Cytoplasmic Molecular Delivery by Hematoporphyrin Derivative-based Photodynamic Treatment Using High-intensity Pulsed Laser Irradiation

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Photosensitized HeLa cells receiving high-intensity pulsed laser irradiation exhibited pores on the membrane surface. We attempted to deliver FITC-labeled dextran (71.6 kDa) into the cell cytoplasm. The ratio of FITC-positive cells in photodynamic-treated HeLa cells was significantly higher than that of non-treated HeLa cells, with no loss in cell viability.

Temporary permeabilization of the plasma membrane has been examined by several methods to enable the delivery of macromolecules into cytoplasm.[1–3] The final goal of these methods is to deliver genes into cells. In particular, since cytoplasmic molecular delivery using physical stresses can limit the location of gene expression sites, all kinds of stresses should be examined extensively.

Photodynamic therapy (PDT) shows potential as a mean of local and selective destruction of malignant tumors.[4] The cytotoxicity of PDT depends on the properties of the photosensitizers, fluence rate, fluence, and irradiation mode. When a high-intensity pulsed laser is used as an excitation light source for PDT, the cytotoxic effect is not necessarily as high as that obtained with the continuous wave (CW) laser.[5,6]

We previously reported that (1) the type of cell death differs between the pulsed and CW forms of laser irradiation; (2) the predominant type of cell death accompanying pulsed laser excitation PDT was cell-cycle-dependent apoptosis; (3) the DNA damage that induces apoptosis was attributable to the photosensitizer entering the cell during pulsed laser irradiation.[7] It follows from these findings that pulsed laser excitation photodynamic therapy could be used for the intracellular delivery of molecules that genes contain. In the present study, we examined whether or not PDT using a high-intensity pulsed laser can temporarily damage the membrane and deliver macromolecules into cytoplasm.

HeLa cells were seeded into a 24-well flat-bottom culture plate at a cell density of 2×10^5 cells/well and incubated at 37 °C overnight. The medium in each well was replaced with 10 μg/mL hematoporphyrin derivative (HpD)-containing medium, and then the cells were subjected to Q-switched Nd:YAG laser (GCR-150, Spectra Physics, USA) pulses. The wavelength and the pulse width of the laser were 532 nm and 10 ns, respectively. PDT was carried out with an average fluence rate of 30 mW/cm^2 (i.e. ≈3 mJ/cm^2 pulse) at light dosages, ranging from 0.5 to 2 J/cm^2. Cells treated with HpD alone and not irradiated were used as the control. Immediately after the irradiation, the HpD-containing medium was changed and the cells were kept in medium including FITC-dextran (FITC-D) (Molecular Probes, USA) at room temperature. The concentration of the FITC-D was 10 μM (MW = 71.6 kDa). After 5 min, the cells were washed twice with the culture medium and then incubated at 37 °C before each assessment. Scanning electron microscopy was performed as follows. The cells were fixed immediately after PDT, then dehydrated and coated with gold. Samples were observed under a scanning electron microscope. The viability of the HeLa cells was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay at 24 h after the pulsed laser irradiation.[8] The cellular uptake of FITC-D was measured by flow cytometry. A total of 10000 events per sample was collected in list mode, and data were analyzed with Multigraph software. Fluorescence data were collected by using 488 nm excitation from an argon-ion laser. The emission was collected through a 530 ± 30 nm band-pass filter.

Figure 1 shows the morphology of a HeLa cell observed by a scanning electron microscope. The membrane surface of the untreated cell was smooth and partly covered with microvilli. In contrast, although the PDT-treated cell remained almost in its original shape, pores ranging from 200 to 1000 nm in diameter were observed on the membrane surface. In the case of laser irradiation alone (absence of HpD), we could not find the pores on the cell membrane. Thus, it would be required for both photosensitizer (HpD) and laser irradiation to form the pores on the cell membrane.

Table 1 shows the effect of the pulsed laser excitation PDT on cell viability as measured by the MTT assay. The cells were exposed to a 30 mW/cm^2 fluence rate and 0.5–2.0 J/cm^2 light. The activities were calibrated with controls containing HpD

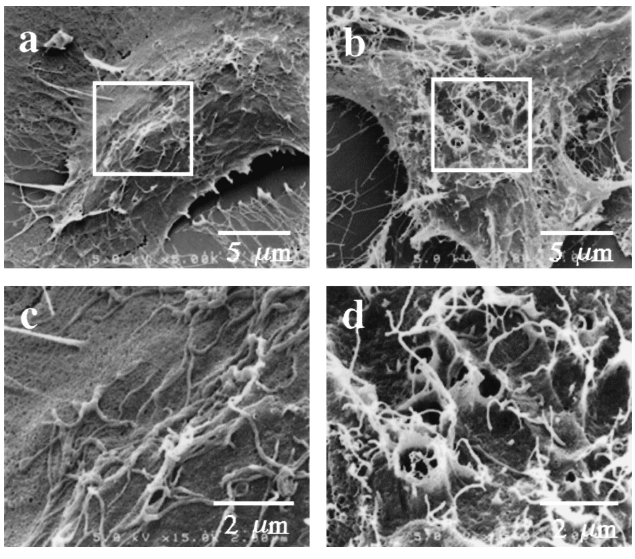

Figure 1. Morphological changes in HeLa cell after the pulsed laser excitation PDT. (a): Normal HeLa cell, (b): PDT-treated HeLa cell. (c) and (d) are high magnification images of (a) and (b), respectively.

Table 1. Cells viability

Light dose (J/cm^2)	Mean normalaized non-treated HeLa cells
Absence of HpD	
0.5	0.99 ± 0.01
1.0	0.99 ± 0.01
1.5	0.98 ± 0.01
2.0	0.98 ± 0.01
Presence of HpD	
0.0	0.99 ± 0.01
0.5	0.97 ± 0.01
1.0	0.96 ± 0.02
1.5	0.95 ± 0.03
2.0	0.78 ± 0.04

Cell viability was assessed by MTT assay. Data represent the mean of at least four experiments (±SD)

and not irradiated. The viability values of the PDT-treated cells were maintained at more than 95% of control viability until the $1.5\,J/cm^2$ light dose was administered. In contrast, the viability of cells that received $2.0\,J/cm^2$ light was less than 80% of the control.

Figure 2 shows the light-dose-dependence of FITC-D uptake. The ratio of viable HeLa cells indicating FITC-D uptake increased with the light dose. Although these values ranged from

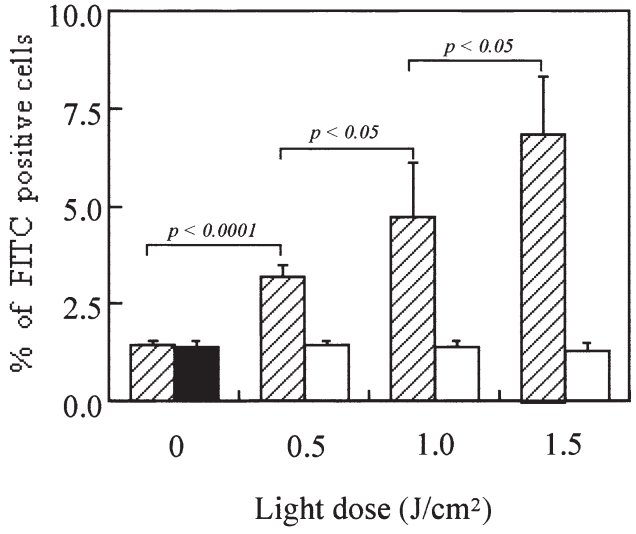

Figure 2. The ratio of FITC-positive cells in viable HeLa cells (Laser light alone, white bars; HpD treated group, hatched bars). The measurement of FITC-positive cells by flow cytometry was assessed at 24 h after PDT. Data represent the mean of at least four experiments (±SD).

3.2 to 7.0% of the total viable cells, every PDT-treated group was significantly higher than that of the control.

The mechanism of reversible membrane injury observed in this treatment applies to the existing PDT. In the case of high-intensity pulsed excitation PDT, the photosensitizer, which localizes at the plasma membrane, tends to produce photobleaching. Thus, the high-toxicity effect is not necessarily acquired. These mild oxidative stresses, however, may have produced the membrane conditions suitable for reversing the membrane injury.[9]

Another point to be discussed is the generation of mechanical stresses. Since PDT makes use of a photosensitizer that absorbs excitation laser light effectively, the mechanical stresses generated by transient heat expansion would not be negligible when a high-intensity pulsed laser is used.[10] It would be considered that the mechanical stresses lower the plasma membrane's fluidity by mild oxidation, resulting in damage to the membrane. At the same time, mechanical factors such as cavitation bubbles induced by the mechanical stresses would damage the membrane if the intensity and frequency of these stresses were high enough.[11] In our speculations, these cavitation bubbles on the cell surface would be formed by the mechanical stresses which are derived from the cell membrane and adjacent cell. Collapse of the cavitation bubbles would generate negative pressure on the cell, which damages the cell membrane.

It will be necessary to define the PDT parameters required for a more effective temporary permeabilization without a loss of cell viability.

References

1 D. J. McAuliffe, S. Lee, T. J. Flotte, and A. G. Doukas, *Lasers Surg. Med.*, **20**, 216 (1997).
2 T. Kodama, M. R. Hamblin, and A. G. Doukas, *Biophys. J.*, **79**, 1821 (2000).
3 Y. Miyamoto, Y. Umebayashi, M. Koyano, M. Wakita, and T. Nishisaka, *Cancer Lett.*, **199**, 45 (2003).
4 T. J. Dougherty, *Photochem. Photobiol.*, **45**, 879 (1987).
5 D. A. Bellunier, C. W. Lin, J. A. Parrish, and P. C. Mock, *Prog. Clin. Biol. Res.*, **170**, 533 (1984).
6 E. Ben-Hur, H. C. Newman, S. W. Crane, and I. Rosenthal, *Proc. SPIE*, **847**, 154 (1987).
7 Y. Miyamoto, Y. Umebayashi, and T. Nishisaka, *J. Photochem. Photobiol., B*, **53**, 53 (1999).
8 T. Mosmann, *J. Immunol. Methods*, **65**, 55 (1983).
9 T. K. Saito, H. Muguruma, and K. Mabuchi, *Biotechnol. Lett.*, **24**, 309 (2002).
10 T. Ninomiya, Y. Miyamoto, T. Ito, A. Yamashita, M. Wakita, and T. Nishisaka, *J. Bone Miner. Metab.*, **21**, 67 (2003).
11 D. Kessel, R. Jeffers, J. B. Fowlkes, and C. Cain, *Int. J. Radiat. Biol.*, **66**, 221 (1994).

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