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Research Article

Photodynamic Therapy Effects of Hybrid Liposomes Including Indocyanine Green against a Xenograft Mouse Model Mice of Colorectal Cancer *in vivo*

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ABSTRACT

Aim: To examine the photodynamic therapy (PDT) effects of hybrid liposomes (HLs) composed of L-α-dimyristoylphosphatidylcholine (DMPC), polyoxyethylene (25) dodecyl ether (C12 (EO) 25), and indocyanine green (ICG) in human colorectal cancer cells (HCT116) *in vitro* and *in vivo*.

Materials and Methods: HL/ICG composed of 89 mol% DMPC, 10 mol% C12 (EO) 25, and 1 mol% ICG were prepared by sonication. The inhibitory effects of PDT-HL/ICG in a subcutaneous xenograft mouse model of colorectal cancer were examined *in vivo*.

Results: The inhibitory effects of HL/ICG irradiated with far-red light laser on the growth of HCT116 cells were observed. A remarkable reduction in tumor weight in xenograft mouse models intravenously treated with PDT-HL/ICG after subcutaneous inoculation of HCT116 cells was verified *in vivo*. The occurrence of Reactive Oxygen Species (ROS) from PDT-HL/ICG treatment was observed in HCT116 cells based on ROS detection using fluorescence microscopy *in vitro*. An increase in peroxidation products in tumor cells of colorectal cancer xenograft model mice intravenously administered with PDT-HL/ICG was observed in micrographs *via* immunostainingusing anti-8-hydroxy-2'-deoxyguanosine (8-OHdG).

Conclusion: ROS-inducing therapeutic effects of PDT-HL/ICG in a xenograft model after subcutaneous inoculation with human colorectal cancer cells were revealed for the first time *in vivo*.

Keywords: Hybrid liposome, Photodynamic therapy, Colorectal cancer, Reactive oxygen species, Indocyanine green

INTRODUCTION

Colorectal cancer is the second leading cause of cancer-related deaths. It occurs in the large intestine, which comprises the colon, rectum, and anus. The most common sites for this cancer to develop are the sigmoid colon and rectum, which account for 70% of all colorectal cancer cases.

Photodynamic Therapy (PDT) involves the administration of a photosensitizer to tumor tissue and new blood vessels *in vivo* [1-3]. This treatment method uses laser light to irradiate the place where photosensitisers accumulate, causing a photochemical reaction in the photosensitisers and generating reactive oxygen species, which denatures and necrotises cancer cells.

Unlike the general liposomal preparation method, Hybrid Liposomes (HLs) can be prepared simply by sonicating vesicular and micellar molecules in a buffer solution [4,5]. Effective drug delivery systems using HL as a drug carrier have been developed for the treatment of brain tumors and duchenne muscular dystrophy [6,7]. High inhibitory effects of HLs on the growth of various tumor cells *in vitro*, along with the induction of apoptosis have been obtained without using drugs [8-11]. Remarkable therapeutic effects of HLs have been also demonstrated in the growth of tumor cells *in vivo* [12-17]. Moreover, no HL toxicity or side-effects were observed in normal rats *in vivo* [12,13]. After receiving approval from the Bioethics Committee, successful chemotherapy with drug-free HL in patients with lymphoma has been reported [13]. Hybrid Liposomes (HL)-23/NBDPC and HL-23 containing a fluorescent lipid (NBDPC) selectively fuse and accumulate in human lung cancer cells [13]; however, NBDPC has low biopermeability. The antitumor effect of HL-induced apoptosis and the long-term accumulation of HL/Indocyanine Green (ICG), an HL containing

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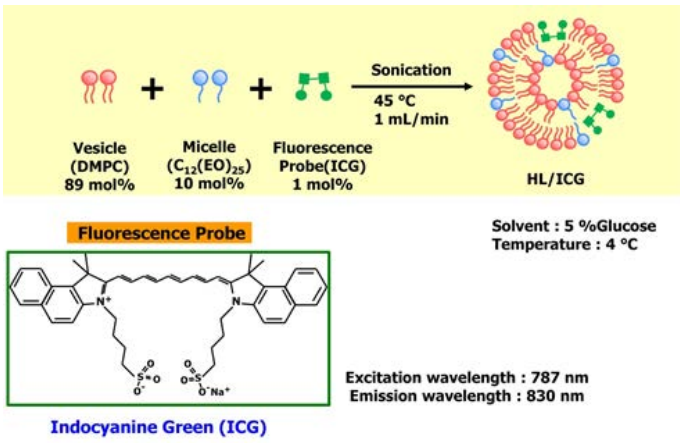
the near-infrared fluorescent reagent ICG, in an orthotopic colorectal cancer transplant mouse model has been demonstrated (Scheme 1) [17]. ICG is a cyanine pigment approved by the Food and Drug Administration (FDA) in 1954. It is used as a contrast medium for the evaluation of liver function in patients and during surgery because it has a low frequency of side effects, such as allergic reactions, and is generally safe. The excitation and emission wavelengths are near-infrared with excellent biopermeability; hence, it is suitable for non-invasive observation.



Scheme 1: An orthotropic colorectal cancer transplant mouse model has been demonstrated.

The purpose of this study was to assess the application of HL/ICG in PDT and investigate the *in vitro* and *in vivo* therapeutic effects of excitation light irradiation on colorectal cancer cells.

MATERIALS AND METHODS

Preparation of HLs and HL/ICG

HLs were prepared by sonication of a mixture containing 90 mol% L-α-dimyristoylphosphatidylcholine (DMPC; NOF, Tokyo, Japan) and 10 mol% polyoxyethylene(25) dodecyl ether (C12(EO)25; Nikko Chemicals, Tokyo, Japan) in a 5% glucose solution using a bath-type sonicator (VS-N300; VELVO-CLEAR, Tokyo, Japan) at 45°C and 300 W and then filtering the mixture through a 0.20 μm cellulose acetate filter (Advantec, Tokyo, Japan). The HL and fluorescent probe, ICG (ICG; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), combination, HL/ICG, was prepared by sonication of a mixture containing 80 mol% DMPC and 10 mol% C12 (EO) 25 in a 5% glucose solution using a bath-type sonicator at 45°C and 300 W and then filtering the mixture with a 0.20 μm cellulose acetate filter.

Dynamic light scattering measurements

The diameter of the HL was measured with a light scattering spectrometer (ELSZ-0; Otsuka Electronics, Osaka, Japan) using a He-Ne laser (633 nm) at a 90° scattering angle. The hydrodynamic diameter (dhy) was calculated using the Stokes-Einstein formula (Equation 1), where κ is the Boltzmann constant, T is the absolute temperature, η is the viscosity, and D is the diffusion coefficient:

$dhy = \kappa T / 3\pi\eta D$ .....(Equation 1)

Cell culture

The human colon carcinoma cell line, HCT116, was purchased from the American Type Culture Collection (Manassas, VA, USA). HCT116 cells were maintained in RPMI 1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 100 U/mL penicillin, 50 μg/mL streptomycin, and 10% foetal bovine serum (FBS, HyClone Laboratories, South Logan, UT, USA). The cells were cultured at 37°C in a 5% CO_2 humidified incubator.

In vitro application of HL/ICG in PDT to suppress the growth of CRC cells

The effect of HL/ICG on HCT116 cells was evaluated using the WST-8 assay, which is a cell viability measurement method. A cell suspension with an initial cell number adjusted to 5.0 × 104 cells/mL was seeded in 0.1 mL on a 96-well plate (Nunc) and cultured in an incubator for 24 h. Approximately 10 μL of HL and HL/ICG were added at 250 μM, and the cells were cultured for 24 h. The excitation light was irradiated using an 808 nm laser diode IN-A (1 W, GIGA LASER, Shanghai, China). The irradiation times for each well were 0, 30, and 60 s. The entire irradiation process was performed on a clean bench, and the distance from the bottom of the 96-well plate to the 808 nm laser diode IN-A was 5 cm. Twenty-four hours after irradiation with an excitation light, the WST-8 solution was added. After 3 h, the absorbance of WST-8 formazan at a wavelength of 450 nm was measured using a spectrophotometre. Cell viability ((Amean)/(Acontrol) × 100) was calculated from the absorbance (Amean) and control absorbance (Acontrol).

PDT with HL/ICG for colorectal cancer in a subcutaneous transplantation mouse model

The mice were handled in accordance with the guidelines for animal experimentation set out in Japanese law. Animal studies were approved by the Committee on Animal Research of Sojo University.

After collecting HCT116 cells, they were suspended in a mixed solution of PBS (-) and Matrigel (1:9) to prepare a cell suspension (1.0 × 108 cells/mL). The backs of BALB/c-R/J mice were shaved, and 0.05 mL (5.0 × 106 cells/body) of cell suspension was subcutaneously transplanted using a 26 G needle. Matrigel begins to gel at a temperature ≥10 oC; hence, ice-cooled syringes, needles, and cell suspensions for transplantation were used. Three days after subcutaneous transplantation, the major and minor diameters of the tumors were measured using a digital caliper (Mitutoyo, Kanagawa, Japan). The tumor volume was calculated using Formula 1 and grouped using a stratified randomisation method. From the day of grouping, HL and HL/ICG were administered through the tail vein at 10 mL/kg (DMPC dose: 136 mg/kg). Administration was performed once every 2 days for 2 weeks (7 times in total). Twenty-four hours after administration, the tumor site was irradiated with near-infrared light for 2 min. On the day after the last administration, the mice were anaesthetised, and the tumor weight was measured. The effect of PDT using HL/ICG was evaluated by determining the relative tumor weight.

Tumor volume (mm^3) = 1/2 × major diameter (mm) × minor diameter (mm) × minor diameter (mm) (Formula 1)

In vitro observation of reactive oxygen species in HCT116 cells subjected to HL/ICG in PDT. HCT116 cells were seeded (1.0 ×105 cells/mL) on a glass bottom dish and pre-cultured for 48 h, followed by the addition of HL and HL/ICG (100 μM). Afterwards, the mixture was incubated for 24 h, and irradiation (60 s) was performed one, three, five, seven, and ten times every

3 min. immediately after the completion of each irradiation, CellROX Green (Thermo, MA, USA) was added. Cells were then incubated for 30 min and observed under a confocal laser scanning microscope (Leica DM IRB, Wetzlar, Germany).

Determining the inhibitory effect of PDT-HL/ICG on oxidative stress using 8-OHdG staining

Tumor sections were washed after deparaffinization and treated with 1% hydrogen peroxide/methanol. After blocking non-specific reactions with skim milk, the sections were incubated with the primary antibody, anti-8-hydroxy-2'deoxyguanosine monoclonal antibody (50-fold dilution) (Japan Institute for the Control of Aging). After washing, the cells were treated with a biotin-labelled secondary antibody, (LSAB2), stained with 3,3'-diaminobenzidine, and then nuclear-stained with haematoxylin. The stained sections were observed under a light microscope.

TUNEL method *in vivo*

Apoptotic cells were detected using the TUNEL method with an in situ apoptosis detection kit (ApopTag plus Peroxidase; Intergen, Burlington, MA, USA) according to the manufacturer's instructions. Subcutaneous tumors were removed from mice under anaesthesia after treatment with HL and fixed in a 10% formalin solution. Paraffin-embedded sections were prepared, and apoptosis was detected using the TUNEL assay according to the conventional procedure.

Statistical analysis

Results are presented as mean ± Standard Error (S.E.). Data were statistically analysed using Student's t-test and the log-rank test. A p-value of less than 0.05 was considered to represent a statistically significant difference.

RESULTS

Physical properties of HL

The physical properties of HL composed of DMPC and 10 mol% C12(EO)25, and HL/ICG composed of DMPC, 10 mol% C12(EO)25, and 1 mol% ICG were examined. The diameter of the HL was measured using dynamic light scattering. The DMPC liposomes were unstable and precipitated after 14 days. In contrast, the hydrodynamic diameters of the HL and HL/ICG were under 100 nm and were maintained for over one month.

Inhibitory effects of PDT-HL/ICG on HCT116 cell growth *in vitro*

We examined the PDT effects of HL/ICG irradiated with far-red light laser on the growth of HCT116 cells using the WST-8 assay. The results are shown in Figure 1. The viability values of the control and PDT-HL treatment on the growth of HCT116 cells were 52%-55%, whereas that of the PDT-HL/ICG-treated cells was 47%-49%. There was a significant difference ($p<0.05$) in IC_{50} values between the control and PDT-HL-treated groups. These results indicate that the PDT effects of irradiated HL/ICG were higher than those of the irradiated HL *in vitro*.

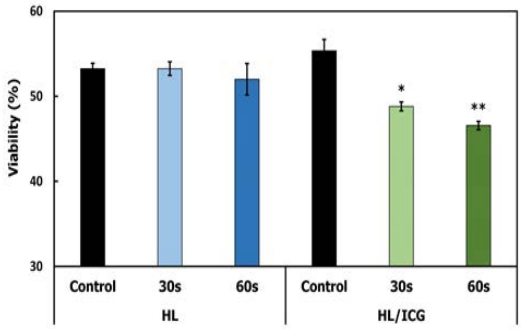


Figure 1: *In vitro* application of HL/ICG in PDT to suppress the growth of colorectal cancer cells. Data presented are mean ± SD. **Note:** Error bar: S.E (n=3); *p<0.05(vs. HL at 30s, HL/ICG at 0); **p<0.05(vs. HL at 60s, HL/ICG at 0).

Therapeutic effects of irradiated PDT-HL/ICG in a subcutaneous xenograft model *in vivo*

We examined the inhibitory effects of PDT-HL/ICG on tumor growth in a mouse xenograft model that was subcutaneously inoculated with HCT116 cells. From the day of grouping, HL and HL/ICG were administered through the tail vein at 10 mL/kg (DMPC dose: 136 mg/kg). Administration was performed once every 2 days for 2 weeks (7 times in total). Twenty-four hours after administration, the tumor site was irradiated with near-infrared light for 2 min. We examined the time course of tumor volume in PDT-HL/ICG-treated xenograft mouse models. The results are shown in Figure 2. The median tumor volumes were 1516 mm^3 and 1510 mm^3 ($p<0.05$, vs. control) in the PDT-HL and PDT-HL/ICG treatment groups, respectively. A reduction in tumor volume (25%) was obtained in the xenograft model that was topically treated with PDT-HL/ICG without drugs after subcutaneous inoculation of HCT116 cells. We next examined the tumor weight in the PDT-HL/ICG-treated xenograft murine model. The results are shown in Figure 3. Mice treated with PDT-HL/ICG (2.0 g, $p<0.05$, vs. control) had a significantly lower tumor weight compared with the animals in the control group (0.39 g-0.05 g). Next, we observed the therapeutic effects of PDT-HL/ICG on this xenograft model by performing an autopsy. As shown in Figure 4, tumor reduction in the PDT-HL/ICG-treated group was observed, although enlargement of the tumor in the control group was confirmed. Moreover, there was little neovascularity in the tumors of the PDT-HL/ICG-treated group. The inside of the tumor was a cavity of the porridge phase due to cell death. These results indicate that PDT-HL/ICG may be effective in a xenograft model *in vivo*.

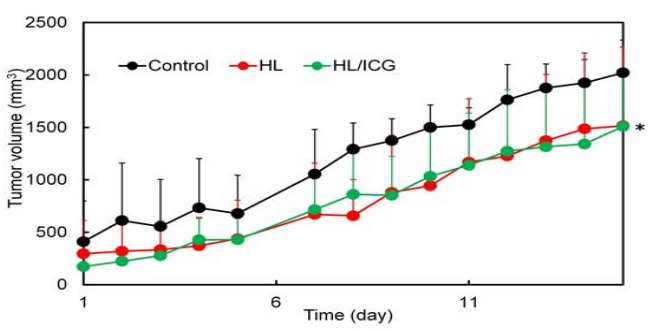


Figure 2: Suppression of tumor volume in the mouse xenograft model topically treated with PDT-HL/ICG after subcutaneous inoculation of HCT116 cells. Data represent the mean (n=7) ± S.E. Dose for DMPC=34 mg/kg, *Significant difference ($p<0.05$) from control, as calculated by the Student's t-test. **Note:** Error bar: S.D. (N=7); *P<0.05(VS control). (—●—) Control, (—●—) HL, (—●—) HL/ICG.

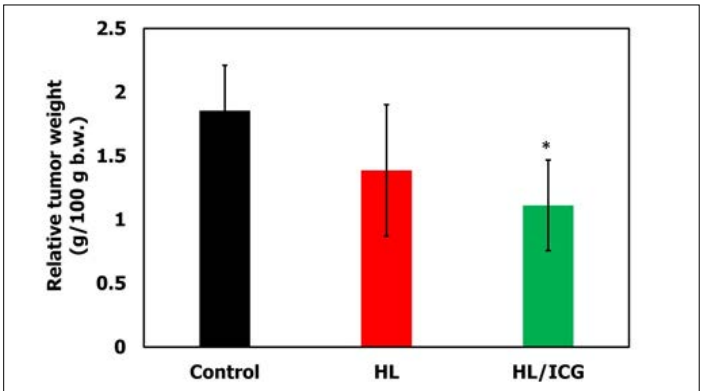


Figure 3: Tumor weight for colorectal cancer in subcutaneous transplantation mice models treated with PDT-HL/ICG. **Note:** Error bar: S.D. (n=7), *p<0.05 (vs. Control).

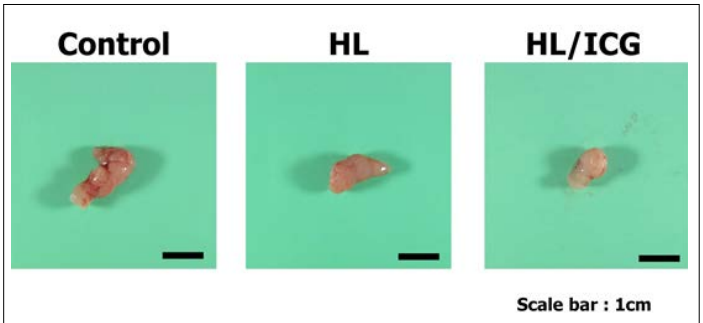


Figure 4: Photographs of tumors in mice topically treated with PDT-HL/ICG after subcutaneous inoculation with HCT116 cells.

Induction of apoptosis by HLs in a mouse xenograft model

We examined the mechanism of the therapeutic effects of PDT-HL/ICG on the subcutaneously-inoculated mice *in vivo* using the TUNEL method. PDT-HL/ICG was topically administered once daily for 14 days from 2 days after the HCT116 cells were subcutaneously inoculated into mice. The tumor was removed from anaesthetised mice immediately after treatment. In contrast to the few apoptotic cells observed in the control group, intense apoptosis was recorded in the PDT-HL/ICG-treated group (Figure 5). These results indicate that PDT-HL/ICG induces apoptosis *in vivo* when using this murine xenograft model.

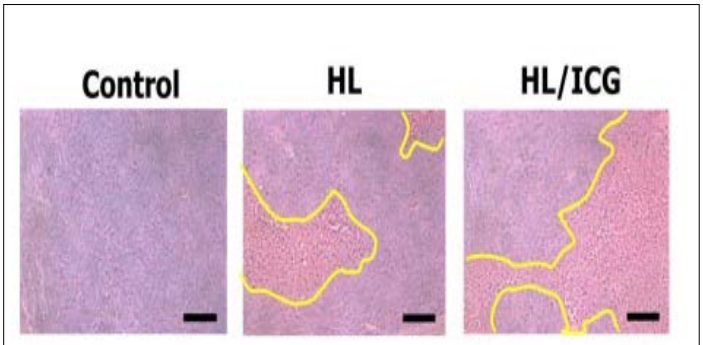


Figure 5: Induction of apoptosis of tumor sections in mice topically treated with PDT-HL/ICG after subcutaneous inoculation with HCT116 cells. **Note:** Arrows indicate apoptotic cells; Scale bar : 500 μm.

Occurrence effects of reactive oxygen species from PDT-HL/ICG in HCT116 cells *in vitro*

We examined ROS production in HCT116 cells subjected to HL/ICG in PDT using a fluorescence microscope *in vitro*. The results are presented in Figure 6. The fluorescence intensity became remarkably stronger as the number of radiations increased. This indicated that PDT-HL/ICG treatment generated ROS.

Effects of PDT-HL/ICG on oxidative stress in xenograft mouse models

We observed oxidative stress in HCT116 cells following PDT-HL/ICG treatment using 8-OHdG staining. The results are presented in Figure 7. Numerous oxidative stress-positive cells (brown color) were observed. The ROS-inducing therapeutic effect of PDT-HL/ICG in a xenograft mouse model after subcutaneous inoculation of human colorectal cancer cells were revealed for the first time *in vivo*.

These results indicate that PDT-HL/ICG could accumulate for a prolonged period of time in tumor cells in a mouse xenograft model due to ROS production and HCT116 cell growth inhibition.

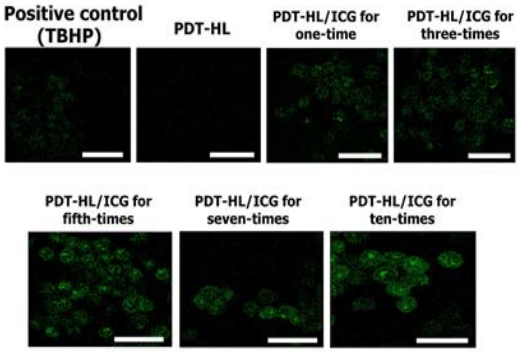


Figure 6: Occurrence effects of reactive oxygen species from PDT-HL/ICG treatment in HCT116 cells *in vitro*. **Note:** Ex : 458 nm; Em : 520 nm; Scale bar : 100 μm.

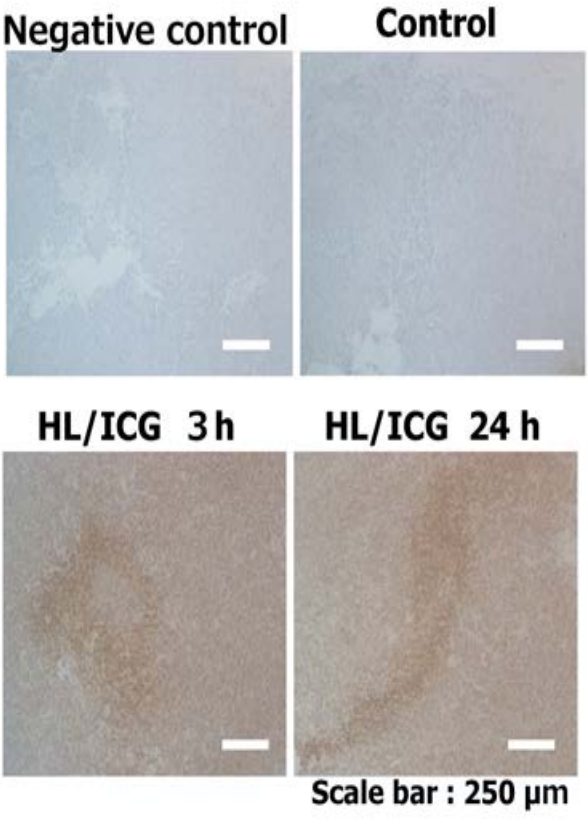


Figure 7: Occurrence effects of PDT-HL/ICG on oxidative stress in xenograft mouse models.

DISCUSSION

Theranostics is a coined word that combines therapeutics and diagnostics. Theranostics can detect and treat very small early stage cancers by simultaneously treating and diagnosing them. Furthermore, it is possible to minimise the physical burden on the patients.

PDT is a laser treatment method that irradiates a low-temperature laser to necrotise tumor cells. Unlike the conventional laser treatment method, which burns the tissue at a high temperature, photosensitization can be performed simply by irradiation with a laser light.

In PDT, a tumor-selective photosensitising agent, which accumulates in cancer cells, is administered to the patient by intravenous injection. After a certain period of time, the area around the cancer tissue is irradiated with a low-temperature laser, causing the accumulated tumor-selective photosensitising agent in the cancer tissue to react and transition from the basal state to the excited state. Reactive oxygen species are generated by the energy produced when the excited tumor-selective photosensitising agent transitions again to the ground state. This reactive oxygen causes the cancer cells to die, and the cancer tissue shrinks. Conventional treatments with PDT, using photofrin and laserphyrin, have been used for early stage lung cancer, gastric cancer, oesophageal cancer, and cervical cancer [1-3].

PDT, using photofrin and laserphyrin, has been approved by the Ministry of Health, Labour and Welfare for the aforementioned cancers and has been covered by the National Health Insurance since 1994. However, their excitation and emission wavelengths are 620 nm and 664 nm, respectively, which are close to the laser absorption region of haemoglobin oxide in the living body. Compared to those of photofrin and laserphyrin, the wavelength of HL/ICG is in the near-infrared region, which is suitable for biopermeability. Furthermore, long-term accumulation of HL/ICG in orthotopic colorectal cancer transplant mouse models has been demonstrated.

Fluctuations in the surface components of tumor cell membranes are very different from those in normal cells. This is because the membranes of tumor cells are generally more fluid than normal cells. HL showed remarkably higher inhibitory effects on the growth of human colorectal cancer cells than DMPC liposomes, in which membrane fluidity was smaller than that of HL [18]. Fusion and accumulation of HL in colorectal cancer cells (WiDr, HCT116) without affecting normal colon cells (CCD33Co) using total reflection fluorescence microscopy has been reported [18]. These results suggest that HL should be distinguished between normal and tumor cells based on the fluidity of the cell membrane.

According to the morphology of HL, dhy of HL has been kept stable for more than one month at room temperature. HL can be stored for a longer time span before its application *in vivo* and clinical field. There is a possibility that HL below 100 nm in diameter might evade Reticular Endothelial System (RES) and also be appropriate for intravenous administration *in vivo* and clinical applications [19].

We examined the PDT effects of Hybrid Liposomes (HL) composed of L-α-dimyristoylphosphatidylcholine (DMPC), polyoxyethylene (25) dodecyl ether (C12(EO)25), and Indocyanine Green (ICG) in human colorectal cancer cells (HCT116) *in vitro* and *in vivo*. The inhibitory effects of PDT-HL/ICG in a subcutaneous xenograft mouse model of colorectal cancer were examined *in vivo*. The inhibitory effects of HL/ICG irradiated with far-red light laser on the growth of HCT116 cells were observed. A remarkable reduction in tumor weight in the xenograft mouse models of colorectal cancer intravenously treated with PDT-HL/ICG after subcutaneous inoculation of HCT116 cells was verified *in vivo*. The occurrence of ROS by PDT-HL/ICG treatment was observed in HCT116 cells *in vitro* using fluorescence microscopy. An increase in peroxidation products in tumor cells of colorectal cancer xenograft model mice intravenously administered with PDT-HL/ICG was observed in micrographs *via* immunostaining with anti-8-OHdG. Hence, the ROS-inducing therapeutic effects of PDT-HL/ICG in a xenograft model after subcutaneous inoculation of human colorectal cancer cells were revealed for the first time *in vivo*.

We examined the PDT effects of HL/ICG irradiated with far-red light laser on the growth of HCT116 cells using the WST-8 assay. The viability values of the control and PDT-HL on the growth of HCT116 cells were 52%-55%, whereas that of PDT-HL/ICG-treated cells was 47%-49%. There was a significant difference ($p<0.05$) in IC_{50} values between the control and PDT-HL-treated groups. These results indicate that the PDT effects of irradiated HL/ICG were higher than those of irradiated HL *in vitro*.

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We examined the mechanism of the therapeutic effects of PDT-HL/ICG on the subcutaneously-inoculated mice *in vivo* using the TUNEL method. In contrast to the few apoptotic cells observed in the control group, intense apoptosis was recorded in the PDT-HL/ICG-treated group, indicating that PDT-HL/ICG exerts therapeutic effects in terms of apoptosis *in vivo* when using this particular murine xenograft model.

We examined ROS in HCT116 cells subjected to HL/ICG treatment in PDT using a fluorescence microscope *in vitro* (Figure 6). The intensity of fluorescence became remarkably strong as the number of radiations increased. The occurrence of ROS in PDT-HL/ICG was observed.

We observed oxidative stress in HCT116 cells treated with PDT-HL/ICG using 8-OHdG staining (Figure 7). Numerous oxidative stress-positive cells (brown color) were observed. The ROS-inducing therapeutic effects of PDT-HL/ICG in a xenograft mouse model

after subcutaneous inoculation with human colorectal cancer cells were revealed for the first time *in vivo*. These results indicate that PDT-HL/ICG could accumulate for a prolonged period of time in tumor cells in a mouse xenograft model, generate ROS, and inhibit HCT116 cell growth.

CONCLUSION

In this study, the inhibitory effect of HL/ICG on the growth of colorectal cancer (HCT116) cells using PDT was determined. The therapeutic effect of HL/ICG in PDT on HCT116 cell subcutaneous transplant model mice was examined. We obtained the following noteworthy findings:

1) The survival rate of HCT116 cells was 53% with HL regardless of the irradiation time. However, in HL/ICG, the survival rate significantly ($p<0.05$) decreased to 49% at an irradiation time of 30 s and 47 % at an irradiation time of 60 s, compared with the HL-treated cells.

2) We compared the tumour volume of the treatment groups on the final day of measurement with that of the control group to which 5% glucose was administered. The tumour volume of the HL and HL/ICG treatment groups indicated an antitumor efficacy of 42%.

3) Compared with those in the control group and HL administration group, the relative tumour weight in the HL/ICG-administered PDT-treated group decreased. Tumour images also showed a reduction in tumour size in the HL/ICG-treated group.

4) Tumour sections of mice treated with HL/ICG-PDT were stained by the TUNEL method, and induction of apoptosis was confirmed.

5) *In vitro* and *in vivo*, oxidative stress due to the development of reactive oxygen species was observed in HCT116 cells treated with HL/ICG-PDT.

These results demonstrated the *in vitro* effect of HL/ICG-PDT on colorectal cancer cells.

Furthermore, we demonstrated the *in vivo* therapeutic effect of PDT through NIR irradiation after the administration of HL/ICG to subcutaneous transplant mice model.

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