developmental toxicity of C60 fullerene should be investigated for applications to new drugs.

Key words: C60 fullerene, embryotoxicity, EL-M3 cell, ES-R1-EGFP B2/EGFP cells

Introduction
Long-term exposure to nanomaterials causes the marked health-related anxiety of society, as observed for asbestos [1-4]. In addition, the effects of these nanomaterials on the next generation remain unknown. The embryotoxicity of nanomaterials in human organs is being investigated using the Embryo Stem Test (EST). However, few embryotoxicity studies have been conducted using cell lines other than ES-D3 cells used for the EST method [5-15]. ES-D3 cells are special ES cells that do not require feeder cells.

In the present study, we considered that the results obtained with other ES cell lines were useful for ensuring data reliability. [9] Thus, the effects of C60 fullerene, mechanically dispersed
in medium, on cell differentiation were examined using EL-M3 and ES-R1-EGFP B2/EGFP cell lines that require feeder cells.

Materials and Methods
1. Culture medium
Dulbecco's Modified Eagle's Medium (DMEM, Nacalai Tesque, Kyoto, Japan) including Non-Essential Amino Acids (NAA, Invitrogen, CA, USA), β-mercaptoethanol (Invitrogen), L-glutamine (Invitrogen), and penicillin / streptomycin (Invitrogen) with 20% Fetal calf serum (FCS, Hyclone®, Utah, USA). Although 1,000 U/mL of LIF (mouse leukemia inhibiting factor, 10^{-7} Units, ESGRO®, Millipore, CA, USA) was added to the medium to prevent differentiation induction, LIF-free medium was prepared for use at the beginning of cell differentiation stage.

2. Preparation of test mediums
Test solutions were prepared by dissolving C60 fullerene (Nanom purple SUH, 99.9% purity, Frontier Carbon, Tokyo) in media for two ES cell lines at 50 mg/mL. These test mediums were ultrasonically cleaned for 2 hours. Subsequently, they were frozen for 8 hours at -20°C, ultrasonically cleaned again, and sterilized through a 0.45-μm membrane filter. The test mediums were diluted eightfold in fresh medium. No aggregation of C60 fullerene was observed on the membrane filter using an inverted phase-contrast microscope. The test solutions at each concentration were cryopreserved at -4°C and thawed before use. A fullerene-free group was used as a control. The experiment was repeated four times.

3. Feeder cell culture
Two mL of filter-sterilized 0.1% gelatin solution (Specialty Media, Lot.00402-3, Millipore) was poured into a plastic culture flask (25mL, Iwaki, Japan) and stood at 37°C for one hour in a 5%CO_{2} incubator (Espec, Osaka, Japan). It was gently washed with PBS(-) after excess gelatin solution was discarded.

Frozen MEF cells (Figure 1, Reprocell Inc., RCHEF0003, Kanagawa, Japan) previously treated with mitomycin were used. Immediately after MEF cells were thawed, DMEM with 5% FCS was added to them to prepare a cell suspension. After the cells were seeded in a flask, they were incubated in a carbon dioxide incubator for 24 hours. It was confirmed with an inverted phase contrast microscope (IX-70, Olympus, Tokyo, Japan) that the morphology and number of cells on the bottom surface of the flask were normal cell shape.

The culture medium was removed from the flask and the feeder cells were gently washed with PBS(-)
4. Preparation of embryoid bodies (EBs)

EL M3 and ES-R1-EGFP B2/EGFP cells (Figure 2a, b) were diluted in each test medium to a final concentration of $3.75 \times 10^4$ cells/mL using a hemacytometer, and a 20μL cell suspension was dropped 40-60 times onto the inside of the lid of a 10 cm diameter Petri dish using a micropipette. Each drop of the cell suspension contained approximately 750 cells. Five mL of sterilized phosphate buffered saline (PBS (-)) was poured into the Petri dish, and the lid was quickly reversed and placed on the dish before the cell suspension on the lid could flow down. Suspension culture was carried out for three days in a CO2 incubator (5% CO2 and 95% air; 37°C). Drops of the cell suspension on the inside of the Petri dish lid were then collected into a dish for germiculture. The test solutions were replaced with new ones using a pipette and each test solution was subjected to reaction for two days in the above listed incubator. Subsequently, two 24-well multidishes were used for every test solution. Each of the one embryoid bodies (EBs) formed were placed in the well with a micropipet, and the EBs were cultured statically for five days. The presence of beating myocardial cells in each well was examined under an inverted phase difference microscope. The number of wells in which beating cells were observed at each concentration was examined, and the ID50 was calculated from the ratio of the number of the above wells to the number of wells in which EBs were successfully disseminated.

5. Statistical analysis

The results are presented as the mean ± S.D. values of five experiments and analyzed by nonpaired Student’s t test. Values between $p<0.05$ were considered significant.

Results

The cell differentiation rates of the control group are shown for both ES cell lines (Figure 3). The cell differentiation rates of EL-M3 and ES-R1-EGFP B2/EGFP cells were 82 and 74%, respectively. For 100% test mediums, the cell differentiation rates of EL-M3 and ES-R1-EGFP B2/EGFP cells were 32 and 18%, respectively. Inverted phase-contrast microscopic images are shown (Figure 4). Differentiated teratomas were significantly smaller than in the control group. The differentiation rates of the twofold diluents of EL-M3 and ES-R1-EGFP B2/EGFP cells were 44 and 31%, respectively. The differentiation rates improved at higher dilution ratios. At the final eightfold dilution, the cell differentiation rates of EL-M3 and ES-R1-EGFP B2/EGFP cells slightly decreased to 67 and 58%, respectively, compared with the control group. The inverted phase-contrast microscope images of

![Figure 3](image_url)  
**Figure 3** The cell differentiation rates of EL-M3 cells and ES-R1-EGFP B2/EGFP cells with test medium in C60 fullerene (Significant difference was observed between all concentrations)
the eightfold dilution group (Figure 5) were not macroscopically different from those of the control group.

**Discussion**

The differentiation disorder levels of ES-D3 cells were examined, demonstrating that C60 fullerene is likely to fall into the category of "non-embryotoxicity." However, according to the EST protocol, a chemical substance should be completely dissolved in culture medium even in the presence of solvent. Thus, the embryotoxicity of C60 fullerenes cannot be completely ruled out because they cannot be dissolved in culture medium. The differentiation disorder of ES cells under the experimental conditions was reconfirmed to be dependent on the concentration of C60 fullerenes. The cell proliferation rates remain the same at a concentration lower than that for cell differentiation. In the present study, the same results as those for ES-D3 cells [9] were obtained for both EL-M3 and ES-R1-EGFP B2/EGFP cells, demonstrating the absence of embryotoxicity of fullerenes.

Fullerenes are nanocarbon molecules smaller than 1 nm. C60 fullerene has a soccer ball-like structure composed of 60 carbon atoms [16-19]. Reportedly, fullerenes are effective for the prevention of cancer metastasis [19-24] and treatment of brain diseases [25-29], HIV [30-32], and...
hepatitis [33], because they detoxify harmful active oxygen. Some fullerenes have already been clinically applied. Fullerenes will be utilized for the development of various new drugs [34-38]. Thus, their biological safety is being actively investigated [39-48].

C60 fullerene may be actively phagocytosed as a nanomaterial, but is less likely to damage cells within tissues than asbestos because it contains no sharp structures. Therefore, it causes no social problems, such as the carcinogenicity of asbestos. In addition, under normal conditions for nanomaterial dispersion in body fluid, most nanomaterials tend to aggregate in micron or submicron sizes, precluding their artificial dispersion in medium. In the present study, C60 fullerene was not uniformly dispersed in spite of mechanical dispersion by sonication. Nanomaterial dispersion in culture medium will be further investigated in vitro.

For the applications of nanomaterials to drugs and biomaterials, multiple materials are generally mixed. Thus, the biological effects of mixing conditions with nano- or submicron-materials will also be examined. The C60 fullerene used in this study was a higher grade product than those actually used. In the future, the relationship between fullerene purity and biological safety will also be further investigated.

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References
25) Tykhomyrov AA, Nedzvetsky VS, Klochkov VK, Andrievsky GV. Nanostructure of hydroxylated C60 fullerene (C60HyFn) protect rat brain against alcohol impact and attenuate behavioral impairments of alcoholized animals. Toxicology 2008; 246: 158-165.
41) Johnston HJ, Huthinson GR, Christensen FM, Peters S, Hankin S, Ascherger K, Stone V. A critical review of the biological mechanisms underlying the in vivo and in vitro toxicity of


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