amino residues introduced increased with the addition ratio of amine compounds, irrespective of the compound type. In this study, the Pronectin F^+ at the molar ratio of amino groups introduced of 26% was chosen for in vitro gene transfection test. On the other hand, our previous study demonstrated that every amine derivative of gelatin at amino residues introduced around 50% was the most effective in enhancing the gene transfection of a plasmid DNA among the derivatives at other percentages [21,22]. Therefore, the gelatin was selected, irrespective of the amine type.

3.2. Characterization of Pronectin F^+ derivatives–plasmid DNA and gelatin derivatives–plasmid DNA complexes

Fig. 1 shows the electrophoretic patterns of Pronectin F^+ derivatives–plasmid DNA and gelatin derivatives–plasmid DNA complexes prepared at different mixing weight ratios of Pronectin F^+ and gelatin to plasmid DNA. Irrespective of the derivative type, the migration of plasmid DNA was retarded with an increase in the mixing ratio, but not observed any more at the ratio higher than a certain

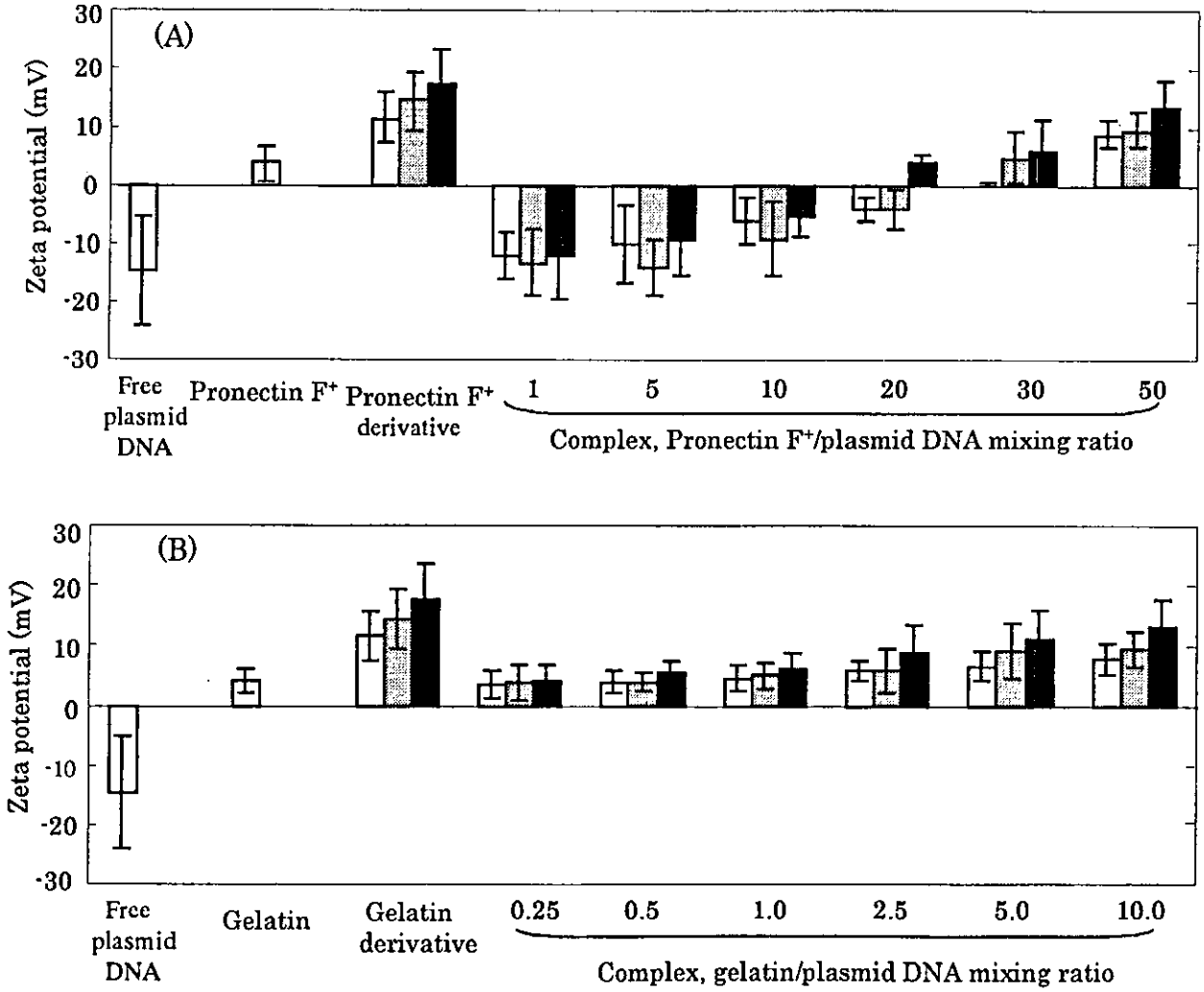

Fig. 2. Zeta potentials of free plasmid DNA, Pronectin F^+, gelatin, Pronectin F^+ or gelatin derivatives, and Pronectin F^+ or gelatin derivatives–plasmid DNA complexes prepared at different mixing weight ratios of Pronectin F^+ (A) or gelatin (B) to plasmid DNA: (□) Ed-, (▨) Sd-, and (■) Sm-derivatives. The molar percentage of amino groups introduced to the hydroxyl groups of Pronectin F^+ is 26%.

value. The Sm derivative of Pronectin F^+ and gelatin enabled the plasmid DNA to firmly prevent the electrophoretic migration at molar ratios lower than other Pronectin F^+ and gelatin derivatives. Neither the original Pronectin F^+ and gelatin without any aminization nor cationized Pronectin F^+ and gelatin with the smallest aminization induced the electrophoretic migration of plasmid DNA.

Figs. 2 and 3 show the surface charge and apparent molecular size of Pronectin F^+ derivatives–plasmid DNA and gelatin derivatives–plasmid DNA complexes prepared at different mixing weight ratios of Pronectin F^+–plasmid DNA and gelatin–plasmid DNA. Free plasmid DNA exhibited a negative ζ potential. However, mixing with the Pronectin F^+ and gelatin derivative changed it to positive values. The value tended to increase as the mixing weight ratio increased, although the increased extent was high for the Sm derivative of Pronectin F^+ and gelatin compared with that of other two types of

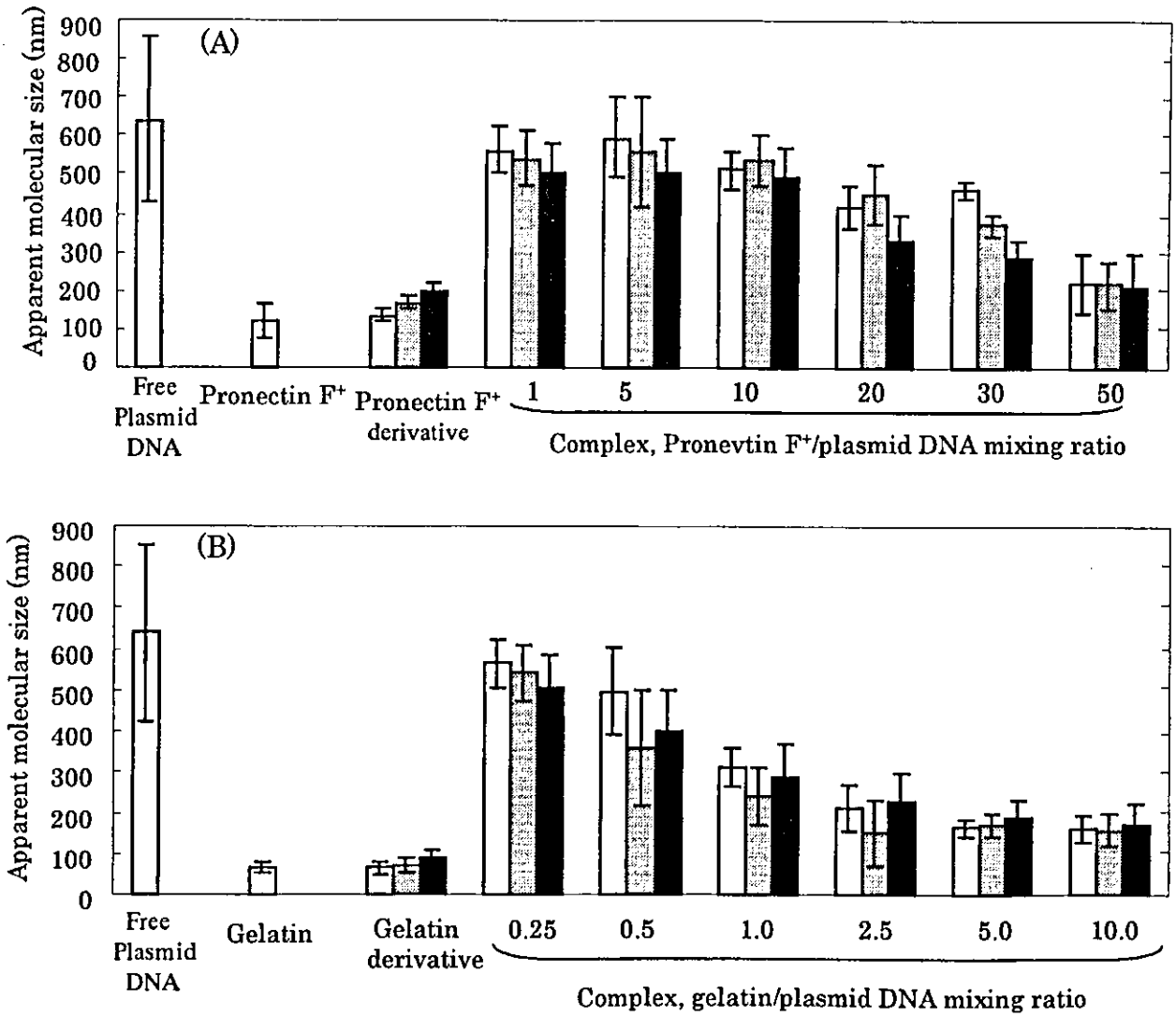

Fig. 3. Apparent molecular sizes of free plasmid DNA, Pronectin F^+, gelatin, Pronectin F^+ or gelatin derivatives, and Pronectin F^+ or gelatin derivatives–plasmid DNA complexes prepared at different mixing weight ratios of Pronectin F^+ (A) or gelatin (B) to plasmid DNA: (□) Ed-, (▨) Sd-, and (■) Sm-derivatives. The molar percentage of amino groups introduced to the hydroxyl groups of Pronectin F^+ is 26%.

Pronectin F^+ and gelatin derivatives. The apparent molecular size of plasmid DNA changed with mixing of the Pronectin F^+ and gelatin derivative and decreased with the increased mixing ratio, irrespective of the Pronectin F^+ and gelatin type.

3.3. Transfection of different Pronectin F^+ derivatives–plasmid DNA and gelatin derivatives–plasmid DNA complexes

Fig. 4 shows the luciferase activity of RGM-1 cells treated with Pronectin F^+ derivative–plasmid DNA complexes at weight mixing ratio of 50 and gelatin derivative–plasmid DNA complexes at weight mixing ratio of 5. Irrespective of the Pronectin F^+ or gelatin, the luciferase activity was enhanced by the incubation of any Pronectin F^+ derivative–plasmid DNA or gelatin derivative–plasmid DNA complex to a significantly higher extent than that of free plasmid DNA. The activity enhancement was higher for the Sm derivative complex than that of the corresponding other complexes. The enhanced level of luciferase activity was significantly much higher for any Pronectin F^+ derivative compared with gelatin derivative. The Pronectin F^+ and gelatin derivative alone was not effective in the activity enhancement and the level was similar to that of PBS treatment.

3.4. Cell attachment

Fig. 5 shows the number of cells attached to tissue culture plate coated with the Pronectin F^+ or gelatin and their derivatives. As expected, cell attachment was significantly much higher for the Pronectin F^+ derivative compared to the gelatin derivative al-

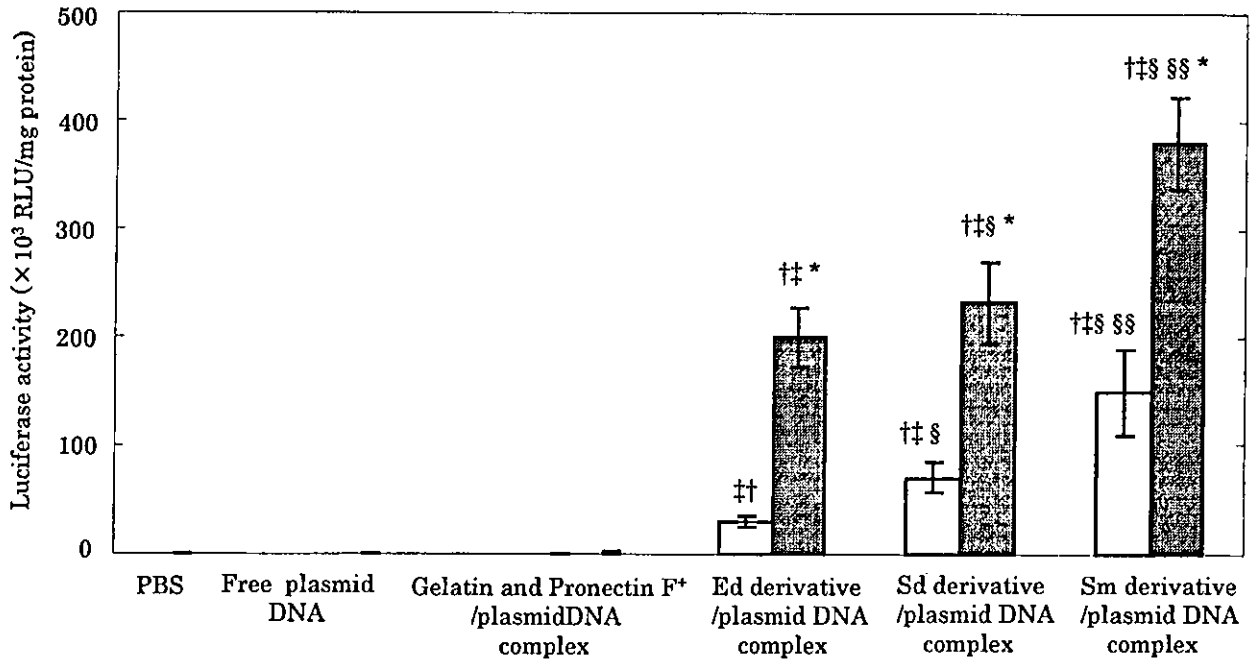

Fig. 4. Luciferase activity of RGM-1 cells 48 h after treatment of plasmid DNA-free PBS, free plasmid DNA, gelatin–plasmid DNA complex, Pronectin F'–plasmid DNA complex, gelatin-derivatives plasmid DNA complex, and Pronectin F' derivatives–plasmid DNA complexes: (□) gelatin and its derivatives and (□) Pronectin F^+ and its derivatives. The dose of plasmid DNA is 0.1 mg/ml and the mixing weight ratio of gelatin and Pronectin F^+ to plasmid DNA is 5 and 50, respectively. The molar percentage of amino groups introduced to the hydroxyl groups of Pronectin F^+ is 26%. $^{\dagger}P<0.05$; significant against the activity of RGM-1 cells after treatment of PBS. $^{\ddagger}P<0.05$; significant against the activity of RGM-1 cells after treatment of free plasmid DNA. $^{\S}P<0.05$; significant against the activity of RGM-1 cells after treatment of plasmid DNA complexes with Ed-50 and Pronectin F^+-Ed. $^{\S\S}P<0.05$; significant against the activity of RGM-1 cells after treatment of plasmid DNA complexes with Sd-50 and Pronectin F^+-Sd. $^{*}P<0.05$; significant against the activity of RGM-1 cells after treatment of plasmid DNA complexes with every gelatin.

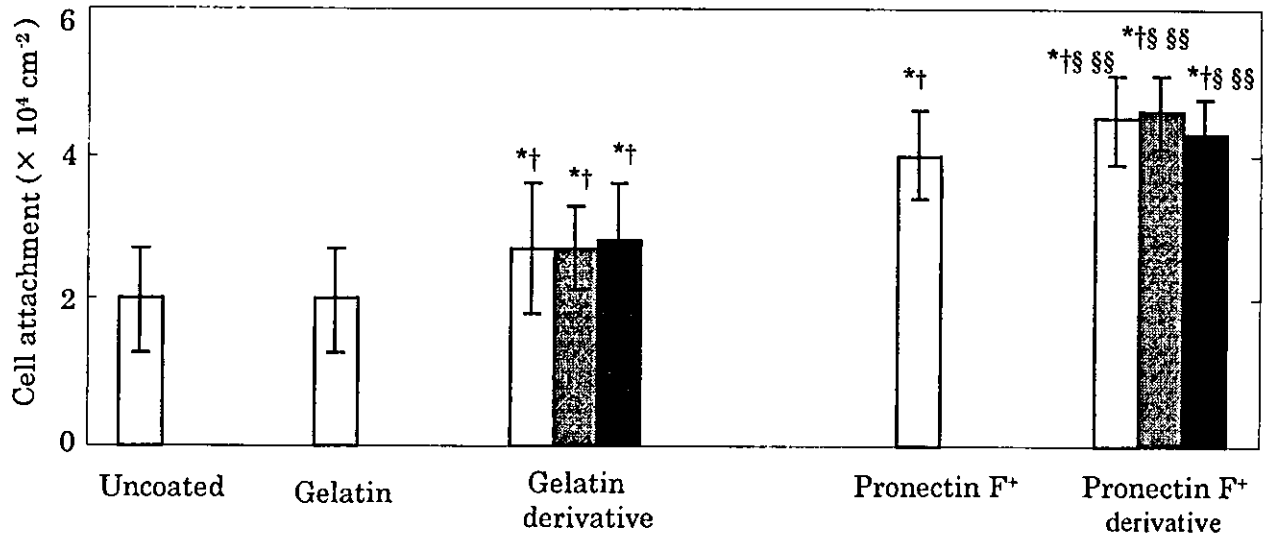

Fig. 5. Cell attachment to a culture dish or that coated with gelatin, Pronectin F$^+$, gelatin or Pronectin F$^+$ derivatives: (□) Ed-, (▨) Sd-, and (■) Sm-derivatives. The concentration of the original gelatin and gelatin derivatives used was 0.1 mg/ml and Pronectin F$^+$ and Pronectin F$^+$ derivatives used was 10 μg/ml. The molar percentage of amino groups introduced to the hydroxyl groups of Pronectin F$^+$ is 26%. *$P<0.05$; significant against the number of cell attached to the uncoated dish. †$P<0.05$; significant against the number of cell attached to the dish coated with gelatin. §$P<0.05$; significant against the number of cell attached to the dish coated with the corresponding gelatin derivative. §§$P<0.05$; significant against the number of cell attached to the dish coated with Pronectin F$^+$.

though there was no difference in the cell attachment between the derivative types. When comparing cell attachment to the plate coated with the original Pronectin F$^+$ and gelatin with the uncoated one, a significant increase in the cell attachment was observed for the original Pronectin F$^+$; however, the original gelatin alone was not effective in the enhanced cell attachment and the level was similar to that of uncoated plate.

3.5. Cell uptake of plasmid DNA

Fig. 6 shows the percent internalized Pronectin F$^+$ derivatives–plasmid DNA and gelatin derivatives–plasmid DNA complexes or other agents into RGM-1 cells. The internalization of plasmid DNA into cells was significantly enhanced by the complexation with every Pronectin F$^+$ and gelatin derivative while it was significantly much higher for Pronectin F$^+$ derivative compared with gelatin derivatives. The internalization level of original Pronectin F$^+$–plasmid DNA and original gelatin–plasmid DNA complexes was similar to that of free plasmid DNA.

3.6. Buffering capacity of Pronectin F$^+$ and gelatin derivatives

Fig. 7 shows the buffering capacity of different Pronectin F$^+$ and gelatin derivatives and PEI. The shape of titration curve for Ed- and Sd-derivatives of Pronectin F$^+$ and gelatin was similar to that of original Pronectin F$^+$ and gelatin without any aminization. On the contrary, the Sm-derivative of Pronectin F$^+$ and gelatin exhibited the titration curve similar to that of PEI and the buffering pH range was wider than that of other derivatives.

4. Discussion

This study demonstrates that the cationized derivative of Pronectin F$^+$ with repeated RGD sequences significantly enhanced the in vitro expression of a plasmid DNA. When compared with the cationized derivatives of gelatin with one RGD sequence per molecule, the gene expression by the Pronectin F$^+$ derivative was significantly higher. The surface charge and molecular size were not different between

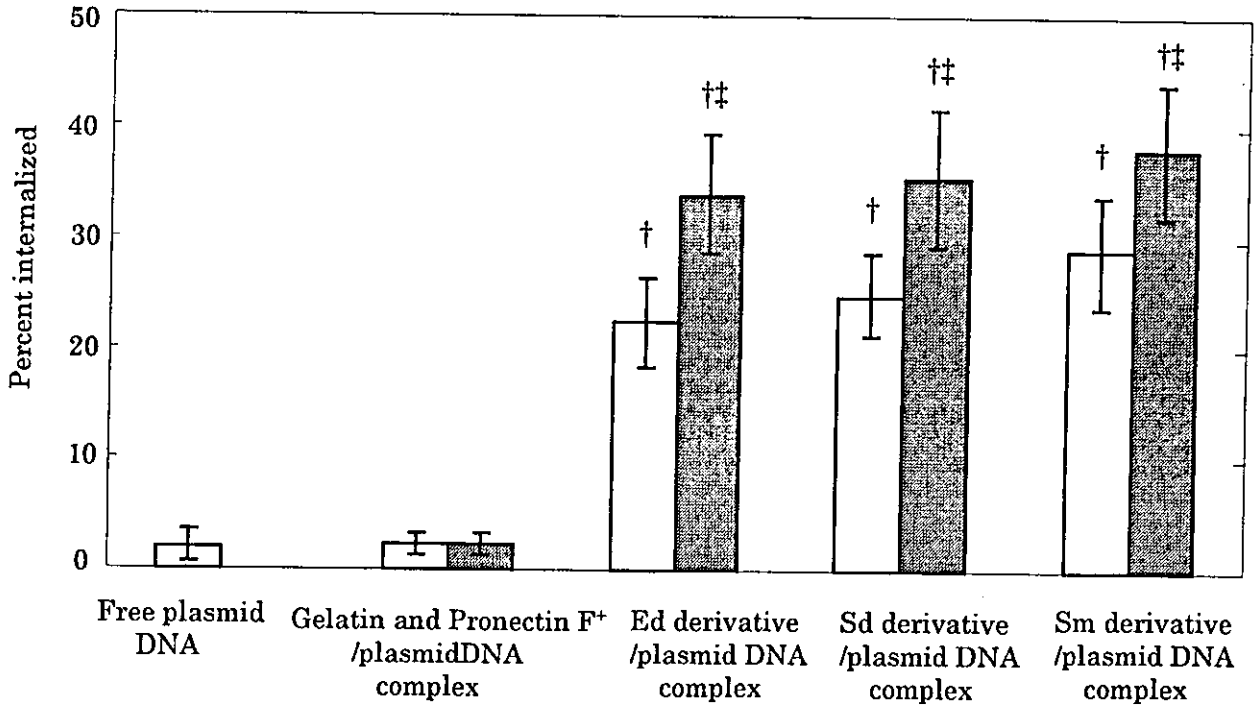

Fig. 6. Internalization of free plasmid DNA, gelatin–plasmid DNA complex, Pronectin F^+–plasmid DNA complex, gelatin derivatives–plasmid DNA complex, and Pronectin F^+ derivatives–plasmid DNA complexes into RGM-1 cells for 48 h: (□) gelatin and its derivatives and (□) Pronectin F^+ and its derivatives. The dose of plasmid DNA was 0.1 mg/ml and the mixing weight ratio of gelatin and Pronectin F^+ to plasmid DNA was 5 and 50, respectively. The molar percentage of amino groups introduced to the hydroxyl groups of Pronectin F^+ is 26%. $^{\dagger}P<0.05$; significant against the internalization percentage of free plasmid DNA. $^{\ddagger}P<0.05$; significant against the internalization percentage of the corresponding gelatin derivative–plasmid DNA complex.

the plasmid DNA complexes with Pronectin F^+ and gelatin derivatives (Figs. 2 and 3). All the Pronectin F^+ and gelatin derivatives were electrostatically interacted with the plasmid DNA (Fig. 1). Retarded or no electrophoretic migration of plasmid DNA complexed with the derivatives is ascribed to the neutralization of DNA negative charge and the positively charging. No electrophoretic migration of Sm Pronectin F^+ and gelatin derivative–plasmid DNA complexes at the lower mixing ratio can be explained by the higher ζ potential of the derivative than other derivatives. Aminization gave both Pronectin F^+ and gelatin-positive charges, irrespective of the type of amine compounds used (Fig. 2). Since the Sm has more amino groups in one molecule than the Ed and Sd, the Sm introduction would enable Pronectin F^+ and gelatin to increase the positive charge upon comparing at the same extent of aminization. The higher ζ potential of Sm Pronectin F^+ and gelatin derivative complexes is due to the higher positive charges. It is possible that this results in larger molecular size of the Sm Pronectin F^+ derivative (Fig. 3), because of the stronger electric repulsion. It is likely that electrostatic interaction with the positively charged Pronectin F^+ and gelatin enabled plasmid DNA of a negative charge to electrically shield the intramolecular repulsion, resulting in formation of condensed complex between the two molecules (Fig. 3). However, the condensation effect on the plasmid DNA was similar among the Pronectin F^+ and gelatin derivatives. This is because the molecular size of amine compounds is not large enough to affect that of complexes.

Complexation enabled the plasmid DNA to significantly enhance the gene expression (Fig. 4). All the Pronectin F^+ derivatives significantly enhanced the level of gene expression compare with gelatin derivatives. This can be explained from the viewpoint of the enhanced cell attachment through the RGD sequence. Here, there is no doubt that the

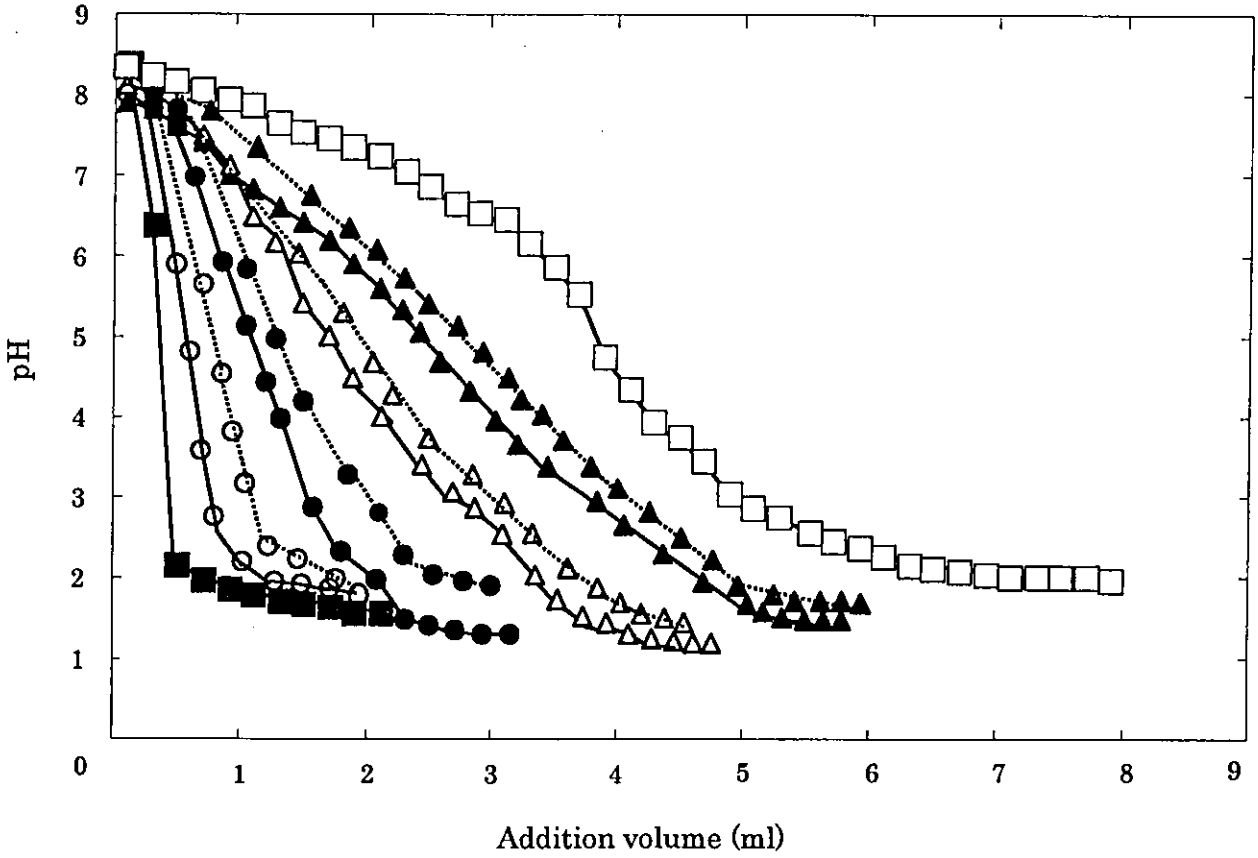

Fig. 7. Fig. 7. Buffering capacity of gelatin, Pronectin F^+, gelatin or Pronectin F^+ derivatives in DDW at 25 °C: (○) original gelatin and Pronectin F^+, (●) Ed-derivatives, (△) Sd-derivatives, (▲) Sm-derivatives, (□) PEI, and (■) no substance. The solid curve represents the data of Pronectin F^+ and its derivatives while the dashed curve represents the data of gelatin and its derivatives. The molar percentage of amino groups introduced to the hydroxyl groups of Pronectin F^+ is 26%.

electrostatic interaction between the cell membrane of negative charge and the Pronectin F^+ and gelatin derivative–plasmid DNA complexes of positive charge is a driving force for the cell attachment of complexes. Both Pronectin F^+ and gelatin derivatives showed higher cell attachment although the former was superior to the latter (Fig. 5). This is because the Pronectin F^+ has larger number of RGD sequences than the gelatin.

The cell uptake of plasmid DNA was enhanced by the complexation with Pronectin F^+ and gelatin derivatives (Fig. 6). This is again due to the existence of RGD sequence. It is likely that the complex is not taken up by cells unless it is attached onto the cells. The enhancement effect of plasmid DNA internalization did not depend on the type of derivatives. This is mainly due to the similarity of Pronectin F^+ and gelatin derivatives in the apparent molecular size and ζ potential. Taken together, it is possible that the existence of RGD sequence is more effective than aminization in enhancing gene expression.

The expression enhancement depended on the type of Pronectin F^+ derivatives used and was higher for the Sm Pronectin F^+ and gelatin derivatives than other Ed and Sd derivatives. This should be considered in terms of the buffering capacity of complexes in addition to their molecular size and charge. Both the condensed formation and a net positive charge of cationized polymer–plasmid DNA complexes have been reported to be key for gene transfection [23]. More importantly, it has been experimentally demonstrated that the buffering capacity of vectors plays an important role in the gene transfection [24–26]. It is

well known that PEI is a representative non-viral vector with a strong buffering effect [23]. The Sm Pronectin F^+ and gelatin derivatives possessed the highest buffering effect among other derivatives and the effect was comparable to that of PEI (Fig. 7). The similarity in the molecular structure between the Sm and PEI may be one of the reasons why their buffering capacity is comparable. Less gene expression of Sm gelatin derivative may be due to the less cell attachment.

In conclusion, the Pronectin F^+ with repeated RGD sequences was a promising non-viral vector of plasmid DNA in gene transfection if it is cationized properly. The RGD sequence enabled the plasmid DNA to effectively internalize into cells, resulting in enhanced gene expression. However, the buffering effect of vector was also another important factor contributing to gene expression. This is the first report to use the Pronectin F^+ with the repeated RGD sequence for gene delivery

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In vivo release and gene expression of plasmid DNA by hydrogels of gelatin with different cationization extents

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Received 13 November 2002; accepted 20 March 2003

Abstract

The objective of this paper is to investigate the in vivo release and gene expression of lacZ plasmid DNA (pSV-lacZ) by the hydrogels of cationized gelatin. Gelatin with different cationization extents was prepared by changing the amount of ethylenediamine added to aminize the carboxyl groups of gelatin with a water-soluble carbodiimide. The cationized gelatin prepared was crosslinked by various concentrations of glutaraldehyde (GA) to obtain cationized gelatin hydrogels with different cationization extents as the release carrier of plasmid DNA. When the cationized gelatin hydrogels incorporating ^{125}I-labeled pSV-lacZ were implanted into the femoral muscle of mice, the radioactivity remaining decreased with time and the retention period was prolonged with an increase in the concentration of GA used for hydrogel preparation. In vivo experiments with ^{125}I-labeled cationized gelatin hydrogels revealed that the higher the GA concentration, the longer the in vivo retention period of radioactivity remaining for every cationized gelatin hydrogel. Only for the hydrogels prepared from gelatin with the aminized percentages of 29.7, 41.6, and 47.8 mol.%, the time profile of pSV-lacZ retention correlated well with that of hydrogel retention. The gene expression by the cationized gelatin hydrogels incorporating pSV-lacZ depended on the aminized percentage of gelatin and was significant at the percentage of 41.6 mol.% or higher. It is possible that the pSV-lacZ was complexed with the degraded fragments of cationized gelatin and released with a positive charge, resulting in enhanced gene expression. We conclude that gelatin with a cationization extent of at least 41.6 mol.% is needed for the enhanced in vivo gene expression of plasmid DNA by the hydrogel release system.

Keywords: Plasmid DNA; Cationized gelatin hydrogel; Controlled release; Hydrogel degradation; Gene expression

1. Introduction

Several viral and non-viral vectors have been explored aimed at the enhancement of gene transfection efficiency [1]. Although the former vectors possess an inherently high efficiency of gene transfection, there are some issues to be resolved for clinical trials, such as immunological and/or toxic responses toward the vectors themselves. To tackle the problems, several genetic modulations have been

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0168-3659/03/$ – see front matter
doi:10.1016/S0168-3659(03)00197-4

attempted for the viral vectors. On the other hand, non-viral vectors have several advantages over viral vectors although the transfection efficiency is much lower than that of viral ones. Their induction nature of toxicity and immune responses are low compared with that of viral vectors and the possible integration of a non-viral vector into the genome will be negligibly small. Many macromolecules and liposomes of positive charges have been designed to demonstrate their feasibility as non-viral vectors in enhancing gene expression [2–4]. However, the transfection efficiency is still low and the duration of the expression period is short compared with those of viral vectors. In addition, the body distribution of non-viral vectors injected should be regulated in terms of gene expression. When the vector in solution form is injected into the body, gene expression cannot always be expected because of the easy diffusion away from the injected site. Thus, one of the possible trials for enhanced gene expression is to achieve controlled release of plasmid DNA over an extended time period by incorporating it into an appropriate carrier. It is possible that the controlled release technology enables the plasmid DNA to enhance the transfection efficiency at the applied site, resulting in promoted gene expression [5]. However, there has been little investigation of the charge effect of the release carriers on that of gene expression.

Gelatin has been extensively used for industrial, pharmaceutical, and medical applications and its biosafety has been proved through its long clinical usage as surgical biomaterial and drug ingredient [6]. Another advantage of gelatin is the ease with which chemical modification of a physicochemical nature takes place. For example, positively charged, cationized gelatin can readily be prepared by introducing amine residues to the carboxyl groups of gelatin. We have experimentally confirmed that hydrogels prepared from the cationized gelatin achieved controlled release of plasmid DNA based on the hydrogel degradation following intramuscular implantation [7]. 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 [7].

The present study was undertaken to investigate how the cationization extent of gelatin constituting hydrogels affects their in vivo gene expression of plasmid DNA. Various biodegradable hydrogels were prepared by chemical crosslinking of gelatin with different cationization extents. Following implantation of the cationized gelatin hydrogels incorporating ^{125}I-labeled plasmid DNA or ^{125}I-labeled cationized gelatin hydrogels into the femoral muscle of mice, the time profile of their radioactivity remaining was compared from the viewpoint of hydrogel biodegradability and gelatin cationization. We examined the effect of the cationization extent on the gene expression level by the cationized gelatin hydrogels incorporating plasmid DNA in the mouse muscle.

2. Materials and methods

2.1. Materials

Gelatin was prepared through an acid process of pig skin type I collagen and kindly supplied by Nitta Gelatin (Osaka, Japan). Ethylenediamine (ED), glutaraldehyde (GA), 2,4,6-trinitrobenzensulfonic acid, β-alanine, and Protein Assay Lowry kit were purchased from Nacalai Tesque (Kyoto, Japan) and used as obtained. As a coupling agent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC) was obtained from Dojindo Laboratories (Kumamoto, Japan). *N*-Succinimidyl-3-(4-hydroxy-3,5-di [^{125}I]iodophenyl) propionate (^{125}I-Bolton-Hunter Reagent, NEX-120H, 147 MBq/ml in anhydrous benzene) was purchased from NEN Research Products (DuPont, Wilmington, DE, USA).

2.2. Preparation of cationized gelatin

ED and EDC were added into 250 ml of 100 mM phosphate-buffered solution (pH 5.0) containing 5 g of gelatin at different molar ratios to the carboxyl groups of gelatin (0, 0.5, 3, 10, and 50). Immediately after that, the pH of the solution was adjusted at 5.0 by adding 5 M HCl aqueous solution. The reaction

mixture was agitated at 37 °C for 18 h and then dialyzed against double-distilled water (DDW) for 48 h at 25 °C. The dialyzed solution was freeze-dried to obtain the samples of cationized gelatin. The percentage of amino groups introduced into gelatin (the cationization extent of gelatin) was determined by the conventional trinitrobenzene sulfonate method [8] based on the calibration curve prepared by using β-alanine.

2.3. DNA isolation

The plasmid DNA was the expression vector consisting of the coding sequence of LacZ and an SV40 promoter inserted at the upstream (pSV-lacZ, 7.9 kbp). The pSV-lacZ was prepared from bacteria cultured with a Qiagen Maxi kit (Qiagen, Tokyo, Japan). Briefly, the *Escherichia coli* transformants containing pSV-lacZ were grown by incubating in Luria-Bertani (LB) medium (Invitrogen Japan, Tokyo, Japan) at 37 °C for 16 h. Following centrifugation (6000×*g*, 15 min, 4 °C), the bacteria pellet was suspended in a resuspension buffer (50 mM Tris(hydroxymethyl)aminomethane (Tris)–HCl, 10 mM EDTA, pH 8.0) in the presence of RNase (100 μg/ml) and lysed in a lysis buffer (200 mM NaOH, 1% sodium dodecylsulfate). The lysate was neutralized by addition of 3.0 M potassium acetate solution (pH 5.5), filtrated, and applied to the Qiagen syringe of anion-exchange resin. The Qiagen syringe was rinsed with a medium-salt buffer (1 M NaCl, 50 mM 2-(*N*-morpholino)-propanesulfonic acid (MOPS) at pH 7.0, and 15% isopropyl alcohol) to remove remaining contaminants, such as the traces of RNA and protein. The plasmid DNA was then eluted with an elution buffer (1.25 M NaCl at pH 8.5, 50 mM Tris–HCl, and 15% isopropyl alcohol) and precipitated by addition of isopropyl alcohol. After centrifugation at 15,000×*g* for 10 min at 4 °C, the pellet was washed with 70% ethanol aqueous solution to remove residual salt and to substitute the solvent. The DNA centrifuged was air-dried and dissolved in a small volume of TE buffer (10 mM Tris–HCl and 1 mM EDTA). When measured at the wavelengths of 260 and 280 nm for the purity evaluation of plasmid DNA, the absorbance ratio was found to be between 1.8 and 2.0.

2.4. Characterization of cationized gelatin and plasmid DNA–cationized gelatin complex

The apparent molecular size and zeta potential of cationized gelatin were measured. Dynamic light scattering (DLS) measurement was carried out using a DLS-7000 (Otsuka Electronic, Osaka, Japan) equipped with an Ar^+ laser at a detection angle of 90° at room temperature and performed three times for every sample. The corresponding hydrodynamic radius, R_s, was analyzed based on the cumulants method and the R_s value was automatically calculated by the equipped computer software and expressed as the apparent molecular size of the sample.

Electrophoretic light scattering (ELS) measurement was carried out on an ELS-7000 (Otsuka Electronic) at room temperature and an electric field strength of 100 V/cm and performed three times for every sample. The zeta potential was automatically calculated using the Smoluchouski equation.

For plasmid DNA–cationized gelatin complex preparation, 0.5 ml of phosphate-buffered saline solution (PBS, pH 7.4) containing 5 mg cationized gelatin was mixed with 0.5 ml of PBS containing 1 mg of plasmid DNA. The solution was gently agitated at 37 °C for 30 min to form plasmid DNA–cationized gelatin complexes. The DLS and ELS measurements were carried out by the procedure described above.

2.5. Preparation of cationized gelatin hydrogels

An aqueous solution of 10 wt.% cationized gelatin (800 μl) was cast into a polytetrafluoroethylene mold (2×2 cm^2, 0.8 mm depth), followed by leaving at 4 °C overnight for gelatin gelation. The cationized gelatin hydrogel sheets were placed in a mixed solution of 0.01 M HCl/acetone (3:7, v/v) containing various amounts of GA. Crosslinking reaction was allowed to proceed for 24 h at 4 °C, and then the resulting hydrogel sheets were immersed in 100 mM glycine aqueous solution at 4 °C for 24 h to block the residual aldehyde groups of GA. The cationized gelatin hydrogel sheets were cut out to obtain cationized gelatin hydrogel (5×5×1 mm^3) and rinsed three times with DDW at 4 °C and freeze-dried. The freeze-dried hydrogels were sterilized by

ethylene oxide gas. No change in hydrogel shape was observed before and after freeze-drying and sterilization processes, irrespective of the crosslinking conditions and the cationization extent of gelatin.

2.6. Radiolabeling of cationized gelatin hydrogels

The cationized gelatin hydrogels prepared were radioiodinated by use of ^{125}I-Bolton-Hunter reagent [9]. Briefly, 100 μl of ^{125}I-Bolton-Hunter reagent solution in anhydrous benzene was bubbled with dry nitrogen gas until benzene evaporation was completed. Then, 125 μl of 0.1 M sodium borate-buffered solution (pH 8.5) were added to the dried reagent, followed by pipetting to prepare aqueous ^{125}I-Bolton-Hunter solution. The aqueous solution prepared was impregnated into the freeze-dried hydrogels of cationized gelatin hydrogels at a volume of 20 μl per hydrogel. The resulting swollen hydrogels were kept at 4 °C for 3 h to introduce ^{125}I residues into the amino groups of gelatin. The radioiodinated cationized gelatin hydrogels were rinsed with DDW by exchanging it periodically at 4 °C for 4 days to exclude non-coupled, free ^{125}I-labeled reagent from ^{125}I-labeled gelatin hydrogels. When measured periodically, the radioactivity of DDW returned to the background level after rinsing for 3 days. No shape change of swollen hydrogels was observed during radiolabeling and the subsequent rinsing process.

2.7. Radiolabeling of pSV-lacZ

An aqueous solution of 2.5 mg/ml of pSV-lacZ in 1.0 ml of PBS (pH 7.4) was added to the ^{125}I-Bolton-Hunter reagent dried by nitrogen-gas bubbling. The resulting solution was kept at 37 °C overnight to introduce ^{125}I residue into the amino groups of pSV-lacZ. Non-coupled, free ^{125}I-labeled reagent was removed from ^{125}I-labeled pSV-lacZ solution by gel filtration with a PD 10 column (Amersham Pharmacia Biotech, Tokyo, Japan).

2.8. Preparation of cationized gelatin hydrogels incorporating pSV-lacZ

For impregnation of pSV-lacZ into freeze-dried cationized gelatin hydrogels, 20 μl of PBS (pH 7.4) with or without 100 μg of pSV-lacZ were dropped onto the freeze-dried hydrogels, followed by leaving at 4 °C overnight to obtain cationized gelatin hydrogels with or without pSV-lacZ incorporation, respectively. The pSV-lacZ solution was completely impregnated into the freeze-dried cationized gelatin hydrogels during the swelling process because the solution volume was much less than that theoretically impregnated into each hydrogel, irrespective of the gelatin type and GA concentration used for hydrogel preparation. Similarly, an aqueous solution of ^{125}I-labeled pSV-lacZ was sorbed into freeze-dried cationized gelatin hydrogels to prepare cationized gelatin hydrogels incorporating ^{125}I-labeled pSV-lacZ. Every hydrogel prepared by this procedure had a similar appearance, irrespective of the radiolabeling, and preparation conditions.

2.9. Estimation of in vivo degradation of cationized gelatin hydrogels

^{125}I-labeled cationized gelatin hydrogels were implanted into the femoral muscle of ddY mice, 6–7 weeks old (Japan SLC, Hamamatsu, Japan). The mouse muscle containing the implanted hydrogel was taken out 1, 3, 7, 10, 14, and 21 days after hydrogel implantation to measure the radioactivity on a gamma counter (ARC-301B, Aloka, Tokyo, Japan). The radioactivity ratio of the muscle sample to the hydrogel implanted initially was calculated to express as the percentage of remaining activity in the hydrogel. For every experimental group, three mice were sacrificed at each time point for in vivo evaluation unless otherwise mentioned. All the animal experiments were done according to the Institutional Guidance of Kyoto University on animal experimentation.

2.10. Estimation of in vivo pSV-lacZ release from cationized gelatin hydrogels incorporating pSV-lacZ

Following implantation of cationized gelatin hydrogels incorporating ^{125}I-labeled pSV-lacZ into the femoral muscle of mice, the mouse muscles containing cationized gelatin hydrogel were taken out at different time intervals. As a control, the solution of ^{125}I-labeled pSV-lacZ in PBS was injected into the femoral muscle (100 μl/site). The radioactivity of

muscle samples was measured on the gamma counter and the radioactivity ratio of the sample to the hydrogel implanted or pSV-lacZ solution injected initially was expressed as the percentage of radioactivity remaining.

2.11. In vivo assessment of gene expression following implantation of cationized gelatin hydrogels incorporating pSV-lacZ

Cationized gelatin hydrogels incorporating pSV-lacZ which showed a similar time profile of biodegradation in the previous in vivo experiments, were implanted into the femoral muscle of mice. As a control, 100 μl of pSV-lacZ solution were intramuscularly injected into the mouse. The pSV-lacZ dose was 100 μg/mouse and six mice were used at each time point for every experimental group. The mice were sacrificed 7 days after the pSV-lacZ application to evaluate the gene expression.

For evaluation of gene expression at the muscle applied by pSV-lacZ, β-galactosidase activity was measured by use of an Invitrogen kit (Invitrogen, USA). Briefly, the muscle samples were immersed and homogenized in the lysis buffer (0.1 M Tris–HCl, 2 mM EDTA, 0.1% Triton X-100) at the lysis buffer volume (ml)/sample weight (mg) ratio of 4 to 1 in order to normalize the influence of weight variance on the β-galactosidase assay. The sample lysate (2 ml) was transferred to a centrifuge tube, followed by the freeze-and-thaw process three times and centrifugation at 15,000×*g* at 4 °C for 5 min. The supernatant (30 μl) was mixed with 70 μl of aqueous solution containing 4 mg/ml of *o*-nitrophenyl β-D-galactopyranoside and 200 μl of a cleavage buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, and 1 mM $MgSO_4 \cdot 7H_2O$, pH 7) in a microcentrifuge tube. After incubation at 37 °C for 30 min, 500 μl of 1 M sodium carbonate solution were added to the solution mixture. The solution absorbance was measured at the wavelength of 420 nm to evaluate the β-galactosidase activity. The number of muscle samples was four for each experimental group.

2.12. Statistical analysis

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

3. Results

3.1. Preparation and characterization of cationized gelatin

Fig. 1 and Table 1 show the cationization extent of gelatin plotted as a function of the molar ratio of ED added to the carboxyl groups of gelatin. The cationization extent was controllable by changing the addition molar ratio. The apparent molecular size of cationized gelatin was hardly influenced by the cationization of gelatin. However, the zeta potential of cationized gelatin increased with an increase in the ED amount.

Table 2 shows the apparent molecular size and surface charge of complexes prepared with pSV-lacZ and cationized gelatin with different extent of cationization. The apparent molecular size of plasmid DNA decreased by mixing with the cationized gelatin and attained around 200 nm when E-10 and E-50 gelatins were used. After mixing with the cationized gelatin of positive charge, the negative charge of plasmid DNA converted to positive, irrespective of the type of gelatin. The zeta potential of complexes tended to increase with an increase in the extent of cationization of gelatin used.

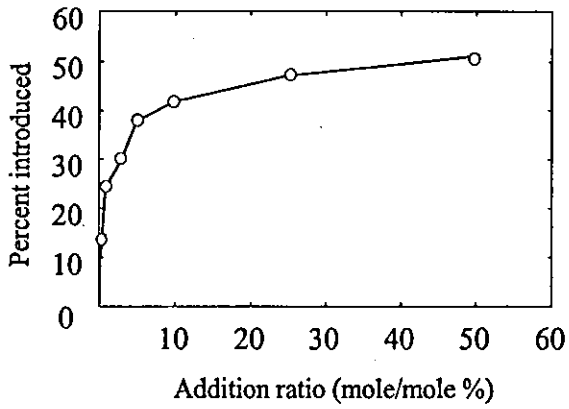

Fig. 1. Influence of the molar ratio of ED added to the carboxyl groups of gelatin on the cationization extent of gelatin.

Table 1
Preparation and characterization of cationized gelatin

Code	Gelatin concentration (g/ml)	ED used		Percent aminized[b] (mol/mol%)	Apparent molecular size (nm)	Zeta potential (mV)
		Concentration (g/ml)	Molar ratio[a]			
E-0	2×10^{-2}	0	0	0	61.7±16.6[c]	4.6±2.2[c]
E-0.5	2×10^{-2}	5.6×10^{-4}	0.5	13.1	63.5±15.2	5.0±2.1
E-3	2×10^{-2}	0.33×10^{-2}	3	29.7	61.4±19.8	5.5±2.9
E-10	2×10^{-2}	1.1×10^{-2}	10	41.6	66.6±15.7	6.7±2.1
E-50	2×10^{-2}	5.6×10^{-2}	50	47.8	65.4±14.2	11.6±4.2

Reaction time=18 h, the ratio of EDC to the carboxyl groups of gelatin=3 mol/mol.
[a] The molar ratio of ED to the carboxyl groups of gelatin.
[b] The molar percentage of amino residues introduced to the carboxyl groups of gelatin.
[c] Mean±S.D.

3.2. In vivo degradation profile of cationized gelatin hydrogels

Fig. 2 shows the time course of radioactivity remaining after implantation of ^{125}I-labeled cationized gelatin hydrogels into the femoral muscle of mice. Irrespective of the cationization extent of gelatin used, the radioactivity remaining of cationized gelatin hydrogels implanted decreased with time for every hydrogel sample, while the hydrogels prepared at the higher concentration of GA retained the radioactivity for longer time periods than the hydrogels at the lower GA concentration. The in vivo degradation period of cationized gelatin hydrogels could be controlled over the time range from 7 to 21 days.

3.3. In vivo release profile of pSV-lacZ from cationized gelatin hydrogels incorporating pSV-lacZ

Fig. 3 shows the decrement patterns of pSV-lacZ radioactivity after implantation of cationized gelatin hydrogels incorporating ^{125}I-labeled pSV-lacZ into the femoral muscle of mice. The residual radioactivity of pSV-lacZ in cationized gelatin hydrogels decreased with implantation time and the decrement pattern of radioactivity depended on the type of cationized gelatin and hydrogel used. For E-3 and E-10 gelatins, the retention period of radioactivity became longer when the concentration of GA used for hydrogel preparation was higher. On the other hand, such a concentration dependence was not observed for hydrogels prepared from other cationized gelatin. The hydrogel of E-0 and E-0.5 gelatins did not exhibit any prolonged retention of plasmid DNA, whereas that of E-50 gelatin showed a prolonged retention without any dependence of GA concentration. For free ^{125}I-labeled pSV-lacZ, the radioactivity rapidly disappeared from the injected site within 3 days.

Table 2
Characterization of plasmid DNA–cationized gelatin complexes

Code	Apparent molecular size (nm)	Zeta potential (mV)
Free plasmid DNA	639±214[a]	−14.7±9.5[a]
E-0	564±57.8	3.6±2.3
E-0.5	530±69.7	3.5±2.0
E-3	404±88.3	4.0±1.8
E-10	311±48.9	4.6±2.0
E-50	164±21.3	6.6±2.5

[a] Mean±S.D.

Fig. 4 shows the relationship of the radioactivity remaining between the pSV-lacZ and cationized gelatin hydrogel which is the carrier of pSV-lacZ release. The hydrogels were prepared at the highest GA concentration. When the hydrogel was prepared from E-3, E-10, and E-50 gelatins, the radioactivity remaining of pSV-lacZ was in good accordance with that of carrier hydrogels. However, such correlation was not observed for cationized gelatin hydrogels prepared at the lower extents of gelatin cationization.

3.4. In vivo gene expression by cationized gelatin hydrogels incorporating pSV-lacZ

Fig. 5 shows the gene expression level at the muscle following intramuscular implantation of cat-

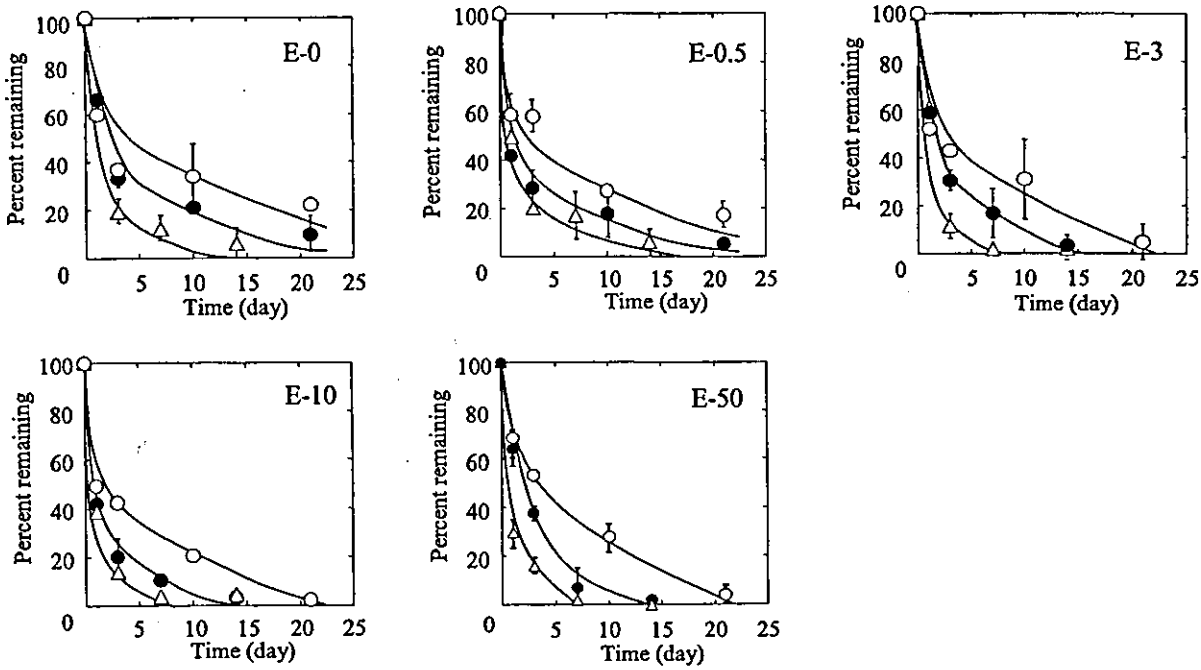

Fig. 2. The time course of radioactivity remaining of ^{125}I-labeled cationized gelatin hydrogels after implantation into the femoral muscle of mice. The cationized hydrogels were prepared from gelatin with different cationization extents at GA concentrations of 0.78 (○), 0.31 (•), and 0.16 (△) μg/ml.

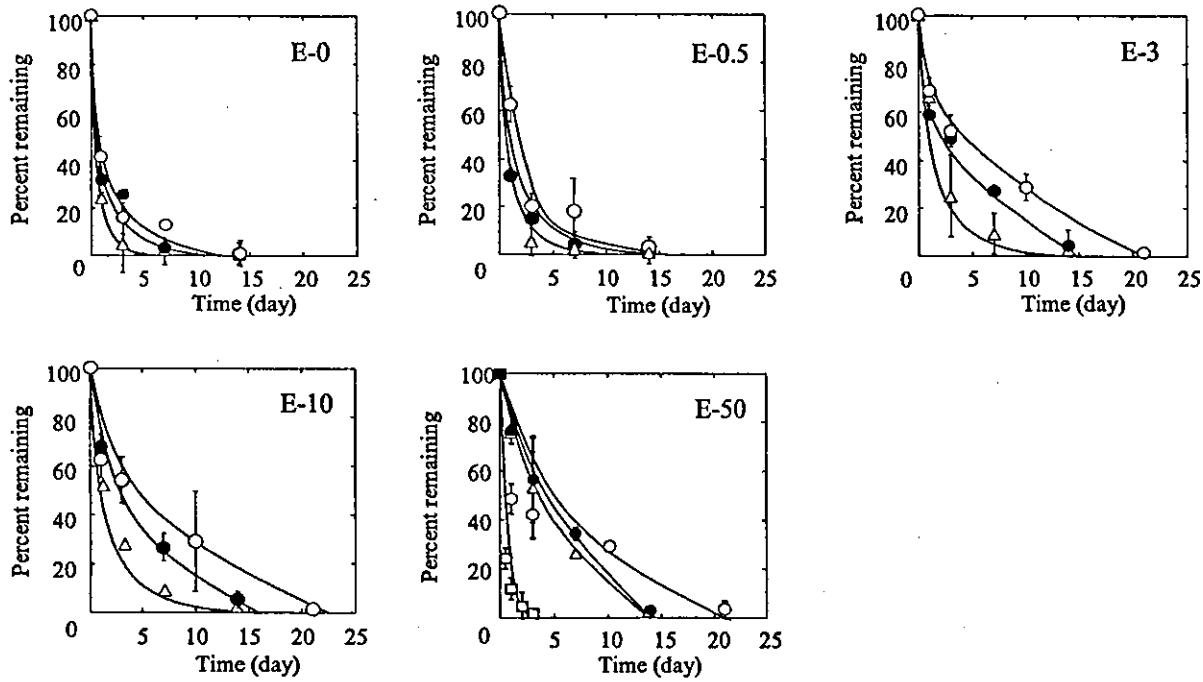

Fig. 3. The time course of radioactivity remaining of cationized gelatin hydrogels incorporating ^{125}I-labeled pSV-lacZ after implantation into the femoral muscle of mice. The cationized hydrogels were prepared from gelatin with different cationization extents at GA concentrations of 0.78 (○), 0.31 (●), and 0.16 (△) μg/ml. □ indicates the radioactivity remaining after the intramuscular injection of ^{125}I-labeled pSV-lacZ in the solution form.

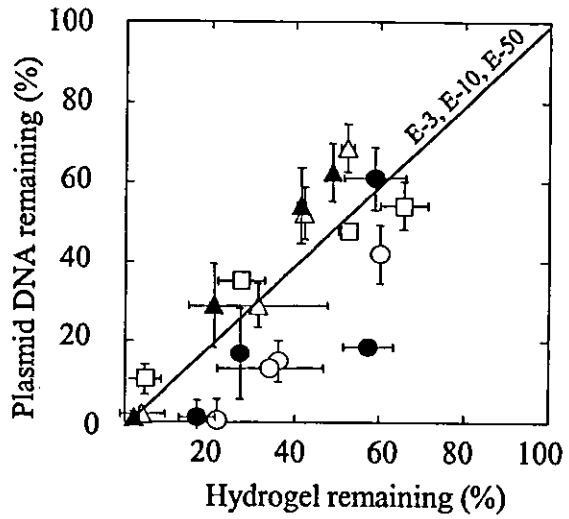

Fig. 4. Relationship of the radioactivity remaining between the pSV-lacZ and cationized gelatin hydrogel after implantation of hydrogels incorporating ^{125}I-labeled pSV-lacZ or ^{125}I-labeled hydrogels into the femoral muscle of mice. The hydrogels were prepared from E-0 (○), E-0.5 (●), E-3 (△), E-10 (▲), and E-50 (□) gelatin at the GA concentration of 0.78 μg/ml.

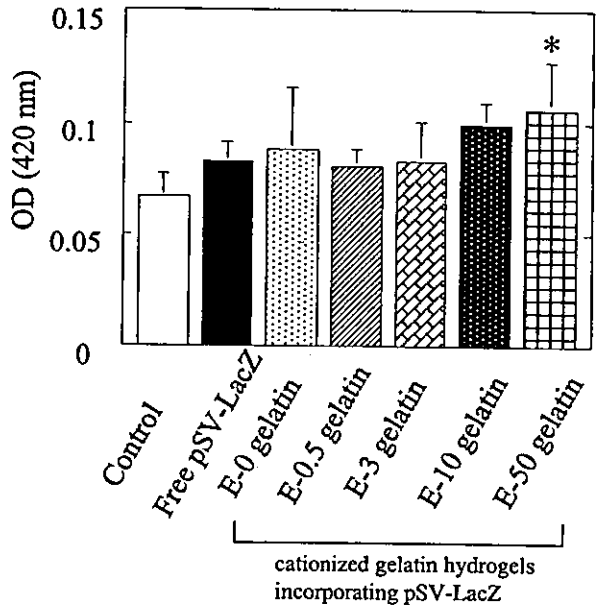

Fig. 5. The gene expression level of pSV-lacZ 7 days after implantation of cationized gelatin hydrogels incorporating pSV-lacZ or injection of free pSV-lacZ into the femoral muscle of mice. Every cationized gelatin hydrogel was prepared at the GA concentrations of 0.78 μg/ml. The pSV-lacZ dose is 100 μg/mouse muscle. *$P<0.05$ significant against the OD value of groups injected with free plasmid DNA.

ionized gelatin hydrogels incorporating pSV-lacZ or injection of free pSV-lacZ. The hydrogels used were prepared from gelatin with different extents of cationization, but their in vivo degradation profile was similar. The injection of the free pSV-lacZ did not induce gene expression 7 days after injection and the level was similar to that of non-treated, controlled muscle. The level of gene expression was not enhanced by implantation of free pSV-lacZ in empty cationized gelatin hydrogels (data not shown). On the contrary, the implantation of cationized gelatin hydrogels incorporating pSV-lacZ enhanced the expression level at the implanted site. The significantly enhanced level of gene expression of pSV-lacZ was observed upon using cationized gelatin hydrogels prepared from E-50 gelatin.

3.5. Time course of gene expression

Fig. 6 shows the time course of gene expression following intramuscular implantation of cationized gelatin hydrogels incorporating pSV-lacZ or injection of PBS containing pSV-lacZ. The water contents of cationized gelatin hydrogels used were 97.4 wt.%. The injection of the pSV-lacZ solution showed significant gene expression only at 3 days after injection and thereafter the expression return to the basal level. On the contrary, the cationized gelatin hydrogels incorporating pSV-lacZ significantly en-

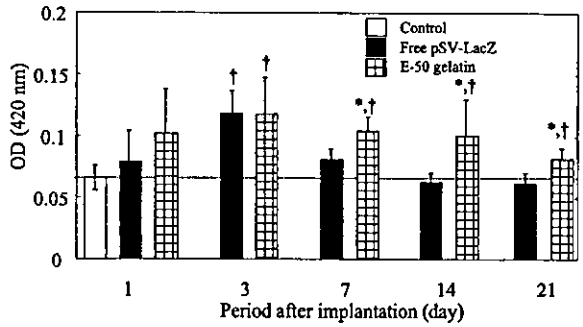

Fig. 6. The time course of lacZ gene expression after implantation of cationized gelatin hydrogels incorporating pSV-lacZ into the femoral muscle of mice. The pSV-lacZ dose is 100 μg/mouse muscle. A dotted line indicates the level of gene expression in the femoral muscle of untreated, normal mice. *$P<0.05$ significant against the OD value of groups injected with free plasmid DNA. †$P<0.05$ significant against the OD value of non-treated, control group.

hanced the level of gene expression as well as prolonging the duration. The level of gene expression significantly increased within 3 days after implantation of the hydrogels and the significant enhancement was observed even on day 21.

4. Discussion

Generally, gelatin is not degraded by simple hydrolysis but by proteolysis. Therefore, in this study, the degradation test of cationized gelatin hydrogels was carried out in the mouse muscle to allow evaluation of the time profile of hydrogel degradation. As is apparent from Fig. 2, the in vivo degradation profile of cationized gelatin hydrogels could be changed by altering the concentration of GA used to crosslink the cationized gelatin, irrespective of the cationization extent. On the contrary, the in vivo retention profile of pSV-lacZ incorporated in cationized gelatin hydrogels could be also changed by the GA concentration. The time profile of in vivo pSV-lacZ retention was prolonged with an increase in the GA concentration used for cationized gelatin hydrogel preparation. For hydrogels prepared from E-3, E-10, and E-50 gelatin, the time profile of pSV-lacZ retention was in good accordance with that of hydrogel retention (Fig. 4). This finding suggests that pSV-lacZ was released from the cationized gelatin hydrogel in the body as a result of hydrogel biodegradation. It seems likely that pSV-lacZ molecules, once ionically complexed with the cationized gelatin, cannot be released from the cationized gelatin hydrogel unless hydrogel degradation takes place. It is possible from the present data that the pSV-lacZ molecules are released from the hydrogels ionically complexed with the water-soluble cationized gelatin fragments of positive charge which are generated with hydrogel degradation. No effect of GA concentration on the remaining amount of pSV-lacZ incorporated in E-50 gelatin hydrogels can be explained in terms of the high positive charge of gelatin. Probably, the positive charge of pSV-lacZ–cationized gelatin complexes released from the E-50 hydrogels with degradation is higher than that of other cationized gelatin hydrogels. It is possible that the higher positive charge enables the complex to interact more strongly with the cell surface and the extracellular matrix of negative charge, resulting in prolonged retention. On the other hand, when the cationization extent of gelatin was low, the percent remaining of pSV-lacZ was always smaller than that of the hydrogel carrier. When compared with a gelatin of higher cationization extent, that of a lower extent will complex with the pSV-lacZ through weaker ionic interaction force. As a result, it is conceivable that pSV-lacZ molecules are detached from the cationized gelatin and released from the hydrogel by simple diffusion.

The present study demonstrated that the controlled release of pSV-lacZ from cationized gelatin hydrogels resulted in enhanced gene expression although the expression level depended on the cationization extent of gelatin for hydrogels. The enhanced level of gene expression was observed for E-50 gelatin hydrogels incorporating pSV-lacZ in contrast to other cationized gelatin hydrogels incorporating pSV-lacZ. The prolonged retention of pSV-lacZ was achieved by the controlled release. It is expected that the continuous presence of complex at a certain body site increases the possibility of complex contact with cells, resulting in promoted gene expression thereat. We can say with fair certainty from our data that the plasmid DNA released is positively charged because it is complexed with cationized gelatin (Table 2). The plasmid DNA–cationized gelatin complex of positive charge will interact with the cell surface of negative charge. Moreover, the apparent molecular size of plasmid DNA decreased by mixing with every cationized gelatin and a large decrease in the size was observed between E-3 and E-10 gelatins. The complex size attained around 200 nm when E-50 gelatin was used. It has been demonstrated that the complex with this size range can be favorably taken up by cells [10,11]. This is also an advantage of hydrogels prepared from E-50 gelatin for enhanced gene expression in terms of efficient DNA packing to nanosize particles. E-3 gelatin hydrogels showed a good profile of pSV-lacZ release in terms of hydrogel degradation-driven release mechanism, but did not enhance the gene expression. This is because the cationization extent of E-3 and E-10 were not high enough to give the pSV-lacZ complex positive charge necessary for gene expression.

In this hydrogel system, the plasmid DNA release can be regulated only by changing the hydrogel

degradability which can be controlled by changing the crosslinking conditions of hydrogels. This hydrogel has an advantage as the release carrier that the profile of controlled release is not influenced by the shape of the release carrier. We can achieve the controlled release of plasmid DNA even from the hydrogel carrier of a small particle. Plasmid DNA is one negatively charged macromolecule with a similar electric nature, irrespective of the type of coding protein. In this sense, the present cationized hydrogel is a universal release system for any type of plasmid DNA. This release system is presently being applied to the plasmid DNA of bioactive molecules like growth factor to demonstrate the in vivo biological functions to be expected.

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TISSUE ENGINEERING
Volume 9, Number 6, 2003

Local Delivery of Matrix Metalloproteinase Gene Prevents the Onset of Renal Sclerosis in Streptozotocin-Induced Diabetic Mice

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ABSTRACT

The present study was undertaken to investigate whether matrix metalloproteinase (MMP) functions to prevent the occurrence of destructive fibrosis in progressive renal disease. As a sustained release carrier of plasmid DNA, biodegradable hydrogels and microspheres were formulated from cationized gelatin prepared through aminization. Plasmid DNA was released from the cationized gelatin hydrogels as a result of hydrogel degradation. A plasmid DNA including a cytomegalovirus promoter and human recombinant MMP-1 gene (pCMV-MMP) was constructed. Gelatin microspheres incorporating pCMV-MMP as well as phosphate-buffered saline (PBS) with or without pCMV-MMP were injected into the renal subcapsule of C57BL/6 mice, which were intraperitoneally injected with streptozotocin (STZ) to induce diabetes 7 days after operation. The mice were killed 4 weeks after STZ injection to sample their blood and kidneys for biochemical and histological examinations. An immunofluorescence study confirmed that MMP protein was expressed around the renal tissue injected with gelatin microspheres incorporating pCMV-MMP. When applied with cationized gelatin microspheres incorporating pCMV-MMP, the mice showed a level of blood urea nitrogen significantly lower than that of other groups. A reduced content of collagen in the kidneys of mice administered gelatin microspheres incorporating pCMV-MMP was histologically observed. Further, the hydroxyproline assay revealed a significantly decreased content of hydroxyproline in kidney. We conclude that sustained release of MMP-1 gene is a promising prophylactic trial for kidney fibrolysis and dysfunction in the STZ-induced diabetic mouse model.

INTRODUCTION

CHRONIC RENAL FAILURE may result from etiologically various causes, including IgA glomerulonephritis, hypertension, diabetes mellitus, or hereditary diseases, although diabetic nephropathy is the most prevalent cause of chronic renal failure and end-stage renal disease all over the world.[1] Irrespective of the initiation events, pathological progression to end-stage renal failure appears to involve a final common pathway represented by glomerulosclerosis and tubulointerstitial fibrosis. This progression is composed of complicated processes involving a multicellular network characterized by the proliferation of cells producing extracellular matrix (ECM) and an excessive accumulation of ECM accompanied by a decreased rate of degradation, leading to tissue fibro-

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sis widespread in the kidney.[2] The pathologically distinctive features of progressive renal disease are the impaired turnover and the interstitial accumulation of type I and III collagen in the lesions of glomerulosclerosis and interstitial fibrosis, which play a central pathogenic role in the expansion and remodeling of ECM.

Therapeutic trials to restrict the progression of renal disease are still limited, although progress has been made in clarifying the cellular and molecular basis of pathogenesis. Conventional therapy for preservation of renal function includes controlling the blood pressure of hypertensive patients and hyperglycemia in diabetic patients. In addition, angiotensin-converting enzyme inhibitors normalize the size selectivity of the glomerular basement membrane, improving proteinuria.[3] However, there is still no specific therapy to halt progressive interstitial scarring.

New therapeutic strategies with endogenous cytokines and growth factors have been advocated in several research reports. Growth factors, such as transforming growth factor (TGF) β, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) 2, and connective tissue growth factor (CTGF), play an important role in renal fibrogenesis.[4,5] In particular, TGF-β and hepatocyte growth factor (HGF) have been demonstrated to be key factors that mutually regulate the accumulation and degradation of ECM associated with pathogenesis of glomerulosclerosis and tubulointerstitial fibrosis.[6–9] TGF-β augments the production of matrix protein, while it simultaneously abrogates the matrix degradation.[6] On the other hand, HGF functions suppressively to compensate the fibrogenetic action of TGF-β to allow balance in the accumulation and degradation of ECM. The relationship of the two growth factors against fibrosis has been extensively investigated.[6,8] However, it is difficult to control their actions only through systemic administration, which may suppress useful actions or cause unexpected detrimental effects. Because of difficulty in the therapeutic use of growth factors, the downstream or parallel mediators of tissue fibrosis have been examined, aiming at pharmacological therapy without any adverse effects on the cell cycle.[8] Matrix homeostasis is generally maintained ultimately through the balance of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs). Degradation in the connective tissue depends mostly on the activity of MMPs. Among various MMPs, MMP-1 is produced by a wide variety of normal cells, for example, stromal fibroblasts, macrophages, endothelial cells, and epithelial cells or numerous types of tumors, and has the potential to degrade collagen type I and III. Therefore, MMP-1 may be an effective agent for fibrolysis therapy because the impaired matrix is occupied mainly by collagen type I.

Iimuro and co-workers demonstrated that induction of pro-MMP-1 gene into the rat liver cirrhosis model, using adenovirus vector, improved the state of fibrous tissue. They suggested that the mechanism was associated with suppression of hepatic stellate cells and promotion of hepatocytes.[10] Other research using the MMP-8 gene and adenovirus vector also reported evidence of liver cirrhosis reversion.[11] However, vectors based on viruses are in limited use because of several problems not yet solved. Their immunogenicity and the possibility of mutanogenesis are considered serious impediments to facile application to patients.[12] On the other hand, nonviral vectors are advantageous with respect to biological safety, ease of large-scale production, and versatility.

It has been recognized that protein and plasmid DNA are quite unstable in the body and have short half-lives in the blood circulation because of liver metabolism or other degradation pathways.[9] It is, thus, difficult to maintain a body level therapeutically acceptable only by their injection in solution. To break through this problem, the technology of drug delivery systems is promising. We have explored biodegradable hydrogels for the sustained release of growth factors or plasmid DNA, and succeeded in enhancing their biological functions. The hydrogel system enabled a plasmid DNA to enhance *in vivo* expression and prolong the expression time period.[13] In this study, plasmid DNA including the MMP-1 gene was applied to the hydrogel release system and the prophylactic potential was investigated for chronic renal fibrosis.

Plasmid DNA was incorporated into the biodegradable microspheres of cationized gelatin for local delivery. Microspheres incorporating MMP-1 plasmid DNA was injected into the subcapsule of mouse kidney in advance, and then the mice received streptozotocin (STZ) to induce diabetic renal disease. The advanced lesions of STZ-induced diabetic kidney were reported to mimic some findings of early-stage clinical diabetic nephropathy.[14] Mouse kidney was histologically and biochemically examined to evaluate the suppressive effect of plasmid DNA on renal fibrosis progression.

MATERIALS AND METHODS

Materials

Gelatin (isoelectric point, 9.0), prepared through acidic treatment of pig skin type I collagen, was kindly supplied by Nitta Gelatin (Osaka, Japan). The dialysis membrane used was Seamless Cellulose Tubing (size 30/32, lot 900201; Viskase, Willowbrook, IL). Ethylenediamine-2,4,6-trinitrobezenesulfonic acid (TNBS), chloramine-T, *p*-dimethylbenzaldehyde, β-alanine, fluorescein isothiocyanate (FITC), goat anti-mouse IgG conjugate (F0479; DakoCytomation, Carpinteria, CA), rhodamine isothiocyanate (RITC)–goat anti-rabbit IgG conjugate (R-6394; Molecular Probes, Eugene, OR), anti-human MMP-1 antibody (210-752-R500; Alexis, Carlsbad, CA), and the

expression vector consisting of the coding sequence of *lacZ* and a simian virus 40 (SV40) promoter inserted upstream (pCH110, 7931 bp; Amersham Biosciences, Piscataway, NJ) (pSV-LacZ) were obtained from Nacalai Tesque (Kyoto, Japan), while 1-ethyl-3-(3-dimethylaminopropyl) carbodimide, hydrochloride (EDC) was purchased from Dojindo (Kumamoto, Japan). *N*-Succinimidyl-3-(4-hydroxy-3,5-di[^{125}I]iodophenyl)propionate (^{125}I-Bolton–Hunter reagent, NEX-120H, 147 MBq/ml in anhydrous benzene) was purchased from PerkinElmer Life Sciences (Boston, MA).

A FLAG-tagging mammalian transient expression system kit (E7773, pFLAG-CMV-5a; Sigma, St. Louis, MO) and mouse anti-FLAG antibody (F3165; Sigma) were purchased from Wakenyaku (Kyoto, Japan).

Preparation of cationized gelatin

Gelatin was cationized through aminization. Briefly, 5 g of gelatin was dissolved in 125 mL of double-distilled water (DDW) containing ethylenediamine and EDC, both at molar quantities of 50 per carboxyl group of gelatin. The pH of the solution mixture was adjusted to pH 5.0 by adding 5 M hydrochloride solution, while the solution volume was made up to 250 mL with DDW. The resulting solution was stirred at 37°C for 18 h to allow the introduction of amine residues to the gelatin. The reaction product was dialyzed against DDW for 48 h at 25°C to remove unreacted reagents and by-products, followed by freeze-drying to obtain a cationized gelatin. When determined by the trinitrobenzene sulfonate method based on the standard curve prepared with β-alanine, the percentage of amine groups introduced was 47.8 mol% of total carboxyl groups of gelatin.[15]

Preparation of cationized gelatin hydrogel granules

An aqueous solution of 10wt% cationized gelatin (800 μL) was cast into a polytetrafluoroethylene mold (2 × 2 cm^2, 0.8 mm depth), followed by leaving at 4°C overnight for gelation. Cationized gelatin hydrogels were cross-linked in HCl-acetone (3:7, vol/vol) containing glutaraldehyde (60 μg/mL). The cross-linking reaction was performed for 24 h at 4°C, and then the resulting hydrogels were stirred in 100 mM glycine aqueous solution at 4°C for 24 h to block the residual aldehyde groups of glutaraldehyde. The hydrogel sheets were cut out to obtain hydrogel granules and rinsed three times with DDW at 4°C and freeze-dried.

Radiolabeling of cationized gelatin hydrogels

Cationized gelatin hydrogels were radioiodinated by use of the ^{125}I-Bolton–Hunter reagent. Briefly, 100 μL of ^{125}I-Bolton–Hunter reagent solution in anhydrous benzene was bubbled with dry nitrogen gas until benzene evaporation was completed. Then, 125 μL of 0.1 M sodium borate-buffered solution (pH 8.5) was added to the dried reagent, followed by pipetting to prepare aqueous ^{125}I-Bolton–Hunter solution. The freeze-dried cationized gelatin hydrogels were impregnated with prepared aqueous solution at a volume of 20 μL per hydrogel. The resulting swollen hydrogel granules were kept at 4°C for 3 h to introduce ^{125}I residues into the amino groups of gelatin. The radioiodinated cationized gelatin hydrogel granules were rinsed with DDW by exchanging it periodically at 4°C for 4 days to exclude noncoupled, free ^{125}I-labeled reagent from ^{125}I-labeled gelatin hydrogels. The resulting swollen hydrogel granules were freeze-dried.

Plasmid construct

DNA fragment containing the MMP-1 gene was obtained by polymerase chain reaction (PCR) with a forward primer (*Hin*dIII5′-CCCAAGCTTAAGGCCAGTATGCACAGCTTTCC-3′) and a reverse primer [p*Sal*Iflag(–)5′-CCCGTCGACTCAATTTTTCCTGCAGTTGAACC-3′ or p*Sal*Iflag(+)5′-CCCGTCGACATTTTTCCTGCAGTTGAACC-3′], using a human fibroblast cDNA library (kindly given by Dr. Takahashi, Department of Dermatology, Kyoto University) as a template. The amplified DNA fragment was digested with *Hin*dIII and *Sal*I. The *Hin*dIII–*Sal*I fragment was verified by DNA sequencing and then subcloned into the multicloning site of the pFLAG-CMV-5a expression vector with or without FLAG-tagged protein to the C terminus of the MMP-1 construct, according to its possession of the stop codon downstream or upstream of the FLAG sequence. The resulting MMP–FLAG fusion proteins contained an additional 21-amino acid sequence derived from the multicloning site of the vector (pCMV-MMP-FLAG). All the experiments, except the immunofluorescence examination concerning the colocalization of MMP protein and FLAG antigen, were performed by a plasmid DNA without the FLAG tag simultaneously prepared (pCMV-MMP).

Preparation of plasmid DNA

Plasmid DNA was prepared from bacterial cultures with a Qiagen Maxi kit (Qiagen, Tokyo, Japan) according to the product manual. The DNA, after centrifugation, was briefly air dried and dissolved in a small volume of TE buffer (10 mM Tris-HCl and 1 mM EDTA). The absorbance ratio (wavelengths of 260 to 280 nm) was measured for purification evaluation and was found to be between 1.8 and 2.0.

Radiolabeling of pSV-LacZ

Phosphate-buffered saline (PBS) containing a 2.5-mg/mL concentration of pSV-LacZ (1.0 mL) was added to the dried ^{125}I-Bolton–Hunter reagent prepared by the method mentioned previously.[13] The resulting solution