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Abstract

 This study was conducted to evaluate the frequency of DNA transfection into human cells following X-ray irradiation. We transfected plasmid DNA (pSV2neo) into human cells, HeLa and PA-1, by either calcium phosphate precipitation or the lipofection method immediately after irradiating the cells with various doses of X-rays. The transfection frequency was evaluated by counting the number of G418-resistant colonies. When circular plasmid DNA was used, irradiation up to a dose of 2 Gy dose-dependently increased the transfection frequency, which reached a maximum of 5 to 10-fold that of the control unirradiated cells. When linear plasmid DNA was used, the transfection frequency was 2 times higher than that of circular DNA. All five of the clones that were randomly chosen expreed the transfected neo gene. In addition, the pSV2neo gene was randomly integrated into the genomic DNA of each clone. These findings indicate that X-ray treatment can facilitate foreign DNA transfer into human cells and that radiation-induced DNA breaks may promote the insertion of foreign DNA into host DNA. The enhancement of DNA transfection with X-rays may be instrumental in practicing gene therapy.

KEYWORDS: DNA transfection, neo gene, X-ray irradiation

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Enhancement of DNA-Transfection Frequency by X-Rays

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This study was conducted to evaluate the frequency of DNA transfection into human cells following X-ray irradiation. We transfected plasmid DNA (pSV2neo) into human cells, HeLa and PA-1, by either calcium phosphate precipitation or the lipofection method immediately after irradiating the cells with various doses of X-rays. The transfection frequency was evaluated by counting the number of G418-resistant colonies. When circular plasmid DNA was used, irradiation up to a dose of 2 Gy dose-dependently increased the transfection frequency, which reached a maximum of 5 to 10-fold that of the control unirradiated cells. When linear plasmid DNA was used, the transfection frequency was 2 times higher than that of circular DNA. All five of the clones that were randomly chosen expressed the transfected neo gene. In addition, the pSV2neo gene was randomly integrated into the genomic DNA of each clone. These findings indicate that X-ray treatment can facilitate foreign DNA transfer into human cells and that radiation-induced DNA breaks may promote the insertion of foreign DNA into host DNA. The enhancement of DNA transfection with X-rays may be instrumental in practicing gene therapy.

Key words: DNA transfection, neo gene, X-ray irradiation

Gene therapy consists of two steps, targeting the appropriate organ and effective transfection of DNA into cells. Since gene transfection into cells is one of the rate-limiting steps of gene therapy, there is a need for simple and effective transfection methods. Several methods of introducing DNA into cells have been reported: DEAE-dextran (1), calcium phosphate (2), electroporation (3), microinjection (4), lipofection (5), retrovirus vectors (6) and adenovirus vectors (7).

For clinical application, high efficiency of gene transfer, technical simplicity, cost effectiveness and safety are prerequisites for gene transfer procedures. To establish the optimum conditions for transfection, various kinds of transfection agents, transfection time, concentration of agents and incubation time were examined (8–11).

To facilitate the delivery of foreign DNA into the nucleus of target cells, it must be possible for the DNA to enter the cytoplasm through the plasma membrane, move from the cytoplasm to the nucleus without being degraded by intracellular nucleases, and become integrated into the host DNA. Thus, the increase in gene transfer frequency largely depends on how these barriers are overcome. For example, DMSO and glycerol, which are well known to stimulate DNA transfer frequency, may improve DNA transfer by increasing the permeability of the cell membrane. X-rays and UV light also reportedly increase DNA transfer frequency (12).

X-rays introduce DNA base damage, DNA strand breaks, and crosslinks within DNA or between DNA and nuclear proteins (13, 14). Radiation-induced DNA strand breaks are generally believed to arise mainly from the action of reactive oxygen species (12). Most strand breaks are rapidly repaired within about 24 h of irradiation (15–18), and among the repair processes of radiation-induced DNA damage, rejoining of the strand breaks is the most important event (19).

X-ray-induced DNA strand breaks cause local denaturation which may allow for additional exposure of bases in the interior of the DNA duplex to various molecules (20). In the excision repair pathway of UV light-induced pyrimidine dimers, damaged parts of the DNA strand are excised, resulting in single strand gaps (21). Under these circumstances, increases in the transfection frequency of thymidine kinase, neo and ecogpt genes have been report-

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ed (22-24). Furthermore, it is well known that ionizing radiation induces single-and double-strand breaks in DNA (19). Thus, we expected that foreign DNA might be integrated into the host DNA during the repair process of radiation-induced strand gaps and that the transfection frequency of foreign DNA into the irradiated cells might be enhanced. In this study, we describe how the transfection frequency of foreign DNA increases in human cells exposed to X-rays.

Materials and Methods

Cells and irradiation. Two human cell lines, HeLa and PA-1, were used. HeLa was derived from cervical cancer (25) and PA-1 from ovarian cancer (26). Cells were maintained in minimum essential medium (MEM) with 10 % fetal bovine serum (FBS). We seeded 5×10^5 cells into a 10-cm Corning plastic plate and 24 h later we irradiated them at room temperature with a Torrex 150D X-ray machine (EG & G, Nashua, NH USA) operating at 120 kV at 3 mA with a 1.2 mm Be filter. The dose rate was 0.3 Gy/min.

Transfection and survival assay. After irradiation, the cells were transfected with 10 μg circular or BamH1 (Boehringer, Mannheim, Germany) digested linear pSV2neo DNA by calcium phosphate precipitation method or liposome preparation method (Gibco BRL, Grand Island, NY, USA). BamH1 digestion was performed according to the manufacturer's specifications and confirmed by electrophoresis. After the cells were incubated at 37°C for 15-20 h with the DNA, they were collected by trypsinization and 1×10^5 cells/6-cm dish were inoculated in MEM + 10 % FBS containing 400 μg/ml G418. At the same time, 200 cells/dish were seeded, and cultured for 1 week. Then the cultures were fixed with 100 % ethanol, stained with 3 % Giemsa solution, and the colonies consisting of more than 20 cells were counted under a microscope to determine the surviving fraction. Alternatively, two weeks later, the G418-selection cultures were fixed with ethanol and stained with 3 % Giemsa solution, and G418-resistant colonies consisting of more than 20 cells were counted under a microscope. The transfection frequency of the neomycin-containing vector was measured by counting the number of G418-resistant colonies expressed as a fraction of 1×10^5 surviving cells. All experiments were repeated three times.

Northern blot analysis. Cytoplasmic RNA was extracted from neo^r HeLa cells and 5 μg RNA per lane was separated by electrophoresis on 1 % agarose-formaldehyde gel. Then, RNA was blotted onto a positively charged nylon membrane and hybridized with a ^{32}P-labeled pSV2neo DNA probe which was prepared using a random-primed DNA labeling kit (Amersham, Buckinghamshire, England). Autoradiography was performed according to standard procedures.

Southern blot analysis. Episomal DNA and genomic DNA were fractionated from exponentially growing cells using the method described by Hirt (27). Each DNA fraction was purified by phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol extraction, followed by precipitation with isopropanol. Purified DNA was digested with Bam H1 as described above, and 10 μg of each DNA fraction was electrophoresed in 0.8 % agarose gel. After the DNA samples were transferred onto a positively charged nylon membrane, the membrane was hybridized with the ^{32}P-labeled pSV2neo probe. The filter was then exposed to X-ray film at − 80°C between two intensifying screens.

Results

The cell survival curve of irradiated HeLa cells is shown in Fig. 1. The cells irradiated at various doses were replated to determine X-ray sensitivity. The plating efficiency of the unirradiated cells was 50-60 %. The D_0

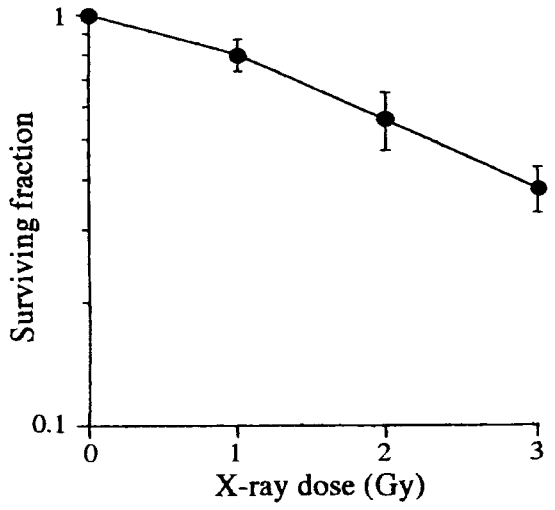

Fig. 1 Dose response curve of HeLa cells. The cells were irradiated at each dose and replated for colony formation. Each point represents the mean of three experiments with standard deviation.

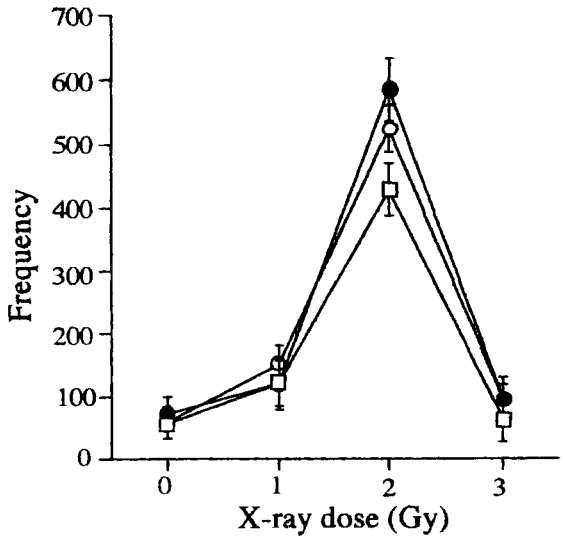

Fig. 2 Frequency of transfection of pSV2neo genes. HeLa (○) and PA-1 (□) cells were irradiated at each dose and immediately transfected with 10 μg pSV2neo DNA by the calcium phosphate precipitation method. HeLa cells were also transfected with DNA by the lipofection preparation method (●). The transfection frequency was measured by counting the number of G418-resistant colonies and expressed as a fraction of 1×10^5 surviving cells after transfection. Each point represents the mean of three experiments with standard deviation.

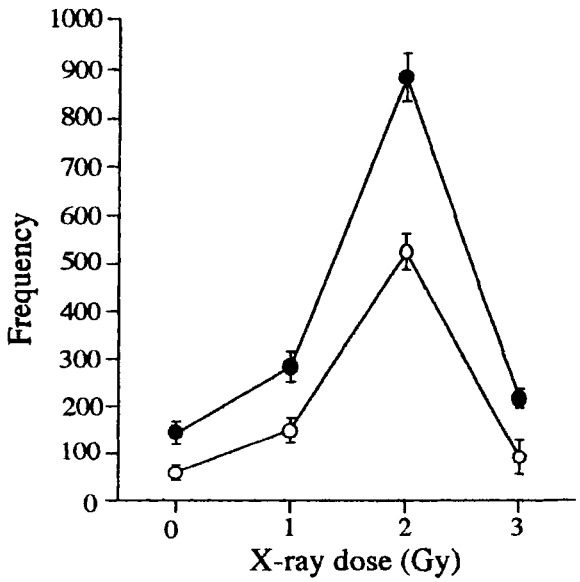

Fig. 3 Difference in the transfection frequency of circular and linear DNA. HeLa cells with circular (○) and linear DNA (●). Circular DNA was digested into linear DNA with BamHI restriction enzyme. The transfection frequency was measured by counting the number of G418-resistant colonies and expressed as a fraction of 1×10^5 surviving cells after transfection. Each point represents the mean of three experiments with standard deviation.

and D_q were calculated to be 4.8 Gy and 0.58 Gy, respectively. The frequency of transfection of the pSV2neo gene into X-ray-irradiated cells is shown in Fig. 2. The frequency reached a maximum at 2 Gy, 5 to 10-fold that of the control (0 Gy). Similar enhancement of the transfection frequency was obtained with PA-1 cells (Fig. 2). All these experiments were done using the calcium phosphate precipitation method. On the other hand, when the pSV2neo gene was transfected into HeLa cells by the lipofection method, the transfection frequency was enhanced by X-rays to almost the same degree (Fig. 2).

The above experiments were done using circular DNA. Therefore, to investigate the influence of the difference in DNA structure, circular pSV2neo DNA was digested into linear DNA by the restriction enzyme and transfected into the cells. As a result, the frequency of transfection with linear DNA increased about 2-fold as compared with circular DNA (Fig. 3).

Then we investigated whether the transfected pSV2neo gene was present in the host cells. Northern blot analyses showed that neo mRNA was well expressed in 5 randomly selected clones obtained by transfection with pSV2neo (Fig. 4). Furthermore, Southern blot

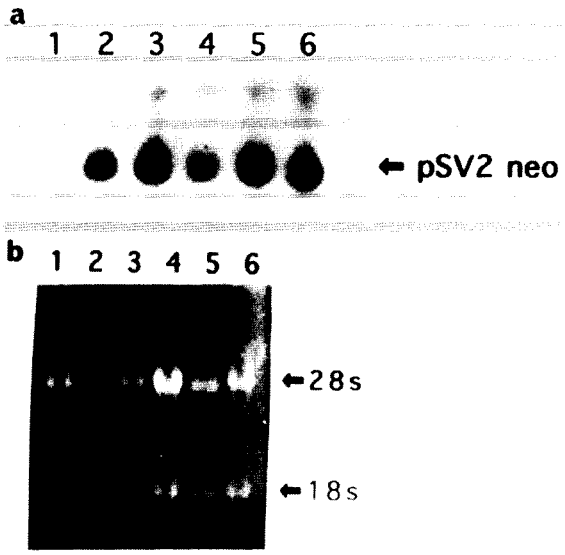

Fig. 4 Northern blot analysis of pSV2neo gene. **a**) Five neo^r HeLa clones (5 μg RNA/lane) were hybridized with a ^{32}P-labeled pSV2neo probe. The five clones were chosen at random from neo^r transformants from 2 Gy-irradiated cells. Lane 1: control HeLa cells; Lanes 2-6: neo^r clones from 2 Gy-irradiated cells. **b**) The amount of RNA/lane was tested by the internal control of ribosomal RNA.

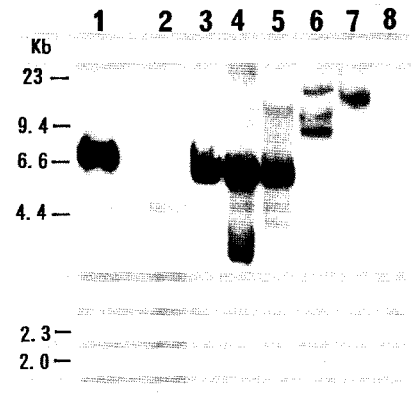

Fig. 5 Southern blot analysis of G-418 resistant clones (10 μg DNA/lane). DNA was digested with Bam HI. Lanes: 1, pSV2neo plasmid; 2, Hirt fraction of HeLa cells transfected with pSV2neo; 3, DNA fraction of HeLa cells plus pSV2neo gene; 4-7, G-418 resistant HeLa clones; 8, genomic DNA fraction of HeLa cells.

analysis revealed that the pSV2neo gene was randomly integrated into the genomic DNA of each clone (Fig. 5). No examples of the free unintegrated neo gene were observed, indicating that the neo gene is not present as an extra-chromosomal form in cells (Fig. 5).

Discussion

Gene transfection frequency into cells is one of the rate-limiting steps for the development of gene therapy. In this study, we investigated the effect of X-ray irradiation on the transfection frequency of the pSV2neo gene into human cells. We transfected plasmid DNA (pSV2neo), circular or linear, into HeLa and PA-1 cells immediately after irradiation and found that the transfection frequency was increased by X-rays. In addition, the transfection frequency with the linear DNA was 2 times higher than that with the circular DNA.

The detailed mechanisms by which external DNA molecules cross the cell membrane, move from the cytoplasm to the nucleus, and are integrated into the host DNA are as yet poorly understood. However, it is supposed that the repair process of radiation induced DNA damage is associated with the introduction of foreign DNA into host DNA.

It has been reported that UV light and X-rays increase the frequency of DNA transfer (22, 23), although the type of DNA damage induced by the two agents is qualitatively different. UV light induces mainly pyrimidine dimers, resulting in DNA strand breaks in the excision repair process of the damaged parts (28). The damaged DNA strand is excised and then the deleted stretch is reconstituted utilizing the intact complementary strand as a template (21, 28). Presumably, foreign DNA could be integrated into this single strand gap, resulting in the enhancement of the transfection frequency. Otherwise, foreign DNA could be integrated into the gap of the local denaturation caused by single-strand breaks directly induced by X-rays, followed by an increase in the transfection frequency. Besides radiation-induced DNA strand gaps, there are several other factors which may increase the transfection frequency. For example, DNA ligase, which repairs breaks in a damaged strand DNA, may also increase the transfection frequency.

In this study, although X-rays enhanced the transfection frequency, the pattern of the enhancement is different from that described by Debenham and Webb (12). They reported that there was an approximately linear increase in the DNA transfer frequency with increasing X-ray doses up to 10 Gy, while we observed that the frequency did not increase dose-dependently. In fact, the transfection frequency in the cells exposed to 3 Gy was lower than that to 2 Gy. This difference, however, may be due to the cell types used. Debenham and Webb used V79 Chinese hamster cells whereas we used HeLa and PA-1 cells, which are human cell lines. Also the time interval between their X-ray treatment and transfection was differrent from ours. Since we transfected immediately after X-ray treatment, the cytotoxity of the transfection reagents could retard or otherwise affect the repair of the potentially lethal damage induced by X-rays.

In conclusion, we showed that X-rays stimulated the transfer of foreign DNA into human cells. Interestingly, linear DNA was more easily integrated into host DNA than circular DNA. This simple and efficient method to introduce foreign genes into cells will be useful in various experiments involving manipulation of genes. If we could enhance the transfection frequency of genes such as the wild-type p53 and IL-2 genes into *in vivo* tumors by irradiation, more efficient treatment of tumors would be possible. It is well known that X-rays are mutagenic to cells, but since they are given locally to a tumor as a routine clinical procedure, the combined treatment of tumors with X-rays and gene transfection into tumor cells will be beneficial for cancer therapy.

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