Novel Method for Substance Injection into the Cell by Laser Beam

—A Study of the Injection Volume—

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ABSTRACT. A DNA transfection method by laser microbeam pricking has been recently reported (Kurata, S. *et al.* Exp. Cell Res. **162**, 372 (1986)). The volume of external fluid transferred into the cell by the method was determined through the injection of diphtheria toxin fragment A (Yamaizumi, M. *et al.* Cell **15**, 245 (1978)). Using these results and the results on laser DNA transfection efficiency (Kurata, S. *et al.* Exp. Cell Res. **162**, 372 (1986)), the approximate number of DNA molecules necessary to transform the recipient cell was estimated.

Diphtheria toxin is separated into two fragments (fragment A: M.W. 21,500, fragment B: 40,000) by trypsin treatment. Fragment A kills cells by preventing protein synthesis (1). On the other hand, fragment B has the conjugating ability to attach to the diphtheria toxin receptors on the surface of the cell (2, 3). Therefore, even if fragment A is dissolved into culture medium it is not toxic, because fragment A can not penetrate the cell without fragment B. If fragment A is introduced into the cell, however the cell is destroyed. Previously, Yamaizumi *et al.* proved that a cell can be killed by one molecule of fragment A (4). After pricking cells with a laser beam in various concentrations of fragment A solution, it is possible to determine the survival rate of the cell and this easily infer the volume of external fluid incorporated by the irradiation. The calcium phosphate method—the most widely employed DNA transfection method—involves making a precipitate of DNA and calcium phosphate and then incorporating it into the cell so it is impossible to specify the number of DNA molecules introduced into the cell. The laser pricking method presents an easier way to estimate the injected volume and thereby estimate the number of DNA molecules injected into the cell.

MATERIALS AND METHODS

Cell line. The cell line used in this study was the NRK (normal rat kidney) and was maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) under 37°C at 5% CO₂. Five hours prior to laser irradiation,

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10^4 cells were inoculated onto quartz plates (for details see Ref. 5).

Injection of diphtheria toxin fragment A. The solution of diphtheria toxin fragment A was repeatedly diluted by half with culture medium to produce a series from the concentration of $1 \mu\text{g/ml}$ to $2^{-10} \mu\text{g/ml}$. All the 1×10^4 NRK cells were next irradiated at cytoplasm through the quartz plate with a 5 nsec pulse at 355 nm. The energy was set at 1 mJ/pulse.

Colony formation ratio. After applying laser microbeam irradiation, the NRK cells were immediately removed from the quartz plate with trypsin and inoculated again onto a 3 cm dish. Six days after, the colonies which appeared were counted. Setting the colony formation ratio at 100% for the control (cells laser-irradiated without diphtheria toxin fragment A), the survival rates of cells treated with various concentrations of diphtheria toxin fragment A were determined.

RESULTS AND DISCUSSION

The figure shows the colony formation ratio of laser-irradiated cells (NRK) in graduated dilutions of diphtheria toxin fragment A. It is apparent that the relationship between colony formation ratio and the concentration of diphtheria toxin indicates poison distribution. When the concentration of diphtheria toxin was $2^{-4} \times 10^{-3}$ (g/l) or greater, a sharp decline in the colony formation ratio was observed. Since it has been reported that one molecule of diphtheria toxin fragment A kills a cell. It is conceivable that around concentration, approximately 1 molecule was transferred into the cell. The injected volume, I (1), can be expressed in the following formula:

Fig.: Correlation between concentration of diphtherotoxin fragment A and colony formation ratio. The colony formation ratio of cells laser-irradiated without diphtherotoxin was defined to be 100%.

$$I(1) = \frac{21,500}{(2^{-4} \times 10^{-3}) \times (6 \times 10^{23})} \dots\dots\dots (I)$$

Where 21,500 is the molecular weight of diphtheria toxin fragment A and 6×10^{23} is Avogadro's number and $2^{-4} \times 10^{-3}$ (g/l) is the initial fatal concentration of fragment A. The injected volume I would then be 1.15×10^{-15} (5). This is very close to the value reported by Yamamoto *et al.* for glass needle pricking (6). Using this value, it is easy to infer how many DNA molecules was transferred during the DNA transfection process. In the previous study, the transformation ratio reached a plateau when the concentration of DNA was over 10 mg/l (5). Therefore the weight of transferred DNA V(g) per cell is expressed in the following equation:

$$V(g) = I(1) \times 0.01 \text{ (g)} \dots\dots\dots (II)$$

If 1.15×10^{-15} is substituted for I, V (the weight of transferred DNA) is 1.15×10^{-17} g. The length of the transferred DNA (Eco-gpt) was approximately 2.5 kb (cf. Ref. 1) and the molecular weight was 1.5×10^6 . In this case (Eco-gpt DNA: 10 mg/l by laser transfection), the number of DNA molecules transferred (N) can be expressed by the following equation:

$$N = \frac{V}{1.5 \times 10^6} \times 6 \times 10^{23} \dots\dots\dots (III)$$

If 1.15×10^{-17} is substituted for V, N is 4.5. Transformation occurred even though the DNA concentration was 2 μ g/ml (cf. Ref. 5, Fig. 4). This result strongly suggests that transformation may occur even if only one molecule DNA is transferred into cell. Same results were obtained by Yamamoto *et al.* (7) by their glass needle pricking method.

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