Cell Membrane Perforation with Photosensitizer and a Brush-shaped Soft-polymer Sheet Using a Malignant Glioma Cell Line

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Abstract. *Background: Transduction of foreign molecules into cells is an important technique to investigate the functions of corresponding molecules and/or targets. Recently, a mass-producible nanoprinting perforator was devised enabling for large-scale, high-performance drug or nucleic-acid transfer into cells without cell damage. Since little is known on the performance of the system, we investigated its effects on a malignant glioma cell line. Materials and Methods: Photosensitization was performed by the Cell Stamper CP-01. The malignant U373MG glioma cell line was used for transduction. Results: Photosensitization transduced FITC-conjugated albumin into cells. Trypan blue inclusion test demonstrated membrane disintegration by the procedure and scanning electron microscopy disclosed perforation of the cell membrane. Conclusion: Local oxidation reaction during the nanoprinting caused reversible membrane perforation. Morphological findings from the current study support the above mechanism, therefore the specific printing system might be convenient for transduction of foreign molecules into malignant glioma cells.*

One method currently used to characterize the behavior of a malignant tumor in the living body is observation of activities of the constitutive cells. With advances in molecular cell biology, several technologies to investigate cell activities have been developed. Among these technologies, transduction or transfer of chemicals, nucleic acids, proteins, or other bioactive molecules into the cell body is an especially attractive method, since such an approach can directly modify cellular functions and activities and lead to elucidation of the roles of corresponding molecules. We transduced the cDNA of functional genes and ribozymes into malignant glioma cells and tried to determine their function and develop a therapeutic strategy through the use of viral vectors (5, 9-12, 18). In the process, we have also studied non-viral methods, including acoustic transduction, in order to avoid immunological or biological responses, and these techniques demonstrated efficacy (7, 8). However, two issues need to be addressed: efficiency and toxicity. Both issues have been proven a bottleneck in the development of such technology, while many researchers have attempted to solve these problems. Recently, a mass-producible nanoprinting perforator was devised, enabling for large-scale, high-performance drug or nucleic-acid transfer into cells without cell damage (16). The mechanism is postulated to be transient perforation of the cell membrane by photo-oxidation. However little is known on its performance. We conducted a morphological survey using a malignant glioma cell line.

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Key Words: Cell stamper, nanoprinting perforator, malignant glioma.

Materials and Methods

The human malignant glioma U373MG cell line (American Type Culture Collection, Rockville, MD, USA) was cultivated in Dulbecco's modified Eagle's medium with 4.5 g/l glucose supplemented with 10% fetal bovine serum. Cells were dispersed by trypsin and cultured on a 6-well culture plate (Multi-well 6-well, Becton Dickinson, Franklin Lakes, NJ, USA.) for 12 h before use. Fine morphology and three-dimensional behavior of the cell line previously were reported (6).

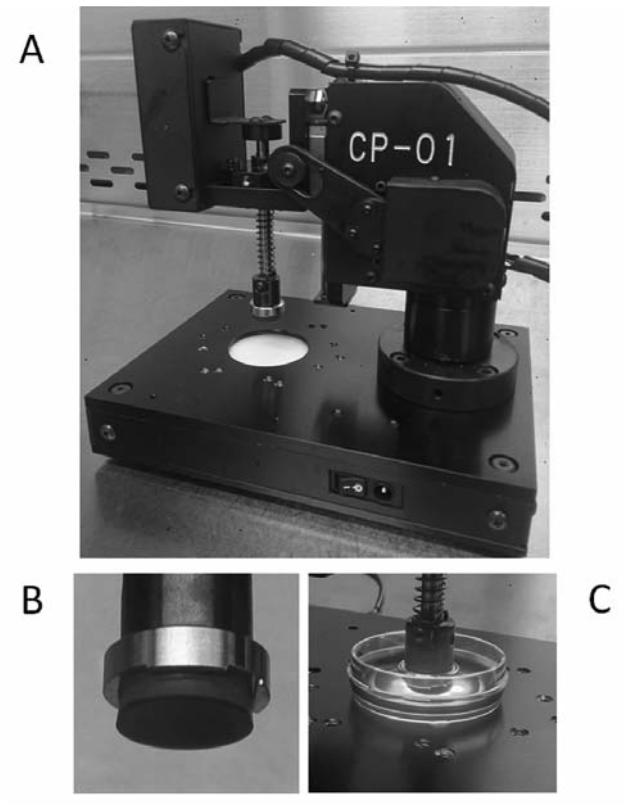

Figure 1. *Printing system with a photosensitizer and brush-shaped soft-polymer sheet. The whole system (A) and soft-polymer sheet (B) are shown. A sterilized sheet was applied directly on top of the culture dish by the cylinder and activated by illumination of a light-emitting diode (C).*

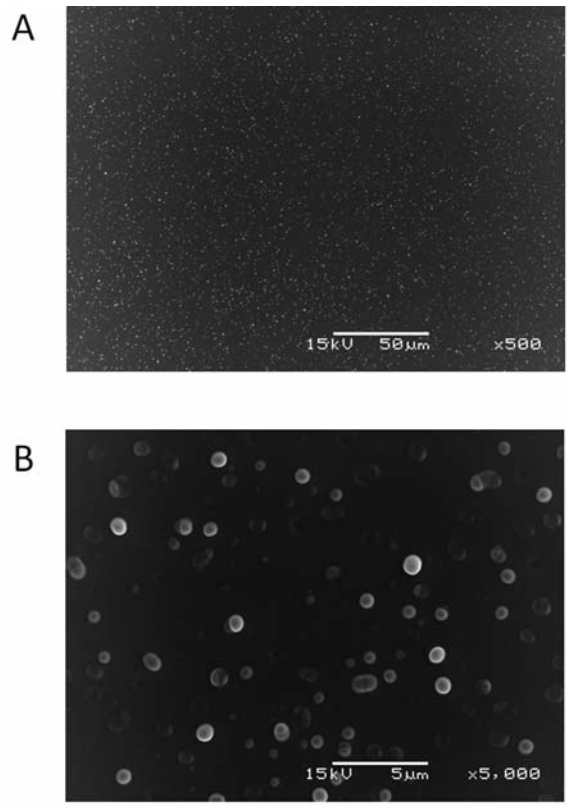

Figure 2. *Surface of soft-polymer sheet for photosensitization. Close-up of the surface of the polymer (A) and higher magnification (B). The polymer sheet contained photosensitizer and the protruding brushes.*

Fluorescein isothiocyanate (FITC) conjugated bovine albumin and trypan blue solution were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Photosensitization with a brush-shaped soft-polymer sheet was performed by the Cell Stamper (CP-01, Marudaikiko Co., Nikaho, Akita, Japan). In the system, a poly-dimethylpolysiloxane sheet was used as brush for perforation of the cell membrane. The sheet contained photosensitizer Hematoporphyrin Dihydrochloride and brush with a 1-μm diameter, 2-μm height and 3-μm pitch, was used in the study. The cells were washed with phosphate-buffered saline (PBS), and then photosensitized for 30 sec under a pressure of 200 hPa with 2 ml of 0.05% trypan blue or 2.5 μM of FITC-conjugated albumin, and subjected to microscopic observation. All experiments were performed under a controlled room temperature (26˚C). Light and fluorescent microscopic images of living cells were obtained by the Olympus IX71 (Olympus, Tokyo, Japan) with cooled CCD (Keyence VB-7010, Osaka, Japan). For scanning electron microscopy (SEM), cells were fixed at a final concentration of 1.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3, 400 mOsm) during photosensitization and further used as specimens. These samples were further treated with serial dilutions of ethanol, dehydrated by a critical point dryer (Hitachi-Hightec, Tokyo, Japan), coated by Au-Pd magnetron sputter (MSP-10, Vacuum Device Co., Ibaragi, Japan), and examined at 15 kV under a JSM-5800LV scanning electron microscope (JEOL Ltd., Tokyo, Japan).

Results

An outline of the cell stamper system used in the study is shown in Figure 1. An autoclaved at 120.85˚C for 20 min, round polymer sheet with a 12-mm diameter and 3-mm thickness, was attached to the bottom of the cylinder of the device by a magnet. After preparation of cells on wells of a 6-multiwell plate, the culture plate was set on the stage of the stamper. The brush-shaped polymer sheet was then pressed on the cell surface by pushing down the cylinder. Duration of the treatment and pressure were adjustable, but in this experiment, treatment with 30 sec of illumination was suitable for the malignant glioma cell line. A gross and fine surface of the polymer sheet are demonstrated in Figure 2.

Photosensitization by the system temporally perforates the membrane of living cells and transduces the extracellular agent. To confirm the effect, FITC-conjugated albumin was applied to the system and localization observed. After the cells were photosensitized for 30 sec and washed twice with PBS, fluorescent microscopy revealed re-distribution of FITC into the cells (Figure 3). Phase-contrast imaging

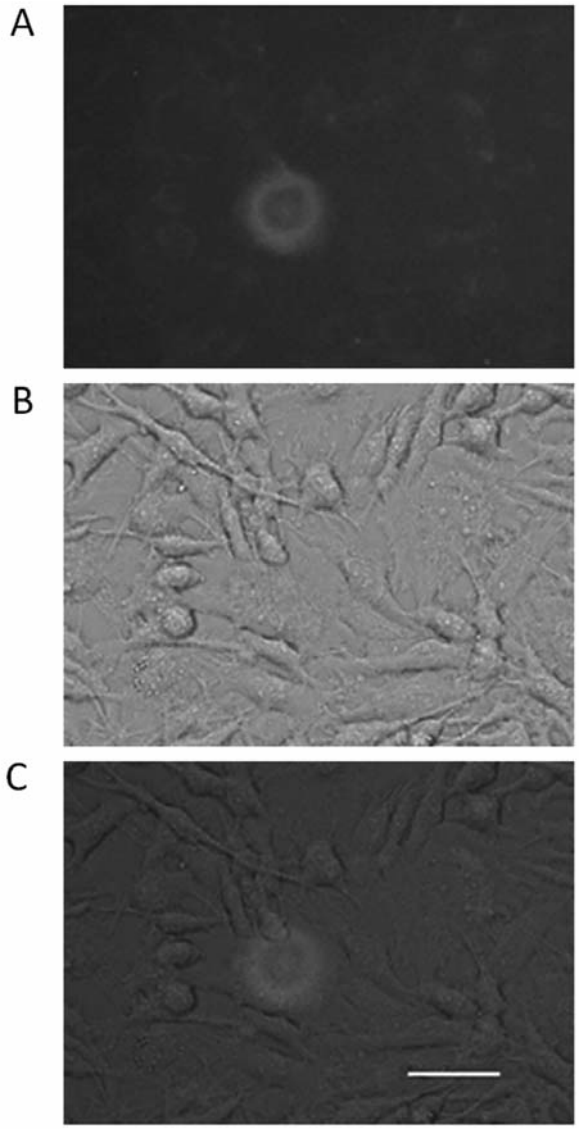

Figure 3. *Transduction of FITC-conjugated albumin to the cells. Intake of albumin (A) and differential interference contrast (DIC) image (B). After treatment, FITC-conjugated albumin was introduced into the cell. Surface of the cell was restored after the stimulation (C: merged image). Note the distribution of FITC in the cell. Bar: 50 μm.*

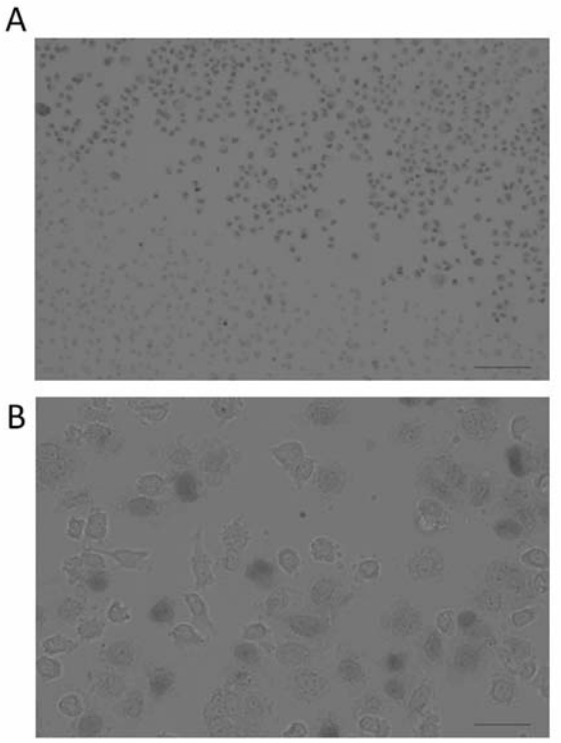

Figure 4. *Trypan blue inclusion after photosensitization. Low- (A) and high- (B) power fields. A: The boundary of the fog is demonstrated. In the photosensitized region (upper half area), more than 90% of cells contained trypan blue. These cells were viable after treatment. Bar: 500 μm. B: Intensity of trypan blue varied in each cell. Some cells were strongly strained, while others less stained. Bar: 50 μm.*

demonstrated that the cell surface had recovered from stimulation at the time of observation. This finding was consistent with a previous report that the perforation was transient and almost all cells were viable after photosensitization (16).

While FITC-conjugated albumin was transduced into the cells, the mechanism of albumin transmigration remained unclear. To understand the route of redistribution by the procedure, the trypan blue inclusion test was performed. Trypan blue is widely used for the identification of dead cells or those with a disintegrated membrane. Living cells with an intact cell membrane effectively exclude the dye and cells take-up trypan blue only when their membrane is impaired. Cells were incubated with trypan blue at a final concentration of 0.05% in PBS and were photosensitized for 30 sec. More than 90% of the cells demonstrated uptake of the dye and were stained (Figure 4). These results contrasted to cells without photosensitization. Less than 1% of cells contained trypan blue when not stimulated by illumination (Figure 4A). The difference suggested disintegration of the cell membrane by photosensitization. The degree and density of staining was different in each cell (Figure 4B) and this difference might have arisen from a variation in the amount of included dye.

As shown above, photosensitization by the system implied transient membrane disruption of cells. For confirmation, morphology of cells during photosensitization was investigated by SEM. Cells were exposed to light illumination under pressure of 200 hPa and fixed during the exposure. The treated cells revealed perforations in the cell membrane (Figure 5). The size varied according to the

localizations of the holes, contact with the photosensitizer, and probably also the duration, pressure, and shape or size of the brush.

Discussion

A photosensitization system with a brush-shaped soft-polymer sheet, or Cell Stamper, was developed as a drug delivery tool to transduce chemical agents or nucleic acid into the cytoplasm from outside the cell. The specific device is commercially available. The system exerts pressure from above the cells, and the photosensitizer in the brush-shaped soft-polymer sheet is activated by a light-emitting diode illuminating from the bottom of the plate. The mechanism is considered to be the generation of reactive oxygen species, which then oxygenates the membrane causing transient perforation of the cell membrane. One advantage of the device is utilization of a polymer-sheet with appropriate intervals of brush surface containing photosensitizer. This enables for simultaneous treatment of multiple cells. According to manufacturer's brochure, the device can perforate 2.5×10^6 cells at the same time. Another advantage is the size of the perforation. The size of the hole produced by the method is 500-1,000 nm and is larger than that achieved by other methods, hence various types of materials can be transduced into cells (16). Perforation was self-restored within several tens of seconds or minutes, and the cells survived after the procedure. In the study, cells that contained FITC-labeled albumin in cytoplasm were confirmed. In these cells, the cell membrane was restored and damage was not observed superficially. However, in the trypan blue experiment, the dye was observed in the cells suggesting transient disintegration of the cell membrane. In fact, cracks and holes on the cell surface were observed by SEM when cells were fixed during photosensitization. These images were not observed in non-photosensitized cells. The size of perforation was larger than reported and the procedure also damaged the cell body and processes of some cells. This finding was attributable to the fixation process of cells. Since the cells were fixed in the middle of photosensitization, most were unable to recover from the impairment.

As for cell manipulation, it is important to have reliable technologies to introduce external foreign materials into cells. For this purpose, various methods to transduce drugs, proteins, nucleic acids or other molecules through the cell membrane have been devised. Protein and nucleic acids are anionically-charged macromolecules. For that reason, some sort of physical or mechanical force is required to pass the molecules through the membrane since the surface of the cell membrane is also negatively-charged and the repellent force must be overcome. Electroporation, sonoporation, magnetofection, or gene guns are examples of such techniques. Gene guns have been used to deliver genes into local tissue using a DNA-loaded micro-particle discharge gun. The effects depend on the type of gun, the type of driving gas, pressure as well as particle size, velocity and density. One of the problems is that a gene gun requires a high functional pressure, which leads to direct or indirect tissue damage (1). Electroporation also became popular when non-thermal low voltage electroporation was proposed (2). A higher voltage of electroporation irreversibly destroyed the target cell membrane and caused unrepairable damage and death due to a loss of homeostasis. A recent system utilized small electrodes with a diameter of about 1 mm. These electrodes were placed either inside or surrounding the target tissue to apply short, repetitive bursts of electricity at a pre-determined voltage and frequency; the bursts of electricity increase the resting transmembrane potential so that nanopores form in the plasma membrane (20). Magnetofection (3) and sonoporation are relatively milder

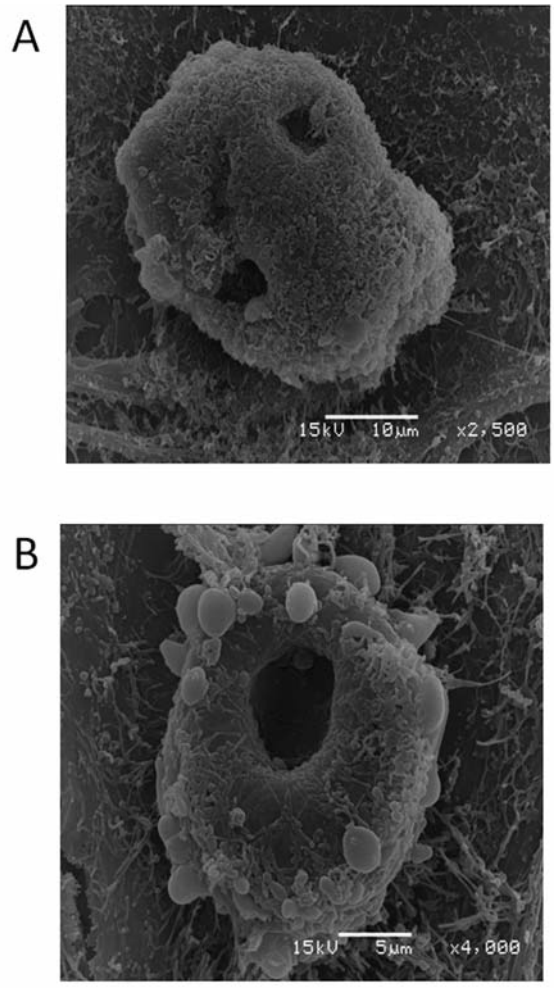

Figure 5. *SEM pictures of the cell surface. Cells were perforated by the procedure. The location and size of the holes varied in each cell (A and B).*

methods to transduce molecules into cells. Like other methods, insonation also perforates the cell membrane; most cells tolerate this stimulation and can survive. This has been demonstrated especially by protein-coding gene transfer, because protein is produced from transduced DNA and this can only occur in living cells. In sonoporation, survival rates have been unclear, but process of the phenomenon was observed with electron microscopy (8, 17, 19).

As for light energy, a laser was developed for perforation of the cell membrane (4). Utilization of light energy has a merit in terms of reduced cytotoxicity. Saito *et al*. examined the light dose and time dependency of a photodynamic cell membrane (13). The laser was used for rapid light activation (<1 s) and the subsequent membrane damage was monitored in the presence of the photosensitizer of 5',5''-bis (aminomethyl)-2,2':5',2''-terthiophene dihydrochloride (BAT). At 49 μM BAT, light levels less than 0.94 J/cm2 led to a reversible membrane de-polarization (20 mV) within 3 sec of illumination while higher intensities of illumination (1.57 J/cm^2) caused a complete and irreversible loss of membrane potential and cell membrane resistance within 8 sec of illumination (13) and that less than 9% of treated cells were detached even after perforation (14). Furthermore, a local oxidation reaction caused by zinc oxide on the cell membrane that could produce submicron-sized reversible membrane perforations in cells, was also found (15). The conventional photochemical cell membrane perforation was achieved under the pressure of 68 hPa and an illumination intensity of 0.82 W/cm^2 for 30 sec (16). These findings were the foundation for the current system and conditions.

In the current system, local oxidation reaction in the cell membrane could produce reversible cell membrane perforation, and our results supported such reversibility of the membrane. While sub-micron sizes of reversible membrane perforations had been previously specified, we found holes of larger sizes in the cells. Regardless of differences in the size of the perforations, the process of transduction was clarified morphologically, indicating that the printing system might be convenient for transduction of foreign agents into malignant glioma cells.

Acknowledgements

The Authors acknowledge Mayumi Nomura and Hideki Saito for their expert assistance with the experiments.

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Received July 24, 2015
Revised September 1, 2015
Accepted September 3, 2015