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Abstract

We evaluated the effects of hyperthermia on the efficiency of gene transduction by using a cationic liposome to develop an efficient method for lipofection. We used Lewis lung carcinoma (LLC), NIH3T3, and A549 cell lines, with Lipofectamine reagent as the cationic liposome and the LacZ gene as the reporter gene. In LLC, co-incubation of the cationic liposome and plasmid DNA complex (lipoplex) with the cells for 2 h at 41 degrees C enhanced the efficiency of gene transduction approximately 1.4-fold compared to incubation for 2 h at 37 degrees C, as measured by X-gal staining and beta-galactosidase activity. In cell lines NIH3T3 and A549, the efficiency of gene transduction showed a tendency toward enhancement after 2 h co-incubation with lipoplex at 41 degrees C compared to that at 37 degrees C, as measured by X-gal staining. This is the first study to demonstrate the enhancement of gene transduction efficiency achieved by using a cationic liposome under conditions of hyperthermia. This method should prove useful for lipofection in other cancer cells.

KEYWORDS: lipofection, gene transduction efficiency, hyperthermia

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Original Article

Enhancement of Gene Transduction Efficiency in Cancer Cells Using Cationic Liposome with Hyperthermia

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We evaluated the effects of hyperthermia on the efficiency of gene transduction by using a cationic liposome to develop an efficient method for lipofection. We used Lewis lung carcinoma (LLC), NIH3T3, and A549 cell lines, with Lipofectamine reagent as the cationic liposome and the LacZ gene as the reporter gene. In LLC, co-incubation of the cationic liposome and plasmid DNA complex (lipoplex) with the cells for 2 h at 41 °C enhanced the efficiency of gene transduction approximately 1.4-fold compared to incubation for 2 h at 37 °C, as measured by X-gal staining and β-galactosidase activity. In cell lines NIH3T3 and A549, the efficiency of gene transduction showed a tendency toward enhancement after 2 h co-incubation with lipoplex at 41 °C compared to that at 37 °C, as measured by X-gal staining. This is the first study to demonstrate the enhancement of gene transduction efficiency achieved by using a cationic liposome under conditions of hyperthermia. This method should prove useful for lipofection in other cancer cells.

Key words: lipofection, gene transduction efficiency, hyperthermia

The use of cationic liposomes as a gene delivery system is attractive because of their low immunogenicity, ease of use, and safety [1, 7-9]. Nonetheless, the low gene transduction efficiency of such liposomes is a major problem that remains to be resolved [1, 5, 6]. There have been many studies of liposomes used as a delivery system for drugs, primarily anti-cancer agents, and hyperthermia has been shown to enhance the effectiveness of several anti-cancer agents by increasing the permeability of the cell membrane or improving drug uptake or release [10-13].

However, there have been no investigations of the possible correlation between cationic liposomal gene delivery and hyperthermia. Hyperthermia is well known to have a cytotoxic or cytostatic effect [15-17, 21-24]. If hyperthermia also enhances cationic liposomal gene transduction, it may present an attractive addition to liposomal gene delivery protocols for patients with cancer. This investigation was conducted in order to evaluate whether or not the efficiency of gene transduction is enhanced by combining the use of cationic liposomes and hyperthermia.

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Materials and methods

Cell lines and culture conditions. The Lewis lung carcinoma (LLC) murine lung cancer cell line, NIH3T3 murine fibroblasts, and the A549 human lung cancer cell line were used in this investigation. The LLC cell line was cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS, USA) and penicillin/streptomycin (SIGMA, St. Louis, MO, USA). NIH3T3 and A549

were cultured in Dulbecco's modified Eagle's medium (GIBCO-BRL, Life Technologies, Gaithersburg, MD, USA) supplemented with 10% FBS (SIGMA) and penicillin/streptomycin (SIGMA). Cultures were maintained in a humidified atmosphere at 37 °C and 5% CO_2. The cultured medium was replaced with fresh medium on days 4 and 6 after the plating.

Cell growth curve. Cells were plated at 5×10^4 in 35 mm culture dishes (CORNING, Corning, NY, USA). The cells were harvested and counted every 24 h using a Burker's hemocytometer (Higaki Medical Science, Tokyo, Japan) with 0.5% Trypan blue staining (GIBCO-BRL).

Hyperthermia treatment. Forty-eight hours after plating, the cells were incubated in another humidified incubator (ASTEC, Fukuoka, Japan) at 40, 41, and 42 °C and 5% CO_2 for 2 and 6 h. Then, the cells were returned to a humidified atmosphere at 37 °C and 5% CO_2. The cultured medium was replaced with fresh medium on days 4 and 6 after the plating. The temperature of the medium reached the indicated level within 20 min.

Lipofection. In brief, 48 h after the plating, the LLC and A549 cells were co-incubated with 8ul of Lipofectamine (GIBCO-BRL) and 2ug of the pCMV・SPORT・LacZ (GIBCO-BRL) complex in serum-free medium (Opti-MEM, GIBCO-BRL) for 2 and 6 h in a humidified atmosphere at 37 °C and 5% CO_2. The NIH3T3 cells were co-incubated with 6ul of Lipofectamine and 2ug of the pCMV・SPORT・LacZ complex in serum-free medium (Opti-MEM, GIBCO-BRL) for 2 and 6 h in a humidified atmosphere at 37 °C and 5% CO_2. After co-incubation, the medium was supplemented with 20% FBS to create a medium containing 10% FBS.

Lipofection with hyperthermia at 40, 41, and 42 °C. Lipofection with hyperthermia was carried out in the same manner as that conducted at 37 °C. In brief, 48 h after plating, the cells were co-incubated with the cationic liposomes and plasmid DNA complex (lipoplex) in serum-free medium for 2 and 6 h in another humidified incubator (ASTEC) at 40, 41, and 42 °C and 5% CO_2. After co-incubation, the medium was supplemented with 20% FBS pre-warmed at 37 °C to create a medium containing 10% FBS. Then, the cells were returned to a humidified atmosphere at 37 °C and 5% CO_2.

Quantification of X-gal staining. Forty-eight hours after the start of lipofection, X-gal staining was performed by using X-gal (5-bromo-4-chloro-3-indoil-β-D-galactopyranoside; Nacarai Tesque, Kyoto, Japan) as described previously [19, 33]. In brief, cells were rinsed twice with phosphate-buffered saline (PBS), and then fixed with 0.25% glutaraldehyde in PBS at 4 °C for 10 min. The cells were rinsed with PBS 4 times, and then stained with the X-gal in PBS containing 5 mM $K_4[Fe(CN)_6]$, 5 mM $K_3[Fe(CN)_6]$, 2 mM $MgCl_2$, and 1 mg/ml X-gal at 37 °C for 8 to 12 h. Transduction efficiencies were evaluated as the X-gal-positive cell percentage in more than 1,000 cells, which was counted under × 100 magnification in random fields.

Quantification of β-galactosidase activity. Forty-eight hours after the start of lipofection, the β-galactosidase activity was evaluated by using a β-galactosidase assay kit (Stratagene, La Jolla, CA, USA), which was read at 420 nm absorption [34]. To minimize the effects of differing cell numbers, the quantification of the β-galactosidase activity was standardized according to the protein in the cell lysate. The protein was assayed by using the Bradford method read at 595 nm (Bio-Rad, Hercules, CA, USA) [35]. Absorption readings were converted to micrograms of protein by using a bovine serum-albumin standard curve, and data were expressed as the unit of β-galactosidase/ug of protein.

Statistical analysis. All cell numbers and transduction efficiencies are presented as the mean ± the standard deviation (SD). Paired t tests were performed to evaluate significant values. Differences were considered statistically significant when $P < 0.05$. SPSS Medical Pack 7.5J software (SPSS, Tokyo, Japan) was used for the data analysis.

Results

Cell growth curve and effects of hyperthermia. After 2 h of hyperthermia, the LLC cell line demonstrated almost the same growth at 37, 40, and 41 °C. However, the growth was significantly inhibited at 42 °C compared to that at 37 °C ($P < 0.05$) (Fig. 1a). After 6 h of hyperthermia, the growth of LLC was significantly inhibited at 42 °C. Cell growth was moderately inhibited at 41 °C compared to that at 37 °C, but hyperthermia at 40 °C did not have an inhibitory effect on growth (Fig. 1b).

The NIH3T3 and A549 cell lines showed no growth

inhibition with either 2 or 6 h of hyperthermia at 41 °C compared to the effect of incubation at 37 °C (Fig. 2a, b).

The cytotoxicity of the Lipofectamine was increased according to the co-incubation time and hyperthermia at 41 °C. Under co-incubation with Lipofectamine at 37 °C, the LLC demonstrated growth inhibition that correlated with the duration of incubation. With hyperthermia co-incubated with Lipofectamine, the cell growth was significantly inhibited at 41 °C compared to that at 37 °C; this effect also correlated with co-incubation time (Fig. 1c). However, cell lines NIH3T3 and A549 showed no evidence of cytotoxicity under co-incubation with Lipofectamine for 2 and 6 h of hyperthermia at 41 °C (data not shown).

Gene transduction efficiency evaluated with X-gal staining. In the LLC cells co-incubated for 2 h with lipoplex, the positive cell percentage on X-gal staining was 23.0 at 37 °C, 19.2 at 40 °C, 29.1 at 41 °C, and 23.3 at 42 °C. At 41 °C, the percentage of positive cells was enhanced 1.27-fold that of cells kept at 37 °C ($P = 0.03$) (Fig. 3, Table 1). For LLC cells in 6 h of co-incubation with lipoplex, the X-gal-positive cell percentage was 24.0 at 37 °C, 24.7 at 40 °C, 26.9 at 41 °C, and 11.9 at 42 °C. The X-gal-positive cell percentage at 41 °C was enhanced 1.12-fold that of cells kept at 37 °C ($P = 0.36$) (Table 1).

In NIH3T3, the X-gal-positive cell percentage was 4.1 at 37 °C and 4.3 at 41 °C with 2 h of co-incubation. The X-gal-positive cell percentage at 41 °C was enhanced 1.04-fold that of cells kept at 37 °C ($P = 0.54$). With 6 h of co-incubation, the X-gal-positive cell percentage was 7.2 at 37 °C and 3.8 at 41 °C. The X-gal-positive cell percentage at 41 °C was reduced 0.53-fold that of cells kept at 37 °C ($P = 0.02$) (Table 2).

In A549, the X-gal-positive cell percentage was 1.5 at 37 °C and 2.2 at 41 °C with 2 h of co-incubation. The X-gal-positive cell percentage at 41 °C was enhanced 1.47-fold that of cells kept at 37 °C ($P = 0.13$). With 6 h of co-incubation, the X-gal-positive percentage was 6.7 at 37 °C and 7.4 at 41 °C. The X-gal-positive cell percentage at 41 °C was enhanced 1.12-fold that of cells kept at 37 °C ($P = 0.70$) (Table 3).

Gene transduction efficiency evaluated according to β-galactosidase activity. In the

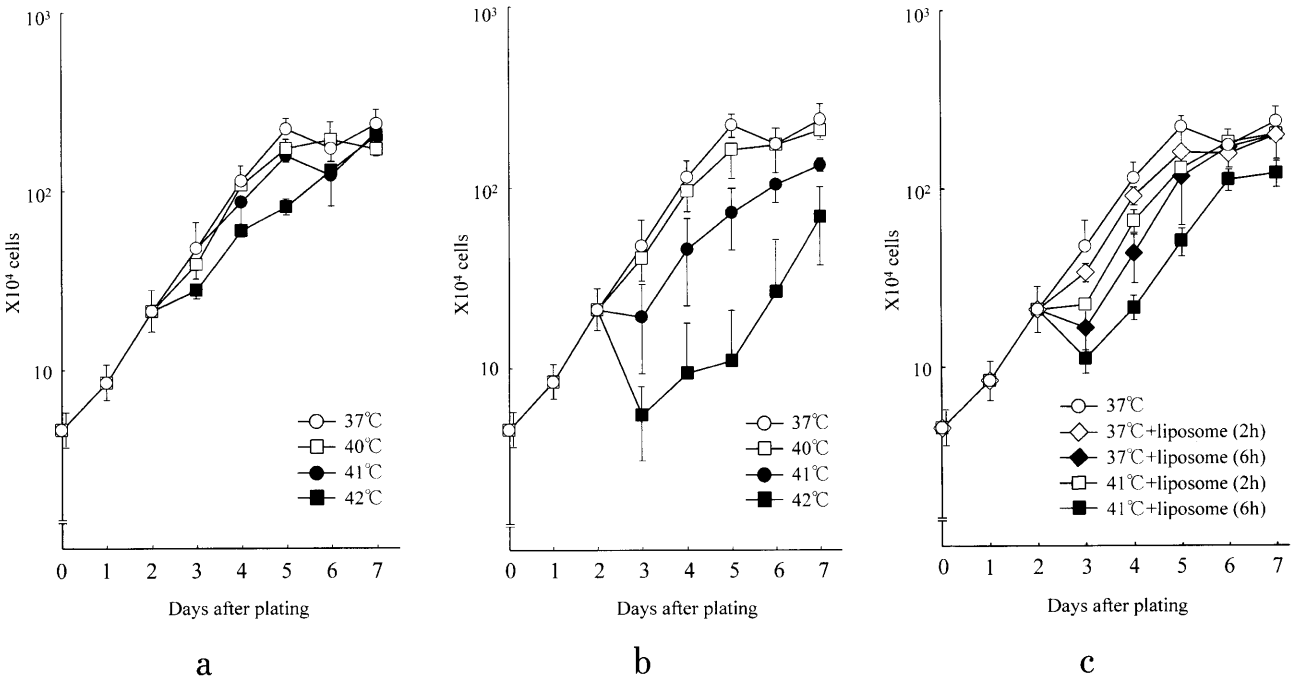

Fig. 1 Growth curves for the LLC murine lung cancer cell line. Cells in 35 mm dishes were incubated at 37 °C and were subjected to hyperthermia at 40, 41 and 42 °C from 48 h after plating. (a) After 2 h of hyperthermia, (b) after 6 h of hyperthermia. (c) Cells were co-incubated with Lipofectamine at 37 and 41 °C for 2 and 6 h from 48 h after plating. The cell number was counted with a hemocytometer. Data are shown as the mean ± SD (n = 4 or more separate experiments).

LLC cells placed in 2 h of co-incubation with lipoplex, the β-galactosidase activity was 86.0 units/ug of lysate at 37 °C, 112.6 units/ug of lysate at 40 °C, 139.2 units/ug of lysate at 41 °C, and 129.1 units/ug of lysate at 42 °C. At 41 °C, the activity was significantly enhanced 1.62-fold that of cells kept at 37 °C ($P = 0.004$). With 6 h of co-incubation, the β-galactosidase activity was 108.0 units/ug of lysate at 37 °C, 114.7 units/ug of lysate at 40 °C, 144.3 units/ug of lysate at 41 °C, and 95.3 units/ug of lysate at 42 °C. The β-galactosidase activity at 41 °C was enhanced 1.34-fold that of cells kept at 37 °C ($P = 0.02$) (Table 1).

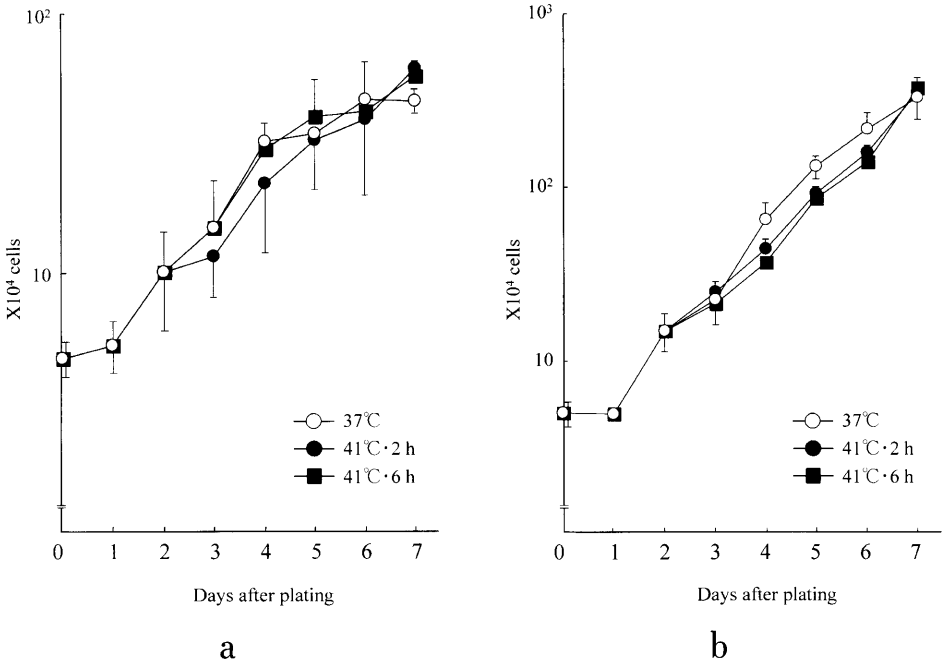

Fig. 2 Growth curves for (a) NIH3T3 murine fibroblasts and (b) A549 human lung cancer cell line. Cells were incubated at 37 and 41 °C for 2 and 6 h beginning 48 h after plating. Data are shown as the mean ± SD (n = 3 or more separate experiments).

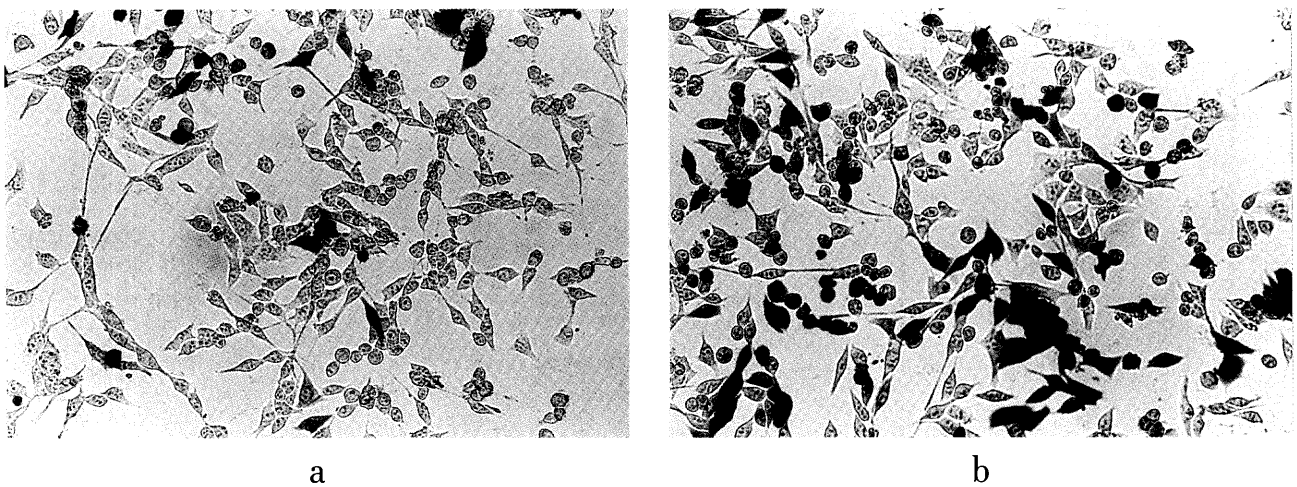

Fig. 3 Representative pictures of X-gal staining of LLC following transduction with pCMV-SPORT-LacZ. LLC cells were co-incubated with lipoplex (a) for 2 h at 37 °C and (b) for 2 h at 41 °C. Original magnification was × 100.

Table 1 X-gal staining and β-galactosidase activity of LLC following transduction with pCMV-SPORT-LacZ at different temperatures

Temp (°C)	2 h		6 h	
	X-gal (% of positive cell)	β-galactosidase activity (Units/ug of lysate)	X-gal (% of positive cell)	β-galactosidase activity (Units/ug of lysate)
37	23.0±5.5	86.0±44.2	24.0±4.2	108.0±29.0
40	19.2±5.9 *	112.6±45.3 **	24.7±2.7 ***	114.7±33.8 ****
41	29.1±9.6	139.2±67.3	26.9±4.8	144.3±39.1
42	23.3±1.9	129.1±17.2	11.9±1.7	95.3±76.4

Cells were co-incubated with lipoplex at 37, 40, 41, and 42 °C beginning 48 h after plating for 2 and 6 h. The X-gal staining was evaluated as the percent of positive cells, and β-galactosidase activity was evaluated as units/ug of lysate protein for standardization, as described in Materials and Methods. Data are shown as the mean ± SD (n = 4 or more separate experiments).
*$P = 0.03$, **$P = 0.004$, ***$P = 0.36$, ****$P = 0.02$

Table 2 X-gal staining and β-galactosidase activity of NIH3T3 following transduction with pCMV-SPORT-LacZ at different temperatures

Temp (°C)	2 h		6 h	
	X-gal (% of positive cell)	β-galactosidase activity (Units/ug of lysate)	X-gal (% of positive cell)	β-galactosidase activity (Units/ug of lysate)
37	4.1±2.1 N.S.	111.0±43.7 N.S.	7.2±0.3 *	290.0±112.0 **
41	4.3±1.9	135.1±65.7	3.8±1.4	79.8± 12.8

Cells were co-incubated with lipoplex at 37 and 41 °C beginning 48 h after plating for 2 and 6 h. The X-gal staining was evaluated as the positive cell percentage in more than 1,000 cells, and β-galactosidase activity was evaluated as units/ug of lysate protein for standardization, as described in Materials and Methods. Data are shown as the mean ± SD (n = 3 or more separate experiments).
*$P = 0.02$, **$P = 0.002$

Table 3 X-gal staining and β-galactosidase activity of A549 following transduction with pCMV-SPORT-LacZ at different temperatures

Temp (°C)	2 h		6 h	
	X-gal (% of positive cell)	β-galactosidase activity (Units/ug of lysate)	X-gal (% of positive cell)	β-galactosidase activity (Units/ug of lysate)
37	1.5±0.8 N.S.	41.8±15.5 N.S.	6.7±2.1 N.S.	144.4±32.6 *
41	2.2±0.5	54.0±39.2	7.4±3.2	65.4±20.0

Cells were co-incubated with lipoplex at 37 and 41 °C beginning 48 h after plating for 2 and 6 h. The X-gal staining was evaluated as the positive cell percentage in more than 1,000 cells, and β-galactosidase activity was evaluated as units/ug of lysate protein for standardization, as described in Materials and Methods. Data are shown as the mean ± SD (n = 3 or more separate experiments).
*$P = 0.04$

In the NIH3T3 cell line with 2 h of co-incubation, the β-galactosidase activity was 111.0 units/ug of lysate at 37 °C and 135.1 units/ug of lysate at 41 °C. At 41 °C, the activity was enhanced 1.22-fold that of cells kept at 37 °C ($P = 0.39$). With 6 h of co-incubation, the β-galactosidase activity was 290.0 units/ug of lysate at 37 °C and 79.8 units/ug of lysate at 41 °C. The β-galactosidase activity at 41 °C was reduced 0.28-fold that of cells kept at 37 °C ($P = 0.002$) (Table 2).

In the A549 cells with 2 h of co-incubation, the β-galactosidase activity was 41.8 units/ug of lysate at 37 °C and 54.0 units/ug of lysate at 41 °C. At 41 °C, the activity was enhanced 1.29-fold that of cells kept at 37 °C ($P = 0.65$). With 6 h of co-incubation, the β-galactosidase activity was 144.4 units/ug of lysate at 37 °C and 65.4 units/ug of lysate at 41 °C. The β-

galactosidase activity at 41 °C was reduced 0.45-fold that of cells kept at 37 °C ($P = 0.04$) (Table 3).

Discussion

Currently, major hurdles for successful gene therapy are preserving safety and achieving an efficient method of gene delivery. Replication-defective viral vectors and synthetic nonviral systems are two representative delivery systems for gene therapy, but safety and immunological responses are major concerns when administration of the viral vectors is repeated [1, 7–9, 29, 30]. Synthetic nonviral vectors, represented by the cationic liposome, are advantageous as regards repeated administration due to their safety, low toxicity, and no (or low) immunogenicity [1, 6–9]. However, the low efficiency of gene transduction is a major problem that remains to be solved [1, 4–6, 8, 9].

As drug-delivery systems for anti-cancer drugs, liposomes are effective in regions subjected to local hyperthermia, which is believed to increase the permeability of the cell membrane and improve drug uptake and release [10–13]. Moreover, hyperthermia is known to have certain cytotoxic effects, such as apoptosis, necrosis, cell-cycle disturbance, and damage to the membrane [15, 18–24]. We investigated the effect of hyperthermia on gene transduction using a cationic liposome, expecting that damage to the cell membrane by hyperthermia would allow the cationic liposome to promote membrane fusion, endocytosis, or both.

In this investigation, we demonstrated that gene transduction efficiency could be enhanced with mild hyperthermia by using a cationic liposome in the LLC, NIH3T3, and A549 cell lines. LLC is a murine and relatively heat-sensitive cancer cell line (Fig. 1). X-gal staining and a β-galactosidase assay showed that gene transduction efficiency could be gradually enhanced in the LLC cell line through co-incubation with lipoplex for 2 and 6 h at 37 to 41 °C (Table 1). But at 42 °C, the gene transduction efficiency was reduced because of the cytotoxic effect of hyperthermia. In the LLC cells, the transduction efficiency was more enhanced at 41 °C than at 37 °C (Table 1). We further investigated this phenomenon in a variety of mammalian cells, specifically NIH3T3 murine fibroblast cells and A549 human lung cancer cells. Neither cell line showed inhibited growth at 41 °C with either 2 or 6 h of incubation (Figs. 2a, b). These data suggest that murine cancer cells are more sensitive to hyperthermia than are human cancer cells, and cancer cells are more sensitive to hyperthermia than normal cells [15–17]. X-gal staining and β-galactosidase activity were also enhanced in the A549 and NIH3T3 cell lines undergoing 2-hour co-incubation with lipoplex at 41 °C, in contrast to the effects of incubation at 37 °C (Tables 2, 3). However, it should be noted that although X-gal staining showed an enhancement of gene transduction efficiency in A549 cells after 6 h of co-incubation (Table 3), a reduction of transduction efficiency in NIH3T3 cells (Table 2) was observed. In both A549 and NIH3T3 cells, β-galactosidase activity was not enhanced after 6 h of co-incubation (Tables 2, 3).

There may be several explanations for the discrepancies between X-gal staining and β-galactosidase activity. X-gal staining may not be useful for evaluating such expression levels and β-galactosidase activity may not be useful for reliably evaluating the number of cells expressing β-galactosidase. In other words, X-gal staining indicates the transduction frequency, and β-galactosidase activity indicates the transduction quantity [31–34]. These possibilities suggest that 2 h of mild hyperthermia enhanced both the transduction frequency and quantity; however, 6 h of mild hyperthermia enhanced only the frequency.

These results should be interpreted cautiously. In comparison to incubation at 37 °C, the cytotoxicity of the cationic liposome was enhanced by mild hyperthermia even at a non-lethal temperature (Fig. 1c). This effect was enhanced with increasing temperatures [14]. However, it is difficult to believe that transduced cells showed more resistance to hyperthermia than non-transduced cells because LacZ does not interfere with either cell growth or differentiation [32, 33].

The mechanisms of gene transduction mediated by cationic liposomes are believed to be membrane fusion, direct penetration, and endocytosis [2–4]. Most of the lipoplex degrades in the endosomes to lysosomes of the transduced cell, and transduced cells require that sufficient amounts of DNA reach the nucleus in a functional form. In this study, we showed that hyperthermia enhanced the cationic liposomal gene transduction efficiency. However, the mechanisms by which it does so remain unclear. Hyperthermia may simply damage the cell membrane, cause other morphological changes, or alter cell function [18–21]. We speculate that hyperthermia has several effects. For example, it may induce plasma membrane damage, thus promoting fusion with the lipo-

plex, as the formation of blebs was shown to increase cell surface area as the duration of heating was increased [19, 20]. Hyperthermia may also promote endocytosis or the turnover of protein via some types of heat-shock proteins [27]. Hyperthermia may accelerate the maturation of lipoplex [25], and may inhibit the degradation of lipoplex in lysosomes [26].

In our knowledge, this is the first study to demonstrate an enhancement of gene transduction efficiency by using a cationic liposome combined with mild hyperthermia. This method should prove useful for lipofection in other cell types. However, additional definitive studies are needed to show a more direct correlation between cationic liposomal gene delivery and hyperthermia. We are currently applying this technique to the pleural dissemination of lung cancer in a mouse model.

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