synthetic cationized polymers often show considerable cytotoxicity.

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. Another unique advantage is the electrical nature of gelatin which can be readily changed by the processing method of collagen for preparation [36]. For example, an alkaline processing allows collagen to structurally denature and hydrolyze the side chain of glutamine and asparagine residue. This result in generation of "acidic" gelatin with an isoelectric point (IEP) of 5.0. On the other hand, an acidic processing of collagen produces "basic" gelatin with an IEP of 9.0. We have explored cationized gelatin for the local delivery of plasmid DNA [37–40]. Complexation with the biodegradable cationized gelatin enables plasmid DNAs to enhance their level of gene transfection in vivo. Complexation also prolongs the in vivo remaining time period of plasmid DNA by reduced DNase digestion, while it decreases the apparent molecular size of plasmid DNA and makes the surface charge of plasmid DNA positive, which accelerates gene expression [37].

This study was undertaken to evaluate the blockade effect of TGF-β type II receptor (TGF-βRII) by the plasmid DNA of TGF-βRII siRNA expression vector on the prevention of renal fibrosis for a mouse model of unilateral ureteral obstruction (UUO). Following the injection of TGF-βRII siRNA expression vector complexed with the cationized gelatin to the mouse kidney via the ureter, the level of TGF-βRII, the collagen content, and the fibrotic histology in the renal cortex were examined to evaluate the anti-fibrotic effect.

2. Materials and methods

2.1. Preparation of pSUPER-TGF-βRII and plasmid DNA of TGF-βRII expression

pSUPER-TGF-βRII was prepared according to the manufacturer's specifications (#VEC-PBS-0002, OligoEngine Inc., USA). We designed two kinds of pSUPER-TGF-βRII against different target sites of TGF-βRII mRNA (accession number; AK043619). Briefly, to insert the target sequence that encodes the TGF-βRII siRNA, the custom ordered oligonucleotides (Insert #1: 5′-gatccccGAAAGATGCATCCATCCACttcaagagaGTGGATGGATGCATCTTTCtttttggaaa-3′ and 5′-tcgatttccaaaaaGAAAGATGCATCCATCCACtctcttgaaGTGGATGGATGCATCTTTCggg-3′, Insert #2: 5′-gatccccGAAGTCCTGCATGAGCAACttcaagagaGTTGCTCATGCAGGACTTCtttttggaaa-3′ and 5′-tcgatttccaaaaaGAAGTCCTGCATGAGCAACtctcttgaaGTTGCTCATGCAGGACTTCggg-3′, the 19-nucleotide TGF-βRII target sequences are indicated in capitals in these sequences) were dissolve in sterilized nuclease-free water to give the concentration of 3 mg/ml. The annealing reaction was performed by mixing 1 μl of each oligonucleotide with 48 μl of an annealing buffer (100 mM NaCl and 50 mM HEPES pH 7.4). The mixture was incubated at 90 °C for 4 min, and then at 70 °C for 10 min. The annealed oligonucleotides were slowly cooled at 10 °C. The pSUPER™ vector was linearized with the XhoI and BglII restriction enzymes. Prior to the cloning reaction, the concentration of digested plasmid was normalized at 0.2–0.5 mg/ml. The linearized vector and each oligonucleotide were ligated by DNA Ligation Kit® Ver.2.1 (#6022, Takara, Japan). The pSUPER-TGF-βRII was transformed into the competent bacterial cells of an appropriate host strain (DH5α). After the cells were grown in ampicillin-agarose plates overnight (16–24 h), the cell colonies were picked up and further grown in an ampicillin broth for an additional cycle. The plasmid DNA was purified by column chromatography with the Qiagen EndoFree™ plasmid kit (#12362, Qiagen, USA). When the purity of plasmid DNA prepared was ascertained by UV spectroscopy, the 260 nm/280 nm absorption ratio was 1.8–1.9. In addition, pSUPER™ without any insertion of oligonucleotide (empty pSUPER) and that with a two-base-pair change in the middle of inserted sequence coding for siRNA (mismatch pSUPER-TGF-βRII) were used as negative controls.

To prepare the plasmid DNA of TGF-βRII expression (pTGF-βRII), the TGF-βRII coding region was generated by PCR using the primers as below [41], 5′-TGTGGACGCGCATCGCCAGC-3′ and 5′-ACACGGTAGCAGTAGAAGAT-3′ and mouse cDNA as template. A full-length TGF-βRII cDNA was inserted into a pRK5 (#556104, BD PharMingen, USA).

2.2. Suppression of TGF-βRII over-expression by pSUPER-TGF-βRII in vitro

293T cells seeded in each well of 6-well multi well culture plates (#3516, Corstar, USA) at 60–70% confluence were co-transfected with pTGF-βRII (5 μg) and each pSUPER-TGF-βRII (Insert #1 or #2, 5 μg), pTGF-βRII (5 μg) and empty pSUPER (pSUPER without any insertion of oligonucleotide, 5 μg) or pTGF-βRII (5 μg) and mismatch pSUPER-TGF-βRII (a two-base-pair change in the middle of inserted sequence coding for siRNA, 5 μg) by Lipofectamine™ reagent (#18324-111, Invitrogen, USA). As a reference plasmid DNA to normalize the transfection efficiency, firefly luciferase plasmid DNA (pLuc, 5 μg) (#E1741, Luciferase Reporter Vectors-pGL3, Promega, USA) was co-transfected for all the experimental groups. After 48 h, the transfected 293T cells were washed twice in ice-cold phosphate-buffered saline solution (PBS, pH 7.4), collected, and resuspended in 1 ml of ice-cold buffer (#E1531,Cell Culture Lysis Reagent, Promega, USA). After centrifugation (15,000 g, 10 min), the supernatants were used for the following Western blotting analysis. To measure the luciferase activity, the lysate was mixed with 50 μl of a reconstituted luciferase assay solution (#E2610, Luciferase Assay System, Promega, USA) and the relative light unit (RLU) of the solution mixture was determined by a luminometer (MicroLumatPlus LB 96V, Berthold, Japan). The total protein of each supernatant was determined by BCA Protein Assay Reagent (#23235, Pierce, USA). Each experimental group was carried out three times independently.

2.3. Preparation of cationized gelatin and the complex with pSUPER-TGF-βRII

A gelatin sample with an isoelectric point 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 [37–40]. Briefly, ethylenediamine (#053-00936, Wako Pure Chemical, Japan) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC, #15022-02, 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 to 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 = 12,000–14,000, 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 [42], 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 pSUPER-TGF-βRII, 0.1 ml of PBS (pH 7.4) containing 2 mg of cationized gelatin was mixed with 0.1 ml of PBS containing 0.4 mg of pSUPER-TGF-βRII. The solution was gently agitated at 37 °C for 30 min to form their complexes [37].

2.4. Preparation of a mouse model with interstitial renal fibrosis and evaluation of the anti-fibrotic effect of pSUPER-TGF-βRII-cationized gelatin complex

As an interstitial renal fibrosis to evaluate the therapeutic effect of pSUPER-TGF-βRII, UUO model was employed [43,44]. The experimental design was shown in Fig. 1. Male C57BL/6 mice, 6-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. Various dosage formulations 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/experimental group). Immediately after injection of the solution, the mouse ureter was completely obstructed by a silk thread. The formulation includes 1) a complex of pSUPER-TGF-βRII (insert #1, 100 μg/mouse) and the cationized gelatin (500 μg/mouse), 2) free pSUPER-TGF-βRII (insert #1, 100 μg/mouse), 3) empty pSUPER (100 μg/mouse), 4) mismatch pSUPER-TGF-βRII (100 μg/mouse), and 5) saline or 6) nothing (sham operation, normal group). Mice of all the groups, except for normal group, were

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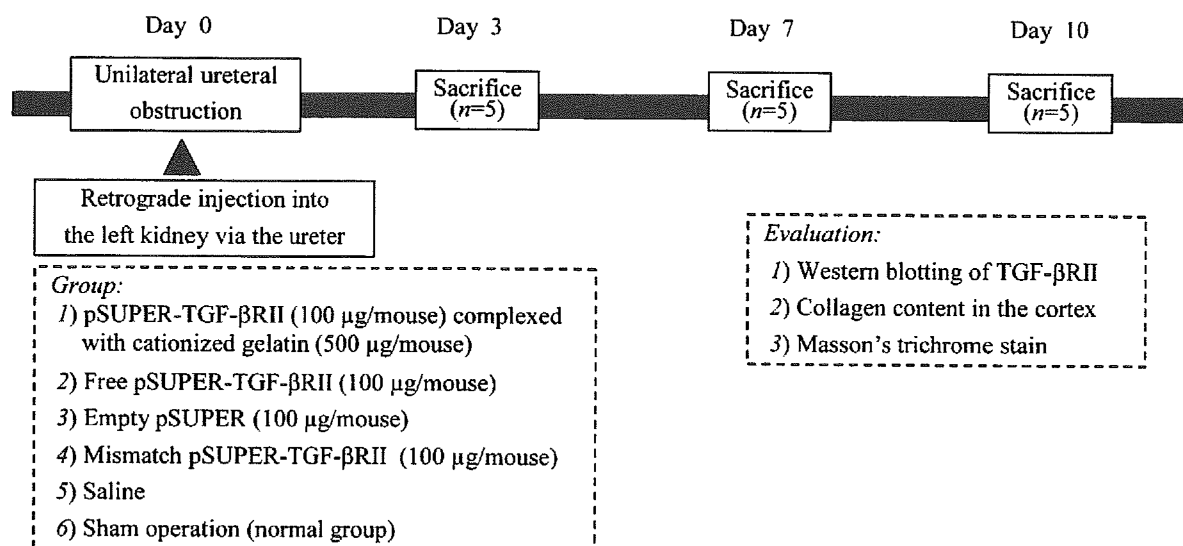

Fig. 1. Experimental protocol of siRNA-based anti-fibrotic therapy for mice receiving unilateral ureteral obstruction (UUO). A complex of pSUPER-TGF-βRII (100 μg/mouse) with the cationized gelatin (500 μg/mouse), free pSUPER-TGF-βRII (100 μg/mouse), empty pSUPER (100 μg/mouse), mismatch pSUPER-TGF-βRII (100 μg/mouse) or saline was retrogradely injected via the ureter of mice, and then their ureter was obstructed. The protein expression of TGF-βRII was evaluated by Western blotting 3, 7, and 10 days after injection. In addition, the collagen content of renal cortex was evaluated while the Masson's trichrome stain was performed.

subjected to the UUO procedure. The mouse kidneys were perfused with cold autoclaved PBS, and the cortex samples were taken and homogenized in 300 μl of cold PBS 3, 7, and 10 days later. All the experimental procedures were handled in accordance with the guidelines of the Animal Committee of Kyoto University. After centrifugation at 13,000 g for 10 min, the supernatant was used for measurement of renal collagen content and Western blotting of TGF-βRII. The collagen content of cortex was measured by a quantitative dye-binding method with Sircol™ collagen assay kit (#S1111, Biocolor, Northern Ireland). Total protein amount was determined by BCA Protein Assay kit (#23235, Pierce, USA) to express the collagen content as μg/mg total protein. For Western blotting analysis, the protein sample (50 μg per lane) was applied to a sodium dodecyl sulfate-polyacrylamide gel and electrophoresed (SDS-PAGE) for 1 h at 40 mA on the vertical gel apparatus (Bio-Rad Laboratories, Japan). The proteins separated on the gel were electrophoretically blotted to a polyvinylidine difluoride transfer membrane (Immobilon-P™, Millipore, Japan). The membrane was washed in PBS containing 0.1 wt.% Tween 20 (PBS-T) and then incubated with a skim milk at room temperature for 2 h on a shaker to block the nonspecific binding site of antibody. The membrane was incubated with a 1/100 diluted biotinylated anti-mouse TGF-βRII antibody (#BAF532, R and D Systems, USA) or anti-mouse α-SMA antibody (#A5691, Sigma, USA) for 1 h at room temperature. After washing with PBS-T, the membrane was incubated with streptavidin-horseradish peroxidase (HRP) conjugate (#438323, ZYMED, USA) or HRP conjugate secondary antibody (Sigma, USA) for 1 h at room temperature. After washing with PBS-T, the antibodies were detected by luminol reagents (ECL Plus Western blotting detection reagents; #RPN2132, Amersham Pharmacia Biotech, UK) and then, the membrane was exposed to the X-ray film (Kodak BioMax ML, Japan) in the dark to visualize the specific protein bands. Kidney tissues were fixed with 4% paraformaldehyde overnight, dehydrated through a graded series of aqueous ethanol solution, and embedded in paraffin. Histological sections (2 μm) of the kidneys were stained by the conventional Masson's trichrome stain method. The area of interstitial fibrosis stained in blue was measured by computerized image analysis using the Image-Pro PLUS™ software (Planetron, Inc., Japan). In brief, cortical fields randomly selected ($n=6$) from each mouse were photographed. The percentage of fibrotic area (blue) to the total field area was calculated. The

image analysis was performed for the microscopic fields other than glomeruli and large vessels.

2.5. Statistical analysis

All the data were expressed as the mean ± the standard derivation of the mean. Statistical significance (defined as p values of less than 0.01) was evaluated based on the unpaired Student's t test (two-tailed).

3. Results

3.1. In vitro inhibition of over-expression of TGF-βRII by pSUPER-TGF-βRII

In this study, to determine the effect of the pSUPER-TGF-βRII on TGF-βRII over-expression, 293T cells were used for in vitro study. We designed two kinds of pSUPER-TGF-βRII (insert #1 and #2) against different target sites of TGF-βRII mRNA. Fig. 2 shows the Western blotting results of inhibited expression of TGF-βRII by the pSUPER-TGF-βRII in vitro. For both the pSUPER-TGF-βRII-transfected groups, TGF-βRII expression was completely inhibited. On the contrary, the TGF-βRII was strongly expressed for the group transfected with the empty pSUPER (pSUPER without any insertion of oligonucleotide) and mismatch pSUPER-TGF-βRII (a two-base-pair change in the middle of inserted sequence coding for siRNA). The transfection efficiency was not influenced by TGF-βRII over-expression considering that the luciferase activity of all the samples was almost the same (data not shown).

3.2. In vivo transfection efficiency of pSUPER complexed with the cationized gelatin

Before therapeutic experiments, to examine the transfection efficiency of gene expression in the renal tissue by the cationized gelatin, the pSUPER vector containing a coding sequence of enhanced green fluorescent protein (EGFP-pSUPER) complexed with the cationized gelatin was retrogradely injected into the left kidney via the ureter. EGFP expression after injection of EGFP-pSUPER complexed with cationized gelatin was observed 3 days after transfection (Fig. 3A). On the contrary, little EGFP-positive cells was observed after injection of free EGFP-pSUPER (Fig. 3B).

3.3. Prevention effect of pSUPER-TGF-βRII injection on the interstitial renal fibrosis

To determine the therapeutic effect of the pSUPER-TGF-βRII on interstitial renal fibrosis, we employed the UUO model mice [43,44] and retrogradely injected of pSUPER-TGF-βRII into the left kidney via the ureter. The experimental design was shown in Fig. 1.

Fig. 4 shows the expression of TGF-βRII protein in the cortex of kidney after ureteral obstruction. Over-

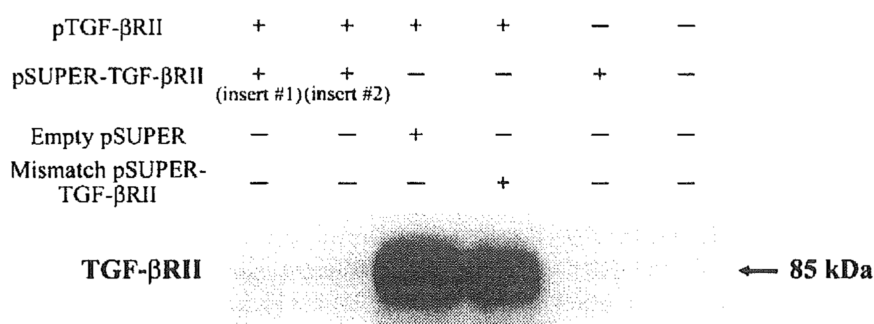

Fig. 2. Western blot analysis of TGF-βRII protein over-expressed in vitro. 293T cells were co-transfected with the plasmid DNA of TGF-βRII expression (pTGF-βRII) and pSUPER-TGF-βRII insert #1 or #2, pTGF-βRII and empty pSUPER or pTGF-βRII and mismatch pSUPER-TGF-βRII by use of Lipofectamine™. As a reference plasmid DNA to normalize the transfection efficiency, luciferase plasmid DNA (pLuc) was co-transfected for all the experimental groups. The cell lysate was prepared 48 h later for Western blotting and the luciferase activity measurement. Each experiment was performed three times independently.

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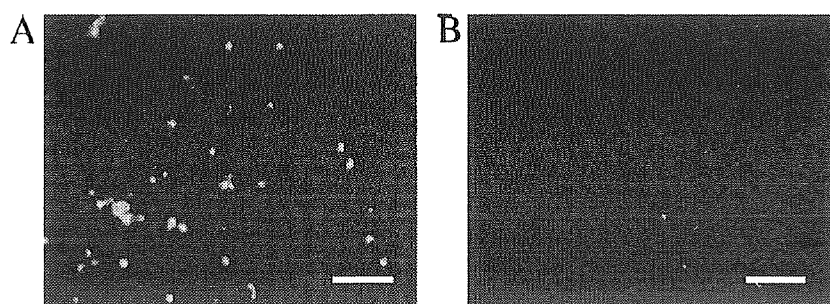

Fig. 3. EGFP expression 3 days after injection of complex of EGFP-pSUPER with cationized gelatin (A) and free EGFP-pSUPER (B) into the left kidney via ureter of UUO mice (magnification; × 100). The bar length is 200 μm. EGFP expression after injection of EGFP-pSUPER complexed with cationized gelatin was observed (A). On the contrary, a few EGFP-positive cells were observed after injection of free EGFP-pSUPER (B).

expression of TGF-βRII was observed for the saline-injected group, in contrast to that of the normal group. Suppression of TGF-βRII over-expression was not observed after injection of free pSUPER-TGF-βRII, empty pSUPER, and mismatch pSUPER-TGF-βRII. On the contrary, TGF-βRII over-expression was effectively suppressed by the injection of pSUPER-TGF-βRII complexed with the cationized gelatin. Interestingly, the suppression effect of TGF-βRII protein expression maintained until to Day 10 after injection.

Fig. 5 shows the expression of α-SMA in the cortex of kidney by Western blot analysis. The α-SMA protein was specifically detected at molecular weights of 40 to 45 kDa. The α-SMA was over-expressed in the saline-injected group in contrast with that of normal group. α-SMA was also expressed after injection of free pSUPER-TGF-βRII, empty pSUPER, and mismatch pSUPER-TGF-βRII compared with that of normal group. On the contrary, the α-SMA over-expression was effectively suppressed by the injection of pSUPER-TGF-βRII complexed with cationized gelatin. Interestingly, the suppression of α-SMA expression continued over 10 days after injection although the effect was not observed for other groups.

Fig. 6 shows the time course of collagen content in the kidney of mice after ureteral obstruction. The collagen content in the kidney of UUO model mice increased after injection of free pSUPER-TGF-βRII, empty pSUPER, mismatch pSUPER-TGF-βRII or saline, which is in contrast to that of normal group of sham operation. However, after injection of pSUPER-TGF-βRII complexed with the cationized gelatin, the increment of collagen content was dramatically suppressed.

Fig. 7A shows histological sections of mouse kidney stained with the Masson's trichrome. The blue staining of the interstitium in the obstructed kidney indicates the interstitial fibrosis. The obstructed kidney of mice injected with the free pSUPER-TGF-βRII, empty pSUPER, mismatch pSUPER-TGF-βRII or saline exhibited increased tubular atropy, in addition to a marked expansion of the interstitium. For the obstructed kidney of mice receiving the injection of pSUPER-TGF-βRII-cationized gelatin complex, a minimal interstitial expansion was observed, although the same extent of tubular atropy was detected. For the control group, obstruction resulted in significant increase in the fibrotic area compared with that of normal group (Fig. 7B). On the contrary, the obstructed kidneys of pSUPER-TGF-βRII-cationized gelatin complex-injected group showed significantly smaller fibrotic area than that of the control group over the time period of 10 days (Fig. 7B).

4. Discussion

TGF-β regulates various cellular processes by binding to three types of high-affinity cell-surface receptors (type I, II, and III) [13]. In the extracellular space, TGF-β binds either to the type III receptor which functions to present to the TGF-βRII or directly to the TGF-βRII on the cell membrane. Then, the (TGF-β)-(TGF-βRII) complex binds to the type I receptor, following by the phosphorylation of the type I receptor [45]. Phosphorylation activates several signaling pathways, including the Smad pathway, and regulates multiple TGF-β functions [46]. In this respect, TGF-βRII is important

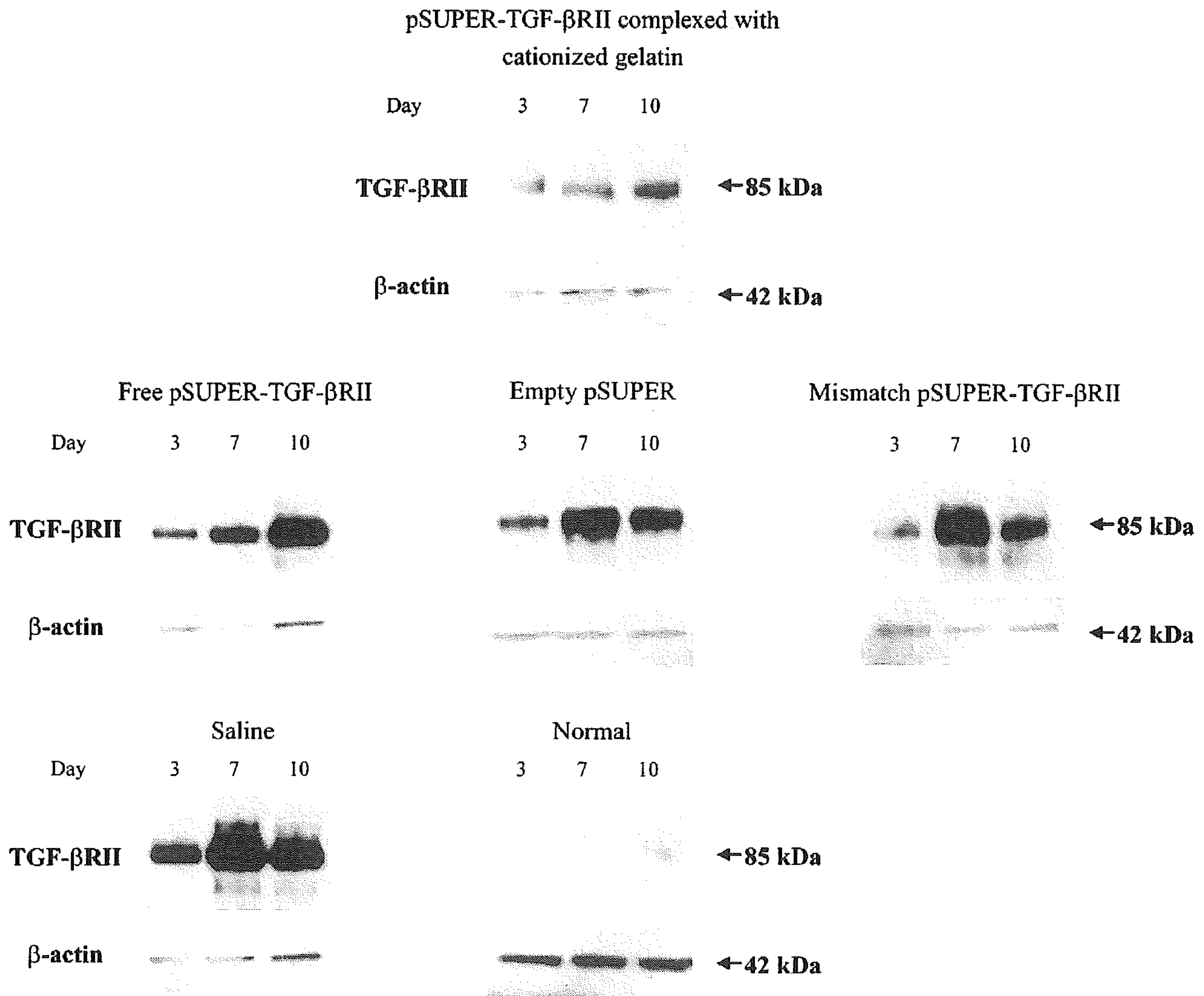

Fig. 4. Western blot analysis of TGF-βRII protein expressed in the renal cortex of mice after pSUPER-TGF-βRII application. A complex of pSUPER-TGF-βRII (100 μg/mouse) with the cationized gelatin (500 μg/mouse), free pSUPER-TGF-βRII (100 μg/mouse), empty pSUPER (100 μg/mouse), mismatch pSUPER-TGF-βRII (100 μg/mouse) or saline was injected into the left kidney via the ureter of mice, followed by their UUO treatment. The renal cortex of mice was sampled for protein analysis 3, 7, and 10 days later. The TGF-βRII protein was over-expressed for the mouse group injected with saline, in contrast with the normal group of sham operation. The expression level of TGF-βRII protein was suppressed by the injection of pSUPER-TGF-βRII with cationized gelatin complexation. The suppression effect was higher and continued for a longer time period by the injection of pSUPER-TGF-βRII complexed with the cationized gelatin than that of free pSUPER-TGF-βRII.

for the TGF-β signaling pathway. Therefore, inhibition or modification of TGF-βRII must be therapeutic strategy promising to inhibit renal fibrosis. The present study is the first clear demonstration that introduction of TGF-βRII siRNA with pSUPER-TGF-βRII into the renal cells by use of non-viral vector and the cationized gelatin can effectively suppress the over-expression of TGF-βRII protein and consequently block interstitial renal fibrosis of mice with UUO. Despite various approaches and techniques, few successful results have been reported on the in vivo transfection for renal cells [47]. Zhu et al. have reported successful transfection of β-galactosidase gene into the interstitial compartment by an adenoviral vector [48]. In addition, Tsujie et al. report that TGF-β1 antisense oligonucleotides transfected could suppress TGF-β1 expression,

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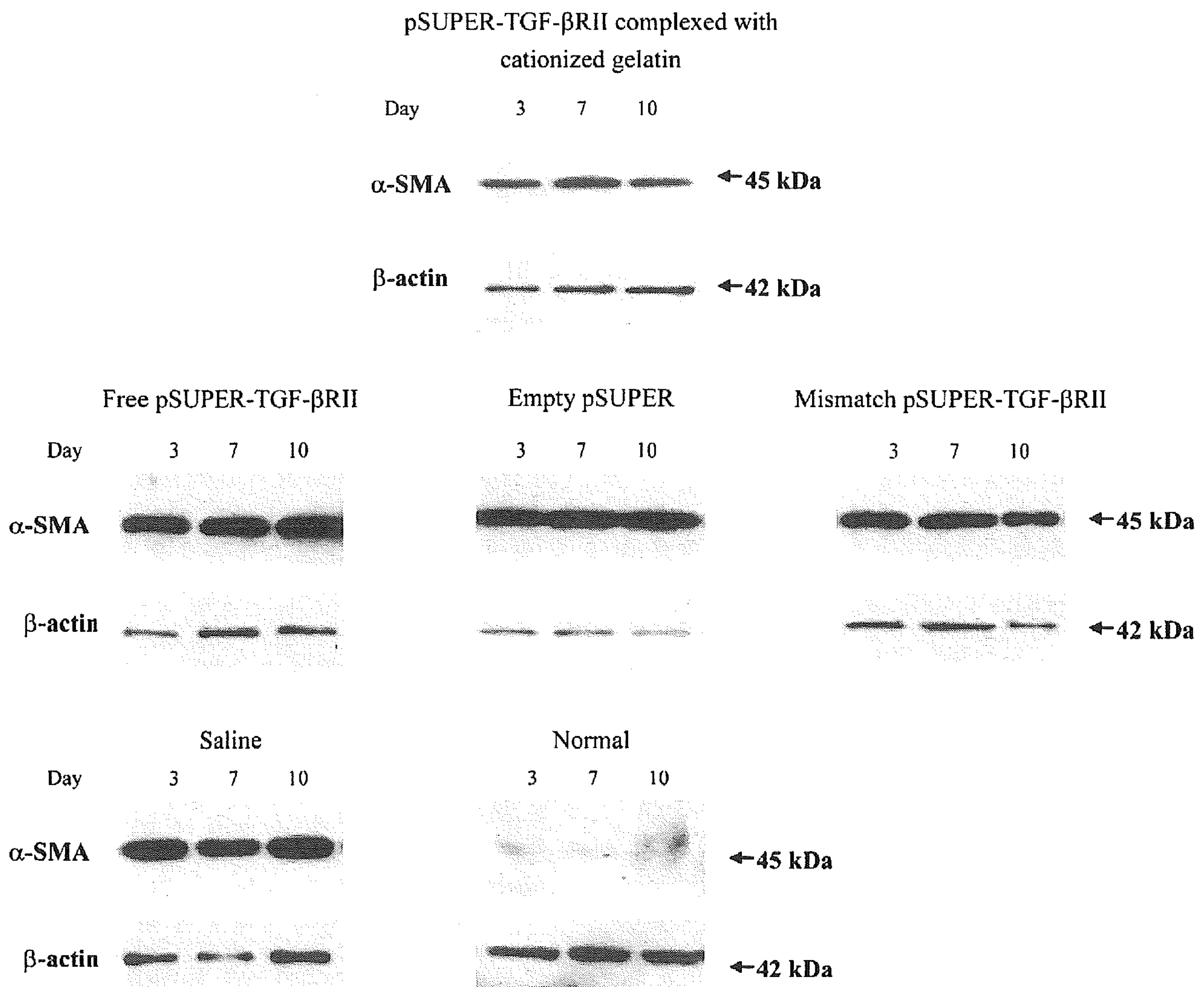

Fig. 5. Western blot analysis of α-SMA protein expressed in the renal cortex of mice after pSUPER-TGF-βRII application. A complex of pSUPER-TGF-βRII (100 μg/mouse) with the cationized gelatin (500 μg/mouse), free pSUPER-TGF-βRII (100 μg/mouse), empty pSUPER (100 μg/mouse), mismatch pSUPER-TGF-βRII (100 μg/mouse) or saline was injected into the left kidney via the ureter of mice, followed by their UUO treatment. The renal cortex of mice was sampled for protein analysis 3, 7, and 10 days later. The α-SMA protein was over-expressed for the mouse group injected with saline, in contrast with the normal group of sham operation. The expression level of α-SMA protein was suppressed by the injection of pSUPER-TGF-βRII with cationized gelatin complexation. The suppression effect was higher and continued for a longer time period by the injection of pSUPER-TGF-βRII complexed with the cationized gelatin than that of free pSUPER-TGF-βRII.

resulting inhibition renal fibrosis [49]. 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. In addition, Song et al. have reported that the efficient suppression of *Fas* expression in the liver after hydrodynamic injection of siRNA protected hepatocytes from *Fas*-mediated apoptosis [50]. *Fas*-mediated hepatocyte apoptosis contributes to the development of liver fibrosis in chronic hepatitis. Hydrodynamic injection of *Fas*-siRNA treatment significantly reduced two chemical indicators of active fibrosis, hepatic hydroxyproline and serum procollagen type III. Although that paper is the first report to show the in vivo efficiency of siRNA on the fibrosis suppression, it is quite questionable whether or not the hydrodynamic injection can be

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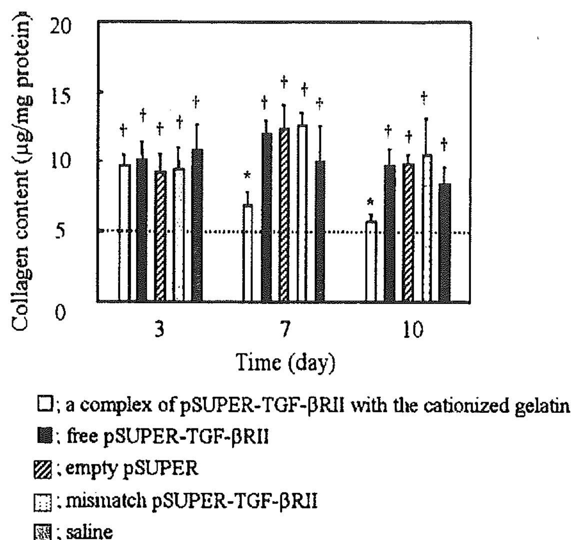

Fig. 6. Time course of collagen content in the kidney of UUO mice after pSUPER-TGF-βRII application. *; $p<0.05$: significant against the collagen content of mouse groups injected with saline. †; $p<0.05$: significant against the collagen content of kidney of normal mice (sham operation). A dotted line indicates the collagen content of kidney of normal mice (sham operation). The injection of pSUPER-TGF-βRII complexed with the cationized gelatin significantly decreased the collagen content in the kidney of UUO mice to the normal level, while that of free pSUPER-TGF-βRII, empty pSUPER, mismatch pSUPER-TGF-βRII or saline was ineffective.

used for tissues and organs other than the liver and human application. Gelatin has been extensively used for industrial, pharmaceutical, and medical applications. The bio-safety has been proved through its long clinical usage as the surgical biomaterials and drug ingredients. Therefore, gelatin is one of the material candidates useful and available for clinical gene therapy. In this study, a non-viral vector was prepared from the gelatin and a common clinical procedure of the retrograde ureteral injection was employed.

It is important to examine the change of several fibrosis parameters for a longer time period and to treat the fibrosis than the prevention for clinical gene therapy. However, we investigate the prophylactic efficiency after injection of the TGF-βRII siRNA to a mice model of acute interstitial renal fibrosis. We are planning to apply this strategy to a chronic renal interstitial model.

The present data demonstrated that the EGFP-pSUPER complexed with cationized gelatin retrogradely injected could transfected into renal cells (Fig. 3). We have reported that the 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 [51]. The intrinsic characteristics of gelatin is low cytotoxicity because it is a denatured form of collagen which is one of the main extracellular matrix proteins and highly compatible to cells. The capacity of cationized gelatin to condense the plasmid DNA may be weaker compared with other cationic polymers and consequently the size of plasmid DNA condensed may be larger. However, the size of plasmid DNA-cationized gelatin complex is small enough to allow the plasmid DNA to internalize into cells for gene transfection. By this cell compatibility feature of gelatin, it is possible that the complex of small size retrogradely injected via ureter may easily infiltrate into the interstitial area by slipping through between ureteric epithelial cells, and subsequently distribute in the cortical interstitial space by simple diffusion. When the pSUPER 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 in between papilla epithelial cells or tubular epithelial cells. The research result of cell transfection after injection of pSUPER complexed with cationized gelatin will be reported in near future. In addition, vascular permeability of macromolecules is facilitated by inflammation of UUO [52]. The pressure stimulation seems important to increase the transfection efficiency to cells. After retrograde injection of EGFP-pSUPER complexed with the cationized gelatin via ureter without ureteral obstraction (normal mice), EGFP expression was not observed (data not shown). These findings suggest that intracellular junction between the epithelial cells of ureter is expanded by UUO. As a result, it is conceivable that the complex easily diffuses into the interstitial area to distribute into the cortical interstitial space. Moreover, the plasmid DNA-cationized gelatin complex of positive charge readily interacted with the cell surface of negative charge [51]. It is demonstrated that the complex with this size can be favorably taken up by cells [53,54]. This is an additional advantage of complex prepared from cationized gelatin for enhanced gene expression in terms of efficient DNA packing to

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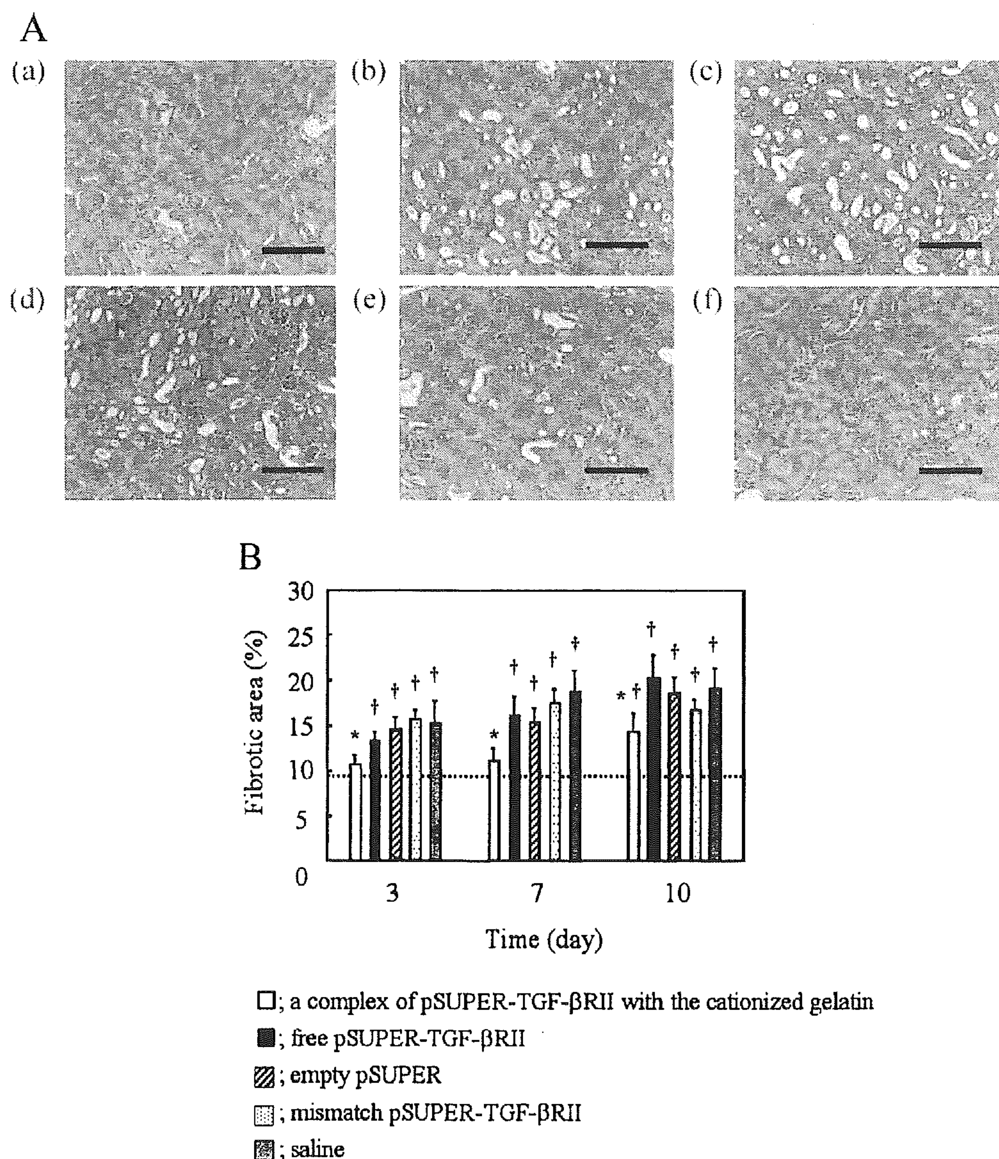

Fig. 7. (A) Histological sections of renal cortex of UUO mice after pSUPER-TGF-βRII application. The section was stained with Masson's trichrome 7 days after injection of (a) pSUPER-TGF-βRII complexed with cationized gelatin, (b) free pSUPER-TGF-βRII, (c) empty pSUPER, (d) mismatch pSUPER-TGF-βRII, (e) saline or (f) nothing (sham operation group) (magnification; $\times 200$). The bar length is 200 μm. The interstitial fibrosis of mice kidney is indicated by blue staining. The kidney of UUO mice receiving the injection of free pSUPER-TGF-βRII, empty pSUPER, mismatch pSUPER-TGF-βRII or saline exhibited increased tubular atropy, with a marked expansion of the interstitium. On the contrary, the renal cortex of UUO mice injected with the complex of pSUPER-TGF-βRII and the cationized gelatin showed a minimal interstitial expansion although the extent of tubular atropy was similar to that of mice injected with control groups. (B) Morphometric analysis of relative interstitial fibrotic area in the kidney of UUO mice after pSUPER-TGF-βRII application. *; $p<0.05$: significant against the fibrotic area of groups injected with saline. †; $p<0.05$: significant against the collagen content of kidney of normal mice (sham operation). A dotted line indicates the fibrotic area of normal (sham operated) kidney of mice. An increase in the fibrotic area of renal cortex with time was observed for mice injected with free pSUPER-TGF-βRII, empty pSUPER, and mismatch pSUPER-TGF-βRII, similarly to the control mice. On the contrary, injection of complex of pSUPER-TGF-βRII and the cationized gelatin significantly reduced the renal fibrotic area to the normal level.

nano-size particles [53,54]. Retrogradely injection of the transgene solution via the ureter with clamping the left renal vein is also overloaded the pressure to increase transfection efficiency. In the clinical setting, it is no practically problem to clamp the renal vein and ureter for a few minutes after injection.

Since the pSUPER™ vector plasmid system is a tool to stably suppress gene expression in mammalian cells [20], it is possible that the siRNA of TGF-βRII was continuously expressed in renal cells after transfection. TGF-βRII and α-SMA over-expression was completely suppressed throughout experimental time period after transfection of pSUPER-TGF-βRII complexed with the cationized gelatin (Figs. 4 and 5). These results indicate that siRNA for TGF-βRII complexed with cationized gelatin was suppressed the increment of ECM. TGF-β is well known as a major promoter of myofibroblast differentiation. In mesangial cells, TGF-β induces α-SMA expression and enhances synthesis of ECM. Increased expression of α-SMA in the interstitium has been noted in several progressive models of renal injury. In addition, it was proposed that up-regulated interstitial expression of α-SMA might serve as a predictor of progressive renal dysfunction. Following the α-SMA expression in the tubulointerstitium, several events occur in parallel, including an influx of monocytes/macrophages and accumulation of various ECM proteins [4–7]. Surprisingly, α-SMA expression was highly progressed and kept at high expression level in the kidney of control group for experimental time period. Because UUO model is acute interstitial renal fibrosis model [53,54], α-SMA expression was immediately increased and kept at high expression level after obstruction.

Moreover, various control experiments were performed to check whether or not the off-target effect or an interferon response occurs. One of the good experimental ways to enhance confidence in the RNAi research is to compare the biological effect with two or more siRNAs targeted to different sites of mRNA sequence under the same experimental conditions. Our data indicate that both the #1 and #2 pSUPER-TGF-βRII samples completely interfered the TGF-βRII over-expression in vitro (Fig. 2). In addition, RNAi feasibility is also confirmed by alternative experimental methods. Since the siRNA of mismatched sequence is not responsive to the 'active' probe, it functions as a truly informative control. A two-base-pair change in the middle of antisense sequence can be useful as a negative control, since the siRNA effect is completely ablated by this sequence change. Our data indicate that the mismatched sequences did not completely interfere the TGF-βRII over-expression in vitro and in vivo (Figs. 2 and 4). The results experimentally confirm that the pSUPER-TGF-βRII induced neither the off-target effect nor the interferon response. These negative controls experimentally neglected the unexpected side-effects of off-target effect and interferon response.

In conclusion, the present data demonstrate that blocking for TGF-βRII by the pSUPER-TGF-βRII-cationized gelatin complex was effective in reducing the deposition of ECM in mice interstitial renal fibrosis without any off-target effect and interferon response. The present concept of transfection of siRNA expression vector complexed with the cationized gelatin will be a new therapeutic strategy to block TGF-β signaling pathway. Moreover, it should be noted that the retrograde ureteral catheterization is a common clinical procedure. Therefore, this new technique of gene transfer to interstitial cells could be a potential therapeutic strategy in the interstitial renal disease.

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Targeting of Plasmid DNA to Renal Interstitial Fibroblasts by Cationized Gelatin

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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 extracelluar 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 extracelluar 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 polyion 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 no-targetability, 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-

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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=100000), 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.[17—20] 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,[21] 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 Smoluchouski 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.[22,23] 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 second 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.[24] 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.[17] 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%.[17] 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)).

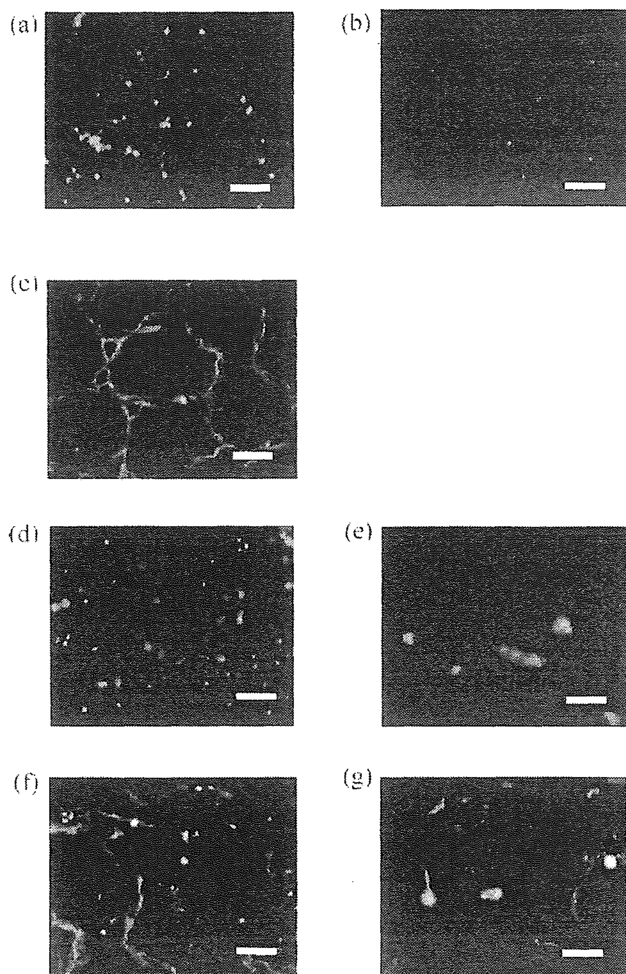

Fig. 1 The Cellular Localization of EGFP Expression 3 d after Injection of Complex of EGFP Plasmid DNA and the Cationized Gelatin into the Left Kidney *via* Ureter of UUO Mice

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 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.[26] 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 were introduced neither into macrophages nor tubular cells, but fibroblasts, suggesting 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 tublar 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 selectively removal of interstitial fibroblasts which produce ECM. From this viewpoint, gene therapy for cell-specific induced apoptosis by use of the cationized gelatin will be useful.

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 clinical procedure. In the clinical setting, it is no practically problematic 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 interstitial renal disease.

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Controlled Release Technology Suppresses The Progression of Disseminated Pancreatic Cancer Cells

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Abstract

NK4, composed of the NH_2-terminal hairpin and subsequent four-kringle domains of hepatocyte growth factor (HGF), acts as a potent angiogenesis inhibitor. This study is an investigation to evaluate the feasibility of controlled release of NK4 plasmid DNA in suppressing tumor growth. Controlled release by a biodegradable hydrogel enabled the NK4 plasmid DNA to enhance the tumor suppression effects. Biodegradable microspheres of cationized gelatin were prepared for the controlled release of a NK4 plasmid DNA. The cationized gelatin microspheres incorporating NK4 plasmid DNA were subcutaneously injected to tumor-bearing mice to evaluate the suppressive effects on tumor angiogenesis and growth. The cationized gelatin microspheres incorporating NK4 plasmid DNA could release over 28 days. When the cationized gelatin microspheres incorporating NK4 plasmid DNA were injected into the subcutaneous tissue of mice intraperitoneally inoculated with pancreatic cancer cells, their survival time period was prolonged. Tumor growth was suppressed to a significantly greater extent than free NK4 plasmid DNA. The controlled release of NK4 plasmid DNA suppressed angiogenesis and increased cell apoptosis in the tumor tissue, while it enhanced and prolonged the serum level of NK4 protein. We conclude that the controlled release technology was promising to enhance the tumor suppression effects of NK4 plasmid DNA.

Introduction

Hepatocyte growth factor (HGF) has been noted as the signal molecule which plays an important role in development, differentiation, and morphogenesis of living systems (1-3). On the other hand, HGF often acts in an autocrine fashion to induce and enhance the invasive, angiogenic, and metastatic functions of malignant tumors by way of the c-Met/HGF receptor (4-7). Therefore, it is highly expected that the molecular blocking of c-Met/HGF receptor effectively suppresses the invasive, angiogenic, and metastatic functions of tumor cells. Based on this concept, Date *et al.* have prepared an antagonist for HGF which composes of the NH_2-terminal hairpin domain of HGF α-subunit and the subsequent four kringles domains (NK4) (8). The NK4 binds to the c-Met/HGF receptor, but does not induce tyrosine phosphorylation of c-Met. NK4 competitively inhibits some biological events driven by the HGF-Met receptor binding, such as the invasion and metastasis of distinct types of tumor cells and angiogenesis (8-10).

Based on recent advent of genomics, new genes have been discovered and will become therapeutically available for various diseases in near future. In this connection, gene therapy is expected as a new and promising therapeutic choice. Presently, several human clinical trials are being proceeded to treat cancer by utilizing the viral vectors of retroviruses, adenoviruses, and adeno-associated viruses. In spite of the high transfection efficiency, the therapeutic trials are limited by the adverse effects of virus itself, such as immunogenicity and toxicity or the possible mutagenesis of cells transfected. As the non-viral vectors, many types of cationized polymers (11) and cationized liposomes (12) have been explored. This approach is to enable the plasmid DNA to neutralize the anionic charge as well as to reduce the molecular size for enhanced efficiency of plasmid DNA transfection which causes an increase in the gene expression. However, the shorter duration and the lower level of gene expression than viral vectors are important issues to be technologically improved. One of the possible ways to tackle the issues is to permit the controlled release of plasmid DNA by combining with an appropriate carrier.

Gelatin has been extensively used for industrial, pharmaceutical, and medical applications and the bio-safety is proved through its long clinical usage as the surgical biomaterials and drug ingredients. Another unique advantage of gelatin is variation in the electrical nature, while the electric nature can be readily changed by the processing method of collagen (13). We have designed and explored the controlled release system of drugs on the basis of drug release governed by degradation of drug carrier. Drugs are immobilized into the biodegradable hydrogel of gelatin on the way of physicochemical interaction forces between the drug

and gelatin molecules. In this release system, the drug immobilized is not released from the hydrogel unless the hydrogel carrier is degraded to generate water-soluble gelatin fragments. The drug release can be controlled only by changing the hydrogel degradation (14). In addition, the cationized gelatin of positive charge can readily be prepared by introducing amine residues to the carboxyl groups of gelatin. The plasmid DNA polyionically immobilized in the cationized gelatin hydrogel is released from the hydrogel only if the hydrogel is degraded to generate the water-soluble gelatin fragments (15, 16). The release mechanism driven by degradation of release carrier is quite different from that of plasmid DNA diffusion from the release carrier which has been reported as the conventional release system of plasmid DNA (17, 18). This study indicates that the cationized gelatin hydrogel enabled a NK4 plasmid DNA to achieve the controlled release and consequently exert the tumor suppressive effects which are not observed for the plasmid DNA solution.

In this study, the cationized gelatin hydrogel was applied to the controlled release of expression plasmid for human NK4, to evaluate the suppressive effects on tumor angiogenesis and growth in tumor-bearing mice.

Results and Discussion

Radiotracing experiment revealed that NK4 plasmid DNA was retained around the injected site of cationized gelatin microspheres incorporating NK4 plasmid DNA over the time period of 28 days, whereas free NK4 plasmid DNA injected was excreted more rapidly (**Figure 1**).

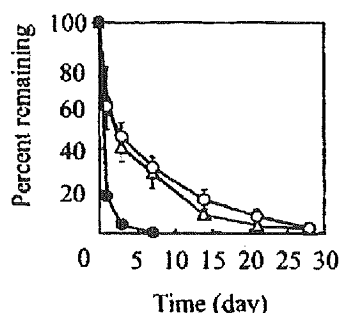

Figure 1
The time course of radioactivity remaining of cationized gelatin microspheres incorporating ^{125}I-labeled NK4 plasmid DNA (O) and free ^{125}I-labeled NK4 plasmid DNA (●) or ^{125}I-labeled cationized gelatin microspheres (△) after the subcutaneous injection into the back of mice. The microspheres enabled NK4 plasmid DNA to retain in the injected site for a longer time period than in the solution form. The in vivo retention profile of NK4 plasmid DNA was in good accordance with that of microspheres as the release carrier, indicating the controlled release of NK4 plasmid DNA accompanied with the carrier degradation.

A good correlation in the time profile of *in vivo* retention was observed between the NK4 plasmid DNA incorporated and microspheres. This indicates that the controlled release of NK4 plasmid DNA was governed by microsphere degradation. When the cationized gelatin microspheres incorporating NK4 plasmid DNA were injected into the subcutaneous tissue of mice intraperitoneally inoculated with pancreatic cancer cells, their survival time period was prolonged (**Figure 2**).

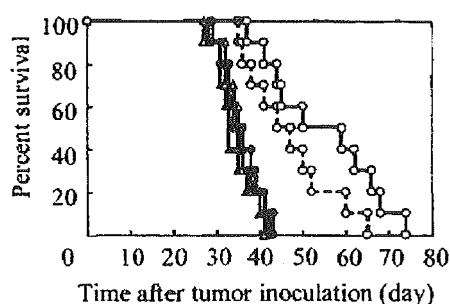

Figure 2
Survival curves of tumor-bearing mice following the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue: cationized gelatin microspheres incorporating 100 (O---) and 200 μg of NK4 plasmid DNA (O—)*, 200 μg of free NK4 plasmid DNA (●—), empty cationized gelatin microspheres (△), and saline (▲). Irrespective of the NK4 plasmid DNA dose, the injection of microspheres incorporating NK4 plasmid DNA significantly prolonged the survival time period of tumor-bearing mice, in contrast to that of free NK4 plasmid DNA. *, $p<0.05$: significant against the survival curve of saline-injected, control mice.*

The subcutaneous injection of cationized gelatin microspheres incorporating NK4 plasmid DNA significantly suppressed the tumor progression of the pancreatic cancer cells disseminated in the peritoneal cavity of nude mice. Generally, it is known that the vessel formation in the tumor tissue is in progress after metastasis of tumor cells. The prolonged expression of NK4 results in significant suppression of increase in the number and total weight of disseminated nodules (**Figures 3A and 3B**). Continuous exposure of NK4 protein to the tumor cells is effective in suppressing the vessel formation. We believe that suppression of angiogenesis at least enables tumor to maintain the dormant state rather than to biologically eradicate. Therefore, in terms of tumor dormancy, tumor gene therapy based on continuous release of NK4 plasmid DNA from cationized gelatin microspheres may be an attractive new approach for treatment of advanced tumor patients.

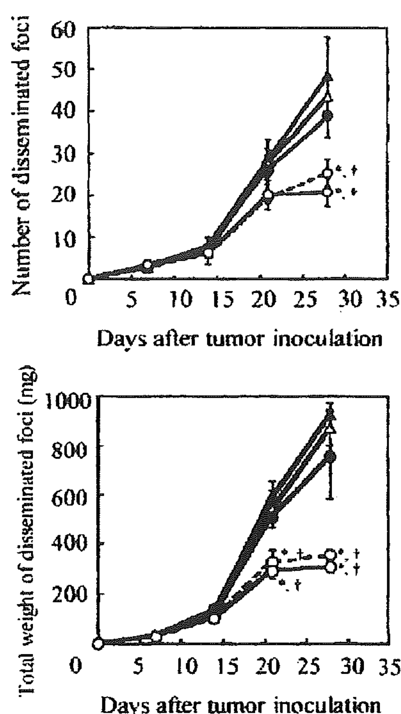

Figure 3

In vivo tumor suppression effects of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA following the single injection into the subcutaneous tissue.

*(A) Time course of tumor number change: cationized gelatin microspheres incorporating 100 (O---) and 200 μg of NK4 plasmid DNA (O—), 200 μg of free NK4 plasmid DNA (●), empty cationized gelatin microspheres (△), and saline (▲). *, $p<0.05$: significant against the tumor number of saline-injected mice at the corresponding day. †, $p<0.05$: significant against the tumor number of mice injected with 200 μg of free NK4 plasmid DNA at the corresponding day.*

(B) Time course of total weight of disseminated nodule: cationized gelatin microspheres incorporating 100 (O---) and 200 μg of NK4 plasmid DNA (O—), 200 μg of free NK4 plasmid DNA (●), empty cationized gelatin microspheres (△), and saline (▲).

**, $p<0.05$: significant against the tumor weight of saline-injected mice at the corresponding day. †, $p<0.05$: significant against the tumor weight of mice injected with 200 μg of free NK4 plasmid DNA at the corresponding day.*

The injection of cationized gelatin microspheres incorporating NK4 plasmid DNA decreased the number of blood vessels in the tumor tissue and the vessel diameter compared with that of other agents. Additionally, the microspheres injection was effective in increasing the number of apoptotic cells. These findings are consistent with previous studies in which angiogenesis inhibitors suppress the tumor growth based on the increasing apoptosis of tumor cells (19, 20). We supposed that NK4 prevented the progression of disseminated tumor cells as an angiogenesis inhibitor in addition to an HGF antagonist. Improved mice survival by the injection of cationized gelatin microspheres incorporating NK4 implies that NK4 also inhibited the further extension of peritoneal dissemination.

Because of the *in vivo* instability and immunogenicity of therapeutic protein itself, it is difficult to induce the biological function and maintain it for a long time period (21). On the other hand, the plasmid DNA may achieve a prolonged biological effect by the transfected cells, although the low transfection efficiency by plasmid DNA should be improved (22). The controlled release enables the plasmid DNA to increase the concentration in the tissue over an extend time period. It is highly conceivable that the enhanced concentration increases the exposure possibility of plasmid DNA to cells, resulting in promoted gene expression. It is likely that the controlled release of the plasmid DNA prevents rapid degradation of DNA and facilitate exposure and transduction of plasmid DNA to cells, thereby increasing gene expression efficiency. Although there are still some rooms to consider as the mechanism of NK4-induced suppression effect of tumor metastasis, the present study indicates the therapeutically positive effect of NK4 plasmid DNA release on tumor suppression.

It is possible that the plasmid DNA released is condensed because of the polyion complexation with the cationized gelatin of degradation product. It has been demonstrated that plasmid DNA can be more readily taken up by cells through condensation in the molecular size of plasmid DNA through polyion complexation with cationized polymers (23, 24). This feature to induce the molecular condensation is also an advantage of the release system to enhance gene expression. Taken together, we can say with certainty that the NK4 plasmid DNA was expressed around the injection site and secreted to the systematic circulation. Our research results demonstrated that it was important for successful tumor therapy to expose NK4 to tumor cells for a long time even at a low concentration by the controlled release system, although there are still unclear points about the mechanism of tumor suppression.

We conclude that controlled release with cationized gelatin microspheres was a promising technology to enhance the *in vivo* tumor suppression effects of NK4 plasmid DNA. This release system is applicable to other types of plasmid DNA and oligonucleotide for enhanced gene expression.

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