Effect of talaporfin sodium-mediated photodynamic therapy on cell death modalities in human glioblastoma T98G cells

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(Received September 25, 2014; Accepted October 2, 2014)

ABSTRACT — While photodynamic therapy (PDT) is an effective treatment for glioma, induction of apoptotic cell death of glioma cells is important for ensuring efficacy and safety of PDT treatment in glioma patients, as necrotic cell death can induce late appearance of obstacles in treatment. Here, we investigated the relationship between type of cell death and PDT treatment conditions involved in laser and photosensitizer dosage in human glioblastoma T98G cells. Photosensitizer talaporfin sodium-mediated PDT (NPe6-PDT) treatment induced laser and NPe6 dose-dependent cell death in T98G cells, whereas almost all cells pretreated with NPe6 at ≥ 30 μg/mL were killed by laser irradiation, regardless of laser dose. Morphological analysis showed that combination of high doses of NPe6 and laser irradiation changes the dominant cell death process from apoptosis to necrosis. Biochemical analysis (detection of caspase-3 activity and staining of cell surface-exposed phosphatidylserine) also showed that increasing laser dose changes the type of cell death from apoptotic to necrotic cell death after high-dose treatment with NPe6. Lactate dehydrogenase leakage assay demonstrated that a laser dose of 5 J/cm² induced less leakage than 30 J/cm². Our results suggested that type of glioma cell death in NPe6-PDT changed with fluctuations in laser and NPe6 dose, and that combination of 30 μg/mL NPe6 with 5 J/cm² laser is the best treatment condition for inducing an increase in apoptotic cells while keeping rate of necrotic cell death low in this in vitro study.

Key words: Apoptosis, Glioma, Necrosis, Photodynamic therapy, Talaporfin sodium

INTRODUCTION

Photodynamic therapy (PDT) is a treatment method that induces selective cell death of neoplastic tissue and connected vasculature by combining a photosensitizer with light. Photosensitizers selectively localize in neoplastic tissues, and subsequent laser irradiation produces reactive oxygen species that injure these tissues (Dougherty et al., 1998; Dolmans et al., 2003). PDT has the potential to become a useful cancer therapy, as tumor cell death can be selectively induced via PDT (Wilson, 1992). We recently examined the safety and efficacy of PDT using talaporfin sodium (mono-L-aspartyl chlorine e6, NPe6) as an additional intraoperative treatment for malignant glioma patients and found that PDT, in addition to surgical resection, achieved better therapeutic results than conventional protocols, particularly in patients with newly diagnosed malignant glioma (Akimoto et al., 2012). We are therefore now investigating useful methods of talaporfin sodium-mediated PDT (NPe6-PDT) to provide more effective and safe treatment for glioma patients.

Cell death modalities have been described at the morphological and biochemical level, including apoptosis and necrosis (Lockshin and Zakeri, 2004). These types of cell death are not mutually exclusive, and previous evidence indicates that they may co-occur (Lockshin and Zakeri, 2004). Apoptosis is characterized by cell shrinkage, membrane blebbing, activation of caspases, and translocation of phosphatidylserine of the external cell surface. Necrosis is characterized by loss of plasma-membrane integrity and swelling of the cytoplasm, resulting in the release of cellular contents such as lactate dehydrogenase (LDH) and induction of inflammation of surrounding tissue. Necrotic cell death has been described as passive, unorganized cell death and difficult to regulate. However, Leist and Nicotera (1997) reported that increa-
ing the intensity (e.g., increasing the exposure time and concentration of chemicals) changes the type of cell death from apoptotic to necrotic cell death. Our recent study demonstrated that NPe6-PDT treatment induces release of cytochrome c from mitochondria and activation of both caspase-9 and caspase-3 in glioma cells, resulting in mitochondrial-mediated apoptotic cell death accompanied by apoptotic morphological changes (Miki et al., 2013). In addition, we have shown that high-dose exposure of NPe6 increases necrotic cell death, whereas NPe6-PDT treatment induces apoptosis superiorly under mild treatment conditions (Miki et al., 2013).

The balance between apoptosis and necrosis is critical for ensuring the safety and efficacy of NPe6-PDT treatment for glioma patients, as necrotic cell death may induce late appearance of obstacles in PDT. Necrotic cells release cytosolic constituents through damaged plasma membrane and induce an inflammatory response, while cellular components in apoptotic cells are safely retained by the plasma membrane until removed by parenchymal cells and phagocytes without inducing inflammation (Fietta, 2006; Hanayama et al., 2004; Maderna and Godson, 2003; Ren and Savill, 1998; Savill and Fadok, 2000). Indeed, a previous study showed that radiation therapy for the brain induces headache, vomiting, loss of consciousness, and hemiplegia after the operation, but these obstacles are improved by surgical excision of the necrotic areas (Ogawa et al., 1979). These observations indicate that necrotic cell death induces delayed obstacles after radiation therapy for the brain. In addition, necrosis may injure normal brain tissue via induction of occlusion or shut down of brain vasculature (Akimoto et al., 2012). Thus, positive induction of apoptotic, and not necrotic, cell death may improve the quality of life of glioma patients who undergo NPe6-PDT.

Although increases in dose of NPe6 have been known to change the type of glioma cell death from apoptosis to necrosis in NPe6-PDT (Miki et al., 2013; Tsutsumi et al., 2013), the relationship between laser dose and type of glioma cell death is poorly understood in NPe6-PDT for glioma. In the present study, to improve safety of this treatment method in glioma patients, we investigated the toxic effects of different combinations of laser and NPe6 dose on the type of cell death in NPe6-PDT.

**MATERIALS AND METHODS**

**Materials**

NPe6 was obtained from Meiji Seika Pharma Co., Ltd. (Tokyo, Japan), a Cell Counting Kit-8 from Dojindo (Kumamoto, Japan), a MEBCYTO apoptosis kit (Annexin V-FITC with propidium iodide [PI] kit) from Medical and Biological Laboratories (Nagoya, Japan), a NUCView™ 488 caspase-3 assay kit for live cells from Biotium, Inc. (Hayward, CA, USA), and a LDH cytotoxicity assay kit from Cayman Chemical Company (Ann Arbor, MI, USA).

**PDT treatment of glioma cells**

Human glioblastoma T98G cells (Riken Cell Bank, Tsukuba, Japan) were seeded at 1 × 10⁵ cells/mL in RPMI 1640 medium supplemented 10% fetal bovine serum (10% FBS-RPMI 1640) at 37°C in 5% CO₂ atmosphere for 24 hr. Cells were then washed with Ca²⁺-, Mg²⁺-free Dulbecco’s phosphate buffered saline (DPBS[-]), and incubated with 0-50 μg/mL NPe6 for 4 hr in 10% FBS-RPMI 1640. After 4 hr, cells were washed with DPBS(-) and incubated for 1 hr in 10% FBS-RPMI 1640. The cells were then immersed in fresh 10% FBS-RPMI 1640 and subjected to laser irradiation (wave length: 664 nm, laser power: 33 mW/cm², total dose of laser irradiation: 0-30 J/cm² [time of irradiation: 0-915 sec]) using a semiconductor laser irradiator (Panasonic Healthcare Co., Ltd., Ehime, Japan). The equation of laser dose ([A] J/cm²) and density of laser power ([B] mW/cm²) and irradiated time ([C] sec) was as follows: A = B × C / 1,000. Therefore, laser dose was controlled by irradiation time.

**Measurement of cell viability**

Twenty-four hours after PDT treatment, viability of the T98G cells was measured using a Cell Counting Kit-8, a commercially available assay kit. Briefly, Cell Counting Kit-8 solution was added to glioma cells in 10% FBS-RPMI 1640 at a final concentration of 10% and then incubated at 37°C for 1 hr. After incubation, the reaction of Cell Counting Kit-8 solution was stopped by adding HCl to the medium at a final concentration of 0.1 M, and absorbance was immediately measured at 450 nm (reference wave-length: 600 nm) using a Varioskan Flash microplate reader (Thermo Scientific, Waltham, MA, USA).

**Detection of apoptosis and necrosis**

Twenty-four hours after PDT treatment, floating and lightly adherent cells were collected by DPBS(-) washing. Adherent cells were then collected by detaching the cells from the bottom of the culture well through incubation with trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO, USA) for 2 min. Activity of caspase-3, cell surface-exposed phosphatidyserine and/or PI staining in glioma cells were measured in these collected cells using commercial assay kits (caspase-3: NucView™ 488 caspase-3 assay kit for live cells, exposed phosphatidyserine...
and PI: MEBCYTO apoptosis kit), as described previously (Miki et al., 2013).

**LDH leakage assay**

Twenty-four hours after PDT treatment, the culture medium was harvested, and an aliquot of the medium was used to measure LDH activity using a LDH cytotoxicity assay kit in accordance with the manufacturer’s instructions. Absorbance of each sample was measured at 480 nm (reference wavelength: 680 nm) using a Varioskan Flash microplate reader (Thermo Scientific).

**Statistical analysis**

Statistical significance of the data was determined using analysis of variance (ANOVA) and Bonferroni’s multiple t-test. P value < 0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

**Effects of different laser doses on T98G cell viability**

We first examined the effect of NPe6-PDT on cell viability in human glioblastoma T98G cells. The cell viability was measured at 24 hr after NPe6-PDT treatment in T98G cells. As shown in Fig. 1A, laser and NPe6 dose-dependent cell death were observed in the cells, whereas no cell death was observed in cells treated with NPe6 without laser irradiation. However, when cells were pretreated with ≥ 30 μg/mL NPe6, almost all cells were killed regardless of irradiated laser dose (5-30 J/cm²).

Fig. 1B shows morphological changes of NPe6-PDT-treated T98G cells. Of note, no morphological changes were observed in cells treated with NPe6 but without laser irradiation. When T98G cells were laser irradiated at 5 J/cm², incidence of plasma membrane-blebbing (black arrows)—a defining characteristic of apoptosis—was increased in both 25 and 30 μg/mL NPe6-pretreated cells, whereas incidence of swelling (necrotic morphology) (white arrows) was significantly increased in 50 μg/mL NPe6-pretreated cells. In 30 J/cm² laser-irradiated cells, plasma membrane-blebbing (apoptotic morphology) (black arrows) was observed in both 20 and 25 μg/mL NPe6-pretreated cells, and swelling (necrotic morphology) (white arrows) was observed in both 30 and 50 μg/mL NPe6-pretreated cells. Taken together, these data indicated that, while NPe6-PDT effectively induced cell death in T98G cells, NPe6 alone did not cause such cytotoxicity. In addition, morphological observations suggested that NPe6-PDT treatment induces both apoptotic and necrotic cell death in T98G cells, and the combination of elevated doses of NPe6 and laser irradiation changes the dominant cell death process from apoptosis to necrosis.

**NPe6-PDT influences cell death modalities in T98G cells**

Using biochemical analysis, we next examined the effect of increasing laser dose on induction of two types of cell death (apoptosis and necrosis) in T98G cells pretreated with NPe6. As an index for apoptosis, we measured caspase-3 activation in the cells (Fig. 2A). Caspase-3 activity was not changed in cells treated with 0-50 μg/mL NPe6 without laser irradiation. However, when cells were pretreated with NPe6 at ≤ 25 μg/mL, caspase-3 activity increased laser dose-dependently. When T98G cells were pretreated with NPe6 at ≥ 30 μg/mL, laser irradiation exerted a stronger effect on caspase-3 activity than did 30 J/cm² laser irradiation. These results suggest that, at low treatment doses of NPe6 (≤ 25 μg/mL), apoptosis may be induced by laser irradiation in a dose-dependent manner, whereas with higher treatment doses of NPe6 (30 and 50 μg/mL), apoptosis may be more strongly induced by low-level laser irradiation (5 J/cm²) than higher-level irradiation (30 J/cm²).

We therefore determined the population of apoptotic and necrotic cells after NPe6-PDT. Apoptosis and necrosis were distinguished by evaluating the number of cells positively stained for cell surface-exposed phosphatidylserine (Annexin V) and PI (Vermes et al., 2000). As shown in Fig. 2B, the populations of annexin V-positive and PI-negative cells (apoptotic cells) and of annexin V-positive and PI-positive cells (necrotic cells) were not increased in cells treated with 0-50 μg/mL NPe6 but without laser irradiation. However, when cells were pretreated with NPe6 at ≤ 25 μg/mL, the population of apoptotic cells (annexin V-positive and PI-negative) increased in a laser dose-dependent manner. When T98G cells were pretreated with NPe6 at 30 μg/mL, 5 J/cm² (but not 30 J/cm²) laser irradiation also increased the population of apoptotic cells (annexin V-positive and PI-negative). However, when cells were pretreated with NPe6 at 50 μg/mL, almost all cells were necrotic (annexin V-positive and PI-positive).

Taken together, these results indicate that increasing laser dose changes the type of cell death from apoptotic to necrotic after high-dose treatment of NPe6 in T98G cells, with laser dose-dependent induction of apoptotic cells also observed in cells exposed to relatively low doses of NPe6. In addition, the optimum combinations for effective induction of apoptotic cell death with NPe6-PDT were suggested to be 25 μg/mL NPe6 with 30 J/cm² irradiation and 30 μg/mL NPe6 with 5 J/cm² irradiation.
Effect NPe6-PDT on LDH leakage
Since suppression of the leakage of cytosolic constituents from NPe6-PDT-treated cells is important for improving safety of PDT, we investigated the effect of NPe6-PDT on LDH leakage through damaged plasma membrane. As shown in Fig. 3, LDH leakage was NPe6- and laser dose-dependently increased during NPe6-PDT. Overall, the effect of 5 J/cm² irradiation on inducing leakage was weaker than that of 30 J/cm², suggesting that, if the cytotoxicity level is the same, a weaker laser dose is safer for patients. On comparing the two optimum combinations described above (Fig. 2), LDH activity was much
Laser level-dependent glioma cell death in NPe6-PDT

Fig. 2. Effect of increasing laser-dose on two types of cell death (apoptosis and necrosis) in T98G cells pretreated with NPe6. T98G cells were pretreated with the indicated concentration of NPe6 and then subjected to laser irradiation (0, 5, or 30 J/cm²). Twenty-four hours after PDT treatment, the indices of apoptosis and necrosis were calculated after measuring fluorescence using a flowcytometer. [A] Caspase-3 activity, an apoptosis index. Mean fluorescence intensity (caspase-3 activity) is displayed in each figure. [B] Staining T98G cells with Annexin V-FITC (exposed phosphatidylserine staining) and PI. The cells showing fluorescence above the basal level were regarded as positive cells. The right lower area (Annexin V-FITC-positive, PI-negative cells) is regarded as apoptotic cells. The right upper area (Annexin V-FITC-positive, PI-positive cells) is regarded as necrotic cells. Proportions of apoptotic and necrotic cells are displayed in the figures.
Keeping surrounding tissue undamaged (Hanayama et al., 2004; Maderna and Godson, 2003; Ren and Savill, 1998; Savill and Fadok, 2000; Miki et al., 2009). Therefore, if equal degrees of cell death effect are achieved, it may be advisable to use a milder laser dose to avoid undesirable necrosis in PDT.

We previously revealed that the mitochondrial apoptotic pathway is induced in NPe6-PDT-treated glioma cells (Miki et al., 2013). Reiners et al. (2002) reported the upstream of mitochondrial apoptotic pathway in mouse hepatoma cells as follows: NPe6 accumulates in lysosomes and laser treatment 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. Liu et al. (2011) also reported that NPe6-PDT produces reactive oxygen species, which causes rapid destruction of lysosomes, leading to release of cathepsins and inducing mitochondrial apoptosis in human lung adenocarcinoma cells. Therefore, the apoptotic pathway induced by NPe6-PDT is well understood in a number of tumor cell types. In contrast, however, the necrotic pathway induced by NPe6-PDT is unclear. Although necrosis has been described as a passive and chaotic cell death, recent evidence suggests that necrotic cell death can be actively propagated as part of a signal transduction pathway (Buytaert et al., 2007). Additionally, necroptosis has recently been suggested as a form of programed necrosis (Degterev et al., 2005; Vandenabeele et al., 2010). While necroptosis was first found as a signaling necrosis induced by tumor necrosis factor (Degterev et al., 2005; Vandenabeele et al., 2010), necroptosis is also induced in photosensitizer 5-aminolevulinic acid mediated PDT (5-ALA-PDT)-treated human glioblastoma LN18 cells (Coupienne et al., 2011). NPe6-PDT may therefore also induce necrosis via a necroptotic signal transduction pathway in glioma cells. Further study is needed to elucidate the mechanism of necrotic cell death pathways and to control the cell death modalities of glioma cells treated with NPe6-PDT.

In conclusion, our results demonstrated that type of glioma cell death in NPe6-PDT changed with differences in exposure dose of laser and NPe6. Combination of 30 μg/mL NPe6 with 5 J/cm² laser irradiation was deemed the best treatment condition for induction of both an increase in incidence of apoptosis and decrease in necrosis without a decrease in overall cell death under present NPe6-PDT treatment modalities. Since a dominant increase in apoptotic cells contributes to the quality of life of glioma patients, we believe that clarifying lower in combination of 30 μg/mL NPe6 + 5 J/cm² irradiation than 25 μg/mL NPe6 + 30 J/cm² irradiation (Fig. 3), despite similar levels of cytotoxicity (Fig. 1). These data indicate that the combination of 30 μg/mL NPe6 with 5 J/cm² laser is the best treatment condition for NPe6-PDT in the present study.

Here, we showed that NPe6-PDT treatment induces laser and NPe6 dose-dependent cell death modalities, including apoptosis and necrosis, in glioblastoma T98G cells. Leist and Nicotera (1997) reported that, upon increasing the intensity of the insult through longer exposure times or higher concentrations of the necrosis inducer, the dominant cell-death process changes from apoptosis to necrosis. Indeed, we compared the proportion of apoptosis and necrosis, and found that combination of high doses of laser with high doses of NPe6 decreased number of apoptotic cells and increased those of necrotic cells (Fig. 2). Apoptosis is a desirable means of inducing cell death because the membrane structures of dead cells are maintained as phagocytes rapidly remove cells, keeping surrounding tissue undamaged (Hanayama et al., 2004; Maderna and Godson, 2003; Ren and Savill, 1998; Savill and Fadok, 2000; Miki et al., 2009). In contrast, membranes of necrotic cells are abrogated, and surrounding tissue is damaged by the release of harmful intracellular contents (Fietta, 2006; Hanayama et al., 2004; Maderna and Godson, 2003; Ren and Savill, 1998; Savill and Fadok, 2000; Miki et al., 2009). Therefore, if equal degrees of cell death effect are achieved, it may be advisable to use a milder laser dose to avoid undesirable necrosis in PDT.

**Fig. 3.** Effect of increasing laser dose on leakage of cellular LDH in T98G cells pretreated with NPe6. T98G cells were pretreated with the indicated concentration of NPe6 and then subjected to laser irradiation (0, 5, or 30 J/cm²). Twenty-four hours after PDT treatment, LDH leakage from the cells into culture medium was evaluated. Open circles: no laser irradiation. Open square: 5 J/cm² laser irradiation. Open triangle: 30 J/cm² laser irradiation. Each value represents the mean ± S.D. from experiments conducted at least in triplicate. Significantly different from corresponding control, **p < 0.01.
the mechanism of NPe6-PDT-induced cell death in glia-
oma would be leading to the potential application of this
treatment in human glioma patients.

ACKNOWLEDGMENTS

Talaporfin sodium (NPe6) was provided by Meiji Seika Pharma Co., Ltd. (Tokyo, Japan). The semiconduc-
tor laser irradiation device was provided by Panasonic Healthcare Co., Ltd. (Ehime, Japan).

Conflict of interest----The authors declare that there is no conflict of interest.

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