The Necessity of Radicals for Gene Transfection by Discharge Plasma Irradiation

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The authors parametrically investigated gene transfection process with a micro capillary discharge. Several factors and the time scale at which they became effective were studied. The conclusion is that half of transfections occur during plasma irradiation and the other half occur after plasma irradiation is stopped. As the electric field and current become zero after plasma irradiation is terminated, we conclude that during that period radical species are necessary for the transfection.

Keywords: gene transfection, atmospheric plasma, micro plasma, radical, plasma gene transfection

1. Introduction

Gene transfection is a technique of deliberately introducing nucleic acids into cells in order to give them specific characteristics. In practice this can be achieved in three different ways: chemical method, physical method and the virus vector method. The chemical method for gene transfection was developed first in 1973 by Graham using calcium phosphate [1]. In 1987 Felgner developed the Lipofection method [2]. As a physical method in 1982, Neumann developed electroporation. With electroporation small holes are opened on the cell membrane by applying an electric pulse to the cell suspension and then the gene passes through those holes [3]. Although these two methods are available only for *in vitro* environments, the virus vector method is available for both *in vivo* and *in vitro* environments, so it is expected to be a practical method for gene therapy.

The electroporation method is the most common technique in molecular biological and medical experiments in laboratories. This method achieves a high transfection rate, but it causes a lot of damage to the cells resulting in more than half of them dying by necrosis or apoptosis. The virus

vector method provides both a high transfection rate and a high cell survival rate. However, the viral method has risks of pathogenicity expression or neoplastic transformation.

Therefore, the development of a new safe and damage-free technique is in demand. Such a technique would be expected to play an important role in advanced medical technologies like gene therapy and regenerative medicine through establishment of iPS (induced pluripotent stem) cells. A technique that uses discharge plasma irradiation was invented by Satoh, who is one of the authors, and his group in 2002 [4] and was reported by Ogawa in 2005 [5]. Since this technique is free from adverse effect associated with viruses, there are no risks as the others mentioned above. The plasma irradiation on genes and cells induces the transfection process in which the genes and cells are exposed to discharge current, that is, ions and electrons fluxes, electric field, chemically active species such as radicals, etc.

We have two main problems with the plasma irradiation method proposed by Ogawa. One is the trade-off relationship between transfection rate and

cell survivability and the other is the issue of

(a) Schematic diagram of the plasma generation and its treatment.

(b) Plasma treatment device.

Fig.1. Plasma treatment device and 96-wells plate.

reproducibility, in other words, the instability of the results. Even if the setting parameters of plasma generation such as applied voltage, waveform, frequency, etc. are kept constant, the transfection rate varies shot by shot.

Since the size and the spatial position of the plasma flare changes randomly in time, in some trials the genes and the cells are heavily damaged and reach necrosis or apoptosis by being exposed to the high density part of plasma, while in some other trials plasma flare does not reach the cells and transfection does not happen. The authors assumed that these problems were due to the instability of the plasma. In order to solve this instability, the authors have been trying various types of plasma such as Atmospheric Plasma Jet (APJ) [6-8] and Dielectric Barrier Discharge (DBD). Finally the authors employed micro capillary discharges [9, 10]. In this method, a small capillary copper tube with outer diameter of 70 μm is used as a HV (high voltage) electrode and a gas nozzle. This structure spatially and temporarily stabilizes a plasma which is generated on a tip of the capillary electrode, and limits plasma size. Consequently high transfection rate and high cell

survivability are achieved simultaneously for plasmid. Since the effects of the

(a) Total period (3 groups of pulses)

(b) One group pulse

Fig.2. Current and voltage waveforms.

electronic structure of the device were discussed in the previous works [11,12], in this paper, the authors selectively investigate the effect of radicals as surface interactions among radicals, plasmid and cell membrane on transfection rate by changing the protocol.

2. Experimental

2.1 Setup

As the target COS7 cells, which were taken from an African green monkey, were used. The COS7 cells were cultured in each well. Then 0.8 μg of pCX-EGFP plasmid diluted with Tris/EDTA (TE) buffer (0.4 $\mu\text{g}/\mu\text{l}$) is used.

The plasma irradiation device is shown in Fig.1. The device consists of a copper electrode which was a capillary with 70 μm outer diameter and 20 μm inner diameter, an XYZ-stage with copper plate electrode on which a 96-wells plate was set with cells and plasmid. The distance from liquid surface of the well to the tip of the capillary electrode was 1 mm.

Fig.3 Sample treatment protocols. A: Standard, B: without After Effect, C: After Effect Only, D: Control

Fig.4. Time chart for each factors in each protocol (factors (a)~(i) are as given in Fig. 3).

A: Standard, B: without After Effect, C: After Effect Only, D: Control

A custom made HV power supply (Pearl Kogyo) was used for discharge initiation. The output voltage had a 20 kHz sinusoidal waveform which was pulse modulated at 25 Hz with a duty ratio of 1%. The voltage and current waveforms are shown in Fig.2.

As shown in this figure, a set of discharge

consisted of 3 groups of pulse, and the length of each group of pulse was 0.4 ms. The applied voltage between electrodes was 14 kV.

2.2 Treatment

In order to investigate the timing of transfection and effective factors (a)~(i), the samples were treated in four different protocols as shown in Fig.3.

Fig.5. Fluorescent microscope image of the GFP plasmid transfected COS7 cells in one of wells in 96wells plate treated by the Protocol A.

Fig.6. Bright field and fluorescent microscope images of the plasma treated COS7 cells with GFP plasmid.

The time chart on which the timing of each process for the factors is active/exists for each Protocol is shown in Fig.4.

The Protocol A is the standard treatment. The Protocol B is the treatment in which there is no effective factor after plasma treatment: “without

Fig.7. Transfection Rate Rt2 for each Protocol. A:Standard, B:without After Effect, C:After Effect Only, D: Control.

After Effect”. The Protocol C is the treatment in which the plasmid does not exist during plasma irradiation and only after the irradiation transfection could occur: “After Effect Only”. The Protocol D is just a “Control”, that is, without plasma irradiation.

The COS7 cells were incubated at 37°C under 5% carbon dioxide in D-MEM medium. Before plasma treatment the medium was removed: Process (a). Then washed by PBS (phosphate buffered saline, 0.1 M, pH 7.4): Process (b).

After the Process (b), in the Protocols A, B and D, GFP plasmid diluted with TE buffer (0.4μg/μl) is poured into each well: Process (c). Then in the Protocols A, C, each well is treated by the plasma irradiation: Process (e). After the Process (e), in the Protocols A the medium is poured into each well: Process (i). In the Protocol B, after the Process (c), GFP plasmid and TE buffer are removed from each well: Process (g), and each well is washed by PBS and PBS is removed: Process (h), finally the medium is poured into each well: Process (i). After the Process (b) in the Protocol C, 4 μl of TE buffer without plasmid is poured into each well: Process (d), and plasma treatment is done: Process (e). Following the plasma treatment in the Protocol C, the medium is poured with the plasmid into each well: Process (f). In the Protocol D, after the Process (c), the medium is poured into each well: Process (i). In each Protocol, after the Process (f) or (i), the samples are incubated 24 hours, then they are observed by fluorescence microscope.

3. Results

The fluorescent image of a whole region of a well

after 24 hours incubation from plasma treatment by the **Protocol A** is shown as an example in Fig.5. As shown in this figure, plasmid is transfected not only in the center area but also in the wide area of the well with high transfection rate.

The bright field and fluorescent microscope images of the plasma treated COS7 cells with GFP plasmid for each protocol are shown in Fig.6. The fluorescent images show that **Protocol A** (Standard) achieves the highest number of transfections and **Protocol B** (w/o After Effect) is the second. The **Protocol C** (only After Effect) and **Protocol D** (Control) show almost no transfections. The bright field images in all the protocols show almost no damages on cells.

In order to estimate quantitatively the differences between the protocols in the transfection, transfection rates were calculated from the fluorescent and bright field images. The number of living cells (n_l) and the number of cells emitting fluorescence (n_f) were counted. A control sample without plasma irradiation was also prepared. After 24 hours incubation, the number of cells of control sample was counted (n_0). Then the transfection rate was calculated as n_f/n_0 .

The average transfection rate was obtained from 15 wells of the results for each protocol. The average transfection rate is 20.1% for **Protocol A** (Standard), 10.3% for **Protocol B** (w/o After Effect), 0.3% for **Protocol C** (only After Effect) and 0.2% for **Protocol D** (Control). In Fig.7 all the transfection rates with the respective average values are shown.

4. Discussion

The transfection rate of **Protocol B** becomes nearly a half of that of **Protocol A**. As shown in Fig.4. the difference between **Protocols A** and **B** is whether the cells, plasmid and radicals exist together after plasma irradiation or not, that is, existence of “After Effect”. This means transfection happens not only during the plasma irradiation time, but also during the time after the plasma irradiation stops.

In the **Protocol A** plasmid is irradiated by plasma. On the other hand in the **Protocol C** plasmid is not irradiated, but the plasmid was poured with medium into the well after the plasma irradiation is stopped. According to this difference the transfection rate of the **Protocol C** becomes almost zero in contrast to the difference between the **Protocols A** and **B**. This means that the plasmid must be with radicals before they are diluted by the

medium in order to make the transfection happen after plasma irradiation stops as “After Effect”.

If the transfection rate of the **Protocol B** is similar to that of the **Protocol A**, it could prove that all the transfections happen during plasma irradiation and the transfection mechanism is the direct action of plasma to cells, plasmid and TE buffer. However, as described above, the transfection rate of the **Protocol B** is only a half of that of the **Protocol A**. This means there are effects which can last or start even after plasma irradiation is stopped. Since the current and the electric field extinguish when the plasma is turned off, the “After Effect” is attributed to the interactions of radicals with cells and plasmid. Consequently, radicals play significant roles in plasma gene transfection.

4. Conclusion

The authors investigated the factors for plasma gene transfection by changing protocols and looked at the time periods the factors become effective. The conclusion is that half of transfections occur during plasma irradiation and that the last half of transfections occur after plasma irradiation is stopped. Since in the period after plasma irradiation, the electric field and current become zero, radicals must be necessary for this second stage of transfection. The plasmid needs to be exposed to plasma and radicals during plasma irradiation.

As a next step, the authors will investigate which radicals are important for plasma gene transfection.

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