basis of the physicochemical interaction forces between the growth factor and gelatin. In this release system, the growth factor immobilized is not released from the hydrogel unless the hydrogel carrier is degraded to generate water-soluble gelatin fragments. The growth factor release can be controlled only by changing the hydrogel degradation.[24] In addition, we have experimentally confirmed that cationized gelatin hydrogels, prepared by introducing amine residues to the carboxyl groups of gelatin, achieved the controlled release of plasmid DNA based on the hydrogel degradation following intramuscular implantation.[28,29] The cationized gelatin hydrogel incorporating plasmid DNA enhanced the gene expression level to a significantly greater extent than the plasmid DNA injected in solution form, while it prolonged the duration period of gene expression which basically depends on the release period of plasmid DNA.[28-29]

This study was undertaken to prepare a micelle from this gelatin by PEG grafting. It is expected that the micelle of PEG and gelatin can retain in the blood circulation. Additionally, this micelle may release the drug immobilized to gelatin molecules in a controlled manner accompanied with gelatin degradation. The release mechanism driven by the degradation of the release carrier is quite different from that of drug or DNA diffusion from the release carrier, such as PLA and PLGA, which has been reported. Gelatin was grafted with PEG, and the micelle formation and body distribution of PEG-grafted gelatin (PEG-gelatin) were evaluated in terms of the molecular weight of PEG grafted and the PEGylation degree.

Materials and Methods

Materials. The gelatin sample with an isoelectric point of 5.0 (M_w = 100 000), prepared by an alkaline process of bovine bone, was kindly supplied from Nitta Gelatin Inc., Osaka, Japan. Succinimidyl succinate-methoxy PEG (M_w = 2000, 5000, and 12 000) was kindly provided by NOF Corp., Tokyo, Japan. *N*-Phenyl-1-naphthylamine (PNA) was obtained from Wako Pure Chemical, Ltd., Osaka, Japan.

PEG Grafting of Gelatin. Gelatin (1.0×10^{-5} mol) was dissolved in anhydrous dimethyl sulfoxide (DMSO, 10 g) at room temperature. Various amounts of succinimidyl succinate-methoxy PEG with molecular weights of 2000, 5000, and 12 000 (0.15, 0.3, 0.6, 1.5, 3.0, and 6.0×10^{-4} mol) were dissolved in 10 g of DMSO, and the solution was slowly added to the gelatin solution, followed by 3 h of stirring at room temperature for PEG grafting. The reaction mixture was dialyzed in a cellulose tube (the cutoff molecular weight = 12 000−14 000, Viskase Companies, Inc) against double-distilled water (DDW) for 48 h at room temperature and freeze-dried to obtain a PEG-gelatin. The percentage of PEG introduced to the amino groups of gelatin (PEGylation degree) was determined by the conventional 2,4,6-trinitrobenzene sulfonic acid (TNBS) method.[30]

Light Scattering Measurements. To investigate the hydrodynamic radius of PEG-gelatin, the dynamic light scattering (DLS) measurement was carried out on a DLS 700 (Otsuka Electronics, Japan) equipped with a He−Ne laser at a detection angle of 30°, 90°, and 120° at room temperature. The gelatin and PEG-gelatin were dissolved in phosphate-buffered saline solution (PBS, pH 7.4) at 37 °C to prepare the respective solutions (10 mg/mL) in the presence or absence of 6 M guanidine hydrochloride as a hydrophobic interaction breaker. The solution was filtered by a disposable syringe filter (pore size: 0.8 μm; Millipore Co.) for DLS measurements. The PEG-gelatin solution (10 mg/mL, 5 mL) was added to the cell at room temperature, and the hydrodynamic diameter of gelatin and PEG-gelatin was analyzed based on the cumulants method and automatically calculated by the computer software equipped to express as the apparent molecular size. Electrophoretic light scattering (ELS) measurements were carried on an ELS-7000 (Otsuka Electronic Co. Ltd., Osaka, Japan) at room temperature and an electric field strength of 100 V/cm. Gelatin and PEG-gelatin solution were prepared by the same procedure as the DLS measurement. The zeta potential was automatically calculated using the Smoluchouski equation. Each experiment was done 10−20 times independently unless otherwise mentioned.

Affinity Assay of Gelatin and PEG-Gelatin. An affinity assay with HiTrap Blue HP column (Amersham Pharmacia Biotech AB), which has an affinity for protein, was performed to structurally characterize the PEG-gelatin. Gelatin and PEG-gelatin with various PEG molecular weights and PEGylation degrees were dissolved in PBS (5 mg/mL) at 37 °C. After the solution (0.5 mL) was applied to the HiTrap column, the column was washed with 5 mL of PBS and 400 μL of each elution fraction was collected by microcentrifuge tubes. Then, the column was washed with 5 mL of 2 M sodium chloride aqueous solution, followed by the fraction collection similarly. After desalting of the fractions by a PD-10 column (Amersham Pharmacia Biotech AB), the gelatin concentration of each fraction was determined by the protein assay Lowry kit (Nacalai tesque, Kyoto, Japan).

Critical Micelle Concentration (CMC) Measurements. The critical micelle concentration (CMC) of PEG-gelatin was measured according to the conventional method of PNA incorporation.[31] Gelatin and the PEG-gelatin solution were prepared by the same procedure as the DLS measurement. The concentration of gelatin and PEG-gelatin ranged from 0.04 to 10 mg/mL. A small aliquot (10 μL) of PNA methanol solution (5 mM) was added dropwise to 1 mL of gelatin or PEG-gelatin solution at room temperature and 37 °C. After 2 min of stirring, the absorbance of the mixed solution was measured at a wavelength of 500 nm (Ultrospec 2000; Amersham Pharmacia Biotech AB). The concentration of PEG-gelatin at which the solution absorbance changed drastically was measured and defined as the CMC.

Body Distribution Studies of PEG-Gelatin and the Pharmacokinetis Analysis. The body distribution of gelatin and PEG-gelatin was evaluated in female ddY mice (16−18 g weight) (Japan SLC, Inc., Hamamatsu, Japan) by the radiotracing procedure. Gelatin and PEG-gelatin were radioiodinated according to the chloramine T method.[32] The ^{125}I-labeled gelatin and PEG-gelatin (2 mg/mouse) were injected into the jugular vein of mice in a PBS volume of 100 μL. At predetermined time intervals, the mice were sacrificed by ether inhalation, and their tissues were excised,

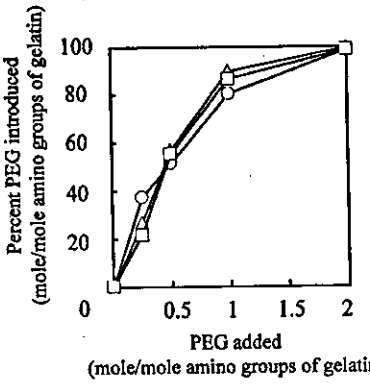

Figure 1. Percentage of PEG introduced to the amino groups of gelatin as a function of the amount of PEG added for grafting reaction. The molecular weight of PEG used was 2000 (○), 5000 (△), and 12 000 (□).

washed quickly with cold saline to remove surface blood, and weighed, and the radioactivity of whole organ was counted on a gamma counter (ARC-301B, Aloka, Tokyo, Japan). Blood samples (100 μL) were obtained in duplicate by cardiac puncture in preweighed tubes. The radioactivity was divided by that of injected initially and the tissue weight to calculate the percentage of injected dose per g of tissue, while the results were normalized to a 20 g mouse to exclude the error by weight variation according to the formula of Sato et al.[33] The percent remaining = [Radioactivity in the tissue/total injected dose/tissue weight (g)] × [body weight (g)/20] × 100. All of the animal experiments were carried out according to the Institutional Guidance of Kyoto University on Animal Experimentation. The mean area under the percent remaining-time curve (AUC) of gelatin and PEG-gelatin in the blood circulation was calculated by the trapezoidal method during the experimental period ($AUC_{[0-24]}$) to compare the stability in the blood circulation between the samples.

Statistical Analysis. All of the data were expressed as the mean ± the standard derivation of the mean. Statistical analysis was performed based on the unpaired Student's t test (two-sided) and significance was accepted at $p < 0.05$.

Results

Characterization of PEG-Gelatin Prepared. PEG was successfully grafted onto gelatin, and nonreacted PEG was completely removed by the dialysis because no peak of PEG was observed by gel permeation chromatography (data not shown). Figure 1 shows the percentage of PEG introduced to the amino groups of gelatin. The number of amino groups of native gelatin was 30 (mol/mol of gelatin). The introduction percentage increased with an increase in the amount of PEG added initially. The PEGylation degree of gelatin could be controlled by changing the addition molar ratio. No effect of the PEG molecular weight on the percentage of PEG introduced was observed.

Table 1 summarizes the apparent molecular size of PEG-gelatin prepared from PEG with molecular weights of 2000, 5000, and 12 000 with various PEGylation degree. The apparent molecular size did not depend on the measurement angle. However, when the guanidine hydrochloride was added, the PEG-gelatin did not show a specific hydrodynamic diameter. Neither gelatin alone nor PEG-gelatin with low degrees of PEGylation exhibited any hydrodynamic diameter. The zeta potential of PEG-gelatin with PEG-introduced percentages of 85 and 100 tended to increased to reach almost 0 mV, in marked contrast to the PEG-gelatin with the PEG molecular weight of 2000 (data not shown). However, there was no significant difference in the zeta potential between the PEG-gelatin samples for any molecular weight of PEG grafted and PEGylation degree.

Figure 2 shows the elution profiles of gelatin and PEG-gelatin prepared from PEG with a molecular weight of 12 000 by HiTrap Blue HP column. The percentage of respective fraction eluted was summarized in Table 1. Because this column has a specific affinity for gelatin, the affinity of gelatin will be reduced by the PEGylation. Gelatin alone and the PEG-gelatin with low PEGylation degrees were adsorbed on the affinity column, whereas the PEG-gelatin with higher PEGylation degrees was not adsorbed.

Figure 3 shows the change in the solution absorbance of PNA incorporated in gelatin grafted with PEG of molecular weights 12 000 as a function of the PEG-gelatin concentra-

Table 1. Physicochemical Properties of PEG-Gelatin

molecular weight of PEG grafted	number of PEG chains grafted (mol/1mol of gelatin)	PEGylation degree[a] (mol/mol%)	percent of eluted[b] PBS (pH 7.4)[c]	percent of eluted[b] 2 M NaCl aq.[d]	hydrodynamic diameter (nm)	CMC[e] (mg/mL)
2000	10	30	0	100	ND[f]	ND[f]
	17	55	34	66	ND	ND
	25	85	58	42	60 ± 10	ND
	30	100	93	7	65 ± 13	0.2
5000	10	30	21	79	ND	ND
	17	55	45	55	ND	ND
	25	85	94	6	78 ± 15	0.3
	30	100	95	5	80 ± 16	0.3
12 000	10	30	13	87	ND	ND
	17	55	73	27	110 ± 17	ND
	25	85	97	3	112 ± 16	1.0
	30	100	95	5	120 ± 27	1.0
gelatin	0	0	0	100	ND	ND

[a] The percentage of PEG introduced to the amino groups of gelatin. [b] Eluted from HiTrapTM Blue HP column which has an affinity for gelatin. [c] The column was washed with PBS and each elution fraction was collected. [d] The column was washed with 2 M sodium chloride aqueous solution, followed by the fraction collection similarly. [e] Critical micelle concentration (CMC) was measured by the *N*-phenyl-1-naphthylamine (PNA) uptake in PEG-gelatin micelle. [f] Not detected.

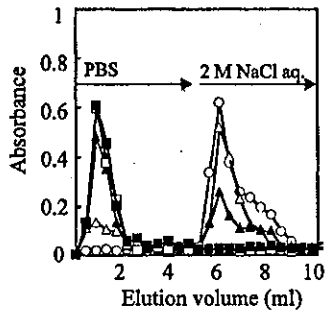

Figure 2. Elution profiles of gelatin and gelatin grafted with PEG of molecular weights 12 000 by HiTrap Blue HP column. The PEGylation degree of PEG-gelatin used was 0 (○) (native gelatin), 30 (△), 55 (▲), 85 (□), and 100 (■) mol/mol%.

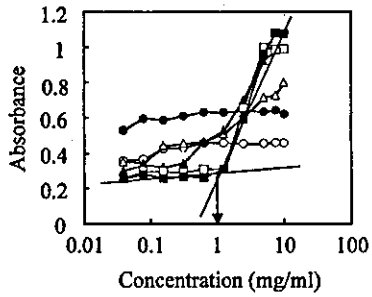

Figure 3. Change in the solution absorbance of PNA incorporated in gelatin grafted with PEG of molecular weights 12 000 as a function of the PEG-gelatin concentration. The PEGylation degree was 0 (○) (native gelatin), 30 (△), 55 (▲), 85 (□), and 100 (■) mol/mol%.

tion. The results of the other PEGs are summarized in Table 1. The solution absorption of PEG-gelatin was drastically increased at concentrations of 0.2, 0.3, and 1.0 mg/mL for the PEG-gelatin with PEG-introduced percentages of 85 and 100. The similar CMC value was observed at room temperature and 37 °C for each PEG molecular weight. The bending point corresponds to the CMC where the intermolecular aggregation of PEG-gelatin takes place. On the other hand, for gelatin and PEG-gelatin with PEG-introduced percentages of 0, 30, and 55, such a drastic increase was not observed.

Body Distribution of PEG-Gelatin Intravenous Administration. Figure 4 shows the percent of remaining of gelatin and PEG-gelatin in the blood circulation following intravenous administration of ^{125}I-labeled PEG-gelatin. The radioactivity of ^{125}I-labeled PEG-gelatin with PEG-introduced percentages of 85 and 100 was retained in the blood circulation compared with that of ^{125}I-labeled gelatin and the PEG-gelatin with PEG-introduced percentages of 0, 30, and 55. At the same PEGylation degree, the blood concentration was significantly higher for the PEG-gelatin prepared from the PEG molecular weight of 12 000 than that of other molecular weights (2000 and 5000).

Figure 5 shows the $AUC_{[0-24]}$ value of gelatin and PEG-gelatin prepared from different molecular weights of PEG at various PEGylation degrees. The $AUC_{[0-24]}$ for the PEG-gelatin with PEG-introduced percentages of 85 and 100 increased with the increasing molecular weight of PEG grafted. On the other hand, the $AUC_{[0-24]}$ for the PEG-gelatin with PEG-introduced percentages of 0, 30, and 55 hardly increased, irrespective of the molecular weight of PEG.

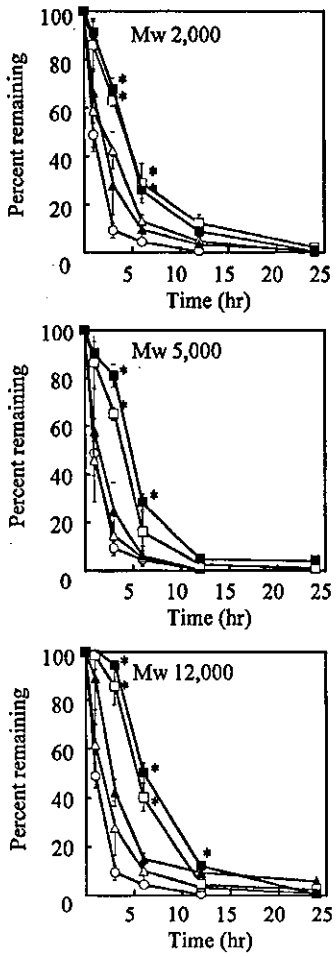

Figure 4. Decrement patterns of gelatin and gelatin grafted with PEG of molecular weights 2000, 5000, and 12 000 in the blood circulation after intravenous administration. The PEGylation degree of PEG-gelatin used was 0 (○) (native gelatin), 30 (△), 55 (▲), 85 (□), and 100 (■) mol/mol%. *, $p < 0.05$: significant against the percent remaining of native gelatin-injected, control mice.

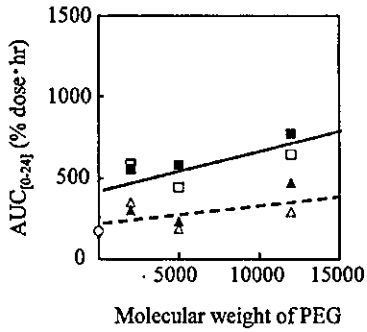

Figure 5. Effect of the molecular weight of PEG grafted on the AUC of PEG-gelatin. The PEGylation degree of PEG-gelatin used was 0 (○) (native gelatin), 30 (△), 55 (▲), 85 (□), and 100 (■) mol/mol%. The solid and dotted lines indicate the PEG-gelatin of micelle and unimer structures, respectively.

Figure 6 shows the mean percent remaining radioactivity in the liver, spleen, and kidney after intravenous administration to mice of gelatin and PEG-gelatin. The PEG-gelatin with PEG-introduced percentages of 85 and 100 tended to accumulate in the liver compared with that with PEG-

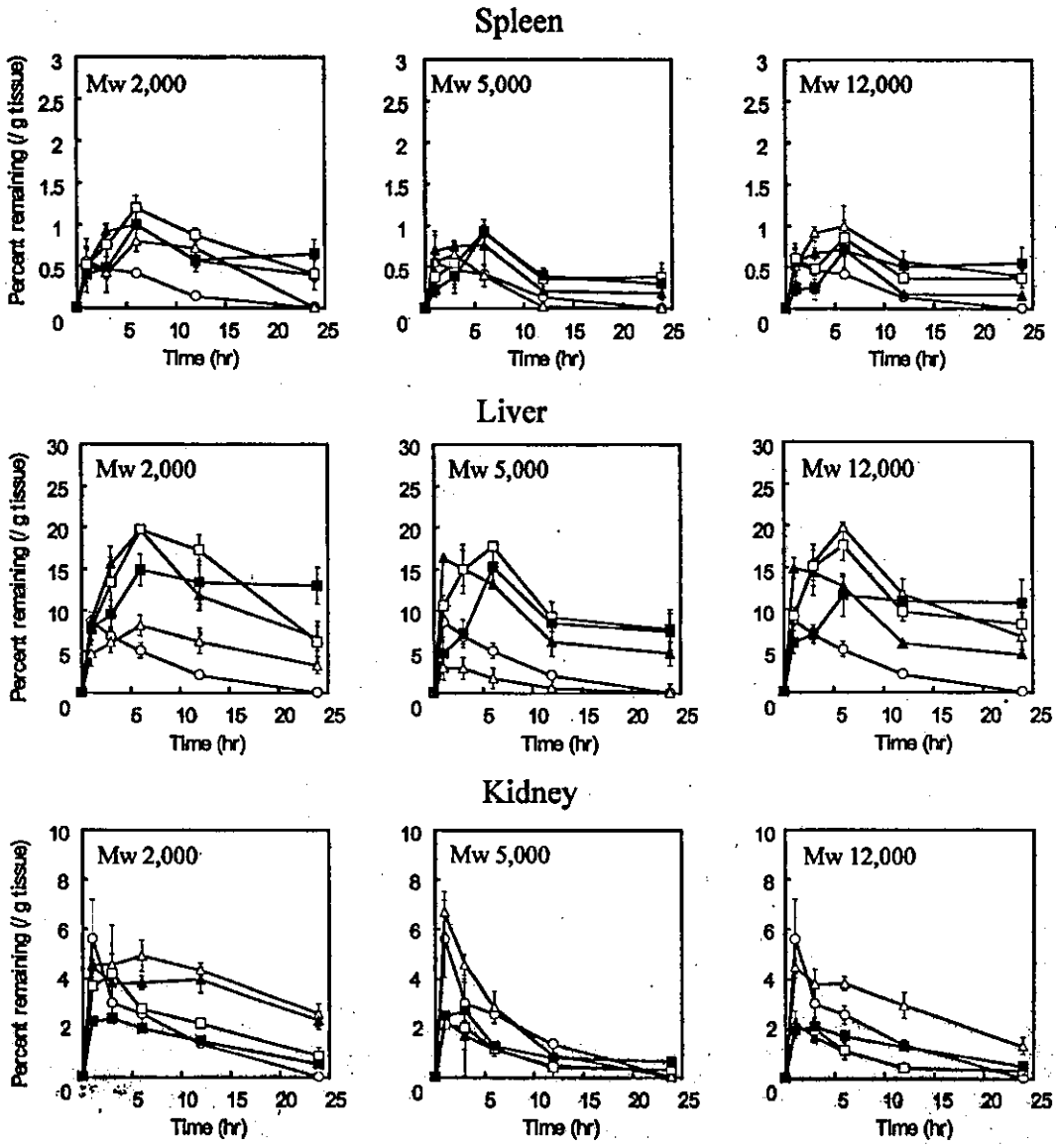

Figure 6. Effect of the molecular weight of PEG grafted and the PEGylation degree of PEG-gelatin on the time profile of PEG-gelatin accumulation in the spleen, liver, and kidney. The PEGylation degree of PEG-gelatin used was 0 (○) (native gelatin), 30 (△), 55 (▲), 85 (□), and 100 (■) mol/mol%.

introduced percentages of 0, 30, and 55. However, there was no significant difference between the PEGylation degree and molecular weight of PEG in the accumulation in the liver, spleen, and kidney.

Discussion

The present study clearly demonstrates that when gelatin was grafted with PEG at the PEG-introduced percentages of 85 and 100 percent the PEG-gelatin showed a micellar structure and tended to circulate in the blood stream for a long time period. No adsorption of the PEG-gelatin to the gelatin affinity column (Table 1 and Figure 2) indicates that the molecular surface of the PEG-gelatin micelle was covered with PEG molecules grafted on gelatin. The PEG-gelatin micelle showed an apparent molecular size in the nanometer range, but the nanomicelle disappeared by the addition of guanidine hydrochloride. Taken together, the PEG-gelatin with PEG-introduced percentages of 85 and 100 built a nanometer-size micelle structure of which the surface is covered with PEG molecules in PBS based on the hydrophobic interaction of gelatin moiety. The nature of PEG-gelatin micelles depended on the molecular weight of PEG grafted. Gelatin grafted with PEG of molecular weight 2000 showed a slightly smaller CMC value than that of PEG with molecular weight 12 000 (Table 1), indicating that the intermolecular interaction force to form the former micelle was stronger than that of the latter micelle. Because the hydrophilicity of PEG was increased with increasing PEG molecular weight, the CMC value was increased with increasing molecular weight of PEG grafted. Moreover, the former micelle showed a smaller slope than the latter one. This means that PEG-gelatin prepared from PEG of high molecular weight has a larger capacity to entrap the

hydrophobic PNA molecules. Some experiments on the molecular structure of PEG-gelatin like the number of molecular aggregation are presently in progress.

The body distribution study indicates that gelatin and PEG-gelatin were readily cleared from the plasma and consequently accumulated in the MPS organs. However, the $AUC_{[0-24]}$ value was increased as the molecular weight of PEG grafted increased at the same PEGylation degree (Figure 6). The diameter of the PEG-gelatin micelle prepared is smaller than 200 nm. The micelle will be promising to prepare a long-circulating drug carrier smaller than 200 nm in diameter being considered optimal for the stealth system to prevent a filtering mechanism in the MPS organs and an increased uptake by macrophage.[16] In fact, it is shown that long-circulating microparticulate drug carriers with sizes of 100−200 nm were effectively accumulated in various tumors tissues based on the EPR effect or "impaired filtration" mechanism.[6,7] This is extremely important from the clinical viewpoint. It is interesting to compare the in vivo behavior of these PEG-gelatin micelles with nanospheres previously reported from PEG-PLGA copolymers which are conventionally prepared as a long circulating carrier.[13] In both the cases, the blood concentration increases as the chain length increases for the same PEG density. It is important to consider that the diameter of PEG-gelatin micelles (65−120 nm) is comparable to that of PEG-PLGA nanospheres (80−150 nm) and that the PEG-gelatin micelles have a higher PEG content: 40−80% of PEG w/w in PEG-gelatin micelles compared to 10% w/w in PEG-PLA nanospheres. Irrespective of PEG molecular weight, the remaining time period of PEG-gelatin micelle in the blood circulation was longer compared with that of PEG-PLGA nanospheres.[34] In addition, the percent remaining of accumulation in the liver or spleen by the MPS was lower than that of the PEG-PLGA nanospheres. Moreover, longer PEG chains can sterically prevent enzymatic attack to gelatin molecules.[34] It is possible that the difference in the blood concentration for PEG-gelatin micelles prepared from PEG of different molecular weights is caused by the difference in their enzymatic degradation rate rather than their susceptibility to removal from the circulation by phagocytic cells.

Another question is about the biological fate of PEG. It has been demonstrated that the urinary clearance of PEG has a pronounced change at a molecular weight around 30 000, suggesting that this value represents the glomerular filtration cutoff for such nonionic polymer.[35] Thus, considering their better efficiency in reducing clearance of micelle in vivo, it is suitable to use PEG with the molecular weights of 12 000 in the micelle composition because the blood concentration was significantly higher for the PEG-gelatin prepared from the PEG molecular weight of 12 000 than that of other molecular weights (2000 and 5000). Nevertheless, the PEG density at the micelle surface could be further optimized by using other amphiphilic biodegradable polymers with more than one PEG chain per gelatin chain to obtain PEG distances as small as used in preparation of stealth liposomes[12] and nanospheres.[17] Furthermore, it would be advantageous to prepare PEG-gelatin micelles smaller than 200 nm, this being considered optimal for stealth systems to prevent a filtering mechanism in the spleen and an increased uptake by macrophages,[17] although this would reduce the payload per particle. Even if gelatin is grafted with PEG, finally the gelatin intravenously administered will be degraded enzymatically in the blood circulation. The PEG-gelatin degraded is removed by the MPS or excreted from the kidney. This is confirmed by the biodistribution result that the radioactivity of PEG-gelatin was accumulated with time in the liver rather than excreted from the kidney (Figure 6). It is well-known that the Kupffer cells play an important role in the MPS uptake in the liver. It is possible that the PEG-gelatin degraded may be taken up by the Kupffer cells.

This study indicates the micelle formation of gelatin by the PEGylation to show a higher residence behavior than gelatin alone. Interaction of this micelle with growth factor, plasmid DNA, and anticancer drugs is underway at present to create the controlled release system of drugs governed by carrier degradation together with the in vivo high residence nature based on the micelle formation. In conclusion, the pharmacokinetics and body distribution profile made this system a new candidate for intravenous administration of drugs which need to remain in the blood compartment and accumulate in the tumor or inflammation site by EPR effect.

References and Notes

(1) Torchilin, V. P.; Trubetskoy, V. S. *Adv. Drug Delivery Rev.* **1995**, *16*, 141−155.
(2) Torchilin, V. P. *J. Liposome Res.* **1996**, *6*, 99−116.
(3) Gbadamosi, J. K.; Hunter, A. C.; Moghimi, S. M. *FEBS Lett.* **2002**, *532*, 338−344.
(4) Torchilin, V. P. *Adv. Drug Delivery Rev.* **2002**, *54*, 235−52.
(5) Palmer, T. N.; Caride, V. J.; Caldecourt, M. A.; Twickler, J.; Abdullah, V. *Biochim. Biophys. Acta* **1984**, *797*, 363−368.
(6) Gabizon, A. A. *Adv. Drug Delivery Rev.* **1995**, *16*, 285−294.
(7) Maeda, H.; Wu, J.; Sawa, T.; Matsumura, Y.; Hori, K. *J. Controlled Release* **2000**, *65*, 271−284.
(8) Klibanov, A. L.; Maruyama, K.; Torchilin, V. P.; Huang, L. *FEBS Lett.* **1990**, *268*, 235−238.
(9) Torchilin, V. P.; Omelyanenko, V. G.; Papisov, M. I.; Bogdanov, A. A., Jr.; Trubetskoy, V. S.; Herron, J. N.; Gentry, C. A. *Biochim. Biophys. Acta* **1994**, *1195*, 11−20.
(10) Torchilin, V. P.; Shtilman, M. I.; Trubetskoy, V. S.; Whiteman, K.; Milstein, A. M. *Biochim. Biophys. Acta* **1994**, *1195*, 181−184.
(11) Stolnik, S.; Illum, L.; Davis, S. S. *Adv. Drug Delivery Rev.* **1995**, *16*, 195−214.
(12) Lasic, D. D. *Trends Biotechnol.* **1998**, *16*, 307−321.
(13) Gref, R.; Minamitake, Y.; Peracchia, M. T.; Trubetskoy, V.; Torchilin, V. P.; Langer, R. *Science* **1994**, *263*, 1600−1603.
(14) Yasugi, K.; Nagasaki, Y.; Kato, M.; Kataoka, K. *J. Controlled Release* **1999**, *62*, 89−100.
(15) Stolnik, S.; Dunn, S. E.; Garnett, M. C.; Davies, M. C.; Coombes, A. G. A.; Taylor, D. C.; Irving, M. P.; Purkiss, S. C.; Tadros, T. F.; Davis, S. S.; Illum, L. *Pharm. Res.* **1994**, *11*, 1800−1808.
(16) Bazile, D.; Prud'Homme, C.; Bassoullet, M. T.; Marlard, M.; Spenlehauer, G.; Veillard, M. *J. Pharm. Sci.* **1995**, *84*, 493−498.
(17) Gref, R.; Domb, A.; Quellec, P.; Blunk, T.; Muller, R. H.; Verbavatz, J. M.; Langer, R. *Adv. Drug Deliv. Rev.* **1995**, *16*, 215−233.
(18) Peracchia, M. T.; Gref, R.; Minamitake, Y.; Domb, A.; Lotan, N.; Langer, R. *J. Controlled Release* **1997**, *46*, 223−231.
(19) Emile, C.; Bazile, D.; Herman, F.; Helene, C.; Veillard, M. *Drug Delivery* **1996**, *3*, 187−195.
(20) Kakizawa, Y.; Kataoka, K. *Adv. Drug Delivery Rev.* **2002**, *54*, 203−222.
(21) Harada-Shiba, M.; Yamauchi, K.; Harada, A.; Takamisawa, I.; Shimokado, K.; Kataoka, K. *Gene Ther.* **2002**, *9*, 407−414.
(22) K Kataoka, A Harada and Y Nagasaki, Block copolymer micelles for drug delivery: design, characterization and biological significance. *Adv. Drug Delivery Rev.* **2001**, *47*, 113−131
(23) Veis, A. *Int. Rev. Connect. Tissue Res.* **1965**, *3*, 113−200.

(24) Tabata, Y.; Ikada, Y. *Adv. Drug Delivery Rev.* **1998**, *31*, 287−301.
(25) Yamamoto, M.; Tabata, Y.; Ikada, Y. *J. Biomater. Sci. Polym. Ed.* **1998**, *9*, 439−458.
(26) Yamamoto, M.; Tabata, Y.; Hong, L.; Miyamoto, S.; Hashimoto, N.; Ikada, Y. *J. Controlled Release* **2000**, *64*, 133−142.
(27) Ozeki, M.; Ishii, T.; Hirano, Y.; Tabata, Y. *J. Drug Target.* **2001**, *9*, 461−471.
(28) Fukunaka, Y.; Iwanaga, K.; Morimoto, K.; Kakemi, M.; Tabata, Y. *J. Controlled Release* **2002** *80*, 333−343.
(29) Kushibiki, T.; Tomoshige, R.; Fukunaka, Y.; Kakemi, M.; Tabata, Y. *J. Controlled Release* **2003**, *90*, 207−216
(30) Snyder, S. L.; Sobocinski, P. Z. *Anal. Biochem.* **1975**, *64*, 284−288.
(31) Ouchi, T.; Nishizawa, H.; Ohya, Y. *Polymer* **1998**, *39*, 5171−5175.
(32) Wilbur, D. S.; Hadley, S. W.; Hylarides, M. D.; Abrams, P. G.; Beaumier, P. A.; Morgan, A. C.; Reno, J. M.; Fritzberg, A. R. *J. Nucl. Med.* **1989**, *30*, 216−226.
(33) Sato, N.; Kobayashi, H.; Saga, T.; Nakamoto, Y.; Ishimori, T.; Togashi, K.; Fujibayashi, Y.; Konishi, J.; Brechbiel, M. W. *Clin. Cancer Res.* **2001**, *7*, 3606−3612.
(34) Mosqueira, V. C. F.; Legrand, P.; Morgat, J.; Vert, M.; Mysiakine, E.; Gref, R.; Devissaguet, J. Barratt, G. *Pharm. Res.* **2001**, *18*, 1411−1419.
(35) Yamaoka, T.; Tabata, Y.; Ikada, Y. *J. Pharm. Sci.* **1994**, *83*, 601−606. Hydrodynamic diameter (nm).

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Efficacy and cytotoxicity of cationic-agent–mediated nonviral gene transfer into osteoblasts

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Abstract: *Ex vivo* gene transfer into osteoblastic cells is an advantageous strategy for bone tissue engineering. This study investigated the efficacy and cytotoxicity of *in vitro* cationic-agent–mediated nonviral gene transfer into osteoblasts. Various cationic agents, lipid, gelatin, and polyethylenimine (PEI) were tested. Each was formulated in various concentrations to form a complex with plasmid DNA encoding red fluorescent protein. The cationic agent/DNA complexes were transfected into human fetal osteoblastic cell line and rat bone-marrow–derived primary osteoblasts, as well as NIH 3T3 fibroblast controls. Rat primary osteoblasts were transfected more with cationic lipid and PEI agents than with gelatin carrier, yielding transfection efficacy up to 18.1% and 12.7 %, respectively. In contrast, human fetal osteoblastic cell line was transfected more with cationic lipid and gelatin than with PEI. There was a positive correlation between the lipid and PEI doses and cytotoxicity. When the lipid and PEI were used to transfect the rat primary osteoblasts in a dose that yielded the highest transfection efficacy, cell survival rates decreased as low as 40%. When their transfection efficacies into primary osteoblasts were compromised at two thirds of the highest value, that is, 12.6% and 8.3% for the lipid and PEI, respectively, the cell survival rate was nearly 80%. Cationic gelatin was associated with cell survival rates over 60 % in any cell type, regardless of the doses tested. These results suggest that different types of osteoblastic cells may possess different ability to the uptake and expression of cationic-agent–bound DNA. There seemed to be agent-specific threshold doses that dropped the cell survival rate. Cationic-agent–mediated nonviral gene transfer into osteoblastic cells may be successful when the agent- and dose-dependent transfection efficacy and cytotoxicity are optimized. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 71A: 308–315, 2004

Key words: bone gene therapy; plasmid gene transfer; polyethylenimine (PEI); cationized gelatin; cationic lipid

INTRODUCTION

Bone tissue engineering offers the prospect of new therapies for clinically significant skeletal morbidity.[1] Tissue replacement strategy in the skeletal system is designed to mimic the components found in the healthy tissue. These components include biologic scaffold, cells, and signaling molecules,[2] among which controlled expression of signaling molecules may ensure and accelerate the tissue engineering process.[3] Delivering growth factors and cytokines into osteoblastic cells may stimulate migration and proliferation,[4–6] promoting differentiation,[5,7–9] and eventually enhancing mineralization. Because osteoblasts can be easily cultivated *in vitro* from bone marrow stromal stem cells and osteoprogenitor cells, *ex vivo* gene transfer is an advantageous strategy for delivering the signaling molecules.[10–13]

Ex vivo viral gene transfer has been used in bone-marrow–derived osteoblasts,[14,15] but the viral recombinant vectors have disadvantages. In addition to the need for specific cell culture conditions,[16] viral transfer induces immune/inflammatory responses that adversely affect the cellular phenotype.[17–19]. Nonviral plasmid DNA transfer overcomes these problems, but gene transfer efficacy is low compared with the viral transfer. One way to bridge the gap in effectiveness between the viral and nonviral systems is to optimize the nonviral system to its full potential.

Carrier systems that modulate the chemical/physi-

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cal properties of nonviral plasmid vector to improve gene transfer efficacy have been investigated. Cationic lipid is the first developed carrier agent to be widely used.[20] Biodegradable cationic gelatin was introduced as a low toxic and manipulable gene transfer agent for liver,[21] mucosa,[22] and muscle[23] cells. An artificial, cationic polymer, polyetheylenimine (PEI), has been demonstrated to be effective for *in vitro* and *in vivo* gene transfer, including liver,[24] brain,[25] and cancer[26] cells. These cationic agents increase gene transfer efficacy consistently compared with naked DNA. However, the degree of cellular uptake and expression of plasmid DNA is associated with the cell type and culture conditions.[27,28]Specific capability of these agents in osteoblastic gene transfer needs to be determined.

This study investigated the potential of cationic carrier agents for nonviral gene transfer into osteoblastic cells. The efficacy and cytotoxicity of nonviral plasmid gene transfection were evaluated using three different cationic agents: lipid, gelatin, and PEI. Transfection into human fetal osteoblastic cell line and rat bone-marrow–derived primary osteoblastic cells was compared with that into clonal fibroblasts. We report that PEI, when it is optimized with a specific P/N ratio (phosphate in DNA/nitrogen in PEI), is as effective as the lipid agent for transfecting the rat primary osteoblasts. We also demonstrate that there are minimal threshold doses for lipid and PEI agents to reduce their cytotoxicity.

MATERIALS AND METHODS

Cell culture

Human fetal osteoblasts, a clonal cell line obtained from American Type Culture Collection (Manassas, VA) were cultured in a 1:1 mixture of Ham's F12 medium and Dulbecco's Modified Eagle's medium with 2.5 m*M* L-gluamine (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Gibco) and 0.3 mg/mL of Geneticin (Sigma, St. Louis, MO).

Primary osteoblasts were cultured from rat bone marrow stromal stem cells. Femurs were excised aseptically from the 6-week-old male Sprague Dawley rats. Soft tissues and muscles were removed and washed with 1× phosphate buffered saline (PBS; Gibco, Carlsbad, CA) several times. The both ends of the femur bone were carefully severed and the bone marrow was flushed out using 3 mL of osteoblastic differentiating medium through a 20-gauge needle. The osteoblastic differentiating medium contained α-modified Eagle's medium, 15% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 0.25 μg/mL of amphotericin B, freshly prepared 50 μg/mL of ascorbic acid (Sigma), 10 m*M* Na-β-glycerophosphate (Sigma), and 10^{-8} *M* dexamethasone (Sigma).

NIH 3T3 fibroblast cells were cultured in Dulbecco's modified Eagle's medium combined with 10% FBS, 100 U/mL of penicillin (Gibco), 100 μg/mL of streptomycin (Gibco), and 0.25 μg/mL of amphotericin B (Sigma). All cells were incubated at 37°C in 5% CO_2 and 95% humidity, and the medium was renewed every 3 days during expansion.

One day prior to gene transfection, the three types of cells were detached with trypsin/ethylenediaminetetraacetic acid (EDTA) solution (Gibco) and seeded in a 12-well culture plate with the concentration of 2×10^4 cells/cm^2, which gave the 70% confluency in 17 to 24 h. When the cells reached 70% confluency, pDsRed2-N1 plasmid vector with CMV promoter encoding red fluorescent protein (Clontech, Palo Alto, CA) was transfected into the cells according to the following three different protocols.

Transfection via cationic lipids

Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used as a cationic lipid. Transfection was performed according to the manufacture's instruction. For each well, 2 μg of plasmid DNA was diluted in 100 μL of the cell-type–specific culture medium. Three different doses of lipofectamine 2000 (2, 4, and 6 μL) were prepared in 100 μL of culture medium. The DNA and lipid mixtures were combined and incubated for 20 min at room temperature. After the existing culture medium was refreshed with medium containing no serum, the DNA/lipid complex was added and mixed gently by agitating the culture dish. Five hours after addition of the complex, the medium was replaced with the fresh serum containing medium specific to each cell type.

Transfection via cationic gelatin

Cationized gelatin was prepared according to a previously reported protocol established by one of the investigators.[22] Briefly, the gelatin was cationized by introduction of 1:50 ethylenediamine isolated from pig skin. A stock solution of gelatin was prepared to a concentration of 2 mg/mL. The stock gelatin solution in four different volumes, 2.5, 5, 10, and 60 μL, was separately diluted to 30 μL of 0.1× PBS, giving the concentration of 5, 10, 20, and 60 μg of the gelatin per 30 μL, respectively. Two micrograms of plasmid DNA was diluted with 30 μL of 0.1× PBS. The DNA solution and the gelatin solution were mixed, vortexed, and incubated at 37°C for 30 min to form gelatin–DNA complex. The culture medium was replaced with serum-free medium before adding the complex, which was added to each well and mixed gently by agitating the culture plate. After 5 h, the medium was replaced with the serum-based medium.

Transfection via cationic polymer

Polyethylenimine (PEI), a synthetic cationic polymer that mediates plasmid gene transfer was prepared as follows:

The stock solution of 10 μ*M* PEI prepared from 50% (w/v) 50 to 60 kD PEI (Sigma) was adjusted to pH 7.0, and filtered through a 0.2-μm Milipore filter system (Milipore, Billerica, MA). Two micrograms of plasmid DNA were diluted with 50 μL of 150 m*M* NaCl. Stock PEI solutions were mixed with 150 m*M* NaCl, giving P/N (phosphate in DNA/nitrogen in polyethylenimine) ratios of 1:3, 1:7, 1:10, and 1:13, to control P/N ratio for the reported P/N ratio effects on gene transfection efficacy.[29–31] The DNA and PEI solutions were separately vortexed and allowed to settle for 10 min, then mixed, briefly vortexed again, and incubated for an additional 10 min at room temperature. Immediately before transfection, the culture medium was replenished with medium containing no serum. The complex was added directly to each well and mixed gently by agitating. After a 5-h incubation period, the medium was replaced with the serum-containing medium.

Transfection efficacy

Viable cells were stained with 5 drops of Calcein, AM (1:5000 in 1× PBS; Molecular Probes, Eugene, OR) 48 h after the commencement of gene transfection, and the total number of the cells was counted. The number of transfected cells (red fluorescent) and total number of viable cells (green fluorescent) were counted under an inverted fluorescence microscope (Leica Microsystems Inc., Chantilly, VA). The counting was carried out in two different microscopic fields, and the average value was calculated. Transfection efficacy was defined as a percentage of number of fluorescing transfected cells to the total number of cells. Experiments were performed in triplicate prior to statistical analysis.

Cell survival rate

Cytotoxicity was evaluated by measuring cell survival rate 24 h after gene transfection. Cells were washed with 1× PBS and stained with 1.4% methylene blue solution (Sigma). The area of the staining was measured using Image-Pro Plus software (Media Cybernetics, Carlsbad, CA). The survival rate was defined as the cell area after gene transfection referenced to the untreated control in triplicate experiments.

Statistical methods

Effects of the cell type and transfection conditions on gene transfection efficacy and cell survival rate was assessed by using analysis of variance (ANOVA) testing at $p < .05$. Bonferroni test was used as a multiple comparison testing.

RESULTS

Cationic lipid-mediated transfection

Transfected cells showed bright red fluorescence, and Calcein fluorescent staining visualized the total number of the cells [Fig. 1(a)]. Transfected cells of all three types appeared morphologically normal. Transfection efficacy was affected by the cell type (two-way ANOVA, $p < .0001$) and dose of cationic lipid agent ($p < .0001$), as shown in Figure 1(b). The higher the dose of lipid used, the higher the transfection rate in the all three cell types (one-way ANOVA, $p < .005$). Rat primary osteoblasts showed higher transfection efficacy than the human fetal osteoblasts and NIH 3T3 fibroblasts at the higher lipid/DNA ratio (4 μL and 6 μL of cationic lipid), yielding the transfection efficacy of 18.1% and 12.6%, respectively (Bonferroni, $p < .001$).

Cationic gelatin-mediated transfection

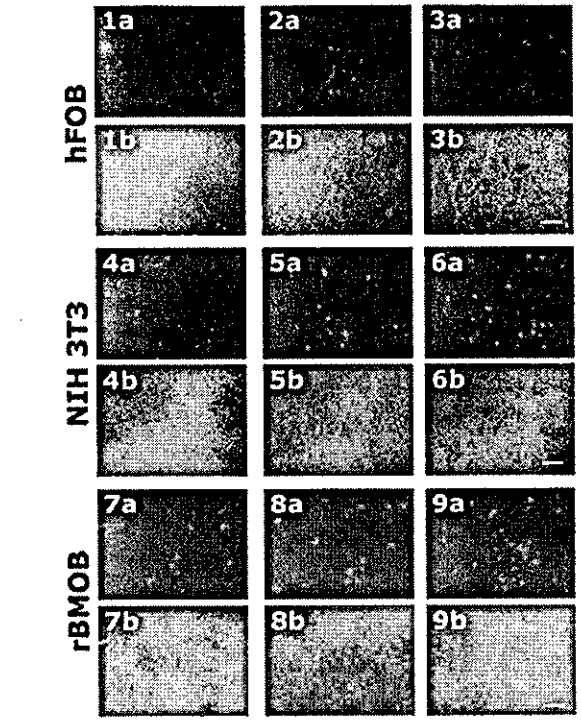
The morphology of the transfected cells appeared normal [Fig. 2(a)]. Two-way ANOVA showed that the transfection efficacy varied significantly with the cell type [$p < .0001$; Fig. 2(b)]. Transfection efficacy into the human fetal osteoblasts was higher than that in NIH 3T3 fibroblasts and rat primary osteoblasts in all conditions tested (Bonferroni, $p < .0001$), differing from that with the lipid-mediated transfection. Human fetal osteoblasts showed increasing transfection efficacy correlated with gelatin concentration (one-way ANOVA, $p < .0001$), for example, 2.9% with 60 μg gelatin. Transfection efficacies for NIH 3T3 fibroblasts and rat primary osteoblasts were less than 0.5% with all 4 doses tested.

Cationic polymer (PEI)-mediated transfection

Some cells showed altered cellular configuration (e.g., rounded, narrowed, and elongated shapes); minor sparkle-like fluorescence was observed predominantly in the primary osteoblasts [Fig. 3(a)], which exhibited remarkably higher transfection efficacy than the human fetal osteoblast and NIH 3T3 fibroblast (Bonferroni, $p < .0001$). Transfection efficacy in rat primary osteoblasts was 12.7 % when P/N ratio was 1:10, which was significantly higher than those with other P/N ratio (Bonferroni, $p < .001$). In NIH 3T3 fibroblasts and human fetal osteoblasts, transfection efficacy was less than 0.5%.

Cell survival rate

Cell survival rate after the lipid-mediated transfection was affected by the cell type and dose of lipid (two-way ANOVA, $p < .0001$; Fig. 4). Although higher doses reduced cell survival rate, there was a signifi-



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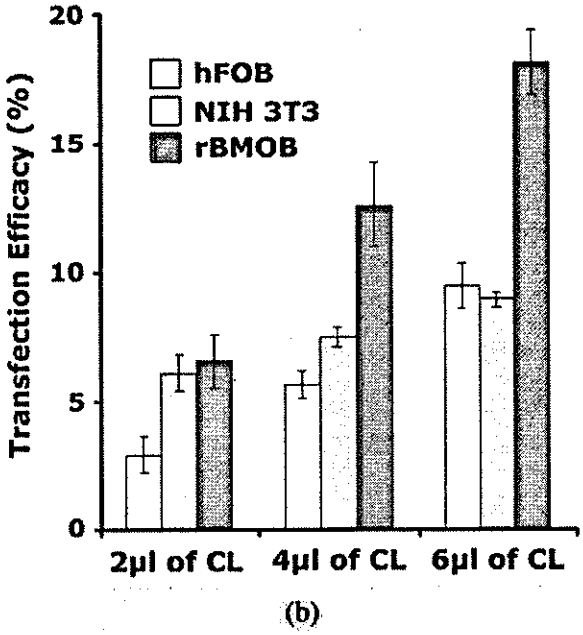
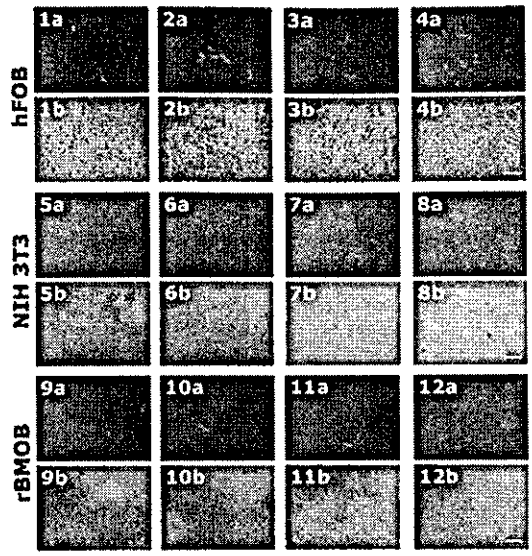
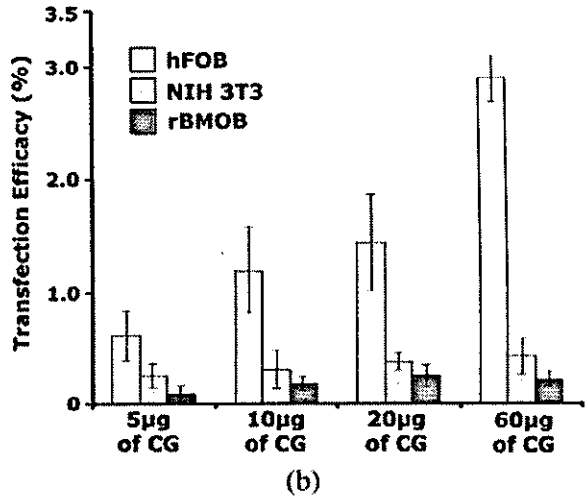


(b)

Figure 1. Cationic-lipid–mediated gene transfection. Clonal human fetal osteoblasts (hFOB), NIH 3T3 fibroblasts (NIH 3T3), and rat bone-marrow–derived primary osteoblasts (rBMOB) are transfected with pDsRed2 plasmid DNA encoding red fluorescent protein using 2 μL, 4 μL, and 6 μL of cationic lipid (CL). (a) Fluorescence staining images. Transfected cells show red fluorescence (1a–9a). Total number of cells was visualized with green Calcein staining (1b–9b). Bar = 40 μμm. (b) Transfection efficacy defined as the number of transfected cells divided by the total number of the cells. Data are means and 1 standard deviation (SD).



(a)



(b)

Figure 2. Cationic-gelatin–mediated gene transfection. Four different doses of cationic gelatin (CG) (5, 10, 20, and 60 μg in 30 μL of 0.1× PBS) were used in order to transfect human fetal osteoblasts (hFOB), NIH 3T3 fibroblasts (NIH 3T3), and rat bone-marrow–derived primary osteoblasts (rBMOB). (a) Fluorescence staining images. Transfected cells showing red fluorescence (1a–12a). Total number of the cells visualized with green Calcein staining (1b–12b). Bar = 40 μm. (b) Transfection efficacy obtained from different dosage of cationic gelatin. Data are means and 1 SD.

cant difference in susceptibility among the cell types (two-way ANOVA, $p = .0002$). Cationic lipid at 6 μL reduced the survival rate below 50% for human fetal osteoblasts and rat primary osteoblasts (Bonferroni, $p < .0001$), but 4 μL of the lipid retained cell survival rate to greater than 75%. In contrast, gelatin dose showed no effect on cell survival rate on any of the cell types tested, maintaining survival rate above 60% in all the cell types (two-way ANOVA, $p > .1$).

Polyethylenimine and cationic polymer maintained

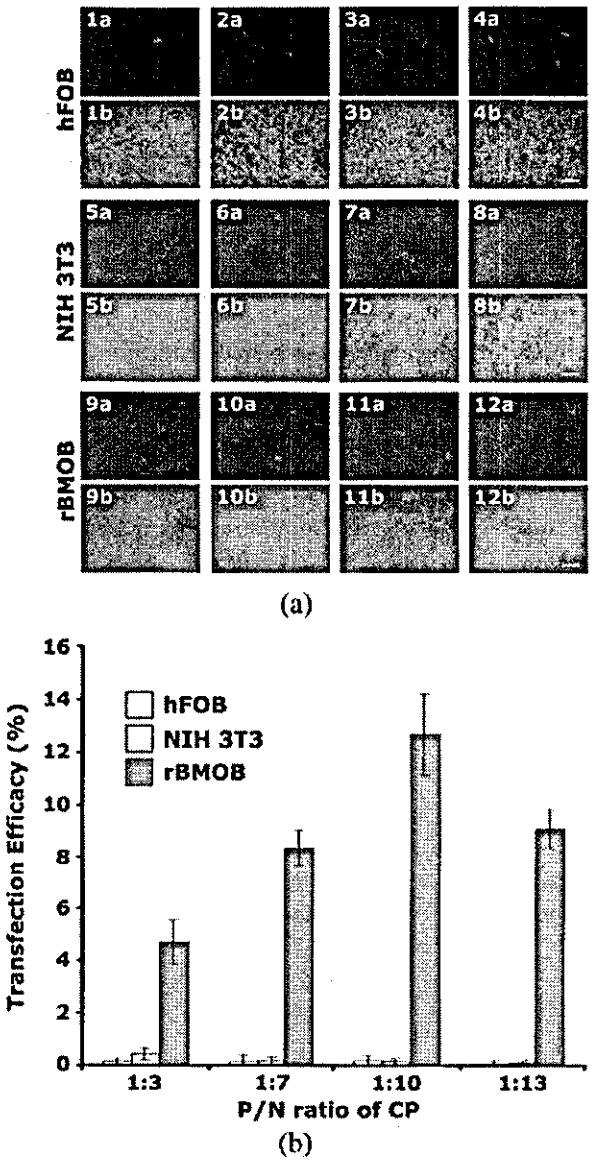


Figure 3. Cationic-polymer–mediated gene transfection. Human fetal osteoblasts (hFOB), NIH 3T3 fibroblasts (NIH 3T3), and rat bone-marrow–derived primary osteoblasts (rBMOB) were transfected with pDsRed2 plasmid DNA using cationic polymer (CP), polyethylenimine (PEI) with P/N (phosphate in DNA/nitrogen in polyethylenimine) ratio of 1:3, 1:7, 1:10, and 1:13 in 150 μ*M* of NaCl. (a) Representative fluorescent images of transfected cells (1a–12a) and total cells (1b–12b). Bar = 40 μm. (b) Transfection efficacy obtained from different P/N ratio of cationic polymer. Data are means and 1 SD from triplicate experiments.

a cell survival rate greater than 70% with a P/N ratio of 1:7, while use in P/N ratios, 1:10 and 1:13, significantly decreased cell survival rate in all cell types tested (Bonferroni, $p < .0001$). In human fetal osteoblasts, PEI with P/N ratio of 1:10 lowered cell survival rate below 20%. Human fetal osteoblasts showed lower cell survival rate than rat primary osteoblasts at higher doses of lipid and PEI agents (Bonferroni, $p < .0001$).

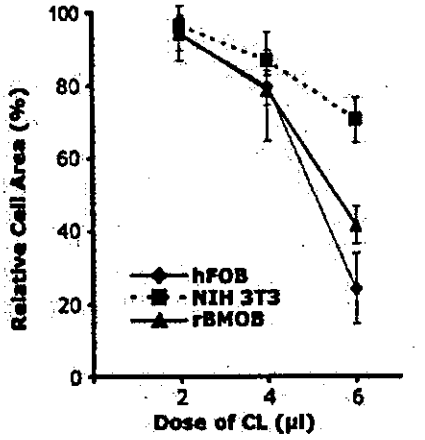
DISCUSSION

Human fetal osteoblasts, rat primary osteoblasts, and clonal fibroblasts showed differential susceptibility to cationic-agent–mediated gene transfer. Rat primary osteoblasts showed higher transfection rates than the other two cells with cationic lipid or PEI agents. Polyethylenimide was specially effective in rat primary osteoblasts. Human fetal osteoblasts were transfected more efficiently via cationic gelatin than either primary osteoblasts or NIH 3T3 fibroblasts.

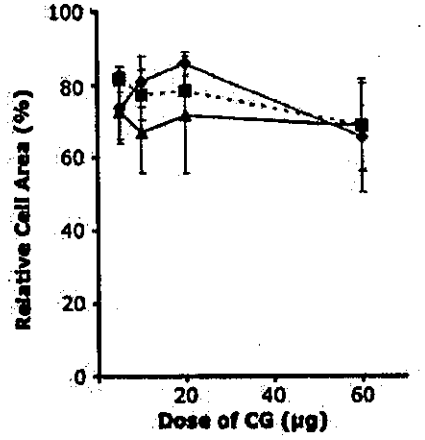
Lipofectamine 2000, a liposome formulation of cationic and neutral lipid, was used as a representative cationic lipid agent in this study. Increase in transfection efficacy with increasing dosage may be due to an increase in the positive charge of the lipid/DNA complex.[32] Interaction between positive complexes and plasma membranes leads to increased endocytosis,[33] but high positive charges may have an adverse effect on the cell viability,[33] as shown in the present results on the cell suvival rate. In general, transfection efficiency into primary cells is lower than that into an established cell line. Also, *in vivo* gene transfer is less efficient than *in vitro* transfer. In particular cell types, such as vascular cells, however, primary cells shows higher transfection efficiency than the cell lines.[34] In this experiment, liposome-mediated gene transfer was approximately 2-fold effective in the primary osteoblasts than in the clonal osteoblasts at all the cationic liposome/DNA ratios tested. Although mechanism of cationic-agent–mediated gene transfer has not been fully elucidated, the formation of stable DNA/agent complexes with a net positive charge associates with the negatively charged surface of the cells.[20] This complex then either fuses with, or causes a transient destabilization of, the cell membrane, resulting in the delivery of the complex into the cytoplasm.[35] Differences in cell surface charge, fusing property of the liposome/DNA complex into the membrane, or translation efficiency into the peptide[36] may have caused the different transfection efficiency. Evaluating other osteoblastic cell lines would be informative in this respect.

The cationic gelatin was generated by introducing ethylenediamine into the carboxyl group of gelatin. The ratio of ethylenediamine to the carboxyl group was 50:1.[22] The transfection efficiency was extremely low for all the cell types tested; it was lower than 0.5% in the primary osteoblasts, while we tested four different doses ranging 12 times. Because the dose–transfection efficacy curve for the human fetal osteoblastic cell line was still increasing at the maximum dose tested, testing even higher doses may lead to higher

transfection efficacy for this particular cell line. However, the low transfection efficacy via cationic gelatin and lack of dose-dependent transfection efficacy in rat primary osteoblasts and NIH 3T3 fibroblasts suggest that use of cationic gelatin may not be suitable for these cell types.



(a)



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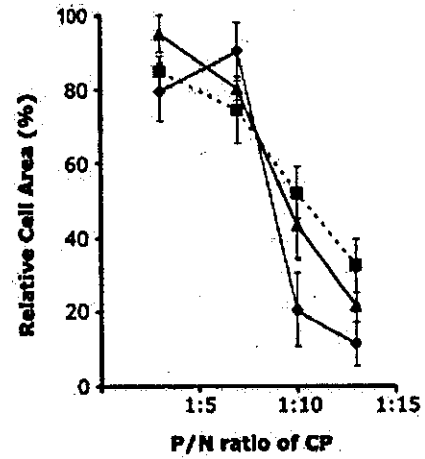

(c)

Polyethylenimine condenses DNA, binds tightly, and encases the condensed DNA.[37] Polyethylenimine has a high cationic charge density[38] and may possess the greatest potential of agents tested to hold DNA molecules. This study showed that the P/N ratio between the DNA and PEI significantly affects transfection efficacy, although the change with P/N ratio was not linear. Each cell type tested showed its own characteristic optimal P/N ratio for maximum transfection efficacy: 1:7 for human fetal osteoblasts, 1:3 for NIH 3T3 fibroblasts, and 1:10 for rat primary osteoblasts. Different optimization of P/N ratio was reported in other cell types.[30,31] These suggest that they each require a specific favorable degree of condensation and electric charge of the DNA/PEI complex.[29] The PEI-mediated transfection was selectively effective in the primary osteoblasts, showing 12.7% transfection efficiency when optimized. To the best of our knowledge, PEI has not been used in osteoblastic cells in the previous literature. The use of PEI, as well as cationic lipid, may be useful for future gene transfer studies in primary osteoblasts. Recently, a strategy on receptor-mediated gene transfer using specific ligand–PEI conjugates was demonstrated to improve the transfection efficiency. RGD peptide–PEI conjugate[39] and nerve growth factor receptor–PEI conjugate[40] effectively target integrin-expressing cells and neuron cells, respectively. The development of PEI combined with a specific ligand to primary osteoblasts would be a next approach for more efficient and targeted gene transfer.

Higher dosage of cationic agents generally increased transfection efficacy, but were associated with low cell survival rate, except in case of the gelatin. Therefore, not only transfection efficacy but also cytotoxicity should be considered to optimize the gene transfection protocol, prioritizing either for transfection efficacy, cytotoxicity, or balancing the two. In case that the effect of gene transfection on tissue phenotype is examined, cellular viability may be critical. Technically, when cells are limited in number and need to be preserved, the cell survival rate may be important. On the other hand, transfection efficacy rather than cell survival rate may be paramount for altering or enhancing intracellular signaling by gene transfection.

Figure 4. Cell survival rate after gene transfection via three cationic agents: (a) cationic lipid (CL), (b) cationic gelatin (CG), and (c) cationic polymer (CP). Twenty-four hours after the transfection, cells were stained with 1.4 % methylene blue solution. The stained cell area in the transfected wells was quantified using image software. The cell survival rate is defined as the cell area after gene transfection normalized by the cell area in the untreated control. The experiment was performed three times. Data showing mean and 1 SD. hFOB, human fetal osteoblasts; NIH 3T3, NIH 3T3 fibroblasts; rBMOB, rat bone-marrow–derived primary osteoblasts.

For instance, when transfection efficacies of the cationic lipid and cationic polymer are maximized for the primary osteoblasts, the cell survival rates are as low as 41.8% and 43.3%, respectively. When the transfection efficacies are compromised at 12.6% and 8.3%, respectively, over 75% cell survival rate can be obtained. Because the cell survival rate dropped between 4 μL and 6 μL of the lipid agent and between 1:7 and 1:10 of PEI P/N ratio, there seemed to be a threshold dose that resulted in critical cytotoxicity. NIH 3T3 fibroblasts seemed to be most resistant to cationic lipid and PEI toxicity; human fetal osteoblast seemed to be the most sensitive. It has been reported that presence of high positive charge in the medium induces adverse effects on the cell viability.[28] Duration and degree of destablization of cell membranes by the positively charged DNA complex may affect the cell viability.[41] Especially, PEI has been concerned for its significant cytotoxicity, which manifests itself by either an immediate (within 2 h) or delayed (7–9 h posttransfection) cell death. The immediate cell death is linked to the membrane destabilizing activity of free PEI that occupies 80% of the PEI/DNA mixture.[42] Additionally, the release of PEI into cytoplasm and subsequent diffusion into the nucleus cause endosomal disruption and the large intranuclear PEI concentration.[43] The cellular deformity observed in the present study may be explained by these potential cytotoxic mechanisms of PEI.

We utilized the expression vector encoding a recombinant peptide, pDsRed2-N1 to detect the occurrence of transfection and obtained cell-number–based transfection efficacy. Because the peptide must be properly folded and form a trimeric molecule to be fluorescent, in order for visible detection, successful transfection, transcription, translation, and proper posttranslational processing are required. Therefore, it is possible that the transfection efficacy obtained in the present study is less than other reports using direct detection techniques such as luciferase activity and immunostaining protocols.

All of the three cationic agents tested yielded gene-transfer–mediating potential into osteoblastic cells. However, the effectiveness and optimization of each cationic agent differed among different cell types. Cationic lipid and cationic polymer (PEI) are more effective than cationic gelatin in rat primary osteoblasts. The cell survival rate decreased as the cationic lipid and cationic polymer were used in a higher dose. The cationic gelatin was more effective in transfecting the human feta osteoblast cell line than transfecting rat primary osteoblasts, and the dose did not affect the cell survival rate. This report provides a basis for selection and optimization of cationic agents for nonviral gene transfer into osteoblastic cells and balancing or prioritizing transfection efficacy and cytotoxicity.

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References

1. Doll B, Sfeir C, Winn S, Huard J, Hollinger J. Critical aspects of tissue-engineered therapy for bone regeneration. Crit Rev Eukaryot Gene Expr 2001;11:173–198.
2. Langer R, Vacanti JP. Tissue engineering. Science 1993;260: 920–926.
3. Chen RR, Mooney DJ. Polymeric growth factor delivery strategies for tissue engineering. Pharm Res 2003;20:1103–1112.
4. Simonsen JL, Rosada C, Serakinci N, Justesen J, Stenderup K, Rattan SI, Jensen TG, Kassem M. Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells. Nat Biotechnol 2002;20:592–596.
5. Shen FH, Visger JM, Balian G, Hurwitz SR, Diduch DR. Systemically administered mesenchymal stromal cells transduced with insulin-like growth factor-I localize to a fracture site and potentiate healing. J Orthop Trauma 2002;16:651–659.
6. Bonadio J, Smiley E, Patil P, Goldstein S. Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. Nat Med 1999;5:753–759.
7. Tsuda H, Wada T, Ito Y, Uchida H, Dehari H, Nakamura K, Sasaki K, Kobune M, Yamashita TH, Yamada H. Efficient BMP2 gene transfer and bone formation of mesenchymal stem cells by a fiber-mutant adenoviral vector. Mol Ther 2003;7:354–365.
8. Peng H, Wright V, Usas A, Gearhart B, Shen HC, Cummins J, Huard J. Synergistic enhancement of bone formation and healing by stem cell-expressed VEGF and bone morphogenetic protein-4. J Clin Invest 2002;110:751–759.
9. Wright V, Peng H, Usas A, Young B, Gearhart B, Cummins J, Huard J. BMP4-expressing muscle-derived stem cells differentiate into osteogenic lineage and improve bone healing in immunocompetent mice. Mol Ther 2002;6:169–178.
10. Huard J, Li Y, Peng H, Fu FH. Gene therapy and tissue engineering for sports medicine. J Gene Med 2003;5:93–108.
11. Lee JY, Peng H, Usas A, Musgrave D, Cummins J, Pelinkovic D, Jankowski R, Ziran B, Robbins P, Huard J. Enhancement of bone healing based on ex vivo gene therapy using human muscle-derived cells expressing bone morphogenetic protein 2. Hum Gene Ther 2002;13:1201–1211.
12. Gysin R, Wergedal JE, Sheng MH, Kasukawa Y, Miyakoshi N, Chen ST, Peng H, Lau KH, Mohan S, Baylink DJ. Ex vivo gene therapy with stromal cells transduced with a retroviral vector containing the BMP4 gene completely heals critical size calvarial defect in rats. Gene Ther 2002;9:991–999.
13. Oakes DA, Lieberman JR. Osteoinductive applications of regional gene therapy: ex vivo gene transfer. Clin Orthop 2000: S101–S112.
14. Levy RJ, Goldstein SA, Bonadio J. Gene therapy for tissue repair and regeneration. Adv Drug Deliv Rev 1998;33:53–69.
15. Lieberman JR, Ghivizzani SC, Evans CH. Gene transfer approaches to the healing of bone and cartilage. Mol Ther 2002; 6:141–147.
16. Navarro J, Oudrhiri N, Fabrega S, Lehn P. Gene delivery systems: bridging the gap between recombinant viruses and artificial vectors. Adv Drug Deliv Rev 1998;30:5–11.
17. Yang Y, Nunes FA, Berencsi K, Furth EE, Gonczol E, Wilson JM. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. Proc Natl Acad Sci U S A 1994; 91:4407–4411.
18. Yang Y, Jooss KU, Su Q, Ertl HC, Wilson JM. Immune responses to viral antigens versus transgene product in the elim-

ination of recombinant adenovirus-infected hepatocytes in vivo. Gene Ther 1996;3:137–144.
19. Ritter T, Lehmann M, Volk HD. Improvements in gene therapy: averting the immune response to adenoviral vectors. BioDrugs 2002;16:3–10.
20. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci U S A 1987;84:7413–7417.
21. Hosseinkhani H, Aoyama T, Ogawa O, Tabata Y. Liver targeting of plasmid DNA by pullulan conjugation based on metal coordination. J Control Release 2002;83:287–302.
22. Hosseinkhani H, Aoyama T, Yamamoto S, Ogawa O, Tabata Y. In vitro transfection of plasmid DNA by amine derivatives of gelatin accompanied with ultrasound irradiation. Pharm Res 2002;19:1471–1479.
23. Aoyama T, Hosseinkhani H, Yamamoto S, Ogawa O, Tabata Y. Enhanced expression of plasmid DNA-cationized gelatin complex by ultrasound in murine muscle. J Control Release 2002; 80:345–356.
24. Gharwan H, Wightman L, Kircheis R, Wagner E, Zatloukal K. Nonviral gene transfer into fetal mouse livers (a comparison between the cationic polymer PEI and naked DNA). Gene Ther 2003;10:810–817.
25. Dolivet G, Merlin JL, Barberi-Heyob M, Ramacci C, Erbacher P, Parache RM, Behr JP, Guillemin F. In vivo growth inhibitory effect of iterative wild-type p53 gene transfer in human head and neck carcinoma xenografts using glucosylated polyethylenimine nonviral vector. Cancer Gene Ther 2002;9:708–714.
26. Wang S, Ma N, Gao SJ, Yu H, Leong KW. Transgene expression in the brain stem effected by intramuscular injection of polyethylenimine/DNA complexes. Mol Ther 2001;3:658–664.
27. Nishikawa M, Huang L. Nonviral vectors in the new millennium: delivery barriers in gene transfer. Hum Gene Ther 2001; 12:861–870.
28. Clark PR, Hersh EM. Cationic lipid-mediated gene transfer: current concepts. Curr Opin Mol Ther 1999;1:158–176.
29. Abdallah B, Sachs L, Demeneix BA. Non-viral gene transfer: applications in developmental biology and gene therapy. Biol Cell 1995;85:1–7.
30. Morimoto K, Nishikawa M, Kawakami S, Nakano T, Hattori Y, Fumoto S, Yamashita F, Hashida M. Molecular weight-dependent gene transfection activity of unmodified and galactosylated polyethyleneimine on hepatoma cells and mouse liver. Mol Ther 2003;7:254–261.
31. Oh YK, Suh D, Kim JM, Choi HG, Shin K, Ko JJ. Polyethylenimine-mediated cellular uptake, nucleus trafficking and expression of cytokine plasmid DNA. Gene Ther 2002;9:1627–1632.
32. Behr JP, Demeneix B, Loeffler JP, Perez-Mutul J. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. Proc Natl Acad Sci U S A 1989;86: 6982–6986.
33. Labat-Moleur F, Steffan AM, Brisson C, Perron H, Feugeas O, Furstenberger P, Oberling F, Brambilla E, Behr JP. An electron microscopy study into the mechanism of gene transfer with lipopolyamines. Gene Ther 1996;3:1010–1017.
34. Keogh MC, Chen D, Lupu F, Shaper N, Schmitt JF, Kakkar VV, Lemoine NR. High efficiency reporter gene transfection of vascular tissue in vitro and in vivo using a cationic lipid-DNA complex. Gene Ther 1997;4:162–171.
35. Felgner PL, Holm M, Chan H. Cationic liposome mediated transfection. Proc West Pharmacol Soc 1989;32:115–121.
36. Lin AJ, Slack NL, Ahmad A, George CX, Samuel CE, Safinya CR. Three-dimensional imaging of lipid gene-carriers: membrane charge density controls universal transfection behavior in lamellar cationic liposome-DNA complexes. Biophys J 2003; 84:3307–3316.
37. Godbey WT, Wu KK, Mikos AG. Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. J Biomed Mater Res 1999;45:268–275.
38. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci U S A 1995;92:7297–7301.
39. Kunath K, Merdan T, Hegener O, Haberlein H, Kissel T. Integrin targeting using RGD-PEI conjugates for in vitro gene transfer. J Gene Med 2003;5:588–599.
40. Ma N, Wu SS, Ma YX, Wang X, Zeng J, Tong G, Huang Y, Wang S. Nerve growth factor receptor-mediated gene transfer. Mol Ther 2004;9:270–281.
41. Godbey WT, Wu KK, Mikos AG. Poly(ethylenimine)-mediated gene delivery affects endothelial cell function and viability. Biomaterials 2001;22:471–480.
42. Clamme JP, Azoulay J, Mely Y. Monitoring of the formation and dissociation of polyethylenimine/DNA complexes by two photon fluorescence correlation spectroscopy. Biophys J 2003; 84:1960–1968.
43. Clamme JP, Krishnamoorthy G, Mely Y. Intracellular dynamics of the gene delivery vehicle polyethylenimine during transfection: investigation by two-photon fluorescence correlation spectroscopy. Biochim Biophys Acta 2003;1617:52–61.

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Novel PVA–DNA nanoparticles prepared by ultra high pressure technology for gene delivery

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Abstract

Polyvinyl alcohol (PVA)–DNA nanoparticles have been developed by ultra high pressure (UHP) technology. Mixture solutions of DNA and PVA having various molecular weights (Mw) and degree of saponifications (DS) were treated under 10,000 atmospheres (981 MPa) condition at 40 °C for 10 min. Agarose gel electrophoresis and scanning electron microscope observation revealed that the PVA–DNA nanoparticles with average diameter of about 200 nm were formed. Using PVA of higher Mw and degree of saponifications, the amount of nanoparticles formed increased. The driving force of nanoparticle formation was the hydrogen bonding between DNA and PVA. In order to apply the PVA–DNA nanoparticles for gene delivery, the cytotoxicity and the cellular uptake of them were investigated using Raw264 cell lines. The cell viability was not influenced whether the presence of the PVA–DNA nanoparticles. Further, the nanoparticles internalized into cells were observed by fluorescent microscope. These results indicates that the PVA–DNA nanoparticles prepared by UHP technology showed be useful as drug carrier, especially for gene delivery.
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Keywords: Ultra high pressure; Hydrogen bond; Nanoparticles; Biocompatibility; Gene delivery; Polyvinyl alcohol

1. Introduction

Pressure processing technology has been used in many fields. The range of pressure is varied in each method from 1 to 100,000 atmosphere (atm) (9810 MPa). In the field of chemistry and biology, the pressure of over 6000 atm is thought as ultra high pressure (UHP). It is well known that the hydrogen bond is strengthens than electrostatic and hydrophobic interactions under the UHP condition [1–3]. From this fact, we recently reported that UHP is one of powerful tools for manipulatory inter- or intra-molecular interaction triggered by hydrogen bond [4]. We have shown some evidence of this hypothesis by using polyvinyl alcohol (PVA), which is synthetic hydrogen bonding polymers having simple hydrogen bonding structure, associated each other to form nanoparticles via hydrogen bond by UHP processing [5]. Among various fields of application, we focused on the usage of the nanoparticle as drug and gene delivery system.

Nanoparticles as gene carrier are able to enhance intracellular gene delivery in vitro and in vivo due to protection of DNA from nuclease cleavage [6–10]. Many types of them, such as cationic compounds [6–8], biodegradable polymers

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[9,10] have been developed. Nanoparticles containing DNA have been formed by electrostatic interaction between negative charge of phosphate groups of DNA and positive charge of cationic compounds or encapsulation. However, it was reported that such cationic substances has the essential problem of the cytotoxicity, and the difficulty of controlling of DNA release from nanoparticles.

In the present study, we report the preparation of novel nanoparticles of plasmid DNA and PVA via hydrogen bond using UHP technology and their application for gene delivery. The interaction force of nanoparticle formation is hydrogen bond between PVA and DNA, because DNA is one of typical hydrogen bonding polymer as well as PVA. Further, the biocompatibility and neutral charge nature of PVA allows the low cytotoxicity. The cellular uptake of them was investigated in order to evaluate the nanoparticles as biocompatible gene carriers.

2. Materials and methods

2.1. Preparation of PVA–DNA nanoparticles by UHP method

PVAs having different molecular weights and degree of saponifications were supplied from Kuraray (Osaka, Japan) (Table 1). Plasmid DNA encoding green fluorescent protein under cytomegalovirus promoter (pEGFP-C1) was obtained from BD Biosciences Clontech (Tokyo, Japan). PVA solutions (0.0001–0.1 w/v%) and pEGFP-C1 solution (0.02 w/v%) were mixed in water and treated under 10,000 atm at 40 °C for 10 min (UHP method) using high-pressure machine (Dr. Chef, Kobe Steel, Kobe, Japan).

2.2. Characteristics of PVA–DNA nanoparticles

At 0.0001–0.01 w/v% of PVA concentration, PVA and pEGFP-C1 mixture solutions treated with UHP were analyzed by agarose gel electrophoresis (1.0 w/v%, 100 V, 1 h). At 0.025–0.1 w/v% of PVA concentration, after centrifugation of the UHP-treated mixture solutions at 5000 rpm for 5 min, the supernatant was collected and the precipitation was washed by water. This procedure was carried out twice. The precipitation was melted by heat treatment for 10 min. They were electrophoresed though 1.0 w/v% agarose gels at 100 V for 1 h. The gels were stained with ethidium bromide. The shape and size of structures were observed by scanning electron microscope (JSM-6301F, JEOL, Tokyo, Japan).

Table 1
Various polyvinyl alcohols used

PVA	DP[a]	DS[b]	Mw
PVA205	500	88	22,000
PVA105	500	98.5	22,000
PVA117	1700	99.3	74,800
PVA140	4000	99.8	176,000

[a] Degree of polymerization.
[b] Degree of saponification.

2.3. Cytotoxicity of PVA–DNA nanoparticles

Mouse macrophage cell lines of Raw264 cells were cultured in a complete modified eagle medium (DMEM, Invitrogen, Tokyo, Japan), supplemented with non-inactivated 10% fetal calf serum (FCS), 50 IU/ml of penicillin, 50 μg/ml of streptomycin (ICN Biomaterials, Ohio, USA). To evaluate the cytotoxicity of PVA–DNA nanoparticles, 2.0×10^4 cells incubated with PVA–DNA nanoparticles at 37 °C for 20 h in the present of FCS and the number of viable cells was assessed using a Cell Counting Kit-8 (Dojindo Laboratory, Tokyo, Japan) according to the manufacturer's instruction.

2.4. Cellular uptake of PVA–DNA nanoparticles

To investigate the cellular uptake of PVA–DNA nanoparticles, pEGFP-C1 labeled with rhodamine by Label It kit (Panvera, WI, USA) was added on 2.5×10^5 cells of Raw264 cells cultured in the present of non-inactivated FCS and incubated at 37 °C for 20 h. The cells were observed under fluorescent microscope.

3. Results and discussion

Fig. 1 shows the microscopic observation of the mixture solutions of pEGFP-C1 and various PVAs at 0.1 w/v% concentration treated with UHP after centrifugation at 5000 rpm for 5 min. The mixture solution of PVA205 remained as clear solution as well as pEGFP-C1. However, a little precipitation was observed in PVA105 and the white precipitation was observed in the case of PVA117 and PVA 140 (Fig. 1(A)). When DNA solution mixed with PVA117 at different concentration were pressurized under UHP condition, the amount of white precipitation was decreased with decreasing PVA concentration, and the precipitation was not observed at 0.01 w/v% of PVA117 (Fig. 1(B)). These results indicate that the size of particle obtained varied in each molecular weight and concentration of PVAs used, and that the higher molecular weights of PVA tended to form particles. This phenomena was observed even when the PVA solution without DNA was treated with UHP (data not shown). Fig. 2 shows SEM images of the UHP treated mixture solutions of DNA in the presence of (A) 0.01 w/v% or (B) 0.025% of PVA. Nanoparticles having average diameter of about 200 nm were observed in 0.01 w/v% of PVA concentration. At 0.025 w/v% concentration, the nanoparticles aggregated each other. It became clear that the precipitation formation at higher PVA concentration under UHP condition due to the aggregation of nanoparticles of PVA or PVA/DNA mixture.

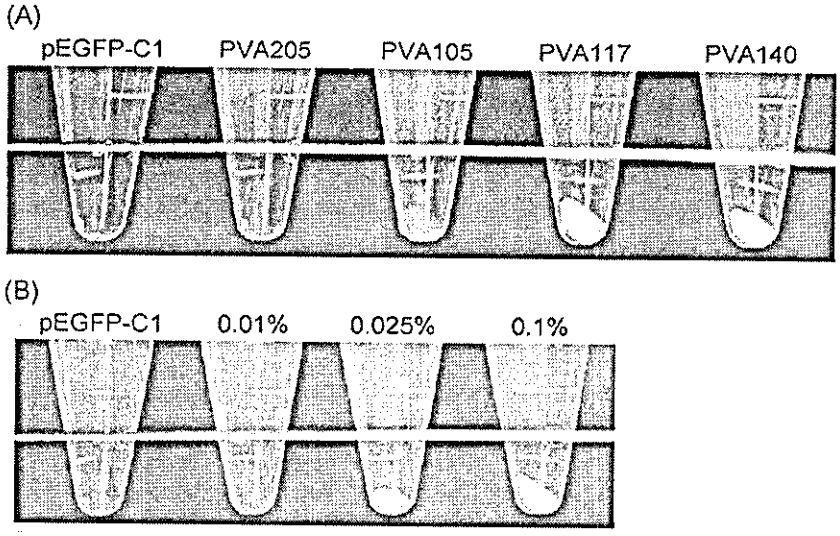

Fig. 1. Microphotographs of mixture solutions of DNA and (A) various PVAs of 0.1% concentration and (B) PVA117 of different concentration treated by UHP.

To confirm whether the nanoparticles contain DNA, the mixture solutions of DNA and PVA140 at less than 0.01% concentration treated with UHP were analyzed by agarose gel electrophoresis (Fig. 3(A)). The DNA bands in the non-treated mixture solutions were observed at the same pattern of pEGFP-C1, which contains circular, linear and super coiled form, irrespective of that concentration. On the other hand, the smear bands of DNA–PVA nanoparticles appeared at each concentration, indicating the nanoparticles consisting of DNA and PVA, but not PVA only. The heat melted aggregates of nanoparticles were

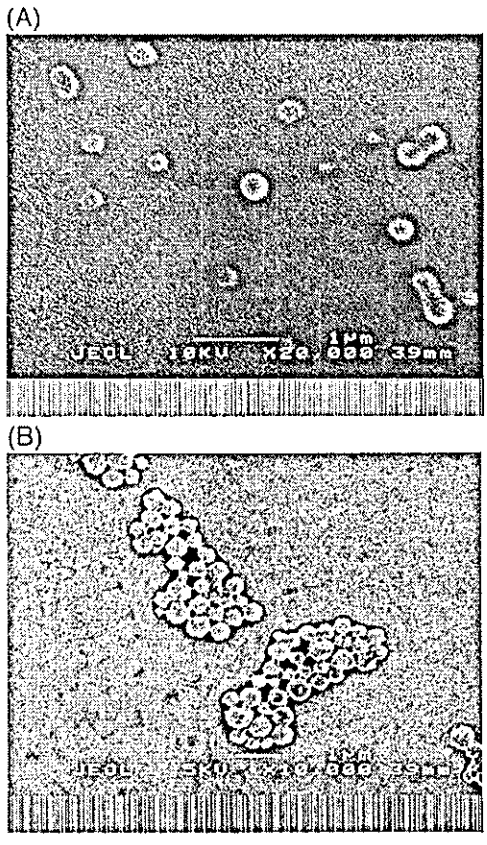

Fig. 2. SEM images of PVA–DNA nanoparticles. PVA117 was used.

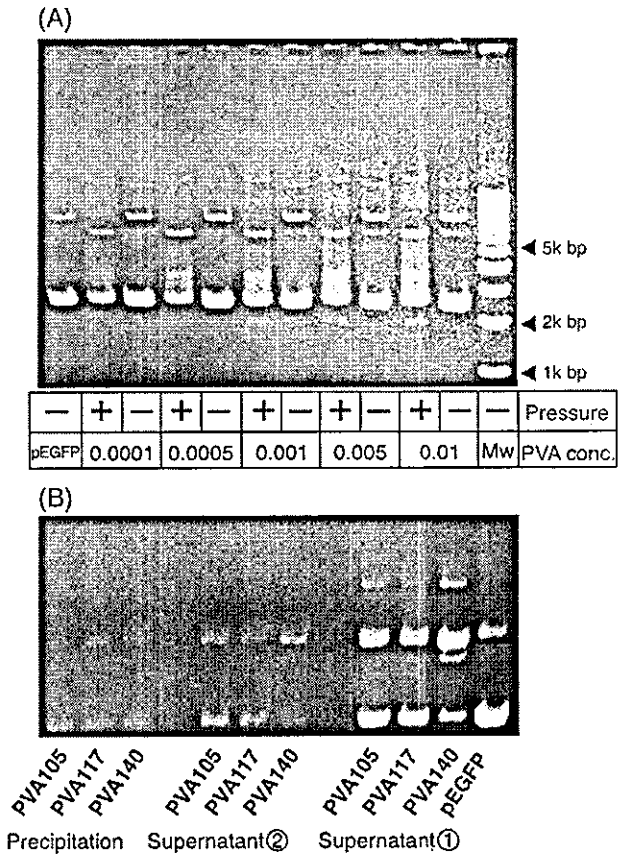

Fig. 3. Agarose gel electrophoresis of (A) PVA117-DNA nanoparticles prepared at 0.01% concentration and (B) the aggregates of PVA–DNA nanoparticles at 0.1% concentration after heat treatment.

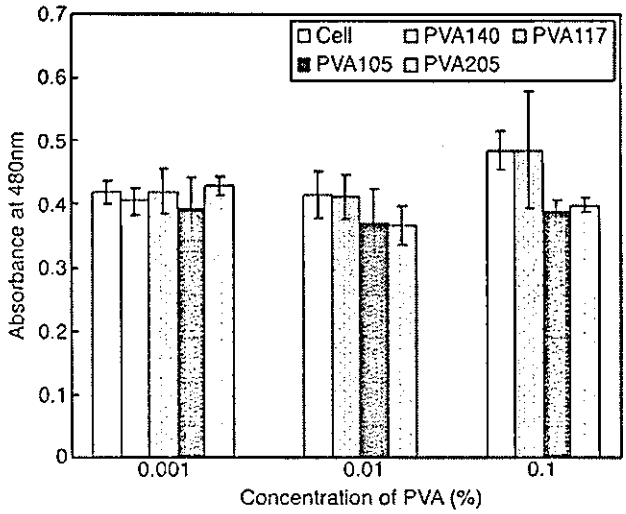

Fig. 4. Cytotoxicity of PVA–DNA nanoparticles.

also electrophoresed with agarose gel after twice washing procedure (Fig. 3(B)). The bands of DNA were observed not only in first and second supernatants but also in the collected precipitation. It was clear that the nanoparticles consisting of PVA and DNA.

Fig. 4 shows the result of the toxicity test of PVA–DNA nanoparticles. The result of high viability of Raw264 cells incubated with PVA–DNA nanoparticles was obtained irrespective of the molecular weights of PVA used. This result indicates that PVA–DNA nanoparticle is non-toxic. Conventionally, cationic polymers were widely used for gene delivery due to complex formation with DNA by electrostatic interaction, however, the cell damage for cationic nature of them was pointed out. Yamaoka et al. [11] reported that the cytotoxicity decreased with decreasing the charge density of polycations. Fischer et al. [12] suggests the necessity of optimizing the balance between the cytotoxicity and the biocompatibility of cationic polymers used as gene carrier. Therefore, it is considered that non-charged PVA permitted the low cytotoxicity of PVA–DNA nanoparticles formed by hydrogen bond.

In order to investigate cellular uptake of the PVA–DNA nanoparticles, the nanoparticles of PVA and pEGFP-C1 labeled with rhodamine were added to Raw264 cells in the present of FCS. In Fig. 5, a lot of red fluorescence spots

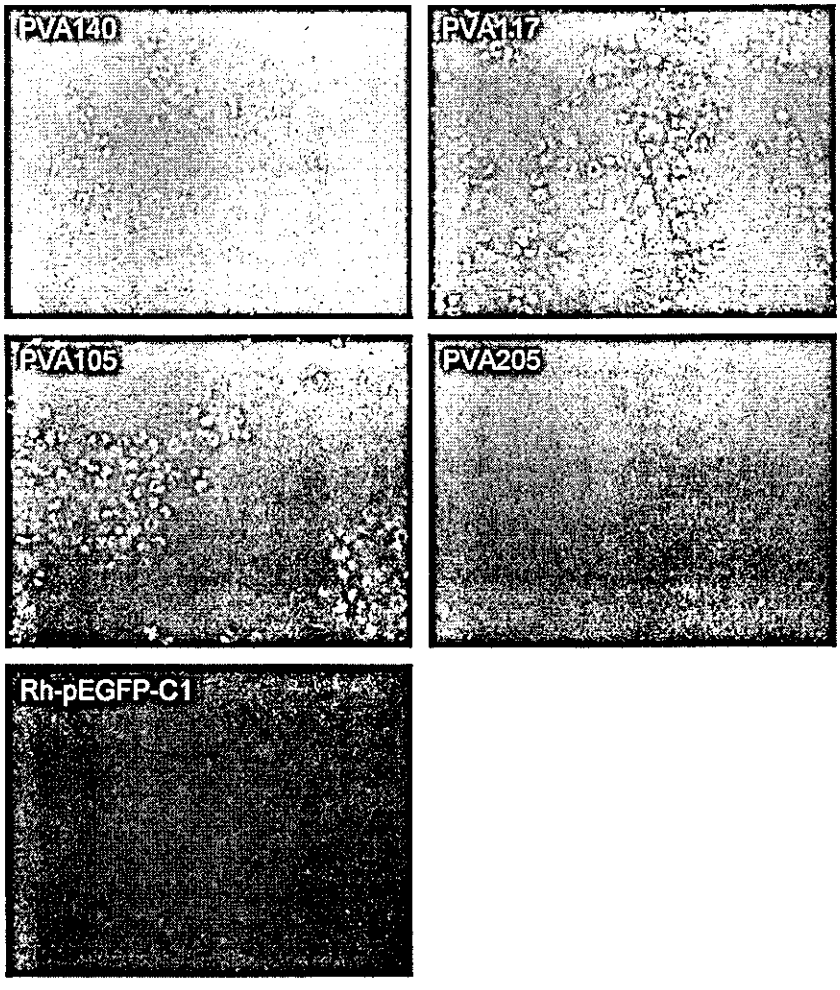

Fig. 5. Fluorescent images of Raw264 incubated with the nanoparticles of PVA and rhodamine-labeled pEGFP-C1 for 24 h.

in many cells were brightly observed in the case of PVA105, PVA117 and PVA140 except for pEGFP-C1 and PVA205. This result suggests that the significant internalization of DNA which means that the PVA–DNA nanoparticle was incorporated into cells. In the case of PVA205, as PVA–DNA particles formation was insufficient, low incorporation result was obtained. These results suggest that PVA–DNA nanoparticles have favorable characteristics for gene delivery system, are non-cytotoxic and high gene transfer into cell. The uptake of PVA–DNA nanoparticles by cells is probably achieved by complement activation because it is well-known fact that PVA activates complement system.

4. Conclusion

We have developed nanoparticles consisting DNA and PVA via hydrogen bonds using UHP technology. The average nanoparticle diameter was 200 nm. The nanoparticle formation could be controlled by the molecular weight of PVA used. Cell viability studies following incubation with the nanoparticles confirmed the lack of toxicity of PVA. The ability of the nanoparticles to delivery DNA into cells was also shown, and PVA–DNA nanoparticles are considered as a potential candidate for a gene carrier.

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References

[1] E. Doi, A. Shimizu, N. Kitabatake, in: R. Hayashi (Ed.), High Pressure Bioscience and Food Science, Sanei Press, 1993, p. 171.
[2] E. Doi, A. Shimizu, N. Kitabatake, Food Hydrocoll. 5 (1991) 409.
[3] S. Sawamura, K. Kitamura, Y. Taniguchi, J. Phys. Chem. 93 (1989) 4931.
[4] K. Yamamoto, A. Kishida, T. Furuzono, S. Mutsuo, H. Yoshizawa, Y. Kitamura, Polymer PrePrints, Japan, vol. 51, 2002.
[5] K. Yamamoto, T. Furuzono, S. Mutsuo, H. Yoshizawa, Y. Kitamura, A. Kishida, in: Meeting Report of the Poval Committee, vol. 121, 2002, p. 25.
[6] S.C. De Smedt, J. Demeester, W.E. Hennink, Pharm. Res. 17 (2000) 113.
[7] Y. Kakizawa, K. Kataoka, Adv. Drug Deliv. Rev. 54 (2002) 203.
[8] X.X. He, K. Wang, W. Tan, B. Liu, X. Lin, C. He, D. Li, S. Huang, J. Li, J. Am. Chem. Soc. 125 (2003) 7168.
[9] C. Perez, A. Sanchez, D. Putnam, D. Ting, R. Langer, M.J. Alonso, J. Control Release 75 (2001) 211.
[10] J. Panyam, V. Labhasetwar, Adv. Drug Deliv. Rev. 55 (2003) 329.
[11] T. Yamaoka, N. Hamada, H. Iwata, A. Murakami, Y. Kimura, Chem. Lett. (1998) 1171.
[12] D. Fischer, Y. Li, B. Ahlemeyer, J. Krieglstein, T. Kissel, Biomaterials 24 (2003) 1121.

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Stem Cell Review Series

Endothelial progenitor cells: past, state of the art, and future

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Abstract

Recent evidences suggest that endothelial progenitor cells (EPCs) derived from bone marrow (BM) contribute to *de novo* vessel formation in adults occurring as physiological and pathological responses. Emerging preclinical trials have shown that EPCs home to sites of neovascularization after ischemic events in limb and myocardium. On the basis of these aspects, EPCs are expected to develop as a key strategy of therapeutic applications for the ischemic organs. Such clinical requirements of EPCs will tentatively accelerate the translational research aiming at the devices to acquire the optimized quality and quantity of EPCs. In this review, we attempt to discuss about biological features of EPCs and speculate on the clinical potential of EPCs for therapeutic neovascularization.

Keywords: endothelial progenitor cell (EPC) - vasculogenesis - angiogenesis - therapeutic neovascularization - cardiovascular disease - cell therapy

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