Comparison of phototoxicity mechanism between pulsed and continuous wave irradiation in photodynamic therapy

Yuuichi Miyamoto *, Yukihiro Umebayashi, Tsuyoshi Nishisaka

School of Materials Science, Japan Advanced Institute of Science and Technology, Ishikawa, 923-1292, Japan

Abstract

A study has been conducted in which HeLa cells are incubated with hematoporphyrin derivative (HpD) for 1 h (1 µg/ml of HpD in PBS) to compare the use of continuous wave (CW) and pulsed laser (10 Hz repetition rate and 7–9 ns pulse width) light for photodynamic therapy. Cytotoxic effects on the cells are evaluated by the 3-(4,5-dimethyl-2-thiazolyl)-2-5-diphenyl-2H-tetrazolium bromide (MTT) assay and the fluorescein diacetate (FDA)/propidium iodide (PI) stain method using a flow cytometer. The type of cell death is estimated by analysis of the DNA content and observation of the nuclear morphology. The cytotoxicity ratio of cells irradiated by pulsed laser light is estimated to be lower than that for CW laser light. The viability of cells that received pulsed laser light gradually decreases, whereas no significant changes are found in the cells irradiated with CW laser light with the elapse of post-irradiation time. The type of cell death differs between the pulsed and CW laser light irradiations. These findings suggest that the cytotoxic efficacy of the excitation light source is displayed by the difference in the type of cell death, namely apoptosis or necrosis.   ©1999 Elsevier Science S.A. All rights reserved.

Keywords: Pulsed laser; HeLa cells; Cell death; Photodynamic therapy

1. Introduction

Photodynamic therapy (PDT) is a treatment for malignant tumors employing the interaction of a photosensitizer with light of the appropriate wavelength in the presence of molecular oxygen [1].

A continuous wave (CW) laser, usually an argon ion-pumped dye laser, has normally been used as the excitation light source in PDT. Recently, some pulsed lasers have become available [2–4]. The effectiveness of PDT using a pulsed laser varies greatly with the operating conditions, for instance, repetition rate, pulse width and pulse energy [2]. Several investigations have reported that by using a pulsed laser of low energy and high repetition rate, the penetration depth of the light and histological findings of the lesions were identical to those of a CW laser [5–9]. In contrast, Okunaka et al. [10] examined the average depth of tumor necrosis in PDT with a hematoporphyrin derivative (HpD) in a BALB/c mouse kidney tumor and achieved a 11 mm increment from the penetration depth of the CW laser light (4 mm) using an excimer pumped dye laser with high energy and low repetition rate. The photodynamic efficacy of the pulsed lasers in PDT is not currently understood in detail. Mathematical models have been adopted to discuss the differences in the efficacy between pulsed and CW laser light. However, the influence of the light source on the actual cytotoxic effects, in particular the mechanism of cell death, is still unclear [11].

On the other hand, the mechanism of cell death has been extensively investigated in the field of cell injury. There are two fundamental types of cell death, namely, apoptosis and necrosis. Apoptosis is characterized by nuclear and cytoplasmic condensation of the cells (shrinkage) followed by loss of the nuclear membrane, fragmentation of the nuclear chromatin, and subsequent formation of multiple fragments of condensed nuclear material and cytoplasm. On the other hand, necrosis is characterized by cell-membrane damage that involves cell swelling rather than cell shrinkage [12].

In this study, we demonstrate the influence of laser sources (pulsed or CW) on the cytotoxic effects of HeLa cells under low-phototoxicity conditions, and discuss cell-killing efficacy when using a pulsed laser. To compare the cytotoxic efficacy of the pulsed (10 Hz repetition rate and 7–9 ns pulse width) and CW lasers, HeLa cells in which HpD localizes at the plasma membrane were employed [13,14]. As HeLa cells are grown in a monolayer, the average fluence rate of the pulsed and CW laser light in the irradiation area can be regarded as being at the same level. Therefore, we do not

---

* Corresponding author. Tel./fax: +81-761-51-1685; e-mail: yuuichi@jaist.ac.jp

have to take into account the difference in the light penetration between both lasers, and can focus the discussion on the effect of the irradiation mode.

2. Materials and methods

2.1. Cells and cell culture

HeLa cells were cultivated at 37°C in Ham's F-10 medium (Cosmo Bio Co., Tokyo, Japan) supplemented with antibiotics and 10% fetal bovine serum. Cells were used in the log phase of growth.

2.2. Chemicals

The HpD was provided by Itoh-ham Laboratory (Tokyo, Japan). 3-(4,5-Dimethyl-2-thiazolyl)-2-5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Dojindo Lab. (Kumamoto, Japan). Fluorescein diacetate (FDA) was from Nacalai Tesque Inc. (Kyoto, Japan) and propidium iodide (PI) from Molecular Probes Inc. (OR, USA).

2.3. PDT experiments

The HeLa cells were seeded in 35 mm Petri dishes (5×10^5 cells) or 96-well microtiter plates (2×10^4 cells/well), which were used for the MTT assay, two days before the laser light irradiation. The cells were incubated for 1 h with HpD (1 μg/ml of HpD in phosphate-buffered saline, PBS), then rinsed with Dulbecco's PBS and irradiated in the buffer using an argon-ion laser (model 165, Spectra Physics, USA) as the CW laser source or an Nd:YAG-pumped optical parametric oscillator, which has a pulse repetition rate of 10 Hz and a pulse width of 7–9 ns (model MOPO-710, Spectra Physics, USA). The wavelengths of these lasers were both 514.5 nm. PDT was carried out with an average fluence rate of 100 mW/cm^2 (i.e., ~ 10 mJ/cm^2 OPO pulse) at light doses ranging from 1 to 10 J/cm^2. Cells treated with HpD alone and not irradiated were used as the control. After the irradiation, PBS was replaced with the culture medium, and the cells were incubated before each assessment.

2.4. Cell survival

The viability of the cells was assessed by the MTT assay [15], which yields results closely correlated with those of clonogenic assays [16]. 10 μl of MTT solution (5 mg MTT/ml in PBS) was added to the wells at each assessment time (3, 6, 24 and 48 h after PDT) and the cells were incubated at 37°C for 4 h. Acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was added to the wells and mixed thoroughly to dissolve the MTT-formazan. After complete solubilization of the dye by lightly vortexing the plate on an orbital shaker, the absorbance was read on a microplate reader (model 550, Bio-Rad Lab., USA) using a wavelength of 570 nm.

2.5. Flow cytometry

HeLa cells in the dishes were trypsinized to obtain a cell suspension. For experiments measuring cell death, the floating cells were also collected. The viable and nonviable cells were identified by simultaneous assessment of PI and FDA fluorescence [17]. At the time appropriate for assessment of the viable cells, an aliquot of each culture was placed in a test tube, and then FDA added to each test tube to a final concentration of 0.5 μg/ml. The tubes were incubated at room temperature in the dark for 30 min, and then PI in isotonic saline was added to each tube to a final concentration of 50 μg/ml. The tubes were then immediately placed in ice, and kept ice-cold during the flow cytometric analysis. These cells were identified by observing the intracellular green (FDA) and red (PI) fluorescence using a flow cytometer (EPICS ELITE, Coulter, USA).

The DNA content of the cells was also analyzed by flow cytometry [18]. HeLa cells were fixed overnight in 70% ethanol (−20°C). The cells were then centrifuged and incubated for 1 h at room temperature in PBS containing 0.1% Triton-X followed by staining with PI (10 μg/ml) and RNase (10 μg/ml). The DNA content of 5×10^3 cells was measured by a flow cytometer (EPICS ELITE, Coulter, USA) and DNA distribution histograms were obtained.

2.6. Nucleus staining

The cells were stained with PI (5 μg /ml in PBS for 15 min), then fixed with glutaraldehyde (usually 2% in PBS, 30 min at 4°C) and washed with PBS. Fluorescence was observed by a confocal scanning microscope (MRC-1000, Bio-Rad Lab., USA).

3. Results

3.1. MTT viability assay

Fig. 1 shows the effect of each light source on the cell viability measured by the MTT assay as a function of time. The cells were exposed to 1–10 J/cm^2 light. The activities were calibrated with controls containing HpD and not irradiated. The viability values of the cells irradiated by the CW laser light (CW group) were not significantly changed, whereas those of the cells irradiated with pulsed laser light (pulsed group) gradually decreased during the post-irradiation time. The viability of the pulsed group was higher than that of the CW group during the experimental period.

3.2. Flow cytometric assessment of the membrane integrity

FDA and PI were utilized to identify the viable and nonviable cells by flow cytometry [17]. FDA quickly permeates all cells. In viable cells, FDA is converted to highly fluorescent fluorescein by intracellular esterases. The fluorescein
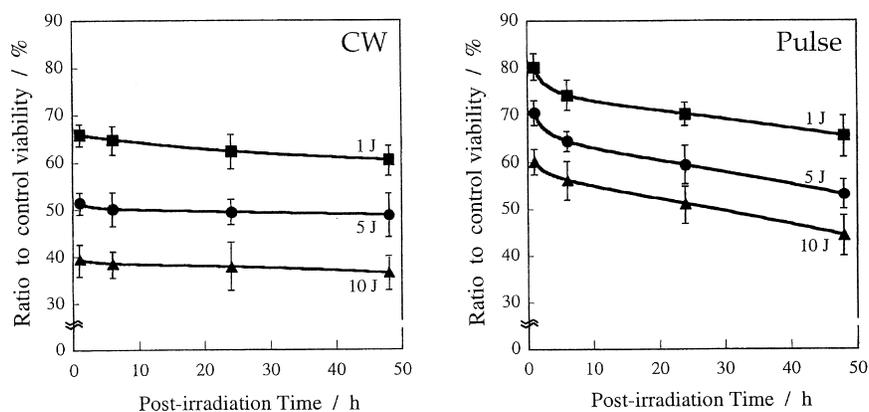
Fig. 1. Changes in the viability of HeLa cells after PDT. Cell viability was assessed by the MTT assay. Original viability was expressed as a percentage of the control. Symbols indicative of the light dose at 514 nm: 1 J/cm^2 (■), 5 J/cm^2 (●) and 10 J/cm^2 (▲). Each data point represents the mean ($n = 4$–6).

diffuses out of cells lacking membrane integrity. PI, which has a red fluorescence, can only permeate cells that lack membrane integrity. Thus, flow cytometric analysis of the cells using FDA and PI can distinguish viable cells with bright green fluorescence from nonviable cells, which show bright red fluorescence. Fig. 2 shows the ratio of nonviable cells (stained by PI) that were irradiated with each type of laser light at 3, 6 and 24 h after PDT. The cells were exposed to 5 J/cm^2 light. As with the results obtained in the MTT assay, the cytotoxicity ratio of the CW group was significantly higher than that of the pulsed group at any assessment point.

3.3. Analysis of DNA content

Fig. 3 shows the DNA distribution histograms at 3, 6 and 24 h after PDT. The cells were exposed to 5 J/cm^2 light. The sub-G1 cell population with a lower DNA stainability than G0 + G1 cells has been suggested as a marker of cell death by apoptosis [18–21]. We compared the ratios of the sub-G1 cell population that was irradiated by each type of laser light (Fig. 4). A significant difference was observed between the CW group and pulsed group at 3 and 6 h after PDT. In the case of the CW group, the ratios of nonviable (membrane damaged) cells at 3 and 6 h after PDT were evaluated to be 22.9 and 23.4%, respectively (Fig. 2), but the ratios of the sub-G1 cell populations were 1.8 and 5.6%. In contrast, the

ratios of the sub-G1 cell populations of the pulsed group at the same assessment points were 10.2 and 14.2%. These values were almost equal to the ratios of nonviable cells (3 h, 9.6%; 6 h, 11.8%). It is suggested that the mechanism of cell death of the pulsed group under the present conditions is mainly apoptosis.

3.4. Effects on the morphology of the nucleus

Fig. 5 shows the nuclei and cell membrane of the PI-labeled HeLa cells observed by a confocal scanning microscope 6 h after PDT. The cells were exposed to 5 J/cm^2 light. Slight fluorescence was observed on the cell surfaces of the control cells (Fig. 5(a)). Upon treatment with CW laser light, we observed the swelling nucleus that is characteristic of necrosis (Fig. 5(b)). In contrast, the dead cells of the pulsed group formed the chromatin condensation and fragmentation typical of apoptosis (Fig. 5(c)). These findings are consistent with the result of the DNA distribution analysis.

4. Discussion

Apoptosis induced by PDT has been mainly observed in lymphocyte and epithelial cell lines, and the apoptotic response often occurred during the early post-irradiation stage [14,22–25]. Therefore, the sudden increase in the sub-G1 population of cells that received CW laser light at 24 h after PDT is unlikely to indicate only apoptotic cells (Figs. 3 and 4). The sub-G1 population seen with flow cytometry could be a necrotic population, as the DNA breaks down when the cell undergoes lysis. Therefore, it is correct to consider that the cell lysis probably occurred 6–24 h post-irradiation time. Conversely, the cell death induced by the pulsed laser irradiation is predominantly apoptosis at 3 and 6 h after PDT (Figs. 3–5). To induce apoptosis by PDT, the light dose and/or concentration of the photosensitizer have to be carefully controlled. The mechanism of cell death induced by PDT has been revealed by recent studies that deal with the intracellular localization of the photosensitizer and the cyto-
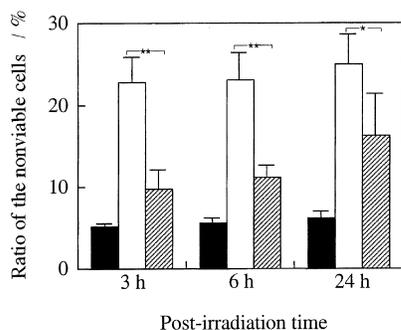

Fig. 2. Ratio of nonviable cells following each treatment at 5 J/cm^2 light irradiation (control, solid bars; CW, white bars; pulse, hatched bars). The FDA/PI viability was assessed at 3, 6 and 24 h after PDT. Data represent the mean of at least three experiments ($\pm$SD). $^*p < 0.05$; $^{**}p < 0.005$.
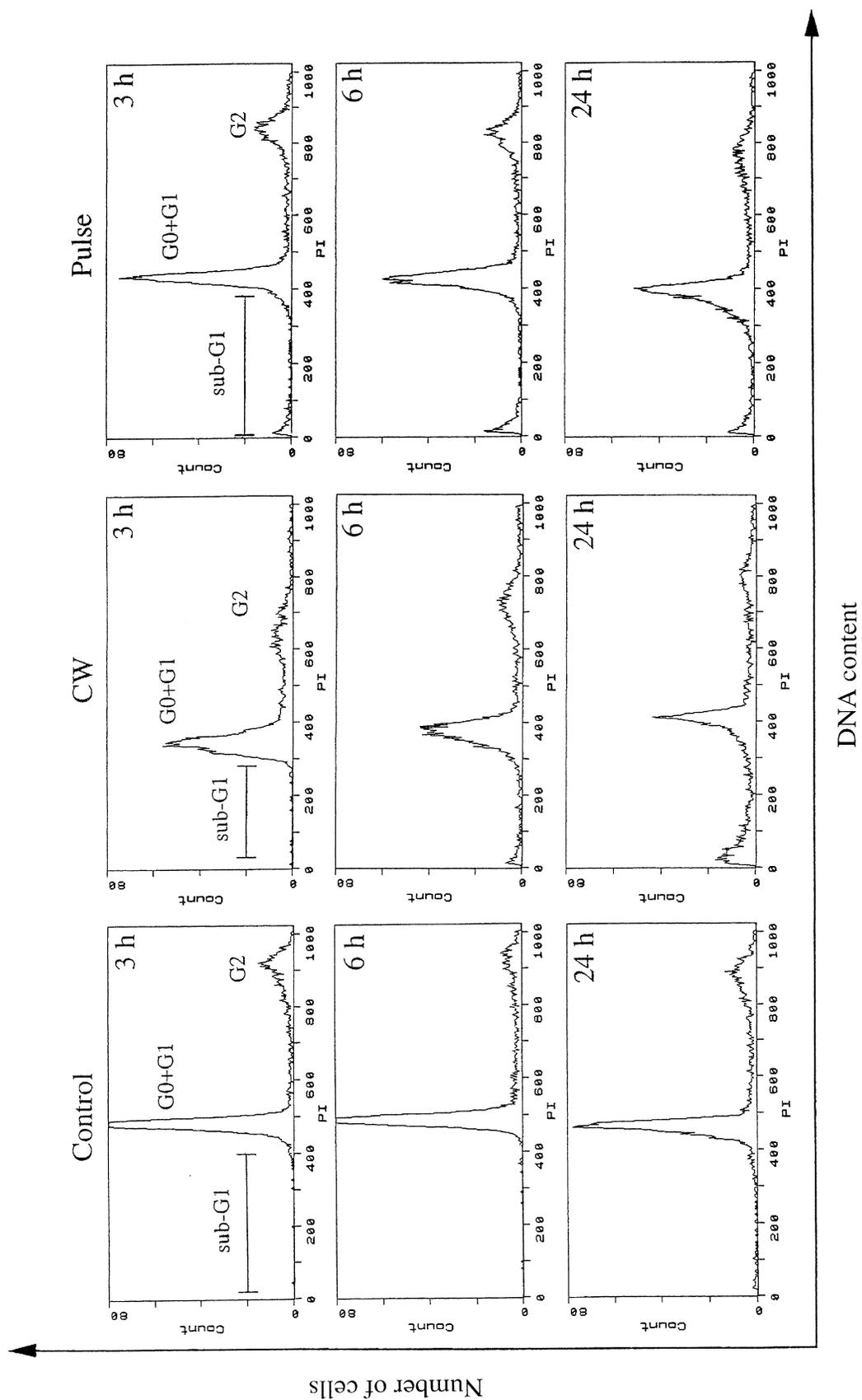
Fig. 3. DNA distribution histograms for HeLa cells following each treatment at 5 J/cm² light irradiation. The analysis was done at 3, 6 and 24 h after PDT.
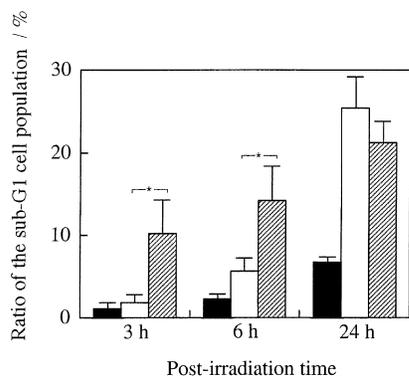
Fig. 4. Ratio of sub-G1 cell population following each treatment at 5 J/cm^2 light irradiation (control, solid bars; CW, white bars; pulse, hatched bars). The data obtained from a region of the apoptotic cells are shown in Fig. 3. Data represent the mean of at least three experiments ($\pm$SD). $^*p < 0.01$.

toxic species [14,22–24]. Dellinger investigated the relation between the intracellular localization of Photofrin® (PF) and cell death after light irradiation with a two-incubation protocol [14]. He showed that cells incubated for a long time with a low PF concentration (1 μg/ml, 24 h) resulted in apoptosis, whereas a short incubation time with a high PF concentration (10 μg/ml, 1 h) induced necrosis of the cells. These results demonstrated that the type of cell death when the cells received a high phototoxic dose was necrosis. Kessel et al. [22] also obtained the same results using two photosensitizers that differed in their intracellular localization characteristics, and concluded that the data are consistent with the hypothesis that cell-membrane lysis causes the inhibition of cellular and DNA fragmentations (arrested apoptosis). This hypothesis means that the cytotoxic level causing necrosis is higher than that inducing apoptosis. Thus, the present results showed that the phototoxic effects of the pulsed group were lower than those of the CW group because necrosis did not

occur. Indeed, the cytotoxic efficacy of the CW laser was estimated to be higher than that of the pulsed laser (Figs. 1 and 2).

In a recent study, Aveline et al. [26] demonstrated saturation of the photosensitizer triplet state using a pulsed laser with another photosensitizer under similar conditions. They reported that radical species were formed by self-quenching of the photosensitizer triplet state when a highly concentrated photosensitizer was localized at the plasma membrane. Based on these phenomena, the predominant cytotoxic species of the pulsed laser would be different from those of the CW laser in that the pulsed laser can cause saturation of the triplet state. In the case of our experimental conditions, as the HpD concentration was comparatively lower (1 μg/ml), electron transfer between the photosensitizer triplet state and the ground state to yield the radical species would be far less efficient. It is considered that the present results were clearly displayed because the phototoxicity was set at a low level. If highly phototoxic conditions were employed in this investigation, we could not identify the type of cell death because the cells receiving laser light would become necrotic independent of the irradiation mode.

Some investigators have discussed the reason for the difference in the cytotoxic effects between pulsed and CW laser light. Ben-Hur et al. [27] showed that the required fluence was about five times greater for the 10% survival level when using a pulsed laser (4.5 kHz repetition rate and 9 ns pulse width) compared with the CW laser. They pointed out that the concentration of the photosensitizer or oxygen molecules in a cell was lower in spite of the excessive input of photons by pulsed laser light. A theoretical analysis performed by Sterenborg and van Gemert [11] demonstrated that the amount of oxygen depletion per pulse became lower with a shorter pulse width. They also concluded that the effective-
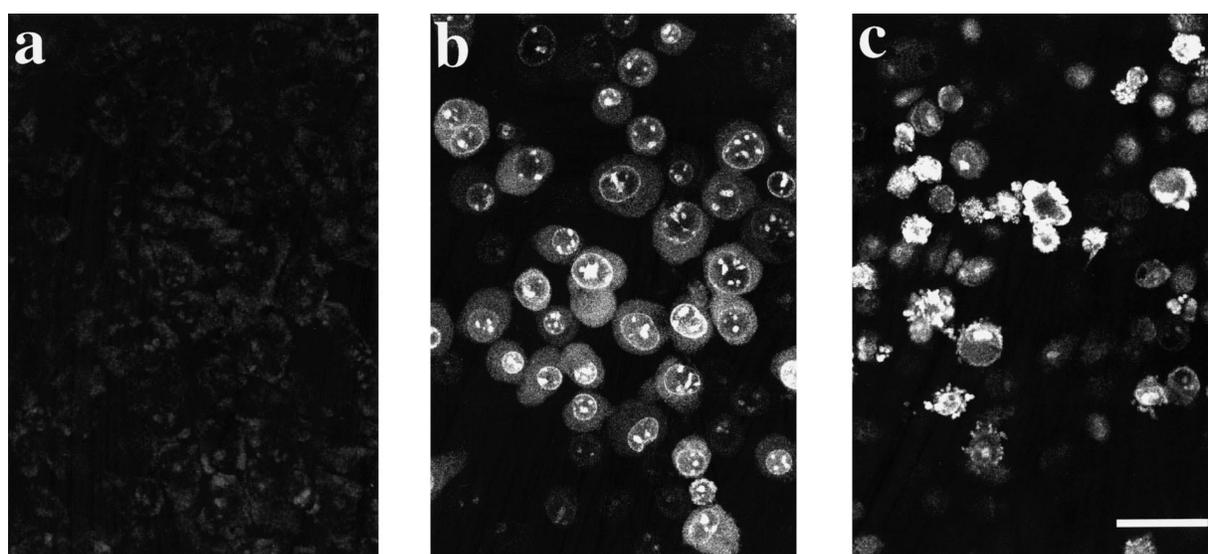

Fig. 5. Confocal fluorescence microscopy showing the nuclei of PI-labeled HeLa cells at 6 h after PDT. The cells were exposed to 5 J/cm^2 light. (a) Control cells (HpD alone, no light irradiation); (b) cells treated with CW laser light irradiation; (c) cells treated with pulsed laser light irradiation. Bar = 15 μm.

ness of pulsed excitation in PDT was identical to that of CW excitation for peak fluence rates below 4×10^8 W/m^2 and that above this threshold, the effectiveness significantly drops, originating in saturation of the triplet to singlet oxygen conversion. In the present study, the pulse width and the peak fluence of the laser were about 10 ns and 10^9 W/m^2, respectively. It seems reasonable to suppose that the photochemical cytotoxicity of the pulsed laser related to the oxygen molecules is inferior to that of the CW laser, but this is not enough to interpret the results of the present study, in particular, for the time-dependent viability studies. We consider that the gradual decrease in the pulsed group, shown in Fig. 1, could be ascribed to the apoptotic response dependent on the cell cycle. It is well known that the apoptosis cell-cycle dependence is triggered by DNA damage [28]. In our experimental model, the HpD is localized at the plasma membrane. It is unlikely that the DNA is damaged by the photochemical reaction, inducing cytotoxic species such as singlet oxygen [29]. Thus the most plausible explanation for the DNA damage is that the sensitizer entered the cell during the laser light irradiation. In the case of pulsed laser irradiation, this effect may appear stronger than for CW laser irradiation.

In conclusion, our results compared the cytotoxic effects between pulsed and CW laser light sources by monitoring cell death. The cytotoxic effect of the pulsed laser was estimated to be lower than that of the CW laser. The dead cells irradiated by the pulsed laser light were mainly induced through apoptosis, whereas CW laser light irradiation led to necrosis. These findings suggest that the type of cell death induced by PDT depends on the irradiation mode of the excitation light source and indicate the difference in the cytotoxic effects between pulsed and CW laser light irradiation.

5. Abbreviations

CW   continuous wave
FDA   fluorescein diacetate
HpD   hematoporphyrin derivative
MTT   3-(4,5-dimethyl-2-thiazolyl)-2-5-diphenyl-2H-tetrazolium bromide
PBS   phosphate-buffered saline
PDT   photodynamic therapy
PI   propidium iodide

References

[1] B.W. Henderson, T.J. Dougherty, How does photodynamic therapy work?, Photochem. Photobiol. 55 (1992) 145–147.

[2] T.J. Dougherty, Photosensitizers: therapy and detection of malignant tumors, Photochem. Photobiol. 45 (1987) 879–889.

[3] H. Yamamoto, H. Kato, T. Okunaka, M.L. Eckhauser, A. Bonaminio, C. Konaka, M. Saito, K. Furukawa, Y. Hayata, Photodynamic therapy with the excimer dye laser in the treatment of respiratory tract malignancies, Lasers Life Sci. 4 (1991) 125–133.

[4] J.S. McCaughan, R.D. Barabash, D.R. Hatch, D.C. McMahon, Gold vapor laser versus tunable argon-dye laser for endobronchial photodynamic therapy, Lasers Surg. Med. 19 (1996) 347–350.

[5] P.A. Cowled, J.R. Grace, I.J. Forbes, Comparison of the efficacy of pulsed and continuous-wave red laser light in induction of photocytotoxicity by haematoporphyrin derivative, Photochem. Photobiol. 39 (1984) 115–117.

[6] M. LaPlant, J. Parker, B. Stewart, M. Waner, R.C .Straight, Comparison of the optical transmission properties of pulsed and continuous wave light in biological tissue, Lasers Surg. Med. 7 (1987) 336–338.

[7] A. Ferrario, N. Rucker, S.W. Ryter, D.R. Doiron, C.J. Gomer, Direct comparison of in-vitro and in-vivo photofrin-II mediated photosensitization during a pulsed KTP pumped dye laser and a continuous wave argon ion pumped dye laser, Lasers Surg. Med. 11 (1991) 404–410.

[8] M. Panjehpour, B.F. Overholt, R.C. DeNovo, M.G. Petersen, R.E. Sneed, Comparative study between pulsed and continuous wave lasers for Photofrin photodynamic therapy, Lasers Surg. Med. 13 (1993) 296–304.

[9] H. Barr, P.B. Boulos, A.J. Macrobert, C.J. Tralau, D. Phillips, S.G. Bown, Comparison of lasers for photodynamic therapy with a phthalocyanine photosensitizer, Lasers Med. Sci. 4 (1989) 7–12.

[10] T. Okunaka, H. Kato, C. Konaka, H. Sakai, H. Kawabe, K. Aizawa, A comparison between argon-dye and excimer-dye laser for photodynamic effect in transplanted mouse tumor, Jpn. J. Cancer Res. 83 (1992) 226–231.

[11] M.J.C.M. Sterenborg, M.J.C. van Gemert, Photodynamic therapy with pulsed light sources: a theoretical analysis, Phys. Med. Biol. 41 (1996) 835–849.

[12] M.L. Buja, M.L. Eigenbrodt, E.H. Eigenbrodt, Apoptosis and encrosis. Basic types and mechanisms of cell death, Arch. Pathol. Lab. Med. 117 (1993) 1208–1214.

[13] D. Kessel, Sites of photosensitization by derivatives of hematoporphyrin, Photochem. Photobiol. 44 (1986) 489–493.

[14] M. Dellinger, Apoptosis or necrosis following photofrin photosensitization: influence of the incubation protocol, Photochem. Photobiol. 64 (1996) 182–187.

[15] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.

[16] J. Charmichael, W.G. DeGraff, A.F. Gazdar, J.D. Minna, J.B. Mitchell, Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing, Cancer Res. 47 (1987) 936–942.

[17] D.D. Ross, C.C. Joneckis, J.V. Ordonez, A.M. Sisk, R.K. Wu, A.W. Hamburger, R.E. Nora, Estimation of cell survival by flow cytometric quantification of fluorescein diacetate/propidium iodide viable cell number, Cancer Res. 49 (1989) 3776–3782.

[18] C. Desaintes, C. Demeret, S. Goyat, M. Yaniv, F. Thierry, Expression of the papillomavirus E2 protein in HeLa cells leads to apoptosis, EMBO J. 16 (1997) 504–514.

[19] Z. Darzynkiewicz, S. Brunko, G. DelBino, M.A. Hotz, P. Lassota, F. Traganos, Features of apoptotic cells measured by flow cytometry, Cytometry 13 (1992) 795–808.

[20] A.B. Lyons, K. Samuel, A. Sanderson, A.H. Maddy, Simultaneous analysis of immunophenotype and apoptosis of murine thymocytes by single laser flow cytometry, Cytometry 13 (1992) 809–821.

[21] H. Kikuchi, S. Ujiie, A. Wakui, A. Yokoyama, R. Kanamaru, Features of DNA oligonucleosomal fragmentation in human tumor cell lines and its detection by flow cytometry: utility and limitations, Jpn. J. Cancer Res. 88 (1997) 56–63.

[22] D. Kessel, Y. Luo, Y. Deng, C.K. Chang, The role of subcellular localization in initiation of apoptosis by photodynamic therapy, Photochem. Photobiol. 65 (1997) 422–426.

[23] Y. Luo, C.K. Chang, D. Kessel, Rapid initiation of apoptosis by photodynamic therapy, Photochem. Photobiol. 63 (1996) 528–534.

[24] D. Kessel, Y. Luo, Delayed oxidative photodamage induced by photodynamic therapy, Photochem. Photobiol. 64 (1996) 601–604.

[25] D. Kessel, Y. Luo, Mitochondrial photodamage and PDT-induced apoptosis, J. Photochem. Photobiol. B: Biol. 42 (1998) 89–95.

[26] B.M. Aveline, R.M. Sattler, R.W. Redmond, Environmental effects on cellular photosensitization: correlation of phototoxicity mechanism with transient absorption spectroscopy measurements, Photochem. Photobiol. 68 (1998) 51–62.

[27] E. Ben-Hur, H.C. Newman, S.W. Crane, I. Rosenthal, Pulsed versus continuous-wave 680 nm laser light in photosensitization by chloro-aluminum phthalocyanine tetrasulfonate, Proc. SPIE 847 (1987) 154–157.

[28] L. Huang, K.C. Clarkin, G.M. Wahl, Sensitivity and selectivity of the DNA damage sensor responsible for activating p53-dependent G1 arrest, Proc. Natl. Acad. Sci. USA 93 (1996) 4827–4832.

[29] J. Moan, On the diffusion length of singlet oxygen in cells and tissues, J .Photochem. Photobiol. B: Biol. 6 (1990) 343–344.