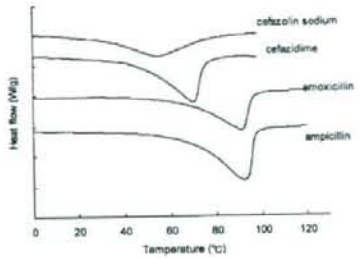

Figure 3. DSC thermograms for four antibiotic hydrates.

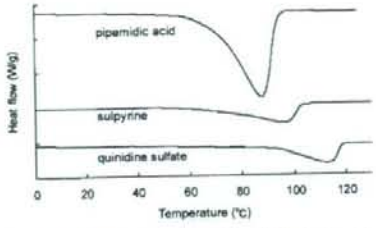

Figure 5. DSC thermograms for API hydrates showing a single endothermic peak.

Thus, determination of the molecular mobility of hydration water in API hydrates using NMR holds some challenges.

However, it is possible to determine the molecular mobility of hydration water in API hydrates by spin–spin relaxation measurement, if the spin–spin relaxation time (T_2) of the water protons is significantly different from that of the API protons. Furthermore, the spin–lattice relaxation time (T_1) of the water protons may be a useful indicator of water mobility, if the ratio of water protons to API protons is sufficiently large, or if the water protons have a correlation time (τ_c) corresponding to the T_1 minimum, such that the T_1 of the water proton is sensitively reflected in the measured T_1 value without being affected by spin diffusion between the water and the API protons. Moreover, even if the ratio of water protons to API protons is not particularly large, and even if water proton does not have a τ_c corresponding to the T_1 minimum, it may be possible to compare the molecular mobility of hydration water in API hydrates based on measured T_1 values, if both of the T_1 of the API proton and the ratio of water protons to API protons are similar for all of the API hydrates compared.

The purpose of this study was to examine the possibility of determining the molecular mobility of hydration water in API hydrates by NMR relaxation measurement. Spin–lattice relaxation, which reflects motions of MHz order, and spin–spin relaxation, which reflects slower motions, were measured for the 11 API hydrates listed in the Japanese Pharmacopeia (JP) using pulsed ^{1}H-NMR, which allows more simplified measurements than high-resolution ^{1}H-NMR. Furthermore, the ease of evaporation of the hydration water was determined under nonisothermal and isothermal conditions using DSC and water vapor sorption isotherm analysis, respectively, and the relationship between the ease of evaporation and the measured values of T_1 and T_2 was examined.

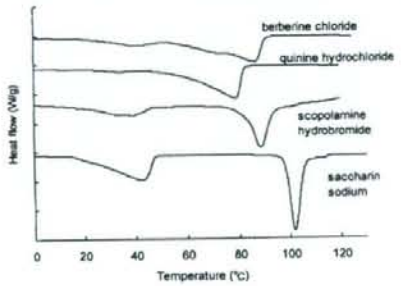

Figure 4. DSC thermograms for API hydrates showing two endothermic peaks.

EXPERIMENTAL

Materials

Cefazolin sodium, ceftazidime, amoxicillin, ampicillin, scopolamine hydrobromide, pipemidic acid, quinidine sulfate hydrates were purchased from Sigma Chemical Co. (St. Louis, MO), and berverine chloride, quinine hydrochloride, saccharin sodium, sulpyrine and di-sodium hydrogen phosphate 12 H_2O were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan), and di-sodium hydrogen phosphate 2 H_2O was from Merck (Darmstadt, Germany).

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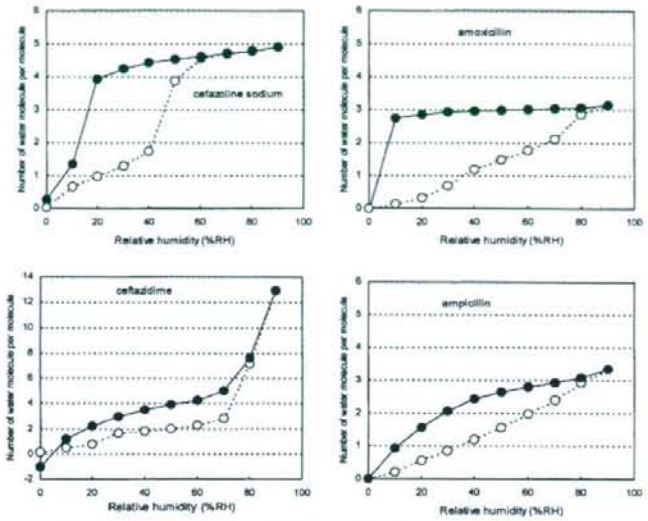

Figure 6. Water sorption isotherms for four antibiotic hydrates.

NMR Relaxation Times

The free induction decay (FID) of protons in API hydrates was obtained using a pulsed NMR spectrometer (25 MHz, JNM-MU25, JEOL, Tokyo, Japan). FID was obtained at 10, 20, 30, and 40°C. The 90° pulses were 2 μs in duration. The "solid echo," with an echo delay of 10 μs, was used in the detection stage of all measurements, in order to overcome the effects of the dead-time.[12] Measurement was repeated four times with a recycling time over five times of the T_1 value measured as described below.

The FID signals obtained between 2.6 and 100 μs that showed only Gaussian-type decay were fitted to Eq. (1) to calculate the T_2 of proton. FID signals obtained for quinidine sulfate and pipemidic acid hydrates showed a small diversion from Gaussian behavior (beat signal) in the final stage of relaxation, suggesting Abragam-type relaxation.[13] However, T_2 was calculated according to Eq. (1) for the purpose of comparison among API hydrates. The FID signals that show both Gaussian and Lorentzian decay patterns were fitted to Eq. (2) representing the summation of the Gaussian and Lorentzian equations.

$$I(t) = I_0 \exp\left[-\left(\frac{t}{T_2}\right)^2\right] \tag{1}$$

$$I(t) = I_0\left[P_G \exp\left(-\left(\frac{t}{T_{2(G)}}\right)^2\right) + P_L \exp\left(-\frac{t}{T_{2(L)}}\right)\right] \tag{2}$$

where $I(t)$ and I_0 are signal intensity at time t and time 0. $T_{2(G)}$ and $T_{2(L)}$ are T_2 for Gaussian decay and Lorentzian decay, respectively, and P_G and P_L are the proportion of protons that show Gaussian decay and Lorentzian decay, respectively.

The T_1 of proton in API hydrates was determined at 30°C by the inversion recovery method. T_1 was calculated according to Eq. (3).

$$I(t) = I_0\left(1 - 2\exp\left(-\frac{t}{T_1}\right)\right) \tag{3}$$

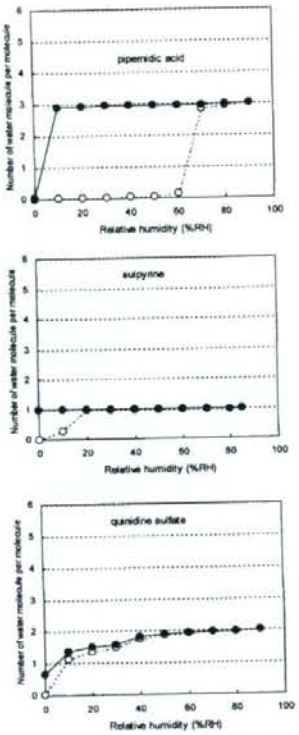

Figure 7. Water sorption isotherms for API hydrates showing a single endothermic peak in DSC thermogram.

Differential Scanning Calorimetry (DSC)

Modulated temperature DSC experiments were performed using a commercial system (2920; TA Instruments, New Castle, DE) attached to a refrigerated cooling accessory. The conditions were as follows: modulation period of 100 s, a modulation amplitude of ±0.5°C, and an underlying heating rate of 1°C/min. Temperature calibration was performed using indium. Samples (approximately 10 mg) were put in a pan without a lid. Nitrogen gas was flowed at 30 mL/min.

Water Sorption Isotherm

Water sorption isotherms were measured gravimetrically at 25°C using the automated sorption analyzer from VTI Corp. (Hialeah, FL). Prior to water sorption and desorption, samples were dried at 60°C and reduced pressure, until the partial vapor pressure became less than 0.0. Equilibrium water content was measured at ascending partial vapor pressures ranging from 0.10 to 0.95, then at descending partial vapor pressures ranging from 0.95 to 0.00 in steps of 0.10 or 0.05. Equilibrium was regarded to have been achieved once the change in sample weight was less than 0.001 mg over 10 min. The limit duration for measurement at a partial vapor pressure was 10 h for scopolamine hydrobromide and 5 h for the others.

RESULTS

NMR Relaxation Times

Figures 1 and 2 show representative examples of the time courses of spin–spin relaxation observed for the 11 API hydrates. Of the four antibiotic hydrates, all exhibited both Gaussian-type decay and Lorentzian decay, as exemplified by ceftazidime and cefazolin sodium hydrates (Fig. 1). The other seven API hydrates exhibited only Gaussian-type decay, as exemplified by quinidine sulfate and scopolamine hydrobromide hydrates (Fig. 2).

In order to calculate the proportion of water protons to API protons, which is required to obtain the T_2 of the water protons by curve-fitting of decay patterns, the number of water molecules per API hydrate molecule was measured by the Karl Fischer method. The results are shown in Table 1, in which the values specified in the JP are also noted for the purpose of comparison. The measured water contents were consistent with those specified in the JP for pipemidic acid, sulpyrine, and quinidine sulfate hydrates, as well as all antibiotic hydrates except for cefazolin sodium hydrate. In contrast, quinine hydrochloride, scopolamine hydrobromide, and saccharin sodium hydrates showed smaller water contents than those specified in the JP.

The time courses of spin–spin relaxation showing both Gaussian decay and Lorentzian decay observed for the four antibiotic hydrates were well fitted to Eq. (2) using the proportion of water protons calculated from the measured water

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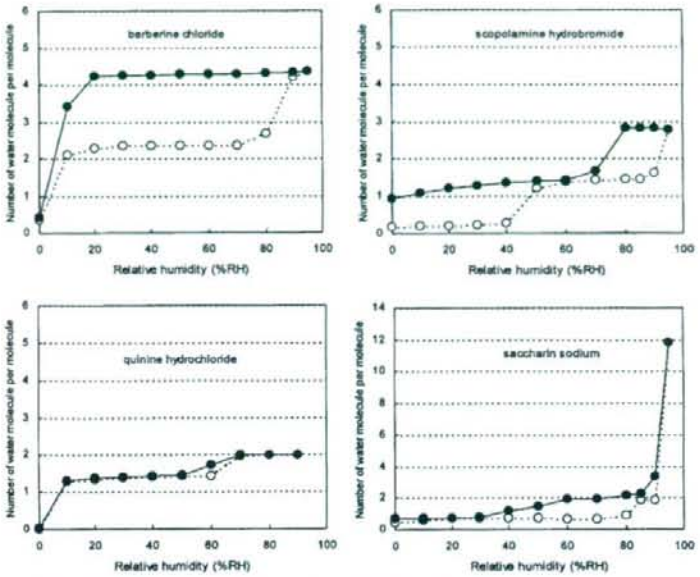

Figure 8. Water sorption isotherms for API hydrates showing two endothermic peaks in DSC thermogram.

content, as shown by the regression curve in Figure 1. Therefore, all of the water protons in the molecule are considered to show Lorentzian decay, and the Gaussian decay is attributed to the drug protons. The T_2 of the Lorentzian decay was calculated according to Eq. (2), and the results will be discussed below. For cefazolin sodium hydrate, better curve-fitting was obtained by regression analysis using a slightly larger value for the proportion of water protons than that calculated from the measured water content. This suggests that a small number of the drug protons exhibit Lorentzian decay; however, it is possible that the water content of the sample used for NMR measurement was different from that of the sample used for Karl Fischer measurements.

The seven API hydrates other than the antibiotic hydrates did not exhibit Lorentzian decay, indicating that all water protons and drug protons in the molecule showed Gaussian decay. The T_2 of the water protons was calculated according to Eq. (1), assuming that the T_2 of the drug protons is similar to that of the water protons. The results will be discussed below.

DSC Thermograms

Figures 3–5 show DSC thermograms measured for the 11 API hydrates. The four antibiotic hydrates, which exhibited Lorentzian decay upon spin–spin relaxation, showed a single endothermic peak due to water evaporation, as shown in Figure 3. In contrast, the API hydrates that did not exhibit Lorentzian decay showed two endothermic peaks (Fig. 4), or one peak (Fig. 5).

The temperature at which an endothermic peak due to water evaporation is observed may be considered to represent the ease of evaporation of hydration water under nonisothermal conditions. The onset temperature was determined as a parameter for approximate comparison of ease of evaporation among the API hydrates, along with ease of evaporation under isothermal conditions as

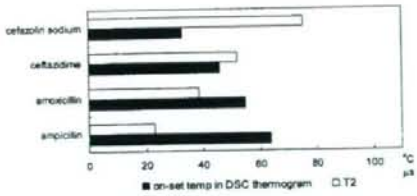

Figure 9. Correlation between onset temperature and T_2 for four antibiotic hydrates.

determined by water vapor sorption analysis. Onset temperature is known to depend on various factors, such as the heating rate, the shapes of the pan and lid, the surface area of the sample, and the flow rate of nitrogen gas. In this study, controllable factors such as the heating rate and the flow rate of nitrogen gas were kept constant, and a pan without a lid was used. The onset temperatures obtained will be discussed below.

Water Vapor Sorption Isotherm

Figures 6–8 show water sorption isotherms observed for the four antibiotic hydrates, the other three API hydrates that exhibited a single endothermic peak due to water evaporation, and the four API hydrates that exhibited two peaks due to water evaporation, respectively. The y-axis represents the number of water molecules per API hydrate molecule, calculated from the water content measured by the Karl Fischer method, assuming that all water molecules present in the sample were evaporated during the drying process

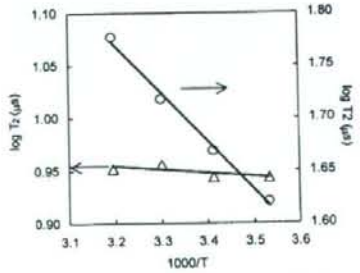

Figure 10. Temperature dependence of T_2 for ceftazidime (circle) and pipemidic acid (triangle) hydrates.

(60°C, reduced pressure) prior to the sorption and desorption processes.

The water sorption isotherms (Fig. 6) observed for the four antibiotic hydrates, which exhibited Lorentzian decay upon spin–spin relaxation, indicate that during the desorption process, the water content decreased with decreasing humidity in the range 90–0% RH, with a significant slope in the water content versus humidity plot.

Among the three API hydrates that did not exhibit Lorentzian decay and showed a single endothermic peak due to water evaporation, pipemidic acid and sulpyrine hydrates gave water desorption isotherms in which the water content was constant over a wide humidity range, as shown in Figure 7. Quinidine sulfate also showed a flat line in the water content versus humidity plot, though it was observed only at high humidities.

The water desorption isotherms observed for the other four API hydrates (except berberine chloride), which did not exhibit Lorentzian decay and showed two endothermic peaks due to water evaporation, indicated that the water content remained approximately constant at two levels (Fig. 8).

DISCUSSION

The molecular mobility of hydration water in API hydrates was found to vary over a wide range; some, such as ceftazidime hydrate, contain hydration water that shows Lorentzian decay upon spin–spin relaxation, while others contain hydration water that shows Gaussian decay.

Hydration Water Showing Lorentzian Decay

All of the water molecules present in the four antibiotic hydrates were found to exhibit Lorentzian decay, because the proportion of Lorentzian decay was consistent with the proportion of water protons calculated from the water content measured by the Karl Fischer method (Fig. 1). The finding that the water molecules in the antibiotic hydrates showed Lorentzian decay rather than Gaussian decay suggests that water molecules are held in voids in the crystal, rather than being firmly trapped in the crystal lattice. These water molecules may evaporate through channels formed in the interior of the crystal.[14] Hydration water that requires more energy to be released

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may exhibit a higher onset temperature of the endothermic peak due to water evaporation in DSC.

The T_2 values determined based on Lorentzian decay is related with τ_c by Eq. (4), such that a smaller value of T_2 represents a larger τ_c (lower mobility).

$$\frac{1}{T_2} = \frac{\gamma^4 \hbar^2 I(I+1)}{5r^6}\left(3\tau_c + \frac{5\tau_c}{1+\omega_0^2\tau_c^2} + \frac{2\tau_c}{1+4\omega_0^2\tau_c^2}\right) \quad (4)$$

where γ, ω_0, I, r, and $\hbar$ are the gyromagnetic ratio, resonance frequency, spin quantum number, spin distance, and the Plank's constant divided by 2π.

As shown in Figure 9, T_2 increased as the onset temperature (Fig. 3) decreased, indicating that hydration water which evaporates at lower temperatures has greater molecular mobility as determined by T_2. This correlation between T_2 and the ease of evaporation under nonisothermal conditions may be explained by assuming that hydration water with a greater T_2 (higher mobility) can escape through channels at a lower temperature.

In order to gain further insight into the correlation between ease of evaporation and the molecular mobility of the hydration water, the ease of evaporation under isothermal conditions was evaluated by water sorption isotherm measurement. Each of the four antibiotic hydrates exhibited a desorption isotherm showing decreases in water content associated with decreases in humidity (Fig. 6). As discussed below, the crystal form of ampicillin hydrate appeared to be altered during the drying process prior to the measurement of water sorption isotherms. Therefore, the isotherm obtained for ampicillin could not be compared with the NMR and DSC data. However, such detrimental effect of predrying was not observed for the other three antibiotic hydrates. The negative water content observed after the desorption process for ceftazidime may be due to chemical degradation occurred under high-humidity conditions or incomplete evaporation of hydration water during predrying. Compared to amoxicillin hydrate, cefazolin sodium hydrate, which has a larger T_2 value, exhibited a greater slope in its water content versus humidity plot. Furthermore, cefazolin sodium exhibited rapid dehydration when humidity was decreased below 20% RH, whereas amoxicillin did not exhibit rapid dehydration until humidity was decreased below 10% RH. These findings suggest that the ease of evaporation of hydration water under isothermal conditions is correlated with molecular mobility as determined by T_2, which supports the conclusion obtained based on DSC measurement. For ampicillin, the slope of the water content versus humidity plot was greater than that of amoxicillin hydrate despite its lower molecular mobility as determined by T_2 and higher onset temperature. This suggests that the drying conditions prior to the sorption and desorption processes were inadequate, which may result in destruction of the crystalline structure. Thus, the isotherm obtained for ampicillin could not be compared with the NMR and DSC data.

As exemplified by ceftazidime hydrate (Fig. 10), T_2 increased significantly with increasing temperature, indicating that T_2 reflects the increases in molecular mobility associated with increases in temperature. Thus, molecular mobility can be considered to correlate with T_2. As shown in Figure 11, antibiotic hydrates with smaller T_2

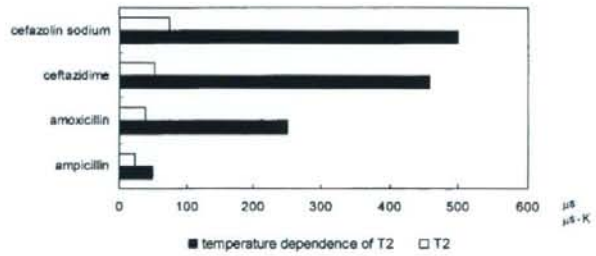

Figure 11. Correlation between T_2 and temperature dependence of T_2 for four antibiotic hydrates.

values showed a smaller change in T_2 with temperature change. This finding suggests that lower values of T_2 reflect a smaller scale of molecular motion, with lower activation energies.

Spin–lattice relaxation time (T_1) is known to reflect molecular mobility, similarly to T_2, but increases with decreasing T_2 (with decreasing molecular mobility) in the slow motional regime. The T_1 values of water protons in the presence of drug protons cannot be determined due to spin diffusion, but an approximate determination of T_1 for water protons is possible if the proportion of water protons is large. For example, in $Na_2HPO_4 \cdot 12H_2O$ and $Na_2HPO_4 \cdot 2H_2O$, water protons are predominant (24/25 and 4/5, respectively). $Na_2HPO_4 \cdot 12H_2O$ exhibits slower spin–spin relaxation (larger T_2) (Fig. 12), and faster spin–lattice relaxation (smaller T_1) (Fig. 13) compared to $Na_2HPO_4 \cdot 2H_2O$, which indicates that both T_1 and T_2 reflect the molecular mobility of hydration water. For the antibiotic hydrates examined, however, correlations between T_1 and T_2 were not observed, as shown in Figure 14. This finding indicates that for API hydrates containing a significant amount of drug protons, such as antibiotic hydrates, the molecular mobility of the hydration water is not reflected in T_1.

Hydration Water Showing Gaussian Decay

As mentioned previously, all of the API hydrates other than the four antibiotic hydrates exhibited only Gaussian decay (Fig. 2). The value of T_2 did not vary significantly among the API hydrates, as shown in Figure 15. Furthermore, the onset temperatures of the single endothermic peaks

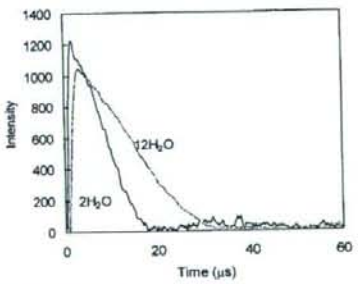

Figure 12. Free induction decay for Na_2HPO_4 $12H_2O$ and $2H_2O$.

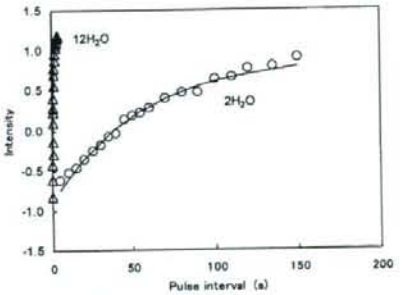

Figure 13. Spin–lattice relaxation for Na_2HPO_4 $12H_2O$ and $2H_2O$.

due to water evaporation for quinidine sulfate, pipemidic acid, and sulpyrine hydrates (Fig. 5), as well as each of the two peaks due to water evaporation observed for quinine hydrochloride, scopolamine hydrobromide, saccharin sodium, and berberine chloride hydrates (Fig. 4), were not correlated with T_2. These findings indicate that the molecular mobility of hydration water that shows Gaussian decay is too low to be reflected in T_2. No correlation between T_2 and molecular mobility is supported by the finding that changes in T_2 associated with changes in temperature were much smaller than those observed for the antibiotic hydrates that exhibited Lorentzian decay, as exemplified by pipemidic acid (Fig. 10). Such low molecular mobility may be attributed to water molecules firmly trapped in the crystal lattice, rather than water molecules trapped in voids in the crystal.

For quinidine sulfate, pipemidic acid, and sulpyrine hydrates, a single endothermic peak was observed in DSC (Fig. 5). The water content versus humidity plots showed a flat line at a certain number of water molecules. Pipemidic acid and sulpyrine showed a flat line at three and one water molecule(s) per hydrate, respectively, and evaporation of these water molecules was observed only under very low humidity (Fig. 7). These findings indicate that water molecules are firmly trapped in the crystal.

For quinine hydrochloride, scopolamine hydrobromide, saccharin sodium, and berberine chloride hydrates, two endothermic peaks were shown in DSC (Fig. 4). The water content versus humidity plots for these hydrates (except for berberine chloride) showed flat lines at two levels

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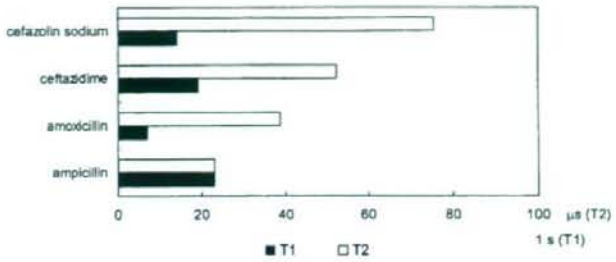

Figure 14. Correlation between T_1 and T_2 for four antibiotic hydrates.

of water content (Fig. 8), suggesting the presence of two water populations: molecules that evaporate at high humidity, and others that evaporate at lower humidity (below 10% RH). This seems to be consistent with the observation of two endothermic peaks in DSC. The endothermic peak observed at a high temperature and the flat line observed at a low humidity may be attributable to hydration water with strong hydrogen-bonding interactions, while the one observed at a lower temperature and higher humidity may be attributable to hydration water with week interactions. The presence of hydration water with week interactions is also supported by the finding that the water contents as measured by the Karl Fischer method were smaller than those specified in the JP (Tab. 1).

CONCLUSION

It was found that spin–spin relaxation time, T_2, is a useful parameter that can indicate the molecular mobility of water of hydration which has relatively high mobility and shows Lorentzian decay upon spin–spin relaxation. For these water molecules, molecular mobility as determined by T_2 is correlated with ease of evaporation both under nonisothermal and isothermal conditions,

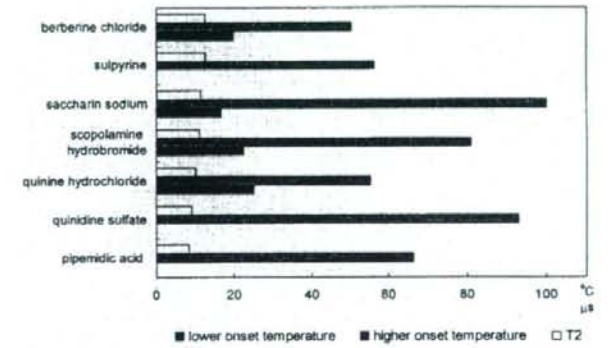

Figure 15. Correlation between onset temperature and T_2 for API hydrates that show Gaussian decay.

such that water molecules with greater ease of evaporation have higher T_2 values.

In contrast, for hydration water that has low mobility and shows Gaussian decay, T_2 was found not to correlate with ease of evaporation under nonisothermal conditions, suggesting that molecular motion that determines the ease of evaporation is not reflected in T_2; in this case, T_2 cannot be used as a parameter to indicate molecular mobility.

The water molecules in the API hydrates studied were found to have wide-ranging molecular mobilities, from low molecular mobility that could not be evaluated by NMR relaxation times, such as the water molecules in pipemidic acid hydrate, to high molecular mobility that could be evaluated by NMR relaxation times, such as the water molecules in ceftazidime hydrate.

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Pharmaceutical Nanotechnology

Hydroxyethylated cationic cholesterol derivatives in liposome vectors promote gene expression in the lung

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Abstract

Three cationic cholesterol derivatives (CCDs), which differ in their types of amine and bear a hydroxyethyl group at the amine group, were synthesized and formulated into liposomes and nanoparticles as gene delivery vectors. *In vitro* transfection into A549 cells proved that liposomes formulated with CCDs and dioleoylphosphatidylethanolamine (DOPE) of 1/2 molar ratio were more effective than the corresponding nanoparticles with CCDs and Tween 80 at charge ratios (+/−) of 1/2, 3/1 and 5/1. Among the liposomal formulations, non-hydroxyethylated CCDs were more effective than hydroxyethylated ones *in vitro*. However, gene transfection in the lung through intratracheal injection showed opposite results to those *in vitro*, with liposomes containing hydroxyethylated CCDs being more potent than those containing non-hydroxyethylated CCDs. Transfection by liposomes with *N*,*N*-methyl hydroxyethyl aminopropane carbamoyl cholesterol iodide (MHAPC) showed the highest luciferase activity, resulting in 2- and 60-fold higher gene expression than jet-PEI and naked DNA, respectively. The distribution of MHAPC lipoplex after intratracheal injection was heterogeneous, and luciferase was expressed in epithelial cells lining the bronchi and bronchioles. All the lipoplexes led to higher TNF-α levels in the lung compared to the nanoplex and jet-PEI, but our findings suggested that modification of the cationic cholesterol with a hydroxyethyl group at the tertiary amine terminal, MHAPC, promoted gene expression in the lung without increasing the toxicity compared with other CCDs. This work firstly proved that liposomes containing hydroxyethylated CCDs could promote gene expression in the lung through intratracheal injection.

Keywords: Cationic liposomes; Nanoparticles; Intratracheal injection; Cationic cholesterol derivatives; Hydroxyethyl group

1. Introduction

Gene therapy in the lung still holds promise as an effective method for treating cystic fibrosis and lung neoplastic disease (Hoag, 2005). Gene delivery vectors are classified into viral and non-viral ones, and both of them have been well studied. Cationic lipids (Miller, 2003) and polymers (Pietersz et al., 2006), which can compact negatively charged genetic materials through electrostatic interaction and transport them into the cells, constitute a large fraction of non-viral vectors. Many cationic lipids have been reported to mediate gene delivery *in vitro* and *in vivo* (Miller, 2003), and more are being reported all the time.

After the discovery of 3β-[*N*-(*N*′,*N*′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) (Gao and Huang, 1991), many effective cationic cholesterol derivatives (CCDs) were soon developed (Ghosh et al., 2002; Hasegawa et al., 2002; Miller, 2003; Nakanishi, 2003; Percot et al., 2004; Bajaj et al., 2007). CCDs are amphiphilic molecules that consist of a cationic headgroup attached via a linker to the cholesteryl skeleton. Clearly, the linker and the cationic headgroup are crucial for the gene transfection ability and toxicity. The inability of CCD-containing cationic liposomes to produce persistent gene expression (Scheule et al., 1997) results in a need for repeated dosing of the liposomes. Therefore, biodegradable CCDs with a linker such as carbamate ester, which can facilitate degradation *in vivo*, are strongly recommended for the design and synthesis of CCDs (Choi et al., 2001). In fact, this strategy has already been verified to be effective by the low toxicity of DC-Chol (Gao and Huang,

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1991) and cationic 3β-[L-lysinamide-carbamoyl]cholesterol derivatives (K-Chol) (Choi et al., 2001; Lee et al., 2006).

Moreover, the amine headgroups of cationic lipids definitely determine their transfection ability (Reynier et al., 2004). Among various amine headgroups, hydroxyethyl group-containing ones have exhibited higher gene transfection than the corresponding hydroxyethyl-lacking ones (Okayama et al., 1997; Venkata Srilakshmi et al., 2002; Arpicco et al., 2004). Cholesteryl-3β-carboxyaminoethylene-*N*-hydroxyethylamine (OH-Chol) is a cationic cholesterol with a hydroxyethyl group at the amine headgroup, linked to the cholesteryl skeleton by an amido bond. Liposomes containing OH-Chol and phosphatidylethanolamine (DOPE) showed high gene transfection ability (Okayama et al., 1997). Furthermore, a nanoparticle formulation with OH-Chol exhibited excellent gene transfection. Their high gene transfection activity was ascribed to the hydroxyethyl group at the cationic headgroup of OH-Chol (Hattori et al., 2007). The combination of a hydroxyethyl group at the headgroup and amine lipids, therefore, will produce effective cationic carbamate-linked lipids for gene delivery vectors.

To develop highly potent and biodegradable CCDs, in this study, we synthesized three types of CCDs bearing secondary, tertiary and quaternary amines, with a carbamate ester linker and a hydroxyethyl group at the amine headgroup. The synthesized CCDs were formulated into nanoparticles and liposomes, and their formulations were optimized for gene transfection of the human lung adenocarcinoma A549 cell line and into the mouse lung through intratracheal injection. The location of luciferase expression was studied by immunohistochemistry. Furthermore, the inflammatory response of the nanoplexes and lipoplexes was evaluated.

2. Materials and methods

2.1. Materials and instrumentation

DC-Chol and cholesterol chloroformate were purchased from Sigma–Aldrich (St. Louis, USA). *N,N*-Dimethyl-1,3-propanediamine; *N*-methyl-1,3-propanediamine; 1,3-propanediamine; 2-iodoethanol were purchased from Wako Pure Chemistry (Osaka, Japan). Tween 80 was obtained from NOF Co. Ltd. (Tokyo, Japan) and DOPE was from Avanti Polar Lipids Inc., (Alabaster, AL, USA). The synthesis of OH-Chol was done as previously reported (Hattori et al., 2005). RPMI-1640 culture medium was purchased from Invitrogen Corp. (Carlsbad, CA, USA). ^{1}H NMR (270 MHz) and ^{13}C NMR (67.8 MHz) spectra were recorded using tetramethylsilane as an internal standard with a JEOL JNM-LA270 spectrometer. Chemical ionization (CI) was carried out on JEOL JMS 600 (JEOL, Tokyo, Japan). The plasmid pCMV-luc encoding the luciferase gene under the control of the CMV promoter was constructed as previously described (Igarashi et al., 2006). A protein-free preparation of the plasmid was purified following alkaline lysis using Maxiprep columns (Qiagen, Hilden, Germany).

2.2. Synthesis of cationic cholesterol derivatives (Fig. 1)

2.2.1. *N,N,N-Dimethyl aminopropane carbamoyl cholesterol iodide (DMAPC, 1) and N,N,N-dimethyl hydroxyethyl aminopropane carbamoyl cholesterol iodide (DMHAPC, 2)*

1 was synthesized as described by Percot et al. (2004) and hydroxyethylated to **2** with some modifications. **1** and iodoethanol were refluxed in toluene (with a catalytic amount of DMF) at 105 °C for 24 h. Silica-gel chromatography with $CHCl_3$/methanol for elution gave **2** (yield 70%), a pale yellow powder.

2.2.2. *N-Hydroxyethyl aminopropane carbamoyl cholesterol iodide (HAPC, 3)*

A solution of cholesterol chloroformate (2.7 g, 6 mmol) in 10 ml of dry dichloride methylene (DCM) was slowly added to a pre-cooled solution of 1,3-propanediamine (5 ml, 60 mmol) in 100 ml of DCM. The mixture was further stirred at RT for 1 h. After the reaction, the solvent was removed by vacuum evaporator and the residue was purified on silica gel to give aminopropane carbamoyl cholesterol (2.8 g, 95%). 2.8 g (5.75 mmol) of aminopropane carbamoyl cholesterol was dissolved in a mixture of DCM/methanol with 1 equiv. of triethylamine, and iodoethanol (1.9 mmol, 150 μl) was added and

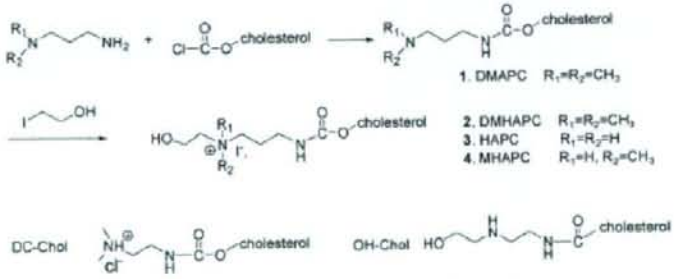

Fig. 1. Chemical structures and synthesis of cationic cholesterol derivatives.

stirred at RT for 24 h. Careful purification on silica-gel chromatography with $CHCl_3$/methanol for elution gave **3** (1.2 g, 31%). ^1H NMR ($CDCl_3$) δ: 7.62 (s, 1H, –CO–NH–C), 5.36 (t, 3H, C=CH–C), 4.41 (m, 1H, CH–O–CO–), 4.1 (m, 2H, –C–CH_2–O). ^{13}C NMR ($CDCl_3$) δ: 157.2 (NH–CO–O), 139.5, 122.5 (C=CH), 74.9 (=CH–O), 64.9 (–CH_2–OH). CI-MS *m/z*: found 658 (Calcd for $C_{33}H_{59}IN_2O_3$, 658.36).

2.2.3. *N,N-Methyl hydroxyethyl aminopropane carbamoyl cholesterol iodide (MHAPC, 4)*

Similarly to **3**, **4** (3.6 g, 94%) was synthesized from *N*-methyl-propanediamine (3.7 ml, 30 mmol) and cholesterol chloroformate (2.7 g, 6 mmol) and subsequently hydroxyethylated with iodoethanol (6.84 mmol, 540 μl). ^1H NMR ($CDCl_3$) δ: 7.91 (s, 1H, –CO–NH–C), 5.36 (t, 3H, C=CH–C), 4.41 (m, 1H, CH–O–CO–), 4.1 (m, 2H, –C–CH_2–O), 2.92 (s, 3H, CH_3–N^+). ^{13}C NMR ($CDCl_3$) δ: 158.1 (NH–CO–O), 139.2, 122.8 (C=CH), 74.8 (=CH–O), 63.2 (–CH_2–OH). CI-MS *m/z*: found 673 (Calcd for $C_{34}H_{61}IN_2O_3$, 672.37).

2.3. Preparation of liposomes/lipoplexes and nanoparticles/nanoplexes

The synthesized CCDs, namely DMAPC, DMHAPC, HAPC and MHAPC, together with DC-Chol and OH-Chol (Fig. 1), were formulated into liposomes with DOPE and into nanoparticles with 5% Tween 80 by a modified ethanol injection method (Hattori et al., 2005). The molar ratio of CCDs to DOPE in the liposomes was varied from 2/1 to 1/1 to 1/2. Each type of liposomes and nanoparticles contained 0.9 mM CCD lipid concentration for *in vitro* transfection and 4.5 mM for *in vivo* experiments.

The CCD liposome/DNA complex (CCD lipoplex) and CCD nanoparticle/DNA complex (CCD nanoplex) for *in vitro* transfection at various charge ratios (+/−) of CCD to DNA were prepared by addition of each liposome or nanoparticle preparations (1.67, 10, 16.7 μl for the charge ratio (+/−) of 1/2, 3/1 and 5/1) to 1 μg of DNA in 5 μl of MilliQ water with 10 rounds of pipetting. After the preparations were left at room temperature for 15 min, the size of each lipoplex/nanoplex in water was measured after incubation with RPMI-1640 medium for a further 15 min. The mean particle size was measured by the dynamic light scanning method (ELS-Z2, Otsuka Electronics Co. Ltd., Osaka, Japan). For the *in vivo* study, the lipoplexes and nanoplexes at charge ratio (+/−) of 3/1 were prepared by the addition of 40 μl of liposomes or nanoparticles, respectively to 20 μg of DNA in 25 μl of MilliQ water. The total injection volume was fixed at 65 μl per mouse.

2.4. Cell culture

The human lung adenocarcinoma A549 cell line was kindly provided by OncoTherapy Science, Inc. (Kanagawa, Japan). The cells were maintained in RPMI-1640 medium supplemented with 10% FBS and kanamycin (100 μg/ml) at 37 °C in a 5% CO_2 humidified incubator.

2.5. Gene transfection in A549 cell line and in the lung

For transfection into A549 cells, the lipoplexes or nanoplexes were diluted in 500 μl of 10% FBS supplemented RPMI-1640 and then incubated with the cells in 12-well plates for 24 h. As a positive control, the Lipofectamine 2000 (LA2000, Invitrogen Corp.)/DNA complex was prepared according to the manufacturer's protocol.

To study the gene expression in the mouse lung, intratracheal injection through the exposed trachea was used as an injection method. Briefly, a ddY mouse (male, 5 weeks of age, Sankyo Lab., Shizuoka, Japan) was anesthetized with phenobarbital sodium (50 μg/g body weight) by intraperitoneal injection (i.p.). Then the mouse was positioned in a vertical position and the trachea was exposed by blunt dissection of the neck. Sixty-five microliters of complex suspension per mouse was bolus injected into the trachea using a 29G injection syringe. The jet-PEI (polyplus-transfection, NY, USA)/DNA complex was prepared at a (+/−) ratio of 5/1 according to the manufacturer's instructions.

2.6. Luciferase assay and TNF-α in the lung tissue

Luciferase expression in A549 cells was measured as counts per second (cps)/μg total protein using the luciferase assay system (Picagene, Tokyo Ink Mfg. Co. Ltd., Tokyo, Japan) and BCA reagent (Pierce, IL, USA) as previously described (Maitani et al., 2007).

The luciferase in the lung was measured 24 h after intratracheal injection. Mice were anesthetized with ethyl ether and the lung was perfused with 10 ml of PBS through the left ventricle to remove the blood. The lung was collected with minimal main bronchi and immediately homogenized in 500 μl of cold lysis buffer (Promega Co., Madison, WI, USA). The homogenate samples were centrifugated at 15,000 rpm for 5 min at 4 °C and the luciferase assay was done as described above.

For TNF-α measurement, lysates were prepared exactly as described for the luciferase assay in the lung. TNF-α levels were determined using a mouse TNF-α ELISA kit (R&D, Minneapolis, MN, USA).

2.7. Immunohistochemistry of luciferase in the lung

0.01% (molar percentage of lipids) rhodamine-DHPE (*N*-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt) labeled MHAPC liposomes (MHAPC/DOPE=1/2, molar ratio) were prepared with MHAPC concentration of 4.5 mM as described in Section 2.3. The lipoplex was prepared with 20 μg of DNA at a charge ratio (+/−) of 3/1. The lipoplexes were intratracheally injected into mouse lung, and the lung was collected at 24 h. The frozen lungs were cryosectioned into 12 μm slices. The sections were fixed with 70% ethanol and washed in PBS before incubation with primary goat anti-luciferase pAb (1:100) (Promega Co.). The sections were then incubated in bovine serum albumin (BSA) to reduce non-specific binding of a secondary antibody. Finally, they were incubated with rabbit

anti-goat IgG–HRP (Santa Cruz Biotech, Santa Cruz, CA, USA) for 2 h. The color was developed using a peroxidase substrate kit DAB SK-400 (Vector Lab, Inc. Burlingame, CA, USA).

3. Results and discussion

3.1. DOPE content in liposomes affected gene transfection

DOPE played a very important role in the destabilization of liposomes upon contact with cellular membranes and/or endosomes. In the formulations of cationic liposomes containing DOPE as a helper lipid, most studies used cationic lipids/DOPE at a molar ratio of 2/1, 3/2 or 1/1 as the optimum formulation (Miller, 2003). Recently we reported that DC-Chol/DOPE (1/2, molar ratio) liposomes, which were prepared by a modified ethanol injection method, were more effective for gene transfection than DC-Chol/DOPE = 1/1 and 3/2 liposomes (Maitani et al., 2007). To optimize the CCD liposomes, therefore, at first the DOPE content in liposomes was investigated in an attempt to determine the most effective formulations. As shown in Fig. 2, all the lipoplexes showed higher gene transfection in A549 cells with increasing molar ratio of DOPE (CCD/DOPE = 1/2, molar ratio) at a charge ratio (+/−) of 3/1, lipoplexes with less DOPE content (CCD/DOPE = 1/1 and CCD/DOPE = 2/1) were not effective enough to transfect A549 cells. Therefore, a liposome formulation rich in DOPE content, CCD/DOPE = 1/2-liposomes, was used for further studies. The lipoplexes were named DC-Chol, DMAPC, DMHAPC, HAPC and MHAPC lipoplexes.

3.2. Characterization of lipoplexes and nanoplexes

Although liposomes rich in DOPE had higher gene transfection ability than those poor in DOPE, they were only 1/10 to 1/50 as effective as LA-2000 (Fig. 2). Since a nanoparticle formulation with OH-Chol and Tween 80 has shown excellent gene transfection (Hattori et al., 2007), we prepared CCD nanoparticles using Tween 80 as well as CCD liposomes and investigated their use for gene transfection in A549 cells at three charge ratios (+/−) of 1/1, 3/1 and 5/1.

The liposomes and nanoparticles prepared by modified ethanol injection had a mean particle size of about 200 nm, with zeta-potential from +40 to +60 mV, except for OH-Chol/DOPE (1/2, molar ratio) liposomes, which were about 400 nm in size.

To establish the relationship between gene transfection and particle size, the size of lipoplexes (Fig. 3A) and nanoplexes (Fig. 3B) was measured after incubation with RPMI-1640 culture medium. CCD lipoplexes or nanoplexes showed a mean

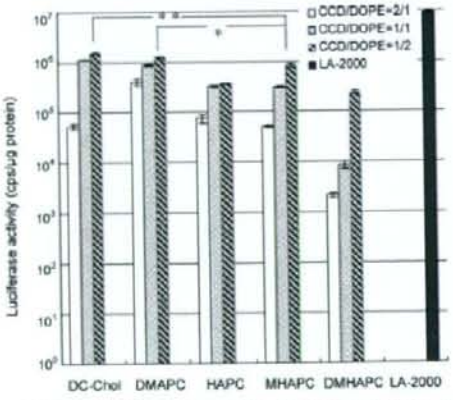

Fig. 2. Effect of DOPE composition in CCD lipoplexes on transfection efficiencies. LA-2000 is lipofectamine-2000. Charge ratio (+/−) was 3/1 and the amount of DNA was 1 μg/well. The values are expressed as mean ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, Student's t test.

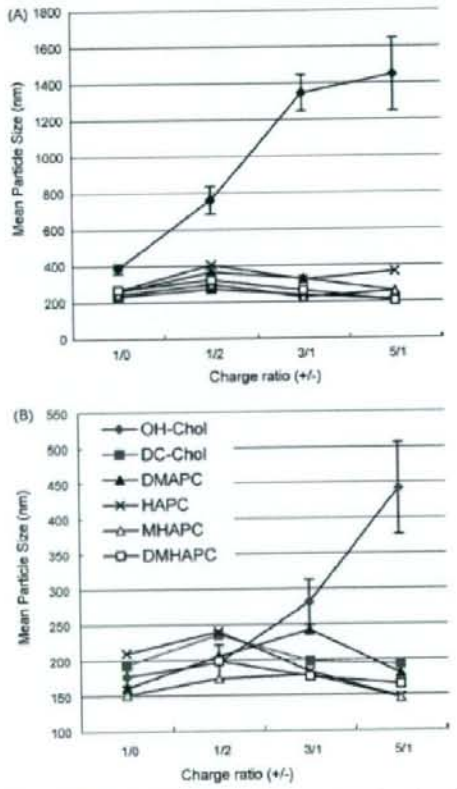

Fig. 3. Particle size of lipoplexes (A) and nanoplexes (B) after incubation with RPMI-1640 medium. The size of each lipoplex and nanoplex in water was measured after incubation with RPMI-1640 medium for 15 min. The values for OH-Chol were expressed as mean ± S.D., other values are expressed as mean values ($n = 3$).

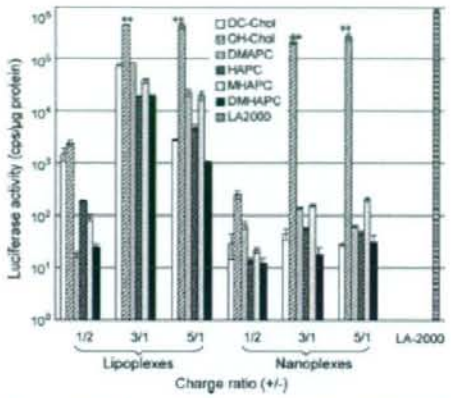

Fig. 4. Transfection efficiencies of lipoplexes and nanoplexes in A549 cells. The values were expressed as mean ± S.D. ($n = 3$). **Significant difference between OH-Chol and other CCDs in the same charge ratio group ($P < 0.01$, Student's t test). The values are expressed as mean ± S.D. ($n = 3$).

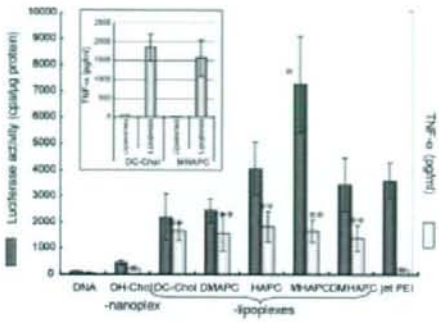

Fig. 5. Luciferase activity (closed columns) and TNF-α (open columns) in the lung at 24 h after intratracheal injection of lipoplexes and nanoplexes (+/− = 3/1). *Significant difference from OH-Chol nanoplex, jet-PEI, DC-Chol and DMHAPC lipoplexes ($P < 0.05$, Student's t test). **Significant difference from OH-Chol nanoplex and jet-PEI ($P < 0.01$, Student's t test). The values are expressed as mean ± S.D. ($n = 3$). The inset showed the TNF-α values of DC-Chol and MHAPC liposomes or lipoplexes in the lung at 24 h after intratracheal injection.

particle size from 150 to 300 nm at a charge ratio (+/−) of 3/1. The OH-Chol liposomes and nanoparticles formed large particles with DNA at charge ratios (+/−) of 3/1 and 5/1, and, therefore, for the *in vivo* study, we selected OH-Chol nanoplexes only at a charge ratio (+/−) of 3/1 as the optimum formulation.

3.3. Comparison between lipoplexes and nanoplexes for gene transfection in A549 cells

As shown in Fig. 4, the lipoplexes were far more effective than nanoplexes at the same charge ratio, especially at charge ratios (+/−) of 3/1 and 5/1. This may be explained by the contribution of the membrane destabilization role of DOPE in the liposomes. Among the six kinds of cationic cholesterol derivatives, OH-Chol lipoplexes and nanoplexes exhibited significantly elevated gene transfection, which may have been due to many factors, such as the amido linker and hydroxyethyl group in the structure of OH-Chol (Okayama et al., 1997), and some physical characteristics of the OH-Chol lipoplex and nanoplex, such as large particle size as shown in Fig. 3. The large particle size of OH-Chol lipoplex and nanoplex contributed partly to the high gene transfection in A549 cells, since large particles were more readily endocytosized into cells.

Furthermore, by comparing various lipoplexes at a charge ratio (+/−) of 3/1, we can see that the non-hydroxyethylated cationic cholesterol derivatives, DC-Chol and DMAPC, were more effective than hydroxyethylated ones (except for OH-Chol), namely DMHAPC, HAPC and MHAPC. Moreover, MHAPC, which bears a tertiary amine in the headgroup, exhibited a little higher transfection ability than HAPC and DMHAPC at the optimized formulation (CCD/DOPE = 1/2, molar ratio). Based on the higher gene transfection of lipoplexes than nanoplexes in A549 cells, we selected lipoplexes at a charge ratio (+/−) of 3/1 for further *in vivo* research.

3.4. Luciferase expression and TNF-α levels in the lung

Bolus intratracheal injection through the exposed bronchui of mouse was employed for all the *in vivo* studies. This administration method guarantees that 100% of the injected solution reaches the lung instantly (Driscoll et al., 2000). As long as the lipoplexes and nanoplexes can be stabilized under the mucus during distribution of the suspension in the lung, they have a chance to transfect the epithelial cells and even the alveolar cells in the lung.

As shown in Fig. 5, use of the lipoplexes resulted in much higher gene expression in the lung than use of OH-Chol nanoplexes, which were the most effective in A549 cells. One possible reason for this may be the insufficient DNA-protecting ability of OH-Chol nanoplexes in the presence of mucus and surfactants in the lung, while the lipoplexes might be able to encapsulate DNA in highly ordered multilammellar structures. Among these lipoplexes, DMHAPC, HAPC, and MHAPC lipoplexes, which were all hydroxyethylated in the cationic terminal, showed higher luciferase level than DC-Chol and DMAPC lipoplexes, which were not hydroxyethylated. In contrast to the *in vitro* data, the lipoplexes containing hydroxyethylated cationic cholesterol derivatives most strongly promoted gene expression in the lung. Although it is unclear how a hydroxyethyl group at the amine headgroup improves transfection, the hydroxyethyl moiety may affect the interaction between DNA and the cationic lipid membrane, and assist cellular association or some steps after internalization into the cells (Nakanishi and Noguchi, 2001).

Interestingly, the use of MHAPC lipoplexes, which contained a hydroxyethylated tertiary amine as the cationic part, resulted in 2- and 60-fold higher gene expression than the use of jet-PEI and naked DNA, respectively. The exact mechanism by which

the hydroxyethyl group in the cationic part and the tertiary amine in MHAPC increased gene expression is not known, but might be related to the stability of MHAPC lipoplexes in the presence of mucus and/or increased release of DNA from the lipoplexes in the acidic endosomal compartment.

Although the use of lipoplexes resulted in much higher gene expression in the lung, the lung seemed to have some inflammatory response to the lipoplex suspensions. The lipoplexes induced higher TNF-α secretion than OH-Chol nanoplexes and jet-PEI. The strong inflammatory response to lipoplexes was thought to be related to the lipoplexes themselves, since only low levels of TNF-α were detected with the DNA alone and DC-Chol and MHAPC liposomes alone (Fig. 5, inset). Furthermore, the high DOPE content in the lipoplexes might also have been responsible for the inflammatory response, since both OH-Chol nanoplexes and MHAPC nanoplexes induced low levels of TNF-α (data not shown). The present data were in accord with a report showing that lung toxicity observed with lipoplexes could be increased by the addition of DOPE, although DOPE suspension alone caused a negligible inflammatory response (Scheule et al., 1997).

3.5. Charge ratio of MHAPC lipoplex affected gene transfection

As shown in Fig. 3, the positively charged (+/− = 3/1) MHAPC lipoplexes were far more effective than negatively charged ones (+/− = 1/2) in A549 cells. Since most reports showed that nearly neutral or negatively charged lipoplexes/nanoplexes can produce higher gene expression in tumor tissues than positively charged ones (Miller, 2003; Hattori et al., 2007), we investigated the effect of the charge ratio (+/−) of MHAPC lipoplexes on gene transfection in the lung. In Fig. 6, it can be seen very clearly that the *in vivo* result corresponded to the *in vitro* one, with positively charged (+/− = 3/1) lipoplexes being significantly more effective than negatively charged ones. Since there are large amounts of surfactants and proteins in the lung lavage fluids and much mucin covers the epithelial cells in the lung, negatively charged lipoplexes might have a small chance of being retained as intact particles to transfect epithelial cells, whereas positively charged lipoplexes might be stable enough to exhibit gene expression ability (Rosenecker et al., 2003).

3.6. Distribution of MHAPC lipoplexes in the lung and localization of luciferase by immunostaining

Since MHAPC lipoplexes at a charge ratio (+/−) of 3/1 produced the highest gene expression in the lung, we investigated the distribution of rhodamine-labeled MHAPC lipoplexes and the location of the luciferase expression they produced after intratracheal injection. By observing the fluorescence of rhodamine in the cryosections, it was clear that rhodamine was mainly distributed throughout the bronchi and bronchioles, and some had even diffused to the alveolar cells (Fig. 7a and b). However, the distribution of lipoplexes was not homogeneous throughout the lungs: no fluorescence of rhodamine was

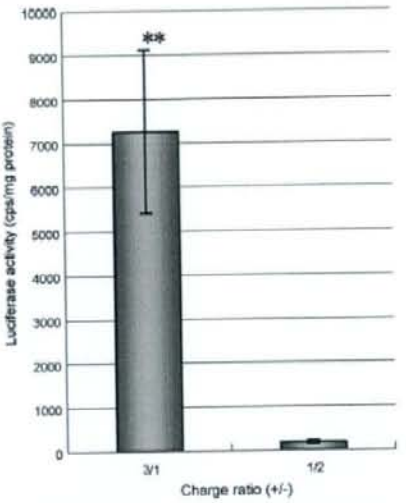

Fig. 6. Effect of charge ratio (+/−) of MHAPC lipoplexes on gene expression in the lung. **, Significant difference ($P < 0.01$, Student's t test). The values are expressed as mean ± S.D. ($n = 3$).

observed in some other regions in the same slice (Fig. 7c and d). From the morphological observation of the lungs which received the MHAPC lipoplex suspension, only the upper and middle lobes exhibited an increased inflammatory response compared to normal lung tissue, indicating that the intratracheally injected lipoplex was mainly located in the upper and middle lobes (photos not shown).

After luciferase immunostaining (Fig. 7e–h), the fluorescence of rhodamine was markedly decreased after many rounds of washing. The fluorescence was only located in the epithelial cells of the bronchi and bronchioles (Fig. 7f and h), and luciferase was also only expressed in the epithelial cells (Fig. 7e, arrows). Although some fluorescence of rhodamine was seen in the alveolar cells (Fig. 7b), there was no luciferase expression in the alveolar cells (Fig. 7e). We suppose that the fluorescence in the alveolar cells was mainly caused by free rhodamine-DHPE that became separated from the lipoplexes in the lung.

Lipoplexes administered by intravenous injection can be captured by the vascular system in the lung and induce gene expression in the lung alveolar region (Scheule et al., 1997). Since intratracheal injection has limited injection volume (1–2 ml/kg weight), the injected lipoplexes are directly exposed to the mucus around the bronchi and bronchioles, resulting in gene expression only in the epithelial cells lining the bronchi and bronchioles. This mode of injection, however, avoids gene expression in other organs, making it possible to decrease the dose of lipoplexes and possibly to decrease toxic side effects, and provides a potentially effective way for gene therapy of cystic fibrosis and other lung diseases.

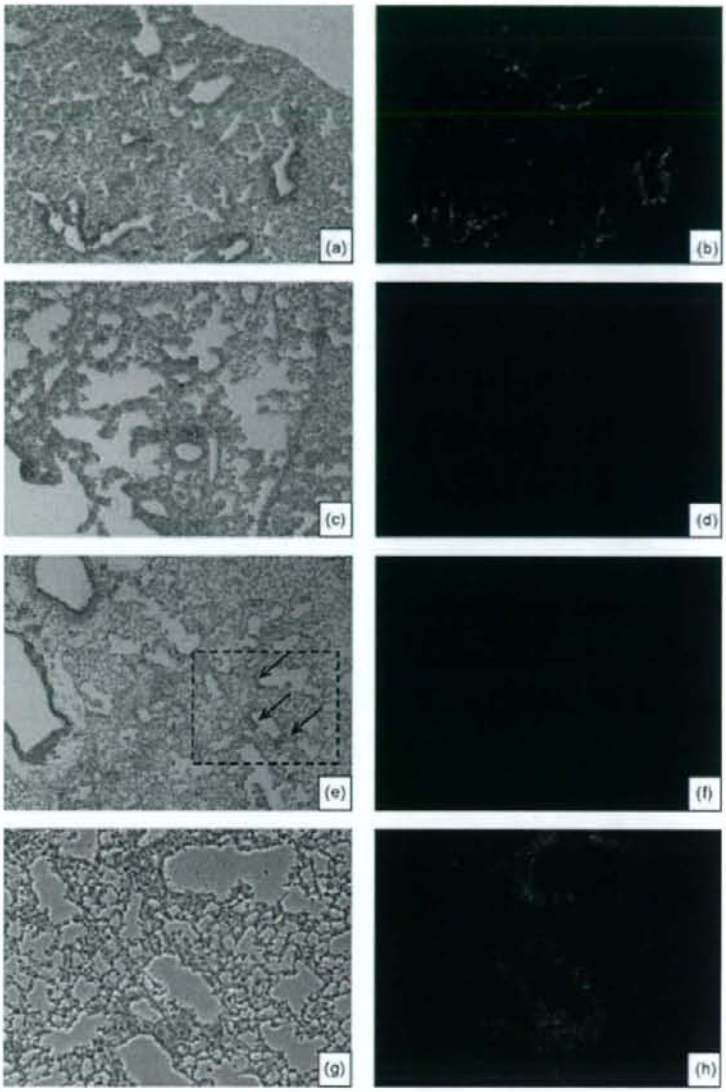

Fig. 7. Distribution of rhodamine-labeled MHAPC lipoplexes (a–d; before immunohistochemistry) and luciferase location in the lungs (e–h; after immunohistochemistry). a–f are shown at 40× magnification; g and h show 100× magnification of the regions in the dashed squares in e and f. Arrows in e indicate the luciferase (dark brown colored).

4. Conclusions

In the present work, three CCDs with a carbamate ester linker and a hydroxyethyl group were synthesized and formulated into liposomes and nanoparticles. In *in vitro* formulations, liposomes formulated with CCDs and DOPE of 1/2 molar ratio were more effective than the corresponding nanoparticles with CCDs and Tween 80 at all charge ratios. Furthermore, among the liposomal formulations, non-hydroxyethylated CCDs such as DC-Chol and DMAPC were more effective than hydroxyethylated ones in A549 cells. Gene transfection in the lung showed opposite results to those *in vitro*, with liposomes containing hydroxyethylated CCDs being more potent than ones containing non-hydroxyethylated CCDS. MHAPC liposomes, which contained a hydroxyethylated tertiary amine as the cationic part, showed the highest gene expression among CCD liposomes. All the lipoplexes caused higher TNF-α levels in the lung than the nanoplexes and jet-PEI and we considered the toxicity was largely caused by the lipoplex formulation, but our findings demonstrated that use of CCD lipoplexes with modification of the cationic cholesterol with a hydroxyethyl group at the tertiary amine headgroup, MHAPC, promoted gene expression in the lung without increasing the toxicity compared to other CCD lipoplexes. However, further efforts should be made to elucidate the mechanism of toxicity in the lung caused by CCD lipoplex and how to minimize the inflammation response of CCD lipoplex by optimizing the formulation.

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Decaarginine-PEG-Artificial Lipid/DNA Complex for Gene Delivery: Nanostructure and Transfection Efficiency

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Oligoarginine conjugates are highly efficient vectors for the delivery of plasmid DNA into cells. Decaarginine-conjugated lipid (Arg10-PEG-lipid) was synthesized and the effects of Arg10-PEG-lipid concentration at a fixed DNA concentration on transfection efficiency and the structure of the complexes were studied below and above critical micelle concentration (CMC), and at the lipid nitrogen/DNA phosphate (N/P) ratio corresponding to transfection, respectively. Arg10-PEG-lipid at the concentration below CMC showed stronger interaction with DNA by fluorescence intensity distribution analysis, and significantly higher luciferase and green fluorescent protein expression than that above CMC. A phase-contrast cryo-transmission electron microscope (cryo-TEM) experiment showed that the morphology of the complexes depended on the N/P ratio. At a low N/P ratio corresponding to that in transfection at a lipid concentration below CMC, a net-like structure developed in which plasmid DNA was involved. A further increase in the N/P ratio, a large fibrous nanostructure of complexes, was also observed. Without DNA, these structures were not obtained. The cellular uptake mechanism of complexes using flow cytometry with inhibitors suggested that complexes with two different morphologies showed similar cellular uptake and uptake mechanism, macropinocytosis. Differences in transfection efficiency of the complexes may be explained by a large fibrous nanostructure inhibiting the cellular internalization of complexes or the release of DNA from macropinosomes into cytoplasm. Arg10-PEG-lipid/DNA complexes formed a favorable nanostructure for gene delivery, depending on the N/P ratio in water.

Keywords: Decaarginine, Cell-Penetrating Peptide, Macropinocytosis, Gene Delivery, Supramolecular Structure.

RESEARCH ARTICLE

1. INTRODUCTION

The development of a gene delivery vector is believed to be a key to the success of gene therapy. Gene delivery vectors are classified into viral and nonviral vectors. Viral vectors provide very high transfection efficiency, but their safety is a great concern because of their immunogenicity and acute toxicity.[1,2] For the future development of gene therapy, a safe and highly effective nonviral gene vector is indispensable, and nonviral vectors such as cationic liposomes and polymers have been developed;[1,3,4] however, their low-level transfection efficiency, compared with viral vectors, is considered to be a major limitation in their application to gene therapy. Poor efficiency is supposed to arise from the endocytic route of internalization, when cationic lipids or polymers form a complex with DNA; therefore, novel and more efficient synthetic vectors, hopefully with a different cell internalization mechanism, are desired.

Oligoarginine is known as a cell-penetrating peptide (CPP),[5–7] and can deliver its associated molecules into cells.[8–11] In our previous work, we reported oligoarginine ((Arg)n; $n = 4, 6, 8, 10$)-conjugated lipids with a poly(ethylene glycol) (PEG) spacer as novel gene vectors.[12] (Arg)n-PEG-lipid provides three characteristic functions: the PEG-lipid part forms micelles, the (Arg)n part can interact with DNA and make it compact, and this part

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also interacts with cells as CPP. Arg10-PEG-lipid showed the highest transfection efficiency among (Arg)*n*-PEG-lipids in human cervical carcinoma HeLa cells when the lipid formed a complex with plasmid DNA at concentrations much higher than their critical micelle concentration (CMC) to ensure the integrity of micelles.[12] Transfection efficiency was comparable to Lipofectamine 2000, a commercial transfection reagent.[12]

Cationic lipids form small particles when they form a complex with DNA molecules, which is an ideal property for efficient internalization of the complexes by endocytosis.[13] A variety of structural models have been proposed based upon electron microscopy studies about cationic liposome complexes with DNA by using a nature of helper lipids. The superior transfection properties of lipoplexes were related to its ability to undergo a lamellar to a nonlamellar phase.[14] About micelles, however, information of morphologies of complexes depending charge ratios of lipid/DNA was poor.[15] Below CMC, the relationship between transfection efficiency and structure of lipid complexes with DNA were not reported to our knowledge.

The purpose of this study was to investigate the relationship of the concentration of Arg10-PEG-lipid to lipid-mediated gene delivery. The interaction between Arg10-PEG-lipid and plasmid DNA was measured by fluorescence intensity distribution analysis (FIDA), and the morphology of Arg10-PEG-lipid/DNA complex was observed by phase contrast cryo-transmission electron microscopy (phase contrast cryo-TEM). Here, we report novel aspects of Arg10-PEG-lipid, i.e., the concentration-dependent transfection efficiency and relationship with nanostructure formation that may cause difference in the ability to deliver DNA into HeLa cells. The lipid showed higher transfection at a concentration below CMC than above CMC.

RESEARCH ARTICLE

2. MATERIALS AND METHODS

2.1. Materials

The Pica gene luciferase assay kit was purchased from Toyo Ink (Tokyo, Japan). Bicinchonic acid (BCA) protein assay reagent was obtained from Pierce (Rockford, IL). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Corp. (Carlsbad, CA). 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) was from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of reagent grade.

Arg10-PEG-lipid (Arg10-PEG-BDB, Fig. 1) and 7-nitrobenz-2-oxa-1,3-diazole (NBD)-labeled Arg10-PEG-BDB (Arg10-PEG-BDB-NBD) were synthesized as described previously.[12] 3,5-Bis(dodecyloxy)benzamide (BDB) was employed as the lipid component, and a PEG (MW = 2 kDa) spacer was introduced between the C-terminal of Arg10 and the amide group of BDB.

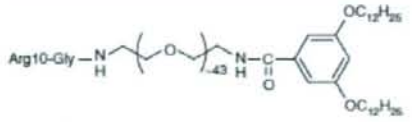

Fig. 1. Chemical structure of Arg10-PEG-BDB.

The plasmid DNA encoding the luciferase gene under the control of the CMV promoter (pCMV-luc) was constructed as previously described.[16] The plasmid pEGFP-C1 encoding the green fluorescent protein (GFP) under the CMV promoter was purchased from Clontech (Palo Alto, CA). A protein-free preparation of pCMV-luc and pEGFP-C1 was purified following alkaline lysis using maxiprep columns (Qiagen, Hilden, Germany). Labeling of pCMV-luc was performed using the protocol of a Label IT TM-rhodamine labeling kit (Mirus, Madison, WI).

2.2. Critical Micelle Concentration of Arg10-PEG-BDB

In order to determine the critical micelle concentration (CMC) of Arg10-PEG-BDB, fluorescence measurements were carried out using pyrene (0.6 μM) as reported in the literature.[17] The fluorescence emission spectra of pyrene were measured at varying Arg10-PEG-BDB concentrations using a fluorescence spectrometer. The concentration of Arg10-PEG-BDB used was within the range from 1×10^{-8} to 1×10^{-3} M. Pyrene was dissolved in acetone and transferred into capped tubes. The acetone as solvent was evaporated off by a stream of dry nitrogen, and subsequently, aqueous solutions of Arg10-PEG-BDB micelles were added. Then, the micellar solutions containing pyrene were heated to 80 °C for 2 h to equilibrate the partitioning of pyrene into the micelles and were allowed to cool over night at room temperature in the dark.

2.3. Formulation of Arg10-PEG-BDB/DNA Complex

Arg10-PEG-BDB stock solutions (20 mg/mL) were prepared by dissolving lipid in MilliQ water. Arg10-PEG-BDB/DNA complexes were formulated by mixing DNA and Arg10-PEG-BDB stock solutions. The number of nitrogen of Arg10-PEG-BDB was defined as 10 and the lipid nitrogen/DNA phosphate (N/P) ratio was calculated. For transfection and FIDA experiments, the following amounts of the Arg10-PEG-BDB to 2 μg of DNA were used to prepare Arg10-PEG-BDB/DNA complexes at various N/P ratios, e.g., N/P = 8.5; 20 μg/mL (5 μM). For transmission electron microscopy experiment, 0.25 mM of Arg10-PEG-BDB/DNA (N/P = 8.5/1) and 1.25 mM of Arg10-PEG-BDB/DNA (N/P = 42.5/1) were used, since higher Arg10-PEG-BDB concentrations are needed for observation.

Particle size and zeta potential of Arg10-PEG-BDB and its DNA complexes were measured 10–15 min after the complex had formed, using dynamic light scattering and electrophoresis method, respectively (ELS-800, Otsuka Electronics Co. Ltd, Osaka, Japan) at 25 °C after the dispersion was diluted to an appropriate volume with water.

2.4. Fluorescence Intensity Distribution Analysis (FIDA)

FIDA was performed with a MF20 microplate reader (Olympus Corp. Tokyo, Japan) using the onboard 543-nm laser at a power of 300 μW for excitation. Experiments were performed in 384-well glass-bottom plates using a sample volume of 50 μL. The FIDA data was analyzed with the MF20 software package. All single-molecule FIDAs were performed under identical conditions with respect to incubation (10 min) at room temperature. The interaction between Arg10-PEG-BDB and DNA experiments were performed using the rhodamine-labeled DNA. The concentration of the labeled DNA was held constant whereas the concentration of Arg10-PEG-BDB was varied in water.

2.5. Phase Contrast Cryo-TEM and Microscopy

Specimens for electron microscopy were prepared on Quantifoil® R1.2/1.3 holed carbon grids at a Leica EM CPC cryo-preparation station. Cryo-electron microscopy was performed on a JEOL JEM-3100FFC TEM equipped with a field emission gun (FEG), helium temperature specimen stage, omega-type energy filter and Gatan Mega-Scan 795 2 K × 2 K CCD camera. For improved contrast of ice-embedded specimens, we employed a novel Zernike-type phase plate at the back focal plane of the objective lens.[18,19] It provides a true phase contrast regime revealing details in the image that are hidden in the conventional defocus phase contrast mode. All images were taken by the CCD camera with the TEM operated at 300 kV acceleration voltage, zero-loss energy filter mode, ×60,000 indicated magnification and employing the phase plate. At that magnification, the specimen resolution at the CCD is 3.0 Å/pix. To minimize electron beam damage, we employed a minimum dose protocol which irradiates the area of interest only during image exposure. The total dose to the specimen was about 6 $e^-/Å^2$.

Arg10-PEG-BDB/DNA complexes were diluted with serum-free DMEM to 1 mL. Incubation with HeLa cells was conducted for 1 h in the absence of serum, and then the cells were washed 5 times with 1 mL of PBS. Unfixed cells were observed with an ECLIPSE TS100/100-F for Epi-fluorescence Observations (Nicon, Tokyo, Japan). The level of contrast and the brightness of the images were adjusted.

2.6. Flow Cytometry

HeLa cells were kindly provided by Toyobo Co., Ltd. (Osaka, Japan). HeLa cells were grown in DMEM supplemented with 10% FBS at 37 °C in a humidified 5% CO_2 atmosphere.

HeLa cells were grown to just before confluence in a 12-well plate. For cellular uptake, Arg10-PEG-BDB/rhodamine-DNA was diluted with DMEM containing 10% FBS to 1 mL. Incubation with HeLa cells was conducted for 3 h in the presence of serum since the cells could not be detached from the wells after incubation in the absence of serum. For inhibition of uptake, cells were preincubated for 30 min at 37 °C with DMEM containing 10% FBS in the presence of EIPA (50 μM). Subsequent incubation of the Arg10-PEG-BDB-NBD/DNA was carried out for 1 h in the presence of EIPA.

At the end of the incubation, the dishes were washed 3 times with 1 mL of PBS, and the cells were detached with 0.05% trypsin and EDTA solution. The cells were centrifuged at 1500 rpm, and the supernatant was discarded. The cells were resuspended with PBS containing 0.1% BSA and 1 mM EDTA, and directly introduced to a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488 nm argon ion laser. Data for 10000 fluorescent events were obtained by recording forward scatter (FSC) and side scatter (SSC) with green (for NBD; 530/30 nm) and red (for rhodamine; 585/42 nm) fluorescences.

2.7. Gene Transfection

Arg10-PEG-BDB/DNA complexes, prepared by mixing 2 μg of pCMV-luc or pEGFP-C1 with various concentrations of Arg10-PEG-BDB, were diluted with serum-free DMEM to 1 mL.[12] Incubation with HeLa cells was conducted for 3 h in the absence of serum, and cells were cultured for another 21 h in the presence of serum.

Luciferase expression was measured according to the instructions accompanying the luciferase assay system. Incubation was terminated by washing the plates three times with cold phosphate-buffered saline (pH 7.4) (PBS). Cell lysis solution (Pica gene) was added to the cell monolayers and subjected to freezing at −80 °C and thawing at 37 °C, followed by centrifugation at 15000 rpm for 5 s. The supernatants were frozen and stored at −80 °C until the assays. Aliquots of 20 μL of the supernatants were mixed with 100 μL of luciferin solution (Pica gene) and counts per second (cps) were measured with a chemoluminometer (Wallac ARVO SX 1420 multilabel counter, Perkin-Elmer Life Science, Japan, Co. Ltd., Kanagawa, Japan). The protein concentration of the supernatants was determined with BCA reagent using bovine serum albumin as a standard and cps/μg protein was calculated.

GFP expression was analyzed by fluorescence microscopy and flow cytometer. For fluorescence microscopy,