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INHIBITION OF CELL CYCLE AND INDUCTION OF APOPTOSIS DUE TO MODIFIED C60 ON HL-60 PROMYELOCYTIC LEUKEMIC CELLS AND IT'S MACROPHAGE-LIKE CELLS

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This report showed an inhibitory effect of cell proliferation on HL-60 human leukemic cells followed by modified C60s. Additionally, those C60s was capable to be phagocytosed by a macrophages differentiated from HL-60 cells. An adequate modification of C60 may become a candidate of therapeutic tools for cancer.

Keywords: C60; modification; phagocytosis; apoptosis; cell proliferation

Malignant tumor is one of major problems in our society even in tremendous development biological knowledge. We tested a possibility that C60 may become one of therapeutic applications for cancer therapy: because of it's attractive attention in recent years due to their unique chemical structure and potential applications. Hence it is of interest to study their biological effects. It has been reported that a biological ability of the most commonly used fullerene, C60, were focused on their photosensitization and radical control to apply a cancer therapy [1]. However, in those repots, those C60s used their experiments did not cause apoptosis (natural cell death). An agent or method inducing this type of cell death has been expected an ideal one for cancer therapy [2,3]. As a preliminary examination, we investigated here by using HL-60 human leukemic cells and demonstrated here this possibility of a C60.

HL-60 human leukemic cells used here were purchased from (American Type Culture Collection, USA). HL60 cells (passage 60) were seeded at a density of 10^6/5 ml/ ϕ 6 cm dish in RPMI1640 medium (Nissui Pharmaceutical Co., Japan) containing 10% heat-inactivated FBS(ICN Biomedicals,

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Inc., USA) and induced to differentiate by treatment with 0.1 μM TPA (Sigma Chemical Co., USA) in a 5% CO_2 atmosphere at 37°C for 2 days. The cells were then washed in Dulbecco's phosphate buffered saline without calcium and magnesium (PBS) once and culture medium were changed to fresh RPMI1640 medium containing 10% fetal bovine serum (FBS) without phorbol 12-myristate 13-acetate (TPA). C60s were added and introduced to the base of dish by pipetting. After incubation for 24 h, cells were washed in PBS and harvested using cell scraper. Cells were fixed with 70% ethanol and treated with 2 mg/ml RNase A (Wako Pure Chemicals, Japan) and 0.1 mg/ml propidium iodide (Sigma, USA) at 37°C for 30 min 10,000 cells filtrated with nylon mesh (mesh size 200 μm, Kyoshin Rikoh Inc., Japan) were analyzed by fluorescence-activated cell sorting flow cytometry ((FACS-FCM, Becton Dickinson, USA). All of C60s were sterilized by conventional formaldehyde gas in the package. Macrophages were differentiated from HL-60 cells following the conventional way [4]. HL60 cells were seeded at a density of 10^6/5 ml/ ϕ 6 cm dish in RPMI1640 medium (Nissui Pharmaceutical Co., Japan) containing 10% heat-inactivated FBS (ICN Biomedicals, Inc., USA) and induced to differentiate by treatment with 0.1 μM 12-*O*-tetradecarnoylphobol 13-acetate (TPA, Sigma Chemical Co., USA) in a 5% CO_2 atmosphere at 37°C for two days. An original C60 (98%, Matsubo Co., Japan) was purchased. From the original C60, modified C60s of SYK-025 and SYK-081 were provided from SSS Co. (Matsumoto, Japan). A modification of original C60 (10 mg) has been made by heat treatment (a range of 200–500°C for 30 min) with 10 μl of 1 M DMSO solution of a single compound of Yoshixol^{TR} family. Original C60 has been received only same amount of DMSO. Those materials have been washed with phosphate buffer solution. Then, those were dried up in the incubator at 37°C overnight. Those materials have been stored at room temperature into the sealed ample until starting the experiment.

After incubation for 24 hours, the culture medium was removed. After 5–10 minutes the plate was dipped several times in phosphate buffer solution (pH 7.4) to remove unattached biological materials and C60s. Then, each sample was prefixed in cold 2% glutaraldehyde in phosphate buffer solution for one hour for an observation by a scanning electron microscopy (SEM). Each sample was subsequently dehydrated with graded ethanol solutions, and dried with carbondioxide by the critical point method. The dried surfaces were mounted and sputter-coated with gold palladium and carbon. The samples were sputter-coated with 50 Å of gold-palladium and observed at 15 keV in a JEOL-JSM6000F scanning electron microscope.

Figure 1 shows a result of changes in cell cycle of HL-60 cells by using of a flow cytometer analysis. An original C60 shows no alteration of cell cycle of Go/G1 phase, S phase and M phase, indicating that an original C60 did

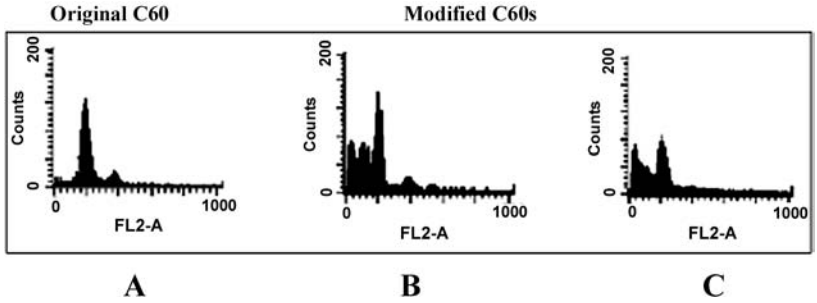

FIGURE 1 Figure shows a graph of flow-cytometer analysis after the treatment with each C60. Panel A, B and C represent graph after treatment with original C60, SYK-025 and SYK-081, respectively.

not influence into a cell proliferation (panel A in Fig. 1). However, a modified C60, SYK-025, caused an increase in apoptotic area that was a left part of G0/G1 peak (panel B of Fig. 1). Additionally, another modified C60, SYK-081, showed an apoptotic pattern and suppression of cell proliferation prior to S phase of cell mitosis (panel C of Fig. 1). Those findings indicate that a modification of C60 may be useful to medical therapeutic application such as malignant tumors.

Figure 2 shows aspects of SEM on macrophage-like cell and an original C60. Macrophage-like cell did not showed a phagocytosis of C60 although a clustering C60 existed near the cell, indicating that when an original C60 may be applied into the blood stream for an application, the removal of C60 along the physiological mechanism from the body could be difficult.

Contrastingly, a phagocytosis could be led by an adequate modification of C60. This possibility is shown in Figure 3. Macrophage-like cell shows a

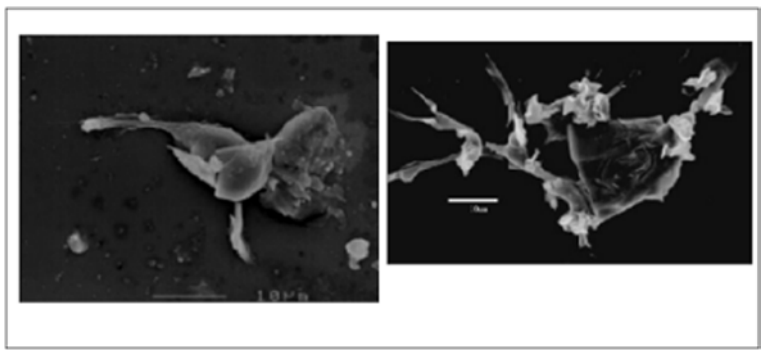

FIGURE 2 Figures show SEM aspects of macrophage-like cell and original C60. Note that original C60 did not phagocytose.

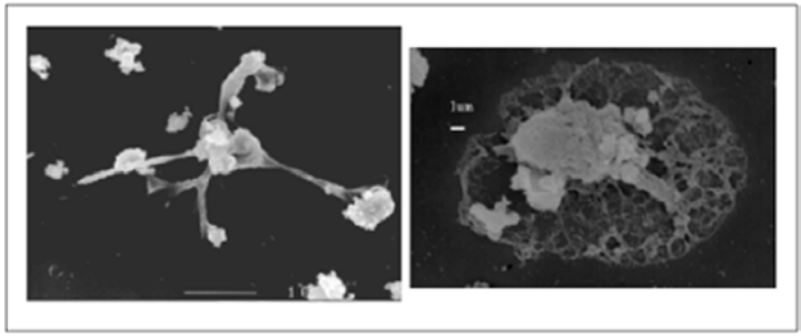

FIGURE 3 Figures show SEM aspects of macrophage-like cell and a modified C60. Note that a modified C60 was engulfed by pseudopodia (left) and was enclosed in a phagocytic vacuole (right). Cell was apoptotic.

well elongation to contact well to C60 materials and a well inclusion into the cell components even in the cell appearance of apoptosis like cell death. Cell death demonstrated here did not need an additional physical force such as an irradiation of photon. Therefore, this kind of modified C60s may induce apoptotic cell death of tumor cells if they may be delivered near the targeted tissues, and modified C60 could be removed by phagocytosis from the body together with an apoptotic fragmented cellular components, following by the physiological mechanism. In future, it is interest in testing this beneficial possibility of the modified C60 in vivo experiments.

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