Targeting of Plasmid DNA to Renal Interstitial Fibroblasts by Cationized Gelatin

Toshihiro KUSHIKIKA, a Natsuki NAGATA-NAKAJIMA, b Manabu SUGAI, b Akira SHIMIZU, b and Yasuhi ko TABATA a, *

* Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University; and b Center for Molecular Biology and Genetics, Kyoto University; 53 Kawara-cho Shogoin, Sakyo-ku, Kyoto 606–8507, Japan.

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Renal interstitial fibrosis is the common pathway of chronic renal disease, while it causes end-stage renal failure. A lot of cytokines and biologically active substances are well recognized to be the candidates of primary mediators to induce accumulation of extracellular matrix (ECM) in the interstitial fibrotic area. Interstitial fibroblasts are played a crucial role in the accumulation of excess ECM during renal interstitial fibrogenesis. Therefore, the targeting of therapeutic drugs and genes to interstitial renal fibroblasts is effective in suppressing the progress of interstitial renal failure. However, despite various approaches and techniques, few successful results have been reported on the in vivo targeting for interstitial fibroblasts. The objective of this study is to deliver an enhanced green fluorescent protein (EGFP) plasmid DNA, as a model plasmid DNA, into renal interstitial space by a cationized gelatin. After the plasmid DNA with or without complexation of the cationized gelatin was injected to the left kidney of mice via the ureter, unilateral ureteral obstruction (UUO) was performed for the mice injected to induce the renal interstitial fibrosis. When the EGFP plasmid DNA complexed with the cationized gelatin was injected, EGFP expression was observed in the fibroblasts in the interstitial area of renal cortex. It is concluded that the retrograde injection of EGFP plasmid DNA complexed with the cationized gelatin is available to target the interstitial renal fibroblasts which are currently considered as the cell source responsible for excessive ECM synthesis.

Key words renal interstitial fibrosis; gelatin; gene targeting

Renal fibrosis is the common pathway of chronic renal disease progressing to end-stage renal failure.1–3 Renal fibrosis is characterized by qualitative and quantitative changes in the composition of tubular basement membranes or interstitial matrices, tubular atrophy, and the accumulation of myofibroblasts.1–3 For chronic renal disease, persistent accumulation and deposition of extracellular matrix (ECM) which lead to widespread tissue fibrosis, are observed.4 Renal interstitial fibrosis is considered to be the commonly converging outcome of chronic renal diseases with a wide spectrum of diverse etiologies. For renal fibrosis, severe accumulation of ECM is observed in the renal interstitial compartment.4–7 It is experimentally confirmed from several animal models of renal fibrosis that transforming growth factor-β (TGF-β), heat shock protein (HSP), and other cytokines function as the primary mediators for ECM accumulation.8–12 Therefore, it has been demonstrated that biological inhibition of TGF-β protein by use of neutralizing antibody,12 antisense oligonucleotide,13 and decorin,14,15 suppressed the accumulation of ECM in the animal models of renal fibrosis. However, there are some therapeutic limitations, for example, because protein or gene is rapidly degraded by enzyme after administration into the body. In addition, despite various approaches and techniques, there is a few trials on the in vivo targeting of plasmid DNA to renal interstitial cells, which have been highlighted as one cell source responsible for expressive ECM synthesis. For one strategy to overcome this problem, it is important to develop the technology and methodology of drug delivery system (DDS) for local delivery of the therapeutic drugs.

We have explored a DDS technique necessary to therapeutic drugs for the long-term and stable expression of biologically active substance.16 As one DDS carrier for the local delivery of plasmid DNA, a cationized gelatin is used.17–20 The electrical nature of gelatin which can be readily changed by the processing method of collagen for preparation. An acidic processing of collagen produces “basic” gelatin with an isoelectric point (IEP) of 9.0. Based on this concept, positively charged gelatin can form a polyelectrolyte complex with DNA because the nature of DNA is a macromolecule of negative charges. Complexation with the biodegradable cationized gelatin enabled some plasmid DNAs to enhance their level of gene transfection in vivo. Complexation also reduced the DNase digestion of plasmid DNA and prolonged the in vivo remaining time period of plasmid DNA. In addition, the apparent molecular size of plasmid DNA was decreased and the surface charge of plasmid DNA became positive by complexation with the cationized gelatin. It is likely that these features resulted in acceleration of in vivo gene expression.17–20 However, unfortunately, the complex of cationized gelatin does not have any inherent natures of targeting to a specific cell. Thus, as one practical possible way to overcome the nontargetability, contriving the administration route will be promising.

This study is a technological trial to deliver the enhanced green fluorescent protein (EGFP) plasmid DNA complexed with the cationized gelatin into renal interstitial fibroblasts which are currently considered as the cell source responsible for excessive ECM synthesis. It is well recognized that the ureteral stenosis is one of the pathogenic characteristics of renal interstitial fibrosis and responsible for the intrinsic renal pressure increase. The objective of this study is to enhance the level of gene transfection in the disease kidney with an increased renal pressure. This study experimentally shows the gene transfection of renal interstitial cells after retrograde injection of plasmid DNA complexed with cation-
ized gelatin via the ureter of unilateral ureteral obstruction (UUO) mouse model. The retrograde ureteral catheterization is one of the procedures clinically capable.

**MATERIALS AND METHODS**

**Preparation of Cationized Gelatin and the Complex with EGFP Plasmid DNA** A gelatin sample with an IEP of 9.0 (MW = 100,000), prepared by an acid process of porcine skin, was kindly supplied from Nitta Gelatin Inc., Japan. The carboxyl groups of gelatin were chemically converted by introducing amino groups for cationization of gelatin.

Briefly ethylenediamine (Wako Pure Chemical, Japan) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC, Nacalai Tesque, Japan) were added at both the molar ratios to the carboxyl groups of gelatin of 50 into 250 ml of 100 mM phosphate-buffered solution (pH 5.0) containing 5 g of gelatin. Immediately after that, the solution pH 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 the reaction mixture was dialyzed in a cellulose tube (the cut-off molecular weight = 12000—14000, Viskase Companies, Inc.) against double-distilled water (DDW) for 48 h at room temperature and freeze-dried to obtain a cationized gelatin. When determined by the conventional 2,4,6-trinitrobenzensulfonic acid (TNBS) method, the percentage of amino groups introduced into gelatin was 50.9 mol% per the carboxyl groups of gelatin. To prepare the complex between the cationized gelatin and the EGFP plasmid DNA (OligoEngine Inc., U.S.A.), 0.1 ml of 100 mM phosphate-buffered saline solution (PBS pH 7.4) containing 2 mg of cationized gelatin was mixed with 0.1 ml of PBS containing 0.4 mg of EGFP plasmid DNA. The solution was gently agitated at 37°C for 30 min to form their complexes.

**Light Scattering Measurement** To investigate the hydrodynamic radius of EGFP plasmid DNA-cationized gelatin complex, the dynamic light scattering (DLS) measurement was carried out on a DLS 700 (Otsuka Electronics Co. Ltd., Japan) equipped with He-Ne laser at a detection angle of 30, 90, and 120° at room temperature. The hydrodynamic diameter of EGFP plasmid DNA-cationized gelatin complex 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) measurement was carried on an ELS-7000 (Otsuka Electronic Co. Ltd., Japan) at room temperature and an electric field strength of 100 V/cm. The complex samples were prepared similarly other than using 10 mM of phosphate-buffered solution (pH 7.4). The zeta potential was automatically calculated using the Smoluchowski equation. Each experiment was done 10—20 times independently.

**Preparation of a Mouse Model with Interstitial Renal Fibrosis and Evaluation of Distribution of EGFP Plasmid DNA-Cationized Gelatin Complex** A mouse model of interstitial renal fibrosis was prepared while the distribution of EGFP plasmid DNA-cationized gelatin complex was evaluated after retrograded injection via the ureter. Male C57BL/6 mice, six-week-old (Nihon SLC, Japan), were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) while the left kidney and ureter were surgically exposed by a mid-line incision. EGFP plasmid DNA-cationized gelatin complex or free EGFP plasmid DNA at the PBS volume of 50 µl were retrogradely injected into the left kidney via the ureter directly by use of HAMILTON™ syringe with 30 G needle while the left renal vein was clamped to apply the pressure aiming at the increasing transfection efficiency (5 mice). Immediately after injection, the mouse ureter was completely obstructed by a silk thread. The kidneys were perfused with cold autoclaved PBS and the cortex samples were taken 3 d later. Frozen sections (5 µm thickness) of cortex samples were incubated with first antibodies for 1 h at room temperature, followed by incubation with a rhodamine isothiocyanate (RITC)-conjugated secondary antibody for 1 h at room temperature. The first antibodies used were a monoclonal antibody to mouse laminin (a marker for tubular basement membrane, Sigma, U.S.A.), MOMA-2 (a marker for macrophages, BMA Biomedicals, Switzerland), and ER-TR7 (a marker for reticular fibroblasts, BMA Biomedicals, Switzerland). The ER-TR7 is an antibody specific for reticular fibroblasts, but not for myofibroblasts, in the kidney. The microphotographs of green fluorescence of EGFP expressed and the red fluorescence of RITC were taken by double exposure (Olympus AX-80, Olympus, Japan).

**RESULTS**

The cationization extent of cationized gelatin prepared was controllable by changing the addition molar ratio of amine molecules to the carboxyl groups of gelatin. We have demonstrated that the gene expression level is influenced by the cationization extent of gelatin complexed with plasmid DNA. The highest level of gene expression by the complex of plasmid DNA with cationized gelatin was observed at a cationization extent of 50.9 mol%. Thus, in this study, the cationization extent for cationized gelatin was selected for all the experiments.

From the DLS measurement, the apparent molecular size of free EGFP plasmid DNA itself was 552±83 nm. On the contrary, that of EGFP plasmid DNA complexed with cationized gelatin was 229±49 nm, because of the condensation of EGFP plasmid DNA in molecular size. The apparent molecular size did not depend on the measurement angle. The zeta potential of free EGFP plasmid DNA was −14.7±2.8 mV, but increased up to 9.3±1.4 mV by complexation with the cationized gelatin.

EGFP expression after injection of EGFP plasmid DNA complexed with cationized gelatin was observed in the interstitial cells 3 d after transfection (Fig. 1a). On the contrary, a few EGFP-positive cells were observed after injection of free EGFP plasmid DNA (Fig. 1b). To examine the cellular localization of EGFP plasmid DNA transfected, the basement membrane was stained by an anti-laminin first antibody and a RITC-conjugated secondary antibody (Fig. 1c). EGFP-positive cells (green) were observed outside the basement membrane (red). When double staining with an antibody of MOMA-2 (a marker for macrophages) or ER-TR7 (a marker for reticular fibroblasts) for cell identification and RITC-conjugated secondary antibody was performed, the gene expression was not observed in macrophages (red, Figs. 1d (×100) and 1e (×400)), but in interstitial reticular fibroblasts (red, Figs. 1f (×100) and 1g (×400)).
was detected in fibroblast-like cells, but not in macrophages. 

Fig. 1. The Cellular Localization of EGFP Expression 3 d after Injection of Complex of EGFP Plasmid DNA and the Cationized Gelatin into the Left Kidney via Ureter of UUO Mice

Magnification; (a, b, d, f)×100, (c)×200, (e, g)×400. The bar length is 200 (a, b, d, f), 100 (c) or 30 (e, g) μm. EGFP expression after injection of EGFP plasmid DNA complexed with cationized gelatin was observed in the interstitial cells 3 d after transfection (a). On the contrary, a few EGFP-positive cells were observed after injection of free EGFP plasmid DNA (b). When the basement membrane was immunologically stained with an anti-mouse laminin antibody (red) (c), EGFP-positive cells (green) were observed in the interstitial side of basement membrane. To investigate the localization of macrophages and interstitial reticular fibroblasts, immunological staining were observed in the interstitial side of basement membrane. To investigate the localization of macrophages and interstitial reticular fibroblasts, immunological staining with MOMA-2 (d, e) and ER-TR7 antibodies (f, g) was performed. EGFP expression was detected in fibroblast-like cells, but not in macrophages.

DISCUSSION

Despite various approaches and techniques, few successful results have been reported on the in vivo transfection for interstitial reticular fibroblasts, which play an important role in the increase of ECM synthesis. 25) The present study is the first clear demonstration that introduction of EGFP plasmid DNA into the interstitial reticular fibroblasts of mice with UUO by use of the cationized gelatin.

Zhu et al. have reported successful transfection into the interstitial compartment by an adenoviral vector. 29) In that work, the expression of β-galactosidase gene was observed in the interstitial vasculature including arteries of the outer medulla in both the outer and inner stripes and in the periglomerular and peritubular capillaries of cortex. In addition, Tsujie et al. report that TGF-β1 antisense oligonucleotides could directly affect TGF-β1 expression in interstitial fibroblasts. 27) However, the clinical trials are quite limited by the adverse effects of virus vector itself, such as immunogenicity and toxicity or the possible mutagenesis of cells transfected. Gelatin has been extensively used for industrial, pharmaceutical, and medical applications. The biosafety has been proved through its long clinical usage as the surgical biomaterials and drug ingredients. Therefore, if gelatin is chemically modified for the carrier of gene transfection, gelatin will be one of the material candidates useful and available for clinical gene therapy.

The present data suggest that the DNA solution retrogradely injected could enter into the interstitial area by slipping through between papilla epithelial cells, and thereafter distribute diffusely into the cortical interstitial space (Fig. 1). We have performed that the EGFP plasmid DNA complexed with the biodegradable cationized gelatin enhanced gene transfection because the apparent molecular size of plasmid DNA decreased to 200 nm by complexation with the cationized gelatin. 28) It is possible that the complex of small size retrogradely injected can easily infiltrate into the interstitial area by slipping through between epithelial cells, and subsequently distribute in the cortical interstitial space by simple diffusion. 29) When the EGFP plasmid DNA complexed with the cationized gelatin was retrogradely injected via the ureter, it is conceivable that the intrinsic renal pressure increases by the injection procedure. The increase in the intrinsic renal or pyelic pressure might enable the complex to penetrate between papilla epithelial cells or tubular epithelial cells. In addition, vascular permeability of macromolecules is facilitated by the pressure. As a result, it is conceivable that the complex easily diffuses into the interstitial area to distribute into the cortical interstitial space. It is demonstrated that the complex with this size can be favorably taken up by cells. 30, 31) This is an additional advantage of complex prepared from cationized gelatin for enhanced gene expression in terms of efficient DNA packing to nano-size particles. Moreover, the plasmid DNA-cationized gelatin complex of positive charge readily interacted with the cell surface of negative charge. It is well recognized that the ureteral stenosis is one of the pathogenic characteristics of renal interstitial fibrosis and responsible for the intrinsic renal pressure increase. The procedure of ureteral ligation in the UUO model is severe for the renal functions compared with the renal stenosis, but can induce the subsequent increase of renal pressure with good reproducibility. However, the reason why the interstitial fibroblasts, but not tubular epithelial cells, were selectively transfected is unclear at present.

In fact, the EGFP plasmid DNA used in this study was a pSUPER™ plasmid DNA system of RNA interference (RNAi) (OligoEngine Inc., U.S.A.) which has been used for efficient and sequence-specific gene silencing, resulting in the functional inactivation of gene targeted. Therefore, the present targeting system of plasmid DNA to interstitial renal reticular fibroblasts by the cationized gelatin may be useful for an efficient silencing of TGF-β, HSP or other cytokines’ function in genetic level which are well known to be the primary mediators for ECM accumulation. In addition, the pro-
gression of renal interstitial fibrosis might be delayed by se-
lectively removal of interstitial fibroblasts which produce
ECM. From this viewpoint, gene therapy for cell-specific in-
duced apoptosis by use of the cationized gelatin will be use-
ful.

In conclusion, the present data demonstrate that plasmid
DNA complexed with cationized gelatin could be delivered
into renal interstitial fibroblasts, which play an important role
in the interstitial renal disease. Moreover, it should be noted
that the retrograde ureteral catheterization is a common clini-
cal procedure. In the clinical setting, it is no practically prob-
lomatic to clamp the ureter for a few minutes after injection.
Therefore, this new technique of gene transfer to interstitial
cells could be a potential therapeutic strategy in the intersti-
tial renal disease.

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