Photodynamic Therapy Using Novel Glucose-conjugated Chlorin Increases Apoptosis of Cholangiocellular Carcinoma in Comparison with Talaporfin Sodium

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Abstract. *Background/Aim:* Photodynamic therapy (PDT) is an effective laser treatment for locally treating advanced bile duct carcinoma (BDC). The study's objective was to evaluate the increased cytotoxic effect by apoptotic PDT using a novel photosensitizer, glucose-conjugated chlorin, by irradiation of light-emitting diode laser (G-PDT) in comparison with conventional PDT using talaporfin sodium (T-PDT). *Materials and Methods:* The cytotoxic effect of G-PDT was compared to that of T-PDT as a control. Tumor viability was determined by an *in vitro* MTS assay. The percentage of apoptosis-positive cells was examined by triple stain flow cytometry (annexin V, ethidium homodimer III and Hoechst 33342) in the BDC cell line (NOZ cell) *in vitro*. The change in transplanted tumor volume *in vivo* (4-week-old male BALB/c mice) was examined 7 days after PDT. *Results:* Cell death was induced in a light dose-dependent manner by PDT. The laser power was set at 5 J/cm² to obtain half maximal inhibitory concentration (IC₅₀) in T-PDT and G-PDT and the concentration of photosensitivity for G-PDT (2.02 µg/ml) was lower than that for T-PDT (4.14 µg/ml).

Both T-PDT and G-PDT showed increased induction rates in comparison to the light only or G-chlorin only. Furthermore, the rate of apoptosis in the G-PDT (92.6%) was increased in comparison to that in the T-PDT (38.9%). The increased rates of tumor volume during the 7 days in both the G-PDT and T-PDT groups were significantly lower than that in the non-PDT group ($p < 0.01$). At day 7, the increased rates of tumor volume in the G-PDT group were significantly lower than that in the T-PDT group ($p < 0.05$). *Conclusion:* The new G-PDT treatment showed a high prevalence of apoptosis and inhibition of tumor growth in treatment of BDC cells.

Because of improvements in early diagnosis, operative procedures and perioperative management, the number of cases of resectable bile duct carcinoma (BDC) have increased and patient survival has improved in recent years (1, 2). Complete resection without cancer exposure as R0 resection is only a curative treatment, leading to long-term patient survival (3). However, curative resection is often difficult because of extensive tumor infiltration, poor physical conditions or multi-organ dysfunction due to obstructive jaundice. In cases of unresectable BDC, remnant cancer cells or recurrence at the bile duct stump after resection, the effective local control for BDC progression is necessary to maintain liver function avoiding occlusion of the biliary stent by the tumor ingrowth and associated patients' survival. Although systemic chemotherapy and radiation therapy are the main treatment options at this stage, significant evidence of clinical effectiveness has not been clarified yet (4). Alternatively, the usefulness of

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photodynamic therapy (PDT) for BDC treatment has been reported worldwide, including Japan (5-8). Therefore, PDT should be a promising treatment modality to augment conventional anticancer chemotherapy and brachytherapy as recommended in the 2009 Japanese BDC treatment guidelines (4). Our institute has been administering PDT for adjuvant local ablation or chemotherapy for the treatment of unresectable or remnant cases of BDC after surgical resection since 2001 as well (8).

PDT, which is a cancer-specific treatment based on using light-activated photosensitizers to induce cytotoxicity as necrosis or apoptosis in targeted cancer cells, has been widely applied in various cancer treatments (9, 10). PDT treatment also induces a powerful anti-tumor immunological response and vascular shutdown effect (11). The first clinically approved photosensitizer, porfimer sodium, is a hematoporphyrin derivative that has a very powerful cytotoxic effect in BDC (5-10, 12). Our experimental study showed a 22.2% induction rate of apoptotic cells by porfimer sodium in a human cholangiocarcinoma cell line, NOZ cell line (13). However, the anti-tumor effect was limited to the shallow bile duct wall because the 630-nm laser used in treatment had a low permeability (7, 14). Furthermore, there was a long period of dark-light management to prevent skin photosensitivity following the porfimer sodium administration (8, 14). We subsequently evaluated a new and effective photosensitizer, mono-L-aspartyl chlorin e6 (talaporfin sodium (NPe6), Laserphyrin), which has been clinically used for treating malignant tumors, such as bronchial cancer (15). The 664-nm semiconductor laser light activates talaporfin sodium and penetrates deep into the tissue to a depth of more than 10 mm (16). Furthermore, talaporfin-PDT (T-PDT) has a lower skin phototoxicity compared with porfimer sodium-PDT (P-PDT) because talaporfin sodium degrades rapidly *in vivo* (17). Based on the demonstrated clinical effectiveness and the photosensitivity principles of T-PDT, our study showed T-PDT had a higher induction rate of apoptosis or vascular endothelial growth factor and a lower tumor proliferation rate when compared with P-PDT in NOZ cell line *in vitro* or *in vivo* (18). Furthermore, the synergistic effect when combining T-PDT and anti-cancer drugs was clarified (19) and we applied T-PDT for BDC as a clinical trial (20). However, the induction rate of apoptosis was still limited within 50% and the complete response could not be obtained with these results (18, 19). The applied laser apparatus is expensive and, thus, a low-cost apparatus is clinically required. To improve the efficacy of PDT, photosensitizers with better cancer cell specificity and selectivity and a low-cost laser apparatus are necessary.

Tanaka and colleagues reported the high effectiveness of PDT using glucose-, mannose- or maltotriose-conjugated chlorin for digestive tract carcinoma by the expected

phenomenon known as the Warburg effect in positron emission tomography, which targets tumor-associated macrophages (21, 22). This newer PDT showed much higher cytotoxicity in comparison with talaporfin sodium (21). These photosensitizers showed water solubility, which may lead to increased uptake in tumor tissue and decreased skin toxicity (22). The light-emitting diode (LED) can be used to activate these photosensitizers. We hypothesize that PDT using glucose-conjugated chlorin (G-PDT) will show a higher induction of apoptosis in BDC cells and a lower toxicity for normal tissues or photosensitivity compared with conventional T-PDT.

As previously described, we have continuously maintained the translational research works with respect to the effects of photosensitizers in PDT and performed clinical trials in patients with cholangiocarcinoma. As the promising new photosensitizer has developed, the cytotoxic effects of G-PDT, in comparison with those of L-PDT, were examined in the NOZ cell line to evaluate our hypothesis. The percent of tumor necrosis, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay and flow cytometry to assess the induced apoptosis of NOZ cells *in vitro* and of tumor tissue *in vivo* were evaluated in the present experimental study.

Materials and Methods

Cancer cell culture. NOZ cells, a human biliary cancer cell line (JCRB1033; Japanese Collection of Research Bioresources, Tokyo, Japan), were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Central Co, Tokyo, Japan) with 10% fetal bovine serum, glutamine (0.6 mg/ml), penicillin (100 units/ml) and streptomycin (100 mg/ml) at 37°C under a humidified atmosphere of 5% CO₂ in air.

Cell viability assay (13, 18, 19). The effect of PDT on NOZ cell viability was investigated by an assay of a novel tetrazolium compound [3- (4,5-dimethylthiazol-2-yl)-5- (3-carboxymethoxyphenyl)-2- (4-sulphophenyl) -2H-tetrazolium, inner salt; MTS] (CellTiter96 AQueous One Solution Cell Proliferation Assay; Promega Co, Madison, WI, USA). Cells were cultured in 96-well microplates and irradiated for 24 h. Subsequently, 20 µl of a MTS solution (317 µg of MTS/ml phosphate-buffered saline (PBS)) was added to each well, followed by a 4-hour incubation. After a complete dye solubilization by vortexing the plate, the absorbance was read on an Immunoreader (Model NJ-2000; Nihon Inter Med, Tokyo, Japan). A cell suspension (1×10⁵ cells/ml) was made and 100 µl/well of this suspension was incubated at 37°C overnight. The viability of each sample was analyzed three times at a 490-nm absorbance. The cells were decanted and washed twice in PBS (19). Cell viability was expressed as a percentage of treated cells *versus* untreated control cells. The half-maximal (50%) inhibitory concentration (IC₅₀) was calculated. The administered solution was incubated at 37°C for 4 hs, later decanted and, then, washed three times in PBS and, subsequently, fetal bovine serum (FBS)-free DMEM was added. A laser power was set to 5 J/cm². The treatment performed at each concentration (between 1 and 20 µg/ml) was calculated to define the IC₅₀.

Photosensitizers. Glucose-conjugated chlorin (chemical structure is $H_2TPPC-SGlc$, 5, 10, 15, 20-tetrakis (4-(β -D-glucopyranosylthio)-2, 3, 5, 6-tetrafluorophenyl)-2, 3-(methano (N-methyl) iminomethano) chlorin) (G-chlorin) was synthesized and provided free of charge by the laboratory of the Research Institute of Natural Sciences, Okayama University of Science (Okayama, Japan) (21, 22). This compound was stored at 4°C in a dark box and, in case of use, dissolved in dimethyl sulfoxide (DMSO) at room temperature. Talaporfin sodium (Laserphyrin (Meiji Seika Pharma Co Ltd., Tokyo, Japan) was used as a control drug (18, 19). The vial was stored at 4°C in the dark box, which was dissolved by the attached lysate.

Photodynamic therapy - In vitro experiments. T-PDT (control) was performed in the same manner and condition as G-PDT, which was different from our previous studies' protocols (18, 19). PDT was performed 4 hours after administration of each photosensitizer on the incubated NOZ cell mixture seeded into the 96-well culture plates at 37°C for 24 hours, which were still incubated with culture medium for another 24 hours. The NOZ cell mixture was exposed for 24 hours to G-chlorin diluted with DMEM and, then, irradiated using the power LED flat light laser apparatus for plant research use (LEDRB-660DL; OptoCode Co, Tokyo, Japan) that was tuned to Red 660 nm. A Select 41-LED light 420-nm (blue) filter (OptoCode Co.) with yellow spectacles was used to observe localization of fluorescence. The G-chlorin dose and energy density were examined by cell viability. Eventually, the adequate combined condition for the main analysis is established in the Results section. PDT was performed in a dark-shielded room under 50 luxes and the distance between the light source and 96-well microplates was fixed as 10 cm in each PDT (Figure 1).

Flow cytometry analysis of apoptotic cells. The Apoptotic/Necrotic/Healthy Cells Detection kit (PK-CA707-30018/D25517; Takara Bio Inc., Kusatsu, Shiga, Japan), including FITC-annexin V (250 μ l in TE buffer containing 0.1% BSA and 0.1% NaN_3 , pH 7.5), ethidium homodimer III (200 μ M in PBS), 5 $\times$ Binding Buffer and Hoechst 33342: 250 μ l (500 μ g/ml in PBS), was used to detect apoptotic cells by a flow cytometry analysis 4 hours after PDT in accordance with the manufacturer's instructions. To induce PDT, cells were washed once with PBS and re-suspended at the concentration of 2×10^6 cells/ml in 1 $\times$ Binding Buffer in which pipetting for 100 μ l of cell suspension in a microcentrifuge tube was performed. A sample with additional reagents of the 5 μ l of FITC-annexin V, 5 μ l of ethidium homodimer III and 5 μ l of Hoechst 33342 mixture was then incubated for 15 min in a dark room. Four hundred μ l 1 $\times$ Biding Buffer was added in each tube and the fluorescence in the FITC-annexin V, propidium iodide and 4',6-diamidino-2-phenylindole (DAPI) channels using flow-cytometer (07B1X000030 00102; BD FACS Canto II (BD Biosciences, Fukushima, Japan)) was measured within 1 h after staining. With this method, triple synchronous fluorescent stain of three types of cells can be simultaneously measured as follows: annexin V gives the early-phase apoptotic cells a green color, ethidium homodimer III stains both the late phase of apoptotic cells and necrotic cells in red and Hoechst 33342 stains the living cells with a blue color (Figure 2) (23). The four fractions were indicated in the dot-plot graph as Q1, Q2 (the late phase of apoptosis), Q3 (non-apoptosis) and Q4 (the early phase of apoptosis). The induction rate of apoptosis was calculated using the formula below:

Number of Q2 plus Q4 / total number of cells (percent)
(2) Animal experiments (*in vivo*)

Figure 1. PDT irradiated by the 660 nm of light-emitting diode (LED) in NOZ cancer cell line in the dark-shielded room *in vitro*. The distance between the light resource and the 96-well microplate was 10 cm.

1) **Tumor xenograft.** In these experiments, 1×10^6 NOZ cells were inoculated subcutaneously into both of the femoral legs of 4-week-old nude mice (BALB/cANcrj nu/nu; Charles River Japan Inc., Yokohama, Japan). The volume of tumors that grew to approximately 100 mm³ in size 21 days after inoculation were used as the experimental models. The volume (mm³) of tumor at 21 days was set as a pre-PDT control and changes of tumor volume at days 1, 3, 5 and 7 after PDT were calculated.

2) **PDT protocol.** Each photosensitizer was intraperitoneally injected into the BALB/cANcrj nu/nu mice. The time interval between photosensitizer injection and light exposure was 4 hours. The accumulated photosensitizer was observed by the Select 41-LED light 420-nm (blue) filter with yellow spectacles (Figure 3). Each tumor received a total energy fluency of 37.5 J/cm² (24) with a LED flat light laser for 12 min and 45 sec according to a previous study (21). During the laser light exposure, mice were anesthetized with an intraperitoneal injection of ketamine (90 mg/kg body weight) and xylazine (10 mg/kg body weight). PDT was directed to the transplanted tumors of the animal. The control animals (n=4) were only treated with the laser light exposure without photosensitizer. The adequate administrative dose of talaporfin sodium and G-chlorin was 6.25 μ mol/kg based on a previous study (24). The change in tumor volume (mm³) and the increased rate of tumor volume (%) (*i.e.*, the volume after PDT / the volume before PDT (day0) $\times 100$) were calculated each day after PDT (n=8, in each).

Statistical analysis. The data for comparison are expressed as the mean $\pm$ the standard deviation (SD). Statistical significance between the two groups was determined by non-parametric Wilcoxon's tests. Statistical significance between multiple groups was determined by one-way factorial ANOVA and the non-parametric Scheffe's multiple comparison tests. The differences in sequential changes between the groups were examined by using repeated measures of ANOVA and Fischer's PLSD multiple comparison test. A *p*-value of less than 0.05 was considered statistically significant. Statistical analyses were performed using the Statistical Package for the Social Science (SPSS) software, Version 18.0 (SPSS, Chicago, IL, USA).

Figure 2. Triple synchronous fluorescent stain of annexin V (green), ethidium homodimer III (red) and Hoechst 33342 (blue) for flow cytometry analysis after PDT in NOZ cells. The white arrow shows the early phase of cell apoptosis with green and blue stains. The black arrow shows the late phase of cell apoptosis with all stains.

Figure 3. The in vivo model. Nude mouse with a transplanted NOZ cell tumor on its femurs. The photosensitizer was administered and followed by laser irradiation 4 h later. The reddish fluorescence that accumulated on the transplanted tumor was observed in purple (arrow).

Results

Establishment of dose of photosensitizer and laser irradiation for PDT. To assess the toxicity of G-chlorin, NOZ cell viability was determined by MTS assay at 24 h after various concentrations of drug administration (Figure 4). Cell viability maintained with up to 15 $\mu\text{g/ml}$ of G-chlorin. Next, G-PDT, by various concentrations of drugs and several energy density

Figure 4. NOZ cell viability in various concentrations of G-chlorin by the MTS assay. Cell viability was maintained with up to 15 $\mu\text{g/ml}$ of G-chlorin.

ranges, was performed and cell viability determined at 24 h after PDT (Figure 5). Cell death was induced in a light dose-dependent manner by PDT and the slopes of decreased cell viability from the 3 and 5 Jules/cm^2 were similar between 2 and 4 $\mu\text{g/ml}$ of G-chlorin, respectively. A decrease in viability was induced by 6 and 7 Jules of energy density of laser. In order to induce IC_{50} PDT conditions, a G-chlorin concentration between 2 and 4 $\mu\text{g/ml}$ and a laser power of 5 Jules/cm^2 was required for NOZ cell. To obtain IC_{50} in the same condition in T-PDT and G-PDT using 5 Jules/cm^2 , the concentration of photosensitivity was 4.14 $\mu\text{g/ml}$ (5.17 $\mu\text{g/mole}$) for talaporfin sodium and 2.02 $\mu\text{g/ml}$ (1.16 $\mu\text{g/mole}$) for G-chlorin, respectively (Figure 6). Thus, the results showed that G-PDT required a lower dose of photosensitizer than that of T-PDT.

Apoptosis in NOZ cells after PDT. Induction of apoptotic cells by PDT was investigated by staining the cells with Hoechst 33342 24 h after treatment under IC_{50} conditions. Figure 7 shows the induction rate of apoptosis in the 5 Jules/cm^2 of light only, 2.02 $\mu\text{g/ml}$ of G-chlorin only, T-PDT and G-PDT. Both T-PDT and G-PDT showed increased induction rates in comparison with the light only or G-chlorin only. The induction rate in the G-PDT (92.6%) showed that the rate increased more when compared with that in the T-PDT (38.9%).

In vivo animal study. The tumor volume change in transplanted NOZ cells on mice is shown in Table I. The actual volume during the 7 days before and after PDT was not significantly different between the three groups (non-PDT, G-PDT and T-PDT). The increased rates of tumor volume at day 0 in both G-PDT and T-PDT groups were significantly lower than those in the non-PDT group at days 3, 5 and 7 after PDT ($p < 0.01$). Increased rates of tumor

Figure 5. NOZ cell viability in various total energy doses of LED and various concentrations of G-chlorin by the MTS assay. The slopes of decreased viability were similar in 3, 4 and 5 Jules/cm² between 2 and 4 μg/ml of G-chlorin.

volume change during the 7 days after PDT in each G-PDT and T-PDT group were significantly lower than those in the non-PDT group as well ($p=0.0011$ and $p=0.0035$, respectively). Seven days after PDT, the increased rates of tumor volume when compared with day 0 in the G-PDT group were significantly lower than those in the T-PDT group ($p<0.05$).

Discussion

The current problem of treating BDC with PDT is the limited access route for laser irradiation, specificity of accumulation or metabolism of the photosensitizer, as well as the high cost of the drug and laser apparatus. Therefore, future advanced endoscopic procedures are anticipated to solve the access route problem (20, 25). To approach this issue, we have applied P-PDT and T-PDT; however, our expectation to prolong patient survival or shorten hospitalization has not been achieved (8, 10, 20). Therefore, increased accumulation of a photosensitizer and a more convenient laser apparatus should be the goal in order to make this local treatment useful.

Previous studies using cultured cells revealed that the main cytotoxic effect of PDT was apoptosis of BDC cells and that the induction rate of apoptosis in T-PDT combined with gemcitabine and oxaliplatin (37%) was significantly increased in comparison with that of porfimer sodium (22.2%) (13, 18, 19). T-PDT has been found clinically effective for lung or esophageal cancer treatment as phototoxicity was dramatically improved (15, 26); therefore, T-PDT is a control method for future experiments or can be manufactured and marketed as a new photosensitizer in Japan. Carbohydrate-chain conjugated chlorin as glucose, mannose or maltotriose has recently been used as a photosensitizer of PDT in animal experiments (21, 22, 34),

Figure 6. NOZ cell viability to measure concentration (μg/ml and μg/mole) of talaporfin sodium and G-chlorin to achieve IC₅₀ by the MTS assay.

which has not been commercially produced. Glyco-conjugated chlorin (H2TFPC-SGlc) was 30 times more cytotoxic to gastric cancer cells than talaporfin (27). Its accumulation was higher in xenograft tumors and it significantly suppressed tumor growth compared to talaporfin. The mechanism of accumulation in cancer cells is similar to the uptake of fludeoxyglucose (¹⁸F) for positron emission tomography, a phenomenon known as the Warburg effect (28); however, detailed mechanisms are still unknown. This drug showed higher water-solubility in comparison with previously developed chlorin-based photosensitizers, which might have advantages of higher cellular uptake and an increase in metabolism, thus leading to reduction of photosensitivity in the normal tissues (27). In the present study, the glucose-conjugated chlorin compounds were provided free of charge by one of the current co-authors (H. A.). Similar to Tanaka *et al.*'s (27), we applied 633 nm of LED light for irradiation for PDT.

Figure 7. Dot-plot analysis of triple staining using annexin V, ethidium homodimer III and Hoechst 33342 by flow cytometry analysis in NOZ cells. The four fractions were divided by stain as Q1, Q2 (the late phase of apoptosis), Q3 (non-apoptosis) and Q4 (the early phase of apoptosis). The induction rate of apoptosis was calculated using the following formula: Number of Q2 plus Q4 / total number of cells (percent). (A) Light irradiation only, (B) G-chlorin administration only, (C) T-PDT and (D) G-PDT.

Our previous studies showed that T-PDT-induced NOZ cell death by apoptosis was in a light dose-dependent manner, as in the current work (13, 18). Only light exposure or photosensitizer administration did not affect the notable cytotoxicity, as observed by viability assays. In the present study, the dose and procedure of laser use in T-PDT were modified compared to previous studies (18, 19) because it appeared reasonable to use the same settings as G-PDT to exclude, except the difference of each photosensitizer, other potentially interfering factors. After setting the same energy irradiation of LED laser to 5 J/cm², the required IC₅₀ concentration of G-chlorin was approximately half the concentration of talaporfin sodium, which meant that it is more convenient for clinical use and formulation of the drug. In a previous study, we assessed apoptosis by histological stain, DNA fragmentation assay and DNA content in pre-G1 fraction using flow cytometry analysis (13, 18, 19). In this work, only flow cytometry measurement *in vitro* was used

and more sensitive procedures of triple staining were additionally applied to detect apoptotic cells. Annexin V has a high affinity against phosphatidylserine (PS) under calcium ions as PS moves outside of the cell membrane from intracellular phospholipids by the asymmetric change of membranes in the early phase of apoptosis (29), demonstrating that it is also detectable in pre-apoptotic cells, while ethidium homodimer III showed apoptotic cells by DNA intercalation, including necrotic and completely apoptotic cells (30, 31). Thus, the early and late phases of apoptotic cells can be shown by measuring chromatin stain with Hoechst 33342. In this method, G-PDT showed extremely higher rates of apoptosis in NOZ cells in comparison to that of T-PDT; more than 2 times under the same PDT conditions as in previous studies (21, 22, 27, 28). The induction power of NOZ cancer cell apoptosis is promising for future use because apoptosis is a relatively acceptable measurement of cell death in comparison with

Table I. Changes of tumor volume and its increased rate after each photodynamic therapy (PDT) in animal model.

	Tumor volume (mm ³)					Increased rate of tumor volume (%)				
	Day 0	Day 1	Day 3	Day 5	Day 7	Day 0	Day 1	Day 3	Day 5	Day 7
Non-PDT (n=4)										
①	0.112	0.16	0.216	0.368	0.51	0	42.9	92.9	228.6	355.4
②	0.28	0.384	0.49	0.88	1.06	0	37.1	75	214.3	278.4
③	0.08	0.1	0.144	0.21	0.41	0	25	80	162	425
④	0.024	0.032	0.06	0.072	0.09	0	33.3	150	200	275
Mean	0.123±0.112	0.168±0.153	0.228±0.187	0.383±0.354	0.518±0.404	0	34.6±7.5	99.5±34.5	201.2±28.6	333.5±71.4
G-PDT (n=8)										
①	0.08	0.075	0.101	0.12	0.17	0	-6.25	26.3	50	112.5
②	0.06	0.105	0.113	0.134	0.14	0	75	88.3	123.3	123.3
③	0.06	0.12	0.13	0.138	0.138	0	100	50	130	130
④	0.21	0.21	0.32	0.342	0.398	0	0	52.4	62.9	89.5
⑤	0.245	0.245	0.31	0.345	0.346	0	0	0	0	-8.6
⑥	0.036	0.048	0.048	0.075	0.12	0	33.3	33.3	108.3	233.3
⑦	0.168	0.28	0.27	0.315	0.365	0	66.7	60.7	87.5	117.3
⑧	0.35	0.48	0.528	0.576	0.602	0	37.1	50.9	64.6	72
Mean	0.153±0.112	0.198±0.141	0.223±0.160	0.258±0.171	0.287±0.172	0	38.2±39.5	45.2±26.0*	78.3±43.2*	108.7±67.4* [#]
T-PDT (n=8)										
①	0.096	0.123	0.153	0.186	0.253	0	28.1	59.4	93.8	163.5
②	0.84	1.092	1.428	1.784	2.436	0	30	70	112.4	190
③	0.084	0.124	0.1468	0.186	0.239	0	47.6	74.8	121.4	184.5
④	0.048	0.072	0.088	0.096	0.129	0	50	83.3	100	168.8
⑤	0.406	0.51	0.651	0.702	0.952	0	25.6	60.3	72.9	134.5
⑥	0.4	0.42	0.586	0.711	0.899	0	7.5	46.5	77.8	124.8
⑦	0.075	0.093	0.122	0.135	0.175	0	24	62.7	80	133.3
⑧	0.253	0.366	0.389	0.401	0.632	0	44.7	53.8	58.5	149.8
Mean	0.277±0.270	0.349±0.344	0.446±0.454	0.527±0.562	0.715±0.769	0	32.2±14.4	63.9±11.8	89.6±21.2*	156.2±24.4*

* $p < 0.01$ vs. control group by the Scheffe's multiple comparison test. [#] $p = 0.015$ vs. L-PDT group.

acute necrosis of the BDC tumor. To clarify this phenomenon, the cytotoxic mechanism or accumulation of G-chlorin PDT should be further examined.

In animal experiments, photodynamic detection (PDD) by red fluorescence was attempted and a purple or reddish change at the tumor site was observed, which was not significant in comparison with Tanaka *et al.*'s study, although a similar fluorescence filter was used (27). PDD is also necessary to find the tumor's extension and to evaluate the treatment response of PDT (32). The normal tissue of mice was also stained by G-chlorin; however, any photoreactions or toxicity after PDT in the normal tissue of mice was not observed. We demonstrated that there was more effective tumor reduction in G-PDT in comparison with T-PDT based on the *in vitro* results. As a result, the increased rate of transplanted tumors was interrupted by both G-PDT and T-PDT in comparison with the non-PDT mice in the present study. Our hypothesis regarding cytotoxic effect superiority by G-PDT was not significantly

proven because the initial tumor volume increased more than twice regardless of PDT. Previous studies showed a notable treatment response reducing tumor volume after PDT (21, 22, 27, 28).

Except of the improved PDT cytotoxic effect in BDC cells using carbohydrate-chain conjugated chlorin of glucose, mannose or maltotriose, our aim was to find alternative effective photosensitizers. By changing the chemical structure of the photosensitizer, using engineering technology, a more water-soluble photosensitizer appeared necessary. Matsumoto *et al.* developed a highly water-soluble phosphorus porphyrin complex, which showed increased bactericidal action (33). Delivery system improvements, that are more specific to cancer cell membrane or receptor, nanoparticles or antibody-conjugated photosensitizers are expected to be of future assistance. In addition, improvement in deeper tissue penetration by the laser apparatus within the safety wavelength between 600 and 700 nm is also necessary.

Conclusion

Tumor viability measured by the MTS assay showed a significantly higher induction of apoptosis in the BDC cell line, NOZ, when treated with G-PDT using G-chlorin in comparison to conventional PDT with talaporfin sodium *in vitro*. In the *in vivo* study using a nude mouse model, the increased rate of tumor volume in both T-PDT and G-PDT was significantly lower in the non-treated group during the first 7 days. At day 7, the increased rate of tumor volume in G-PDT was significantly lower than that in T-PDT. Phototoxicity of G-PDT was not observed in the normal tissue of mice. PDT using G-chlorin appears to induce apoptosis and inhibit tumor growth in BDC cells in the pre-clinical model and represents an important area for future clinical trials.

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