Photodynamic Therapy in Combination with Talaporfin Sodium Induces Mitochondrial Apoptotic Cell Death Accompanied with Necrosis in Glioma Cells

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Photodynamic therapy (PDT) induces selective cell death of neoplastic tissue and connecting vasculature by combining photosensitizers with light. Here we clarified the types of cell death induced by PDT in combination with the photosensitizer talaporfin sodium (mono-1-aspartyl chlorine e6, NPe6) in order to evaluate the potential of this therapy as a treatment for glioma. PDT with NPe6 (NPe6-PDT) induces dose-dependent cell death in human glioblastoma T98G cells. Specifically, cell death modalities were observed in NPe6-PDT treated T98G cells, including signs of apoptosis (activation of caspase-3, expression of phosphatidylserine, and DNA fragmentation) and necrosis (stainability of propidium iodide). In addition, high doses of NPe6-PDT decreased the proportion of apoptotic cell death, while increasing necrosis. Closer examination of apoptotic characteristics revealed release of cytochrome-c from mitochondria as well as activation of both caspase-9 and caspase-3 in cells treated with low doses of NPe6-PDT. Benzoicarboxyl-Leu-Gln(OMe)-His-Asp(OMe)-fluoromethyl-ketone (Z-LEHD-fmk), a caspase-9 specific inhibitor, and benzoicarboxyl-Asp(OMe)-Gln-Met-Asp(OMe)-fluoromethyl-ketone (Z-DQMD-fmk), a caspase-3 specific inhibitor, showed dose-dependent prevention of cell death in NPe6-PDT treated cells, indicating that mitochondrial apoptotic pathway was a factor in the observed cell death. Further, the cell morphology was observed after PDT. Time-and NPe6-dose dependent necrotic features were increased in NPe6-PDT treated cells. These results suggest that NPe6-PDT could be an effective treatment for glioma if used in mild doses to avoid the increased necrosis that may induce undesirable obstacles.

Key words apoptosis; glioma; necrosis; photodynamic therapy; talaporfin sodium

Although neuro-oncologists have striven to improve resectioning techniques, complete excision of glioma cells without injuring normal tissue is still extremely difficult, and malignant glioma often recurs within several months. Indeed, achieving a mean survival period longer than 2 years in cases of glioblastoma remains difficult.1,2)

Major factors hampering curative resectioning of malignant glioma include peritumoral cerebral function and tumor invasiveness.1–4) To overcome these problems, attempts have been made to treat malignant glioma using photodynamic therapy (PDT) to induce selective cell death of tumor cells.5–7) PDT induces selective cell death via selective incorporation of photosensitizers by neoplastic tissue and connected vasculature with subsequent irradiation. Death occurs because the irradiation produces reactive oxygen species that target only those cells that have incorporated the photosensitizers.8,9) PDT has proven useful in treating some cancers, including those of the lung, skin, and other organs.4,10) However, successful treatment of glioma using PDT has yet to be achieved. Establishing PDT as a safe cure for glioma is a major goal, and we therefore investigated the kinetics and types of cell death that are induced by PDT in glioma cells.

Multiple modalities of cell death have been described at the morphological and biochemical level, including apoptosis and necrosis. These forms of cell death are not mutually exclusive, and previous evidence indicates that they may co-occur.11) Apoptosis is characterized as cell death accompanied by DNA fragmentation, membrane-blebbing, and caspase activation. Caspases activation plays a central role in the execution of apoptosis. Caspases are classified as either initiator (2, 8, 9, 10) or executioners/effectors (3, 6, 7), and caspases function mainly through a mitochondrion initiated pathway and a cell-surface death-receptor pathway. Mitochondrial apoptosis is regulated by the release of cytochrome-c from the mitochondria, leading to the activation of initiator caspase-9 which then cleaves and activates downstream effector caspase-3, leading to apoptosis.12)

In cells undergoing necrosis, cytoplasm vacuolization, loss of plasma-membrane integrity, and cell-swelling occur, thereby resulting in the release of cellular contents and inflammation of surrounding tissue.13) Necrotic cell death has been described as passive, unorganized cell death and is therefore difficult to regulate. However, recent advances have facilitated the regulation of necrotic cell death. In particular, Leist et al. reported that on increasing the intensity (necrosis inducer exposure time or concentration) of the insult, cell death changes its type from apoptotic to necrotic.13)

Lysosome-accumulating photosensitizer talaporfin sodium (mono-1-aspartyl chlorine e6, NPe6) is a chlorine compound with an absorption peak at 664 nm and is activated by light with longer wavelengths than conventional photosensitizers. Its light absorption is not affected by hemoglobin and penetrates deeper than other photosensitizers. These advantages were expected to be useful for glioma treatment.14,15) Our previous study revealed that NPe6 was not accumulated in normal brain tissue, but accumulated selectively in the transplanted

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glioma cell area in rats.\textsuperscript{14,15} Histological analysis showed that PDT induced necrotic changes.\textsuperscript{21} Further, observation of nuclei showed apoptotic-like alterations, including a tendency to be condensed, but this change may not have been a sign of apoptosis. Even if it was apoptosis, whether or not this was triggered by hypoxia associated with vascular shut-down or by the cytotoxic effect of NP6-PDT remains unclear.\textsuperscript{21}

Determining the means by which PDT induces cell death is important when considering its use as a medical treatment. While cells dying by necrosis spill cytosolic contents into the extracellular space and provoke inflammatory responses, cells dying by apoptosis are safely consumed by either adjacent parenchymal cells or resident phagocytes without causing inflammation.\textsuperscript{16–18} Although both apoptosis and necrosis have been reported in PDT-induced cell death, the occurrence of each differs depending on the photosensitizer, PDT protocol, and cell type.\textsuperscript{19} The types of cell death induced by NP6-PDT in glioma cells are poorly understood, and the relation between apoptosis and necrosis induced by NP6-PDT remains unknown. To resolve this issue, we investigated the types of cell death and the relation between apoptosis and necrosis induced by NP6-PDT.

\section*{MATERIALS AND METHODS}

\textbf{Materials} NP6 was obtained from Meiji Seika Pharma Co., Ltd. (Tokyo, Japan). Cell Counting Kit-8 was obtained from Dojindo (Kumamoto, Japan). NUCView\textsuperscript{TM} 488 Caspase-3 Assay Kit for Live Cells was obtained from Biotium, Inc. (Hayward, California, U.S.A.). MEBCYT0 apoptosis kit, MEBSTAIN apoptosis kit direct, anti-human caspase-3 mouse monoclonal antibody, and anti-human caspase-3 rat monoclonal antibody were purchased from Medical and Biological Laboratories (Nagoya, Japan). Anti-human \(\beta\)-actin rabbit polyclonal antibody was purchased from Novus Biologicals, LLC (Littleton, Colorado, U.S.A.). Benziloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone (Z-VAD-fmk) was obtained from Peptide Institute (Osaka, Japan). InnoCyte\textsuperscript{TM}; Flow Cytometric Cytochrome-c Release Kit was purchased from Calbiochem (Darmstadt, Germany). Benziloxycarbonyl-Leu-Gln (OMe)-His-Asp (OMe)-fluoromethylketone (Z-LEHD-fmk) and benziloxycarbonyl-Asp (OMe)-Gln-Met-Asp (OMe)-fluoromethylketone (Z-DQMD-fmk) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

\textbf{NP6-PDT Treatment of T98G Cells} Human glioblastoma T98G cells (Riken Cell Bank, Tsukuba, Japan) were detached from culture flask by treatment with trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich) for 3 min at 37°C, and then seeded at 1 \(\times\) 10\(^5\) cells/mL in RPMI 1640 medium containing 10% fetal bovine serum (10% FBS-RPMI 1640), 100 units/mL penicillin, 100 \(\mu\)g/mL streptomycin, 10 mmol/L HEPES, 0.1 mmol/L NaHCO\(_3\), 1 mmol/L glutamine, and 50 mmol/L glucose for 1 h. Then the reaction was terminated by adding 0.01\(\mu\)m HCl to the medium, and absorbance was measured at 450 nm (reference wavelength: 600 nm) using a microspectrophotometer (SAFIRE; Wako, Osaka, Japan) in accordance with the manufacturer’s instructions.

\textbf{Measurement of Caspase-3 Activity, Phosphatidylserine Expression, DNA Fragmentation, and Propidium Iodide Staining} Twenty-four hours after PDT, T98G cells were resuspended in 10% FBS-RPMI 1640. This time, the floating cells and lightly adhering cells were collected following a DPBS(−) washing. Adherent cells were detached from the bottom of the culture well by incubation with Puck’s EDTA solution (5 mmol 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.1 mmol NaCl, 5 mmol KCl, 4 mmol NaHCO\(_3\), 1 mmol EDTA, 5.6 mmol glucose) for 10 min and gentle pipetting. Caspase-3 activity, phosphatidylserine exposure, DNA fragmentation, and propidium iodide (PI)-staining were quantified in these collected cells.

Caspase-3 activity was determined using a commercial assay kit (NuView\textsuperscript{TM} 488 Caspase-3 Assay Kit for Live Cells). Briefly, after treating the cells with appropriate concentrations of caspase-3 substrate for 30 min at room temperature in the dark in accordance with the manufacturer’s instructions, the cell suspension was diluted with 10% FBS-RPMI 1640.

Externalized phosphatidylserine was measured by quantifying the binding of fluorescein isothiocyanate (FITC)-labeled annexin V to cells using a commercial assay kit (MEBCYT0 apoptosis kit) in accordance with the manufacturer’s instructions.

DNA fragmentation was measured by quantifying the binding of deoxyuridine triphosphate (dUTP)-FITC to the nicked DNA of the cells using a commercial assay kit (MEBSTAIN apoptosis kit direct) (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method) in accordance with the manufacturer’s instructions.

Necrosis was also assessed using the MEBCYT0 kit and distinguished from the initial stage of apoptosis by quantifying the number of cells positively stained for PI.

After treatment with these commercial kits, all cells were subjected to analysis by flow cytometry (FACS Calibur, Becton-Dickinson, New Jersey, U.S.A.) with gating for forward scatter and side scatter regions of intact T98G cells.

\textbf{Effect of Caspase Inhibitor Z-VAD-fmk on NP6-PDT-Induced Cell Death} T98G cells were treated with NP6-PDT and subsequently incubated with or without Z-VAD-fmk for 24 h. After 24 h, cell viability was measured as described above.

\textbf{Detection of Cytochrome-c Released from Mitochondria} Twenty-four hours after PDT, T98G cells were collected as described above, and levels of cytochrome-c released from T98G-cell mitochondria were assessed using the InnoCyte\textsuperscript{TM} Flow Cytometric Cytochrome-c Release Kit in accordance with the manufacturer’s instructions. Cellular fluorescence was analyzed using flow cytometry as described above. This kit relies on selectively permeabilizing the cellular membrane for release of cytosolic components while leaving the mitochondrial membrane intact. Positive staining with anti-human cytochrome-c mouse monoclonal antibody and
FITC-conjugated anti-mouse immunoglobulin G (IgG) goat polyclonal antibody revealed viable cells, while cells committed to the apoptotic process remained unstained due to the release of cytochrome-c from the mitochondria into the cytosol.

Identification of Caspase-9 and Caspase-3 in T98G Cells
Twenty-four hours after PDT, T98G cells were lysed with a lysis buffer (20 mM Tris–HCl, 100 mM NaCl, 1% NP-40, 10% glycerol, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 1 mM phenylmethylsulfonylfluoride). Cell lysis that contained 5 µg of protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The gel was transblotted to a polyvinylidenefluoride membrane and incubated with 5% skim milk in DPBS(−) for blocking, after which the membrane was washed and treated with primary antibody for caspase-9, caspase-3 or β-actin, and with a secondary antibody for horseradish peroxidase (HRP)-conjugated anti-mouse IgG sheep polyclonal antibody (GE Healthcare, Tokyo, Japan) (caspase-9), HRP-conjugated anti-rat IgG goat polyclonal antibody (Medical and Biological Laboratories) (caspase-3), or HRP-conjugated anti-rabbit IgG donkey (β-actin) (GE Healthcare). Immunoreactive bands were visualized with an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, New Jersey, U.S.A.).

Observation of Cell Morphology after NPe6-PDT
T98G cells were treated with NPe6-PDT using varying concentrations of NPe6, and then incubated in 10% FBS-RPMI 1640 for indicated hours. Morphology of T98G cells was observed under a phase contrast microscope (µ-Radiance; Bio-Rad, Hercules, CA, U.S.A.).

RESULTS
NPe6-PDT Induces Dose-Dependent Cell Death in T98G Cells
We first examined whether or not NPe6-PDT effectively induced cell death in human glioblastoma T98G cells. T98G cells underwent NPe6-PDT, and cell-viability was ascertained 24 h after the treatment. As shown in Fig. 1, dose-dependent cell death was observed in T98G cells treated with ≥20 µg/mL NPe6. Further, no cell death was observed in cells treated with 20–50 µg/mL NPe6 that were not also subjected to laser irradiation. Taken together, these data indicate that cell death was induced not by toxicity due to NPe6 itself, but rather by activation of NPe6 by laser irradiation.

NPe6-PDT Induces Apoptosis and Necrosis
We examined the type of cell death (apoptosis or necrosis) induced by NPe6-PDT by using T98G cells. As an index for apoptosis, we measured caspase-3 activation, cell-surface exposure of phosphatidylserine, and DNA fragmentation. As an index for necrosis, we measured the number of cells that stained positive for PI.

NPe6-PDT treated cells tested positive on all three indices for apoptosis. As shown in Fig. 2A, caspase-3 activation markedly increased after treatment with ≥25 µg/mL NPe6. The proportion of cells with cell-surface phosphatidylserine increased dose-dependently with NPe6-PDT-treatment (Fig. 2B), and DNA fragmentation markedly increased after treatment with ≥25 µg/mL NPe6 (Fig. 2C). These results strongly indicate that NPe6-PDT induced apoptosis in T98G cells.

Because PI enters necrotic cells through damaged membranes, staining for PI can differentiate necrotic cells from apoptotic ones.20 As shown in Fig. 2D, the population of PI-stained cells increased dose-dependently after NPe6-PDT, indicating that in addition to apoptosis, NPe6-PDT also induced necrosis.

Caspases Are Activated during NPe6-PDT-Induced Cell Death
Caspases are cysteine proteases that are the main executors of the apoptotic process. The involvement of caspases in apoptotic cell death induced by NPe6-PDT was examined. Concentrations of 50–100 µM of pan-caspase inhibitor Z-VAD-fmk is known to completely inhibit apoptosis induced by PDT in conjunction with several different photosensitizers.21,22 Therefore, after NPe6-PDT treatment at different concentrations of NPe6, cells were incubated for 24 h in medium laced with 1–100 µM Z-VAD-fmk, and viability was measured. As shown in Fig. 3A, without Z-VAD-fmk only 9% of T98G cells remained viable after treatment with 25 µg/mL NPe6-PDT. In contrast, cell viability increased dose-dependently after NPe6-PDT treatment with Z-VAD-fmk, with greater effectiveness at lower doses of NPe6. At the highest dose, Z-VAD-fmk (100 µM) inhibited approximately 44% of cell death induced by 25 µg/mL NPe6-PDT, 31% of cell death induced by 30 µg/mL (Fig. 3B), and 4.6% of cell death induced by 50 µg/mL (Fig. 3C).

The Mitochondrial Apoptotic Pathway Is Initiated during NPe6-PDT Induced Cell Death
We further examined the signal for apoptosis resulting from NPe6-PDT treatment. While a report indicates that NPe6-PDT stimulates mitochondrial apoptosis in mouse hepatoma cells,23 the mechanism of cell death resulting from PDT differs by cell type.19 Further, the extent of involvement of mitochondrial apoptosis in NPe6-PDT induced cell death (e.g. rate of cell death) was still unclear. Therefore, we examined the involvement of mitochondrial apoptotic pathway in gliomal cell death induced by NPe6-PDT. First, we investigated whether or not NPe6-PDT caused the release of cytochrome-c from mitochondria. The method employed here measured the amount of mitochondrial cytochrome-c, and cells with less cytochrome-c (due to release) were identified as apoptotic. As shown Fig. 4A, the amount of cytochrome-c in the mitochondria of NPe6-PDT treated T98G cells was less than in NPe6 untreated T98G cells.
Fig. 2. Induction of Apoptosis and Necrosis by NPe6-PDT

T98G cells were treated with NPe6-PDT using varying concentrations of NPe6, and indices of apoptosis (A–C) and necrosis (D) were calculated after measuring fluorescence with a flowcytometer. (A) Caspase-3 activity. (B) Phosphatidylserine expression. (C) DNA fragmentation. (D) PI staining. The cells showing fluorescence above the regular value were regarded as positive cells. Each point represents the mean ± S.D. from experiments conducted at least in triplicate.

Fig. 3. Caspase Activation in NPe6-PDT-Induced Cell Death

T98G cells were treated with PDT using the indicated concentrations of NPe6 and subsequently incubated with or without the indicated concentration of Z-VAD-fmk for 24h. After 24h, cell viability was measured. Viability of 100% was defined as the amount of absorption found in NPe6-untreated cells. Each column represents the mean ± S.D. from experiments conducted at least in triplicate.
We also examined the activation of caspase-9 and caspase-3 using antibodies and detected splicing fragments of both activated caspase-9 (35, 37 kDa bands) (Fig. 4B) and activated caspase-3 (12, 17 kDa bands) (Fig. 4C). These results further suggest that NPe6-PDT induces mitochondrial apoptosis. To determine whether or not activated caspase-9 or caspase-3 were involved in NPe6-PDT induced cell death, we used caspase-9 specific inhibitor Z-LEHD-fmk and caspase-3 specific inhibitor Z-DQMD-fmk. After adding one of the inhibitors to the medium, T98G cells were treated with NPe6-PDT and then incubated for 24 h, after which death was assessed. As shown in Fig. 4D, the addition of each caspase inhibitor caused a dose-dependent increase in cell viability. Z-LEHD-fmk and Z-DQMD-fmk (100 µM) inhibited approximately 27% and 25% of cell death induced by 25 µg/mL NPe6-PDT respectively, indicating that each caspase inhibitor blocked a little more than a quarter of the cell death induced by NPe6-PDT.

Changes of Cell Morphology after NPe6-PDT We observed the morphological changes of NPe6-PDT treated T98G cells. As shown in Fig. 5, no change was observed in NPe6-untreated and 15 µg/mL NPe6-treated cells. In contrast, shrinking cells were increased dose- and time-dependently in 20–50 µg/mL NPe6-treated cells. In addition to cell-shrinkage, vacuolized cells were increased dose- and time-dependently in 25–50 µg/mL NPe6-treated cells. Additionally, swelling cells were also observed in 50 µg/mL NPe6-PDT treated cells with 18 and 24h-incubation after PDT. These results also indicating that necrosis is induced by NPe6-PDT at time- and dose-dependently.

**DISCUSSION**

Here, we revealed that multiple modalities of cell death were observed, including apoptosis and necrosis in NPe6-PDT treated glioma cells, the proportions of which can be influenced by NPe6 concentration. We also confirmed biochemically in vitro that NPe6-PDT induced mitochondrial apoptosis in glioma cells.

We have previously showed that NPe6 was not accumulated in normal brain tissue and cells, but accumulated in glioma tissue and cells. However, a precise mechanism of this...
selectivity cannot be revealed at this time. NPe6 derived from chlorophyll that is a porphrin-related compound.\textsuperscript{14} Porphyrin is amphiphilic and capable of adapting to the micelle formation. Porphyrin has a high affinity with low-density lipoprotein (LDL). To maintain active growth, tumor cells actively takes up LDL and LDL bound porphyrin. However, tumor cells are unable to eliminate porphyrin to which fat-soluble proteins are bound. Disrupting the balance of porphyrin influx and clearance, results in porphyrin-accumulation in tumor cells selectively.\textsuperscript{14,15,24–27} However, hydrophilic property of NPe6 is enhanced by the conjunction with aspartic acid on the side chain of NPe6.\textsuperscript{14,26} Many hydrophilic porphyrin compounds like NPe6 are shown to have a low affinity with LDL, but have a high affinity with albumin.\textsuperscript{15,28,29} Therefore, NPe6-LDL association may not be involved in the selective accumulation in tumor cells. In another possibility, Robey et al. reported that ATP-binding cassette sub-family G member 2 (ABCG2) plays an important role as a factor in cellular-efflux of photosensitizers.\textsuperscript{30} Therefore, ABCG2 may be involved in the selective accumulation of NPe6. Further study is therefore needed to elucidate the precise mechanism for the selective accumulation of NPe6 in tumor cells.

Reiners et al. also reported the mechanism of NPe6-PDT induced mitochondrial apoptosis in mouse hepatoma cells as follows: NPe6-PDT induces lysosomal disruption, resulting in leakage of lysosomal protease cathepsin D. Leaked cathepsin D cleaves Bid, a pro-apoptotic Bcl-2 family member, thereby inducing the release of cytochrome-c from mitochondria, which then leads to apoptosis.\textsuperscript{23} Liu et al. reported that NPe6-PDT produces reactive oxygen species that causes a rapid destruction of lysosomes, leading to release of cathepsins and induces mitochondrial apoptosis in human lung adenocarcinoma cells.\textsuperscript{31} Present study suggesting that a similar mechanism for apoptosis may be responsible, this is not necessarily the only possibility. Caspase-9- and caspase-3-specific inhibitors blocked less than 30% of cell death induced by NPe6-PDT (Fig. 4D), while a pan-caspase inhibitor blocked considerably more (Fig. 3A), implying that while apoptosis induced by NPe6-PDT in glioma cells may be mediated mainly through mitochondrial apoptosis, additional caspase pathways are likely involved.

This study showed that NPe6-PDT induces apoptosis and necrosis in accordance with dose of NPe6. The presence of increased necrotic cells may create several dangerous obstacles. Radiation therapy on the brain induces headache, vomiting, loss of consciousness, and hemiplegia after the operation.\textsuperscript{32} Surgical excision of the necrotic areas induced by radiation therapy can improve these conditions, indicating that necrosis induces delayed obstacles after radiation therapy.\textsuperscript{32} Therefore, to avoid these undesirable effects after NPe6-PDT treatment, it may be important to limit the amount of necrotic cell death while simultaneously increasing apoptotic cell death, because apoptotic cells are consumed by either adjacent parenchymal cells or resident phagocytes without inflammation.\textsuperscript{16–18}

Wilson reported that 92% of glioma cells exist in tumor bulk, 6% in a 2cm-wide margin around the tumor bulk, and
1.8% within an additional 2 cm-wide margin.\textsuperscript{4} The majority of recurrent glioma develop in these marginal areas. Therefore, controlling infiltrating cells in these regions while simultaneously protecting normal brain cells is critical for inhibiting tumor recurrence. We propose the following strategy for glioma treatment: After the tumor bulk is resected as extensively as possible by craniotomy and tumor resection, several sites of probable tumor invasion in the bottom of the resection cavity can be irradiated to induce selective cell death of infiltrating glioma. Recently, we examined the safety and efficacy of PDT using NPe6 as an additional intraoperative treatment for malignant glioma in human patients.\textsuperscript{35} We believe that PDT is useful for the medical treatment of glioma, and clarifying the mechanism of NPe6-PDT induced cell death will contribute to the potential application of this treatment in human glioma cells \textit{in vivo}.

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