GENE DELIVERY

ELSEVIER

Available online at www.sciencedirect.com
SCIENCE DIRECT®

Journal of Controlled Release 110 (2006) 610–617

journal of controlled release
www.elsevier.com/locate/jconrel

Enhanced anti-fibrotic activity of plasmid DNA expressing small interference RNA for TGF-β type II receptor for a mouse model of obstructive nephropathy by cationized gelatin prepared from different amine compounds

Toshihiro Kushibiki [a], Natsuki Nagata-Nakajima [b], Manabu Sugai [b], Akira Shimizu [b], Yasuhiko Tabata [a,*]

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

Received 31 August 2005; accepted 8 November 2005
Available online 27 December 2005

Abstract

The objective of this study is to increase the transfection efficiency of a plasmid DNA expressing small interference RNA (siRNA) for transforming growth factor-β receptor (TGF-βR) by various cationized gelatins of non-viral carrier and evaluate the anti-fibrotic effect with a mouse model of unilateral ureteral obstruction (UUO). Ethylenediamine, putrescine, spermidine or spermine was chemically introduced to the carboxyl groups of gelatin for the cationization. The plasmid DNA of TGF-βR siRNA expression vector with or without complexation of each cationized gelatin was injected to the left kidney of mice via the ureter to prevent the progression of renal fibrosis of UUO mice. Irrespective of the type of cationized gelatin, the injection of plasmid DNA-cationized gelatin complex significantly decreased the renal level of TGF-βR over-expression and the collagen content of mice kidney, in marked contrast to free plasmid DNA injection. It is concluded that retrograde injection of TGF-βR siRNA expression vector plasmid DNA complexed with the cationized gelatin is available to suppress the progression of renal interstitial fibrosis.

Keywords: Small interference RNA (siRNA); Transforming growth factor β (TGF-β) receptor; Cationized gelatin; Gene delivery; Renal interstitial fibrosis

1. Introduction

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, interstitial matrix, tubular atrophy, and the accumulation of myofibroblasts [1–3]. Chronic renal disease is characterized by persistent accumulation and deposition of extracelluar matrix (ECM) which lead to widespread tissue fibrosis [4]. Renal interstitial fibrosis is considered to be the commonly converging outcome of chronic renal diseases with a wide spectrum of diverse etiologies. While tremendous progress has been made in delineating the cellular and molecular pathogenesis during last decades, the clinical therapy of chronic renal fibrosis remains to be extremely challenging. Thus, it is strongly expected to develop a novel therapeutic strategy for anti-fibrotic treatment. For this purpose, drug delivery system (DDS) may open a new pathway for the therapeutic strategy.

For renal fibrosis, severe accumulation of ECM is observed in the renal interstitial compartment [4–7]. It has been demonstrated from several animal models of renal fibrosis that transforming growth factor-β (TGF-β) functions as one of the primary mediators for ECM accumulation [8–12]. TGF-β is multifunctional cytokines acting in many physiologic and pathologic processes, regulates the proliferation and differentiation of cells in various tissues, and plays a central role in fibrogenesis [13]. TGF-β increases the production and deposition of ECM proteins, reduces matrix degradation accompanied with decreased protease production and increased the inhibitors production, and stimulates the synthesis of ECM protein receptors [14]. Therefore, it is possible that block of the

* Corresponding author. Tel.: +81 75 751 4121; fax: +81 75 751 4646.
E-mail address: yasuhiko@frontier.kyoto-u.ac.jp (Y. Tabata).

0168-3659/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2005.11.007

TGF-β action on ECM suppresses tissue fibrosis. It has been demonstrated that biological inhibition of TGF-β protein by use of neutralizing antibody [12], antisense oligonucleotide [15], decorin [16,17], and TGF-β receptor-IgG Fc chimera [18] suppressed the accumulation of ECM in the animal models of renal fibrosis. However, there is therapeutic limitation because protein or gene is rapidly degraded by enzyme after administration in the body. This short-term duration of the TGF-β signaling inhibition is another problem to be solved. As one trial to overcome this problem, in this study, we have explored an RNA interference (RNAi) system to achieve a long-term and stable inhibition of TGF-β signaling. Despite various approaches and techniques, few successful studies have been reported concerning in vivo transfection targeting interstitial cells, which have been highlighted as the source of increased ECM synthesis. Thus, it is important to develop the technology and methodology of DDS for local delivery of therapeutic genes.

RNA interference (RNAi) has been recognized as a phenomenon that mRNA is sequence-specifically degraded and a process that avoids the global depression of protein synthesis induced by the double-stranded RNA in mammalian cells [19]. Selective degradation of target mRNAs in mammalian cells is achieved by transfection with double-stranded, short interfering RNA (siRNA), leading to rapid and efficient degradation of the target [19]. In addition, the pSUPER™ vector plasmid system of RNAi has been used for efficient and sequence-specific down-regulation of gene expression [20], resulting in the functional inactivation of genes targeted. The pSUPER™ RNAi vector plasmid provides a mammalian expression vector that directs intracellular synthesis of siRNA-like transcripts.

To increase the biological activity of siRNA itself or the expressing plasmid DNA, it is necessary to improve their insufficient transfection efficiency to cells in vivo. Several synthetic materials, including cationic liposomes [21–23], poly-L-lysine [24–27], and polyethylenimine [28–33], have been molecularly designed to demonstrate improved transfection of plasmid DNA for mammalian cells both in vitro and in vivo. Generally, since the plasmid DNA is a large and negatively charged molecule, it is impossible to allow the plasmid DNA to internalize into cells even though the attachment onto the cell membrane of negative charges takes place. When the plasmid DNA is polyionically complexed with synthetic cationic polymers, it is well recognized that the molecular size of plasmid DNA decreases by the molecular condensation with the polymers and the complex has a positive charge [34,35]. It is likely that the condensed plasmid DNA-polymer complex of a positive charge can electrostatically interact with the cell membrane for internalization. However, cytotoxicity of the synthetic cationized polymers has been one problem to be improved.

Gelatin has been extensively used for industrial, pharmaceutical, and medical applications. The bio-safety has been proven through its long clinical usage as the surgical biomaterials and drug ingredients. Complexation with a biodegradable cationized gelatin enabled a plasmid DNA to enhance their level of gene transfection in vivo. Complexation also prevented the plasmid DNA from DNase digestion and consequently prolonged their in vivo remaining time period. In addition, it decreased the apparent molecular size of plasmid DNAs and made the net charge of plasmid DNA complex positive, which results in accelerated gene expression [36–39].

The present study was undertaken to investigate the in vivo transfection efficiency of a plasmid DNA expressing TGF-β I receptor type II (TGF-βRII) siRNA by various cationized gelatins of non-viral carrier and compare the prevention effect on renal interstitial fibrosis. Ethylenediamine, putrescine, spermidine or spermine was chemically introduced into the carboxyl groups of gelatin to prepare various cationized gelatins. When the complex of the plasmid DNA expressing TGF-βRII siRNA and the cationized gelatin was injected via the ureter of UUO model mice, the anti-fibrotic effect was assessed in terms of histological and biochemical examinations.

2. Materials and methods

2.1. Construction of pSUPER-TGF-βRII

pSUPER-TGF-βRII was prepared according to the manufacturer's specifications (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 (5′-gatccccGAAAGATGCATCCATCCACttcaagagaGTGGAT GGATGCATCTTTCttttt-ggaaa-3′ and 5′-tcgatttccaaaaaGAAAGATGCATCCATCCACtc tcttgaaGTGGATGGATGCATCTTTCggg-3′, the 19-nucleotide TGF-βRII target sequences are indicated in capitals in these sequences) were dissolve in sterilized nuclease-free water to give a 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 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (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 (Takara, Japan). The pSUPER-TGF-βRII was transformed into the competent bacterial cells of an appropriate host strain (DH5β). After the cells were grown in ampicillin-agarose plates overnight (16–24 h), the cell colonies were picked up and further grown in an ampicilin broth for an additional cycle. The plasmid DNA was purified by column chromatography with the Qiagen EndoFree™ plasmid kit (Qiagen, USA). When measured to ascertain the purity of plasmid DNA prepared by UV spectroscopy, the absorption ratio of wavelength 260 to 280 nm 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.

2.2. Preparation of cationized gelatin prepared from different amine compounds and the complex with pSUPER-TGF-RII

Ethylenediamine [$NH_2(CH_2)_2NH_2$], putrescine [$NH_2(CH_2)_4NH_2$], spermidine [$NH_2(CH_2)_4NH(CH_2)_3NH_2$] or spermine [$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$] was added together with 5 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC, Nacalai Tesque Inc., Japan) into 250 ml of 100 mM phosphate-buffered solution (pH 5.0) containing 5 g of gelatin (from porcine skin, isoelectric point of 9.0, molecular weight of 100,000, Nitta Gelatin Inc., Japan) at different molar ratios to the carboxyl groups of gelatin (0, 1, 10, 25, 50, and 100). Immediately after that, the pH of solution was adjusted at 5.0 by adding 5 M of HCl aqueous solution. The reaction mixture was agitated at 37 °C for 18 h and then dialyzed (the cut-off molecular weight=12,000–14,000, Viskase Companies, Inc.) against double-distilled water (DDW) for 48 h at 25 °C. The dialyzed solution was freeze-dried to obtain various cationized gelatin samples. The percentage of amino groups introduced into the carboxyl groups of gelatin (the cationization extent of gelatin) was determined by the conventional trinitrobenzene sulfonate method [40] based on the calibration curve prepared by using â-alanine at the predetermined concentration. To prepare the complex between the cationized gelatin and pSUPER-TGF-βRII, 0.1 ml of 100 mM phosphate-buffered solution (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.

2.3. 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, an unilateral ureteral obstruction (UUO) model was employed [41,42]. Male C57BL/6 mice,

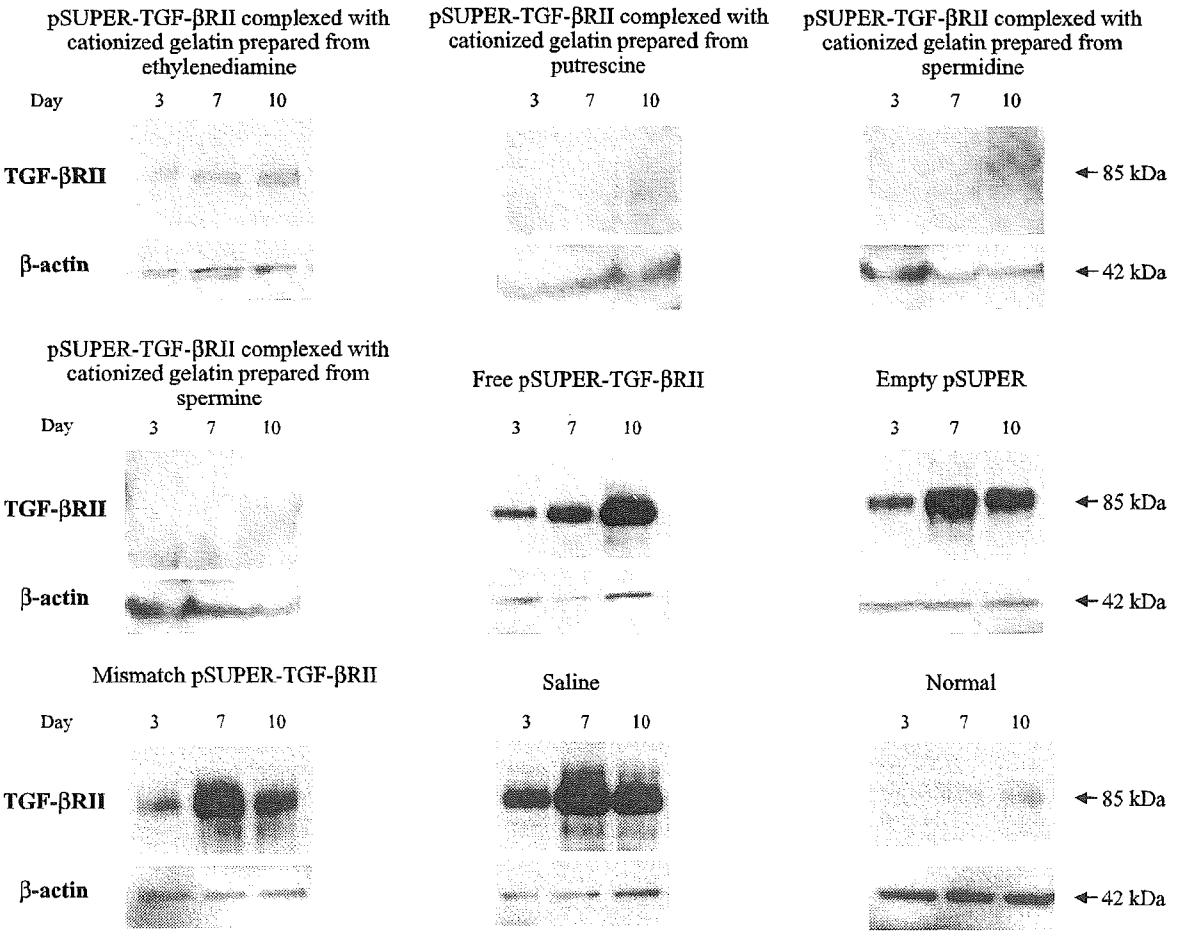
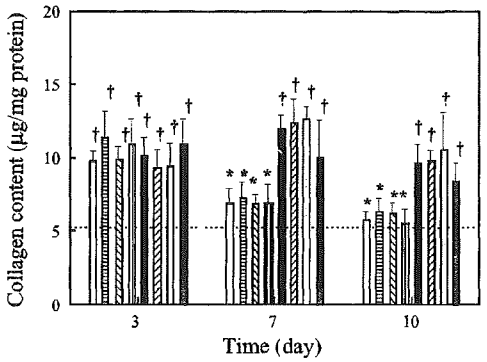


Fig. 1. Western blot analysis of TGF-βRII protein expressed in the renal cortex of mice after pSUPER-TGF-βRII application. A complex of pSUPER-TGF-βRII (100 μg/mouse) with the cationized gelatin prepared from different amine compounds (500 μg/mouse), free pSUPER-TGF-βRII (100 μg/mouse), empty pSUPER (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.

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 test formulations of pSUPER-TGF-βRII at the PBS volume of 50 μl were retrogradely injected into the left kidney via the ureter by use of HAMILTON™ syringe with a 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 test formulations injected included (1) a complex of pSUPER-TGF-βRII (100 μg/mouse) and the cationized gelatin (500 μg/mouse), (2) free pSUPER-TGF-βRII (100 μg/mouse), (3) empty pSUPER (100 μg/mouse), (4) mismatch pSUPER-TGF-μRII (100 μg/mouse), and (5) saline or (6) nothing (sham operation, normal group). Mice of all the groups, except for normal group, were 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 $\times g$ 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 (Biocolor, Northern Ireland). Total protein amount was determined by BCA Protein Assay kit (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 (R&D Systems, USA) for 1 h at room temperature. After washing with PBS-T, the membrane was incubated with streptavidin-horseradish peroxidase (HRP) conjugate (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; 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 (3 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.



□ ; pSUPER-TGF-βRII complexed with the cationized gelatin prepared from ethylenediamine
▤ ; pSUPER-TGF-bRII complexedwith the cationized gelatin prepared from putrescine
▧ ; pSUPER-TGF-bRII complexedwith the cationized gelatin prepared from spermidine
▥ ; pSUPER-TGF-bRII complexedwith the cationized gelatin prepared from spermine
■ ; free pSUPER-TGF-βRII
▨ ; empty pSUPER
□ ; mismatch pSUPER-TGF-βRII
□ ; saline

Fig. 2. Time course of collagen content in the kidney of UUO mice after pSUPER-TGF-βRII application. Mice received the injection of pSUPER-TGF-βRII complexed with the cationized gelatin prepared from ethylenediamine, putrescine, spermidine or spermine, free pSUPER-TGF-βRII, empty pSUPER, mismatch pSUPER-TGF-βRII, and saline $^{*}p<0.05$: significant against the collagen content of mouse groups injected with saline. $^{\dagger}p<0.05$: 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).

3. 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).

4. Results

Fig. 1 shows the expression of TGF-βRII protein in the cortex of kidney after ureteral obstruction. Over-expression of TGF-βRII was observed for the saline-injected group, in contrast to that of the normal group. Suppression of TGF-βRII RII 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 significantly suppressed by the injection of pSUPER-TGF-βRII complexed with the cationized gelatin, irrespective of the type of amine compounds introduced. Interestingly, the suppression effect of TGF-βRII protein expression maintained until to Day 10 after injection of every cationized gelatin.

Fig. 2 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 the normal group. However, for any cationized gelatin, after injection of pSUPER-TGF-βRII complexed with the cationized gelatin, the increment of collagen content was significantly suppressed.

Fig. 3 shows the histological sections of mouse kidney stained with the Masson's trichrome. 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 any 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. 4). On the contrary, irrespective of the gelatin type, 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. 4). It should be noted that the injection of pSUPER-TGF-βRII-cationized gelatin of spermine complex showed a level of fibrotic area similar to that of the sham operation group even on the 10th day (Fig. 4).

5. Discussion

In the present study, the therapeutic efficacy of cationized gelatin prepared from different amine compounds as a non-viral gene delivery system in prevention of renal fibrosis by the plasmid DNA of pSUPER-TGF-βRII was evaluated. We have reported the in vitro transfection efficiency of a luciferase plasmid DNA by the cationized gelatin prepared from different amine compounds [43]. Complexation of the cationized gelatin

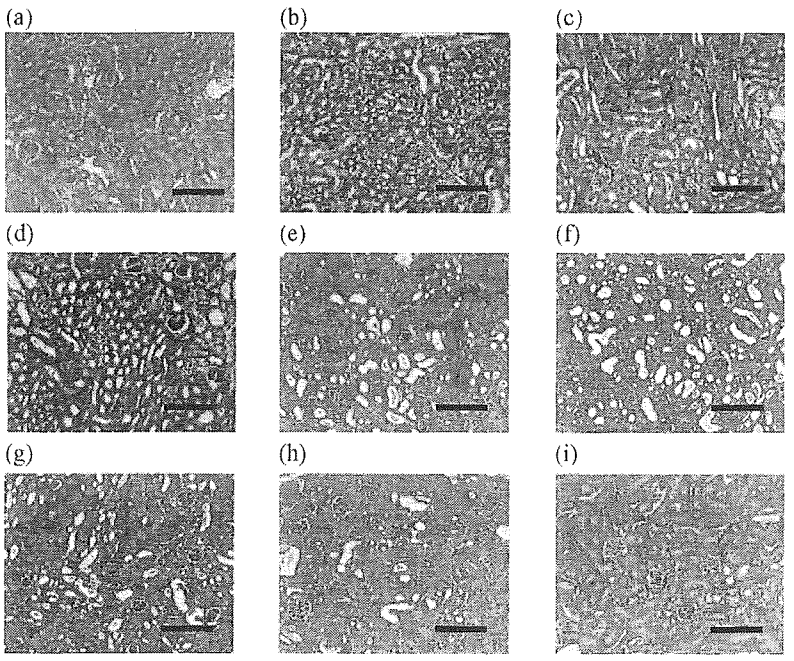
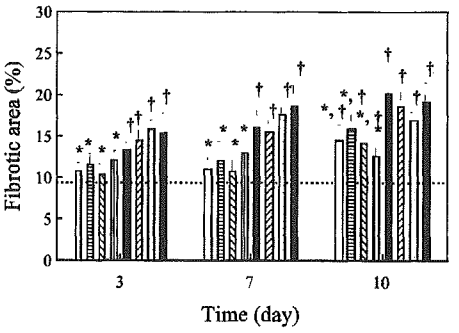


Fig. 3. Histological sections of renal cortex of UUO mice after pSUPER-TGF-βRII application. The section was stained with Masson's trichrome 7 days after injection of pSUPER-TGF-βRII complexed with cationized gelatin prepared from ethylenediamine (a), putrescine (b), spermidine (c), and spermine (d), free pSUPER-TGF-βRII (e), empty pSUPER (f), mismatch pSUPER-TGF-βRII (g), saline (h) or nothing (sham operation group) (i) (magnification: ×200). The bar length is 200 μm. The interstitial fibrosis of mice kidney is indicated by blue staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



□ ; pSUPER-TGF-βRII complexed with the cationized gelatin prepared from ethylenediamine
▤ ; pSUPER-TGF-bRII complexedwith the cationized gelatin prepared from putrescine
▧ ; pSUPER-TGF-bRII complexedwith the cationized gelatin prepared from spermidine
▥ ; pSUPER-TGF-bRII complexedwith the cationized gelatin prepared from spermine
■ ; free pSUPER-TGF-βRII
▨ ; empty pSUPER
⬚ ; mismatch pSUPER-TGF-βRII
□ ; saline

Fig. 4. Morphometric analysis of relative interstitial fibrotic area in the kidney of UUO mice after pSUPER-TGF-βRII application. Mice received the injection of pSUPER-TGF-βRII complexed with the cationized gelatin prepared from ethylenediamine, putrescine, spermidine or spermine, free pSUPER-TGF-βRII, empty pSUPER, mismatch pSUPER-TGF-βRII, and saline $^{*}p<0.05$: significant against the fibrotic area of groups injected with saline. $^{\dagger}p<0.05$: 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.

with the plasmid DNA was performed by simply mixing the two materials at various N/P mixing ratios (the molar number ratio of amino groups of gelatin to the phosphate groups of DNA) in aqueous solution. The complexes prepared had a nano-size structure of which surface is covered with cationized gelatin molecules. When the transfection efficiency of plasmid DNA complexed with the various cationized gelatins at different N/P mixing ratios was evaluated in vitro, the highest transfection efficiency was observed for the complex prepared from the cationized gelatin of spermine at a N/P mixing ratio of 2. This finding indicated that there are an optimal N/P mixing ratio and a type of amine compounds or cationization extent of cationized gelatin to enhance the transfection efficiency of plasmid DNA. However, in this study, there was no difference in the anti-fibrotic activity between the types of cationized gelatin used. This difference can be explained in terms of the reaction mechanism of siRNA. It has been demonstrated that generally siRNA acts in the cytoplasm in a repeated fashion even though the amount is so small [19,20]. It is possible that the siRNA, once expressed from the pSUPER vector, continuously degrades the target mRNA in the cytoplasm. Thus, although the transfection efficiency was different between the cationized gelatins prepared from different amine compounds, a small amount of pSUPER-TGF-βRII transfected would be strong enough to prevent the renal fibrosis.

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 [44]. Phosphorylation activates several signaling pathways, including the Smad pathway, and regulates multiple TGF-β functions [45]. In this respect, TGF-βRII is important 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 [46]. Zhu et al. have reported successful transfection of β-galactosidase gene into the interstitial compartment by an adenoviral vector [47]. In addition, Tsujie et al. report that TGF-β1 antisense oligonucleotides transfected could suppress TGF-β1 expression, resulting inhibition renal fibrosis [48]. 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 [49]. *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 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. However, we have reported that gelatin derivatives cationized by spermine showed considerable cytotoxicity at a large N/P ratio in vitro [43]. In this study, the preparation condition of the pSUPER-cationized gelatin complex was carefully chosen to minimize the cytotoxicity. Although the cytotoxicity was not clearly observed in the level of histological section (Fig. 3), it will be necessary to evaluate the toxicity of cationized gelatins or the complex injected for tissues.

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 and evaluate the therapeutic effect.

We have reported that the plasmid DNA complexed with the biodegradable cationized gelatin prepared from different amine compounds enhanced gene transfection because the apparent molecular size of plasmid DNA decreased to 200 nm by complexation with the cationized gelatin [43]. 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. Based on this cell compatibility nature 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. An 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 results 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 and accelerated by inflammation of UUO. The pressure stimulation seems important to increase the transfection efficiency to cells. As a result, it is conceivable that the complex easily diffuses into the interstitial area and consequently distributes into the cortical interstitial space. Moreover, the plasmid DNA-cationized gelatin complex of positive charge readily interacted with the cell surface of negative charge [50]. It is demonstrated that the complexity with this size range can be favorably taken up by cells [51,52]. This is an additional advantage of complex prepared from the cationized gelatin for enhanced gene expression in terms of efficient DNA packing to form a nano-size particle [51,52]. It is possible that retrograde injection of the transgene solution via the ureter with clamping the left renal vein also enhanced the renal 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 overexpression was completely suppressed over the range of experimental time period after transfection of pSUPER-TGF-βRII complexed with the cationized gelatin (Fig. 1). It is highly conceivable that this suppression resulted in suppressed increase of the collagen content in the kidney (Fig. 2).

In conclusion, the present data demonstrate that blocking for TGF-βRII by the complex of pSUPER-TGF-βRII and cationized gelatin prepared by the chemical introduction of different amine compounds was effective in reducing the deposition of ECM in mice interstitial renal fibrosis, irrespective of the type of amine compounds used. The present concept of transfection of siRNA expression vector complexed with the cationized gelatin will be a new therapeutic strategy to block the TGF-β signaling pathway. This new technique of gene transfer to interstitial cells will be a potential therapeutic strategy in the interstitial renal disease.

References

[1] P.J. Stahl, D. Felsen, Transforming growth factor-β, basement membrane, and epithelial-mesenchymal transdifferentiation: implications for fibrosis in kidney disease, Am. J. Pathol. 159 (2001) 1187–1192.
[2] G.A. Muller, M. Zeisberg, F. Strutz, The importance of tubulointerstitial damage in progressive renal disease, Nephrol. Dial. Transplant. 15 (2000) 76–77.
[3] G. Remuzzi, T. Bertani, Pathophysiology of progressive nephropathies, N. Engl. J. Med. 339 (1998) 1448–1456.
[4] M. Eikmans, J.J. Baelde, E. de Heer, J.A. Bruijn, ECM homeostasis in renal diseases: a genomic approach, J. Pathol. 200 (2003) 526–536.
[5] M. Zeisberg, F. Strutz, G.A. Muller, Renal fibrosis: an update, Curr. Opin. Nephrol. Hypertens. 10 (2001) 315–320.
[6] L.G. Fine, A.C. Ong, J.T. Norman, Mechanisms of tubulo-interstitial injury in progressive renal diseases, Eur. J. Clin. Invest. 23 (1993) 259–265.
[7] G.S. Kuncio, E.G. Neilson, T. Haverty, Mechanisms of tubulointerstitial fibrosis, Kidney Int. 39 (1991) 550–556.
[8] T. Yamamoto, N.A. Noble, A.H. Cohen, C.C. Nast, A. Hishida, L.I. Gold, W.A. Border, Expression of transforming growth factor-β isoforms in human glomerular disease, Kidney Int. 49 (1996) 461–469.
[9] M.V. Rocco, Y. Chen, S. Goldfarb, F.N. Ziyadeh, Elevated glucose stimulates TGF-β gene expression and bioactivity in proximal tubule, Kidney Int. 41 (1992) 107–114.
[10] G. Wolf, K. Sharma, Y. Chen, M. Ericksen, F.N. Ziyadeh, High glucose-induced proliferation in mesangial cells is reversed by autocrine TGF-β, Kidney Int. 42 (1992) 647–656.
[11] S. Okuda, L.R. Languino, E. Ruoslahti, W.A. Border, Elevated expression of transforming growth factor-β and proteoglycan production in experimental glomerulonephritis. Possible role in expansion of the mesangial extracellular matrix, J. Clin. Invest. 86 (1990) 453–462.

[12] W.A. Border, S. Okuda, L.R. Languino, M.B. Sporn, E. Ruoslahti, Suppression of experimental glomerulonephritis by antiserum against transforming growth factor β1, Nature 346 (1990) 371–374.
[13] G.C. Blobe, W.P. Schiemann, H.F. Lodish, Role of transforming growth factor β in human disease, N. Engl. J. Med. 342 (2000) 1350–1358.
[14] R.A. Ignotz, J. Massague, Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix, J. Biol. Chem. 261 (1986) 4337–4345.
[15] Y. Akagi, Y. Isaka, M. Arai, T. Kaneko, M. Takenaka, T. Moriyama, Y. Kaneda, A. Ando, Y. Orita, T. Kamada, N. Ueda, E. Imai, Inhibition of TGF-β1 expression by antisense oligonucleotides suppressed extracellular matrix accumulation in experimental glomerulonephritis, Kidney Int. 50 (1996) 148–155.
[16] W.A. Border, N.A. Noble, T. Yamamoto, J.R. Harper, Y. Yamaguchi, M.D. Pierschbacher, E. Ruoslahti, Natural inhibitor of transforming growth factor- β protects against scarring in experimental kidney disease, Nature 360 (1992) 361–364.
[17] Y. Isaka, D.K. Brees, K. Ikegaya, Y. Kaneda, E. Imai, N.A. Noble, W.A. Border, Gene therapy by skeletal muscle expression of decorin prevents fibrotic disease in rat kidney, Nat. Med. 2 (1996) 418–423.
[18] Y. Isaka, Y. Akagi, Y. Ando, M. Tsujie, T. Sudo, N. Ohno, W.A. Border, N.A. Noble, Y. Kaneda, M. Hori, E. Imai, Gene therapy by transforming growth factor-β receptor-IgG Fc chimera suppressed extracellular matrix accumulation in experimental glomerulonephritis, Kidney Int. 55 (1999) 465–475.
[19] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (2001) 494–498.
[20] T.R. Brummelkamp, R. Bernards, R. Agami, A system for stable expression of short interfering RNAs in mammalian cells, Science 296 (2002) 550–553.
[21] S.A. Audouy, L.F. de Leij, D. Hoekstra, G. Molema, In vivo characteristics of cationic liposomes as delivery vectors for gene therapy, Pharm. Res. 19 (2002) 1599–1605.
[22] M. Voinea, M. Simionescu, Designing of 'intelligent' liposomes for efficient delivery of drugs, J. Cell. Mol. Med. 6 (2002) 465–474.
[23] M.C. Pedroso de Lima, S. Neves, A. Filipe, N. Duzgunes, S. Simoes, Cationic liposomes for gene delivery: from biophysics to biological applications, Curr. Med. Chem. 10 (2003) 1221–1231.
[24] J.W. Nah, L. Yu, S.O. Han, C.H. Ahn, S.W. Kim, Artery wall binding peptide-poly(ethylene glycol)-grafted-poly(L-lysine)-based gene delivery to artery wall cells, J. Control. Release 78 (2002) 273–284.
[25] H. Lee, J.H. Jeong, T.G. Park, PEG grafted polylysine with fusogenic peptide for gene delivery: high transfection efficiency with low cytotoxicity, J. Control. Release 79 (2002) 283–291.
[26] M. Molas, R. Bartrons, J.C. Perales, Single-stranded DNA condensed with poly-L-lysine results in nanometric particles that are significantly smaller, more stable in physiological ionic strength fluids and afford higher efficiency of gene delivery than their double-stranded counterparts, Biochim. Biophys. Acta 1572 (2002) 37–44.
[27] E. Jeon, H.D. Kim, J.S. Kim, Pluronic-grafted poly-(L)-lysine as a new synthetic gene carrier, J. Biomed. Mater. Res. 66 (2003) 854–859.
[28] A. Remy-Kristensen, J.P. Clamme, C. Vuilleumier, J.G. Kuhry, Y. Mely, Role of endocytosis in the transfection of L929 fibroblasts by polyethylenimine/DNA complexes, Biochim. Biophys. Acta 1514 (2001) 21–32.
[29] L. Wightman, R. Kircheis, V. Rossler, S. Carotta, R. Ruzicka, M. Kursa, E. Wagner, Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo, J. Gene Med. 3 (2001) 362–372.
[30] J.M. Benns, A. Maheshwari, D.Y. Furgeson, R.I. Mahato, S.W. Kim, Folate-PEG-folate-graft-polyethylenimine-based gene delivery, J. Drug Target. 9 (2001) 123–139.
[31] K. Sagara, S.W. Kim, A new synthesis of galactose-poly(ethylene glycol)-polyethylenimine for gene delivery to hepatocytes, J. Control. Release 79 (2002) 271–281.
[32] H. Lee, T.H. Kim, T.G. Park, A receptor-mediated gene delivery system using streptavidin and biotin-derivatized, pegylated epidermal growth factor, J. Control. Release 83 (2002) 109–119.
[33] M. Kursa, G.F. Walker, V. Roessler, M. Ogris, W. Roedl, R. Kircheis, E. Wagner, Novel shielded transferrin-polyethylene glycol-polyethylenimine/DNA complexes for systemic tumor-targeted gene transfer, Bioconjug. Chem. 14 (2003) 222–231.
[34] H. Gonzalez, S.J. Hwang, M.E. Davis, New class of polymers for the delivery of macromolecular therapeutics, Bioconjug. Chem. 10 (1999) 1068–1074.
[35] U. Rungsardthong, T. Ehtezazi, L. Bailey, S.P. Armes, M.C. Garnett, S. Stolnik, Effect of polymer ionization on the interaction with DNA in nonviral gene delivery systems, Biomacromolecules 4 (2003) 683–690.
[36] T. Kushibiki, R. Tomoshige, Y. Fukunaka, M. Kakemi, Y. Tabata, In vivo release and gene expression of plasmid DNA by hydrogels of gelatin with different cationization extents, J. Control. Release 90 (2003) 207–216.
[37] Y. Fukunaka, K. Iwanaga, K. Morimoto, M. Kakemi, Y. Tabata, Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation, J. Control. Release 80 (2001) 333–343.
[38] T. Kushibiki, Y. Tabata, A new gene delivery system based on controlled release technology, Curr. Drug Deliv. 1 (2004) 153–163.
[39] T. Kushibiki, K. Matsumoto, T. Nakamura, Y. Tabata, Suppression of tumor metastasis by NK4 plasmid DNA released from cationized gelatin, Gene Ther. 11 (2004) 1205–1214.
[40] S.L. Snyder, P.Z. Sobocinski, An improved 2,4,6-trinitrobenzensulfonic acid method for the determination of amines, Anal. Biochem. 64 (1975) 284–288.
[41] S. Klahr, J. Morrissey, Obstructive nephropathy and renal fibrosis, Am. J. Physiol. Renal Physiol. 283 (2002) F861–F875.
[42] J.R. Diamond, S.D. Ricardo, S. Klahr, Mechanisms of interstitial fibrosis in obstructive nephropathy, Semin. Nephrol. 18 (1998) 594–602.
[43] H. Hosseinkhani, T. Aoyama, O. Ogawa, Y. Tabata, Ultrasound enhancement of in vitro transfection of plasmid DNA by a cationized gelatin, J. Drug Target. 10 (2002) 193–204.
[44] J.L. Wrana, L. Attisano, R. Wieser, F. Ventura, J. Massague, Mechanism of the TGF-β receptor, Nature 370 (1994) 341–347.
[45] A. Nakao, T. Imamura, S. Souchelnytskyi, M. Kawabata, A. Ishisaki, E. Oeda, K. Tamaki, J. Hanai, C.H. Heldin, K. Miyazono, P. ten Dijke, TGF-β receptor-mediated signalling through Smad2, Smad3 and Smad4, EMBO J. 16 (1997) 5353–5362.
[46] A.K. Sharma, S.M. Mauer, Y. Kim, A.F. Michael, Interstitial fibrosis in obstructive nephropathy, Kidney Int. 44 (1993) 774–788.
[47] G. Zhu, A.G. Nicolson, B.D. Cowley, S. Rosen, V.P. Sukhatme, In vivo adenovirus-mediated gene transfer into normal and cystic rat kidneys, Gene Ther. 3 (1996) 298–304.
[48] M. Tsujie, Y. Isaka, Y. Ando, Y. Akagi, Y. Kaneda, N. Ueda, E. Imai, M. Hori, Gene transfer targeting interstitial fibroblasts by the artificial viral envelope type hemagglutinating virus of Japan liposome method, Kidney Int. 58 (2000) 1973–1980.
[49] E. Song, S.K. Lee, J. Wang, N. Ince, N. Ouyang, J. Min, J. Chen, P. Shankar, J. Lieberman, RNA interference targeting Fas protects mice from fulminant hepatitis, Nat. Med. 9 (2003) 347–351.
[50] H. Maeda, J. Fang, T. Inutsuka, Y. Kitamoto, Vascular permeability enhancement in solid tumor: various factors, mechanisms involved and its implications, Int. Immunopharmacol. 3 (2003) 319–328.
[51] W. Zauner, A. Kichler, W. Schmidt, A. Sinski, E. Wagner, Glycerol enhancement of ligand-polylysine/DNA transfection, Biotecniques 20 (1996) 905–913.
[52] P. Midoux, M. Monsigny, Efficient gene transfer by histidylated polylysine/pDNA complexes, Bioconjug. Chem. 10 (1999) 406–411.

J. Biomater. Sci. Polymer Edn, Vol. 16, No. 5, pp. 549–561 (2005)

Also available online - www.vsppub.com

In vivo degradability of hydrogels prepared from different gelatins by various cross-linking methods

MAKOTO OZEKI and YASUHIKO TABATA *

Institute for Frontier Medical Sciences, Kyoto University, 53 Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

Received 25 March 2004; accepted 3 August 2004

Abstract—This study is an investigation to evaluate the *in vivo* degradation of gelatin hydrogels in terms of their number of cross-links. Various hydrogels were prepared from acidic gelatin, extracted from bovine bone, porcine skin or fish scale, and basic gelatin, extracted from porcine skin, through four types of cross-linking methods, i.e., glutaraldehyde (GA) or dehydrothermal treatment and ultraviolet (UV) or electron beam irradiation. The water content of hydrogels and their number of cross-links, calculated from the tensile modulus of hydrogels, were evaluated as the measure of hydrogel cross-linking extent. Following subcutaneous implantation of ^{125}I-labeled gelatin hydrogels into mice, the radioactivity remaining was measured at different time intervals to assess the *in vivo* degradability of hydrogels. Irrespective of the gelatin type and cross-linking method, a good correlation was found between the *in vivo* degradability of hydrogels and their number of cross-links, which is different from the correlation to their water content. This finding indicates that the degradability of hydrogels is governed by their number of cross-links.

Key words: Hydrogel; gelatin; degradability *in vivo*; cross-linking; irradiation.

INTRODUCTION

Growth factors have been widely recognized to act on different types of cells in the body to regulate their proliferation and differentiation by which tissue regeneration is promoted [1]. However, if a growth factor is administered to the body in the solution form, the *in vivo* efficacy of tissue regeneration cannot be always expected because the factor has only a short half-life period. Therefore, it is necessary to develop a drug-delivery system which facilitates the controlled release of growth factors over an extended time period [2, 3].

*To whom correspondence should be addressed. Tel.: (81-75) 751-4121. Fax: (81-75) 751-4646. E-mail: yasuhiko@frontier.kyoto-u.ac.jp

We have explored gelatin hydrogels as delivery system to succeed in the controlled release of biologically active growth factors [3]. Gelatin has been extensively utilized for pharmaceutical and medical purposes. Its biosafety has been proven in various clinical applications over the years [4–6]. For example, it has been used as hard and soft drug capsule [7, 8], sealant for vascular prostheses [9–11] and hemostatic [12–14]. Moreover, gelatin has been studied as a of drug-delivery carrier in the water-soluble [15, 16] or hydrogel form [17–19]. Another advantage of gelatin is the commercial availability of materials with different electric charges [3, 20, 21]. Gelatin is a biodegradable protein prepared by the acid and alkaline processes of collagen. This process allows collagen to denature and change the electrical nature, which provides various types of gelatin with different isoelectric points (IEPs).

We have already demonstrated that the hydrogel prepared from 'acidic' gelatin with IEP 5.0 can release biologically active basic fibroblast growth factor (bFGF) [22, 23], transforming growth factor (TGF)-β1 [24, 25] and hepatocyte growth factor (HGF) *in vivo* [26]. On the other hand, bone morphogenetic protein (BMP)-2 could be released *in vivo* from the hydrogel of 'basic' gelatin with IEP 9.0 [27]. The time period of growth factors remaining in the hydrogels was in good accordance with that of hydrogel remaining, indicating the release mechanism that the growth factor is released accompanied with hydrogel degradation [2, 3, 23]. The *in vivo* degradability of gelatin hydrogels depended on their water content which could be modified by changing the preparation conditions. The hydrogel with higher water contents was degraded for a shorter time period, while the time period of hydrogel degradation became longer with a decrease in the hydrogel water content [22–24, 26, 27].

In this study, hydrogels were prepared from different kinds of gelatin through various cross-linking methods. As a measure of the cross-linking extent of hydrogels, tensile tests were done to calculate the number of hydrogel cross-links, while their water content was also determined. Following implantation of ^{125}I-labeled gelatin hydrogels into the back subcutis of mice, the time profile of radioactivity remaining was evaluated. We examined the relationship between the *in vivo* degradability of hydrogels and their two types of cross-linking measure, such as the water content and the number of cross-links of hydrogels.

MATERIALS AND METHODS

Materials

Gelatin samples with an IEP of 5.0 (MW 99×10^3), prepared by an alkaline process, of the bovine bone, porcine skin and fish scale collagens, or that with an IEP of 9.0 (Mw 99×10^3), prepared through an acidic process, of the porcine skin collagen, were kindly supplied by Nitta Gelatin (Osaka, Japan) and named 'acidic' and 'basic' gelatins, respectively. 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 Perkin-Elmer Life Sciences (Boston, MA, USA). Other chemicals were purchased from Wako Pure Chemical (Kyoto, Japan) and used without further purification.

Preparation of gelatin hydrogels cross-linked by glutaraldehyde treatment

An aqueous solution of 10 wt% gelatin (800 μl) was cast into a polypropylene mold (2 × 2 cm^2), followed by storage at 4°C for 24 h to allow gelatin to set. The gelled gelatin was placed at 4°C in aqueous solution containing 12.4 μg/ml of glutaraldehyde (GA) and cross-linking reaction was allowed to proceed at 4°C for different time periods. The resulting hydrogel sheet was immersed in 100 mM of glycine aqueous solution for 1 h to block residual aldehyde groups of GA. The hydrogel sheets were rinsed 3 times with double distilled water (DDW) at 4°C and stored in DDW at 4°C until use or assay.

Preparation of gelatin hydrogels cross-linked by dehydrothermal treatment

Non-cross-linked gelatin hydrogels, prepared by the same procedure described before, were freeze-dried. The resulting freeze-dried gelatin hydrogel sheets were cross-linked by dehydrothermal treatment at 140°C for 24, 48 and 72 h in vacuum. The hydrogel sheets were swollen in DDW at 37°C for 24 h until equilibrium was reached. The gelatin hydrogel sheets were stored in DDW at 4°C until use or assay.

Preparation of gelatin hydrogels crosslinked by ultraviolet irradiation

Freeze-dried non-cross-linked gelatin hydrogels were prepared as described above. The resulting freeze-dried gelatin hydrogel sheets were cross-linked by dehydrothermal treatment at 140°C for 48 h in vacuum. Further cross-linking was introduced by ultraviolet (UV) irradiation to both sides of gelatin hydrogel sheets in a Funa-UV-Linker FS-800 (5 × 8 W, Funakoshi, Tokyo, Japan) for 10, 20 and 30 min. The hydrogels sheets were swollen in DDW at 37°C for 24 h and stored in DDW at 4°C until use or assay.

Preparation of gelatin hydrogels cross-linked by electron beam irradiation

An aqueous solution of 10 wt% gelatin was cast into a polypropylene mold to give a solution depth of 200 μm. The solution was irradiated by different doses (20, 60 and 100 kGy) of electron beam in an Curetron® Electron Beam Processing System (Nissin High Voltage, Tokyo, Japan). The casting and irradiation operation was repeated five times to obtain 1-mm-thick gelatin hydrogels. The resulting gelatin hydrogel sheets were swollen in DDW at 37°C for 24 h to reach the equilibrium and stored in DDW at 4°C until use or assay.

Evaluation of cross-linking extent of gelatin hydrogels

Two measures to evaluate the extent of hydrogel cross-linking were performed. Gelatin hydrogels, cut with scissors to obtain square sheets ($5 \times 5 \times 1$ mm^3) were swollen in DDW and dried at 60°C under vacuum. The weight of hydrogel sheets before and after drying was measured and the weight ratio of water present in the hydrogel to the DDW-swollen hydrogel was calculated as the water content. The cross-linking density was estimated on the basis of the theory of rubber elasticity [28]. The specimens of gelatin hydrogel, with a length of 20 mm, a central width of 10 mm and a thickness of 1 mm, were subjected to a tensile testing on an AGS-5D Autograph (Shimadzu, Kyoto, Japan) with a low capacity load cell (10 N). The initial slope of the stress–strain curve was calculated from the curve of tensile test to obtain the number of cross-links per gelatin molecule by the following equation on the assumption of an affine deformation

$$N = \frac{2\sigma M}{\rho_g v^{1/3} kT(\alpha - \alpha^{-2})},$$

where N is the number of cross-links per gelatin molecule, ρ_g is the density of gelatin, v is the volume fraction of gelatin in hydrogel, M is the molecular weight of gelatin (99×10^3), k is the Boltzmann constant, T is the absolute temperature, σ is the stress (the applied force per unit area of swollen, unstrained hydrogel) and α is the strain determined by the ratio of sample length at tension to initial length of sample [28, 29]. Four hydrogel sheets were used for each hydrogel type. Figure 1 shows the plots of σ *vs.* $(\alpha - \alpha^{-2})$ on gelatin hydrogel cross-linked by dehydrothermal treatment for 24 h. The number of cross-links per gelatin molecule was calculated from the slope of the σ *vs.* $(\alpha - \alpha^{-2})$ plots.

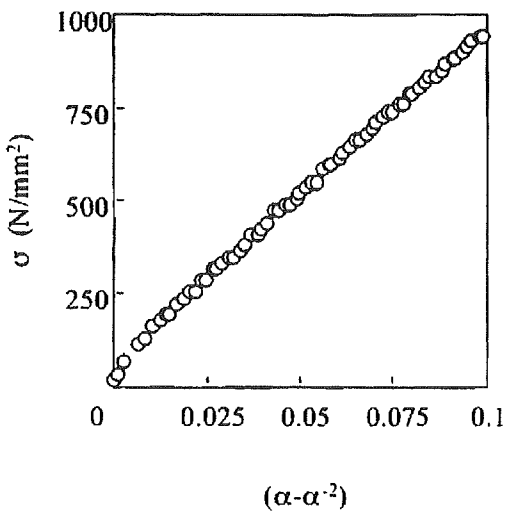


Figure 1. σ *vs.* $(\alpha - \alpha^{-2})$ plots of the gelatin hydrogel cross-linked by dehydrothermal treatment for 24 h.

Radiolabeling of gelatin hydrogels

Gelatin hydrogels were cut with scissors to obtain square sheets ($5 \times 5 \times 1$ mm^3) for the assessment of biodegradability. Gelatin hydrogel sheets were radioiodinated by use of [^{125}I]Bolton-Hunter reagent [30]. Briefly, 20 μl of [^{125}I]Bolton-Hunter reagent solution in anhydrous benzene was bubbled with dry nitrogen gas until benzene was completely evaporated. Then, 1 ml of phosphate-buffered saline solution (PBS, pH 7.5) was added to the dried reagent to prepare an aqueous solution of [^{125}I]Bolton-Hunter reagent. The reagent solution prepared was impregnated into the gelatin hydrogel sheet at a volume of 20 μl per sheet. The resulting hydrogel sheets were kept at 4°C for 3 h to introduce ^{125}I into the amino groups of gelatin. The gelatin hydrogel sheets radioiodinated were rinsed in DDW, which was exchanged periodically at 4°C for 4 days, to exclude non-coupled, free ^{125}I-labeled reagent from ^{125}I-labeled gelatin hydrogel sheets. When measured periodically, the radioactivity of DDW returned to a background level after 3 days rinsing. No shape change of hydrogel sheets was observed during radiolabeling and the subsequent rinsing process, irrespective of hydrogel preparation conditions.

Estimation of in vivo *degradation of gelatin hydrogels*

In vivo degradation of gelatin hydrogels was evaluated in terms of the radioactivity loss of ^{125}I-labeled gelatin hydrogels implanted. Various types of ^{125}I-labeled gelatin hydrogels were implanted in the back subcutis of ddY mice (3 mice per group, 6–7 weeks old, Shimizu Laboratory Supply, Kyoto, Japan) under pentobarbital anesthesia. At 1, 3, 7, 10, 14 and 21 days after hydrogel implantation, the radioactivity of hydrogels implanted was measured on a gamma counter (ARC-301B, Aloka, Tokyo, Japan). Next, the mouse back skin around the hydrogel implanted site was cut into a strip of 3×5 cm^2 and the corresponding facial site was thoroughly wiped off with a filter paper to absorb ^{125}I-labeled gelatin. The radioactivity of the skin strip and the filter paper was measured to evaluate the remaining radioactivity of tissue around the implanted hydrogel. The ratio of total radioactivity measured to the radioactivity of hydrogel implanted initially was expressed as the percentage of remaining activity for hydrogel degradation. The number of mice in each experimental group was 18, while 3 mice were killed at each time point for *in vivo* evaluation. The half-life time periods of gelatin hydrogels were evaluated from the time-course curve of radioactivity remaining of ^{125}I-labeled gelatin hydrogel. All the animal experiments were done according to the Institutional Guidance of Kyoto University on Animal Experimentation.

Statistical analysis

Experimental results were expressed as the mean ± S.D. (standard deviation of the mean). Graphs were drawn based on the least-square approximation method and the correlation coefficient was described as R^2 value.

RESULTS

Cross-linking density and water content of gelatin hydrogels prepared by different gelatin types and cross-linking methods

Figure 2A and 2B shows the number of cross-links per molecule gelatin of hydrogels cross-linked by GA and dehydrothermal treatments for different time periods. Figure 2C and 2D shows the number of cross-links per molecule gelatin of hydrogels prepared by UV irradiation for different time periods after dehydrothermal treatment for 24 h and different doses of electron beam irradiation. Irrespective of the gelatin type, the number of cross-links per gelatin molecule increased with increasing time of GA and dehydrothermal treatments. The number of cross-links of basis gelatin hydrogels increased with UV irradiation time up to 10 min, but leveled

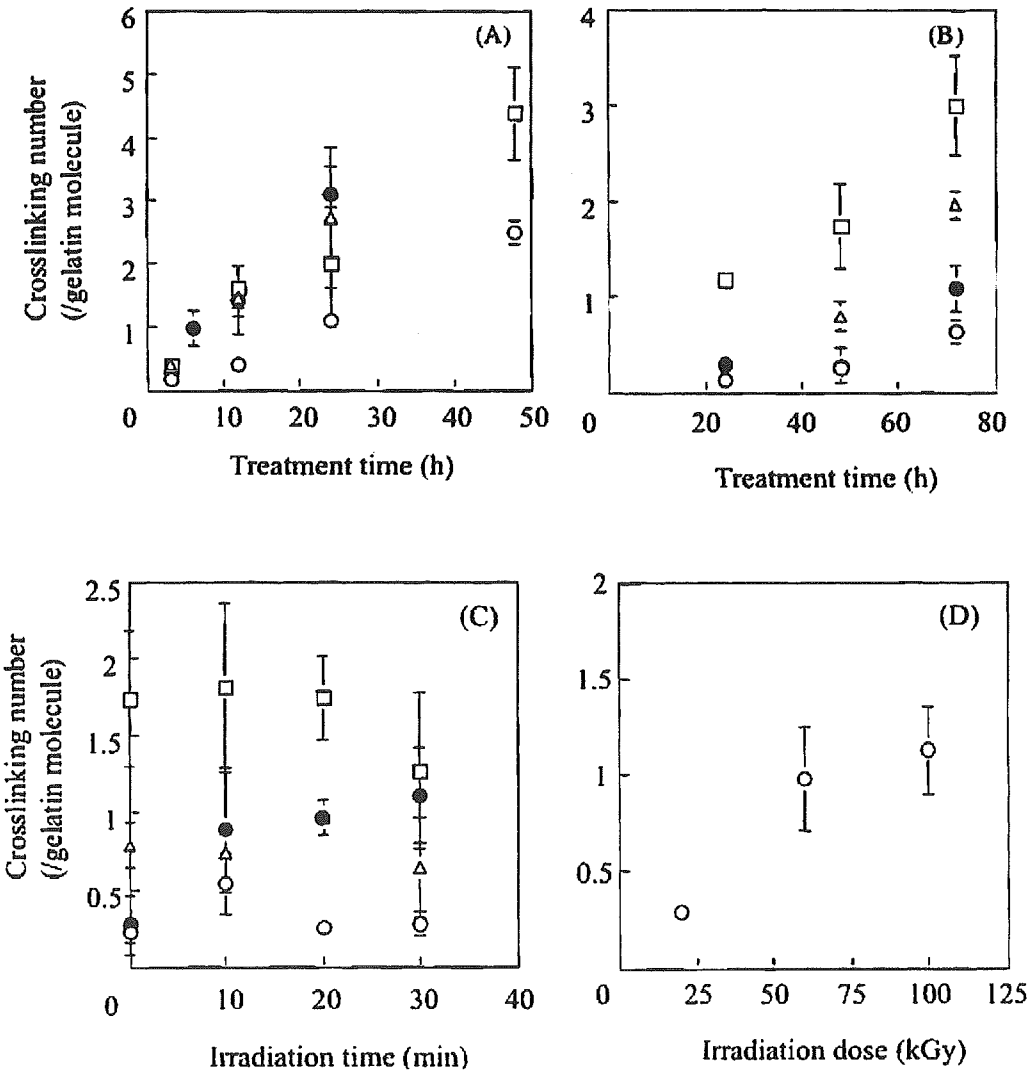


Figure 2. The number of cross-links per gelatin molecule of hydrogels as a function of GA treatment time (A), dehydrothermal treatment time (B), UV irradiation time (C) and electron beam irradiation dose (D). The hydrogels were prepared from acidic gelatin extracted from bovine bone (○), porcine skin (△) or fish scale (□) and basic gelatin extracted from porcine skin (●).

off thereafter. On the other hand, no big influence of irradiation time on the number of cross-links for acidic gelatin hydrogels was observed. The number of cross-links of gelatin hydrogels tended to increase as the irradiation dose increased, although the increment was as large as other cross-linking methods.

Figure 3A and 3B shows the water content of gelatin hydrogels prepared by GA treatment and dehydrothermal treatment. Figure 3C and 3D shows the water content of gelatin hydrogels prepared by UV irradiation after dehydrothermal treatment for 24 h and different dose of electron beam irradiation. The water content decreased with an increase in the time of GA and dehydrothermal treatments. However, the difference of water content between the types of gelatin was bigger than that in the number of cross-links. The water content of gelatin hydrogels rapidly decreased when UV irradiation was done for 10 min, but longer UV irradiation did not

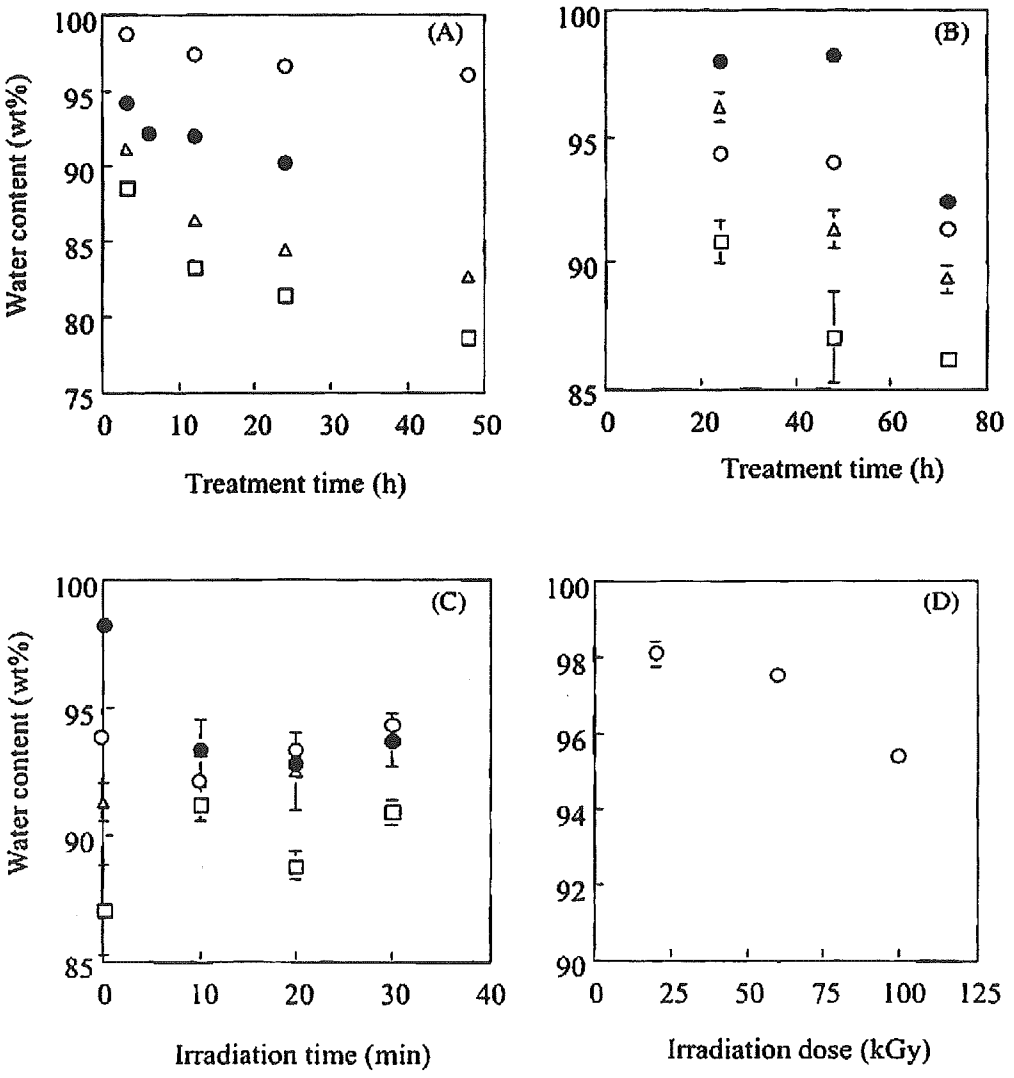


Figure 3. The water content of gelatin hydrogels as a function of GA treatment time (A), dehydrothermal treatment time (B), UV irradiation time (C) and electron beam irradiation dose (D). The hydrogels were prepared from acidic gelatin extracted from bovine bone (○), porcine skin (△) or fish scale (□) and basic gelatin extracted from porcine skin (●).

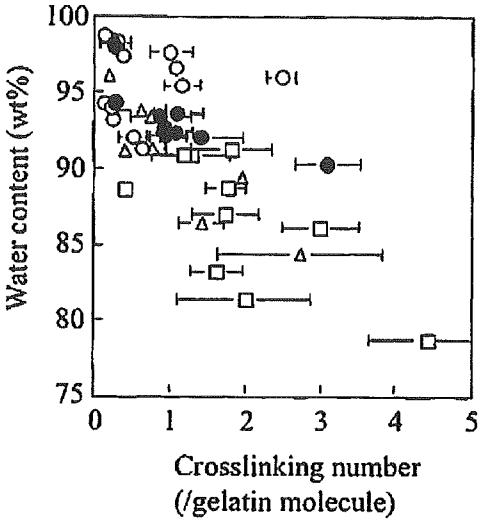


Figure 4. Relationship between the number of cross-links of gelatin molecule of hydrogels and the water content of gelatin hydrogels. The hydrogels were prepared from acidic gelatin extracted from bovine bone (○), porcine skin (△) or fish scale (□) and basic gelatin extracted from porcine skin (●).

contribute to any change in the water content of hydrogels. The water content slightly decreased with the increased dose of electron beam.

Figure 4 shows the relationship between the number of cross-links per molecule gelatin of various hydrogels and their water content. The water content of gelatin hydrogels decreased with an increase in the number of cross-links. This good correlation was observed only for gelatin of the same type. However, the correlation pattern in the water content–cross-linking point relationship was not similar between the different types of gelatin hydrogels.

In vivo *degradability of gelatin hydrogels with different water content and the number of cross-links*

Figure 5 shows the relationship between the water content of hydrogels prepared from each type of gelatin by various cross-linking methods and the hydrogel biodegradability. When compared for the same type of gelatin hydrogels and cross-linking methods, the *in vivo* half-life time of gelatin hydrogel became longer as the water content decreased. However, the correlation of half-life time and water content was different from hydrogel to hydrogel. Figure 6 shows the relationship between the biodegradability of gelatin hydrogels and their number of cross-links. Only hydrogels of fish-scale gelatin, prepared through GA cross-linking, showed slower biodegradability than the hydrogels from other types of gelatin. However, irrespective of the cross-linking methodsand the gelatin type, there was better correlation between the *in vivo* degradability of gelatin hydrogels and their number of cross-links ($R^2 = 0.837$) than that between their degradability and water content ($R^2 = 0.729$).

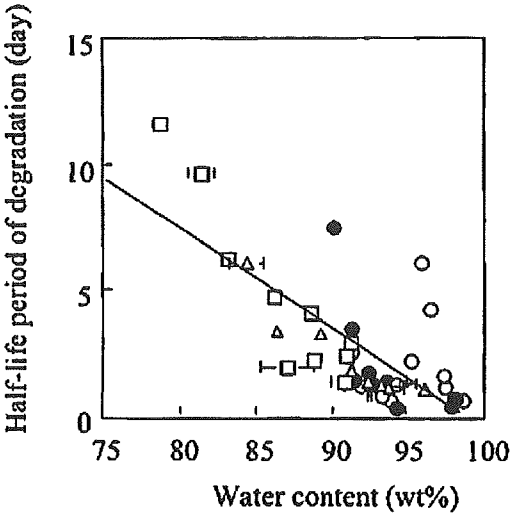


Figure 5. Relationship between the *in vivo* degradation of gelatin hydrogels and water content of the hydrogels. The hydrogels were prepared from acidic gelatin extracted from bovine bone (○), porcine skin (△) or fish scale (□) and basic gelatin extracted from porcine skin (●). The line was drawn based on the least-squares approximation method ($R^2 = 0.729$).

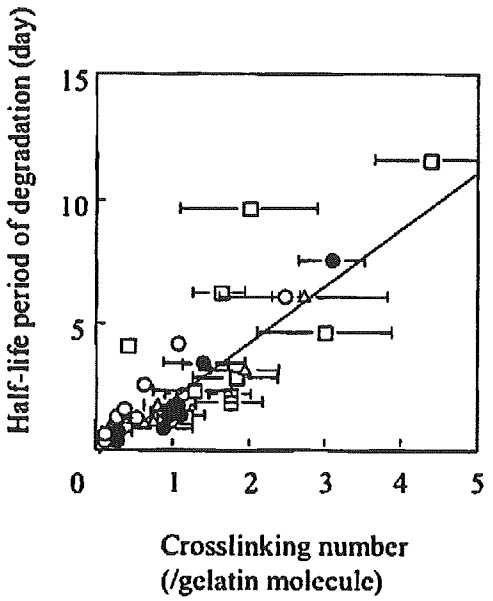


Figure 6. Relationship between the *in vivo* degradation of gelatin hydrogels and the number of hydrogels cross-links. The hydrogels were prepared from acidic gelatin extracted from bovine bone (○), porcine skin (△) or fish scale (□) and basic gelatin extracted from porcine skin (●). The line was drawn based on the least-squares approximation method ($R^2 = 0.837$).

DISCUSSION

In this study, gelatin hydrogels were prepared through different cross-linking methods from various types of gelatin, extracted from several kinds of animal species. The cross-linking density of gelatin hydrogels could be controlled by the time of cross-linking and irradiation. Similar to the water content of hydrogels, the number of cross-links in hydrogels was changed by the conditions of hydrogel preparation.

Gelatin hydrogels were prepared by GA or dehydrothermal treatment and UV or electron beam irradiation. Among the chemical cross-linking agents, GA is most widely used due to its high efficiency in the stabilization of collageneous materials [31]. Cross-linking of collagenous samples with GA involves the reaction of free amino groups of lysine or hydroxylysine residues of polypeptide chains with the aldehyde groups of GA [32]. It is likely that GA can cross-link between the two amino residues of collagen chains. It has been reported that dehydrothermal treatment generates chemical bonding between the amino and carboxyl groups of collagen molecules due to thermal dehydration [33, 34]. Dehydrothermal cross-linking can occur only if the amino and carboxyl groups are close to each other. Thus, it is possible that dehydrothermal treatment allows gelatin molecules to cross-link to a lesser extent than GA treatment. When compared for the gelatin hydrogels cross-linked by GA treatment, the number of cross-links in hydrogels prepared from porcine skin gelatin increased more than that in hydrogels prepared from other types of gelatin. However, when compared for the gelatin hydrogels cross-linked by dehydrothermal treatment, the number of cross-links in hydrogels prepared from fish-scale gelatin was the highest of all. The difference may be due to a difference of the transition temperature. In the case of fish-scale gelatin, the gelatin molecules may be closer to each other because the transition temperature from random coil to helix conformation is higher. The hydrogels prepared from fish-scale gelatin were cross-linked more than other type of gelatin.

It is reported that UV irradiation generates radicals at the aromatic residues of gelatin amino acids, such as tyrosin and phenylalanine. The binding of these radicals will react to each other, resulting in cross-linking formation [34, 35]. However, from Figs 2C and 3C it can be seen that the cross-linking density of gelatin hydrogels largely changed depending on UV irradiation time. When the irradiation time was short, UV irradiation would enable gelatin to cross-link intermolecularly. However, it is possible that irradiation for longer time preferably acts on the chain scission of gelatin molecules. A balance of the cross-linking and chain scission would result in unchanged density of hydrogel cross-linking. This same phenomenon has been reported in the case of UV irradiation to collagen membranes [36].

Electron beam irradiation also produces radicals [37]. The number of cross-links did not change, whereas the water content of gelatin hydrogels deceased, as the dose of electron beam irradiation increased. The number of cross-links was not large and the water content did not decrease very much. This is because the chain scission by the overdose of electron beam also occurs [38].

There are two methods to assess the cross-linking density of hydrogels. One is from the water content of hydrogels. There is one theoretical equation between the water content of hydrogels and the hydrogel density which contains the Flory–Huggins interaction parameter (χ parameter) [38]. Thus, when the cross-linking density is assessed based on water content, the χ parameter is required. The water content of gelatin hydrogels was different between the types of gelatin or cross-

linking methods. It is likely that the χ parameter of gelatin greatly depends on the gelatin type because the amino acid sequences are different among the gelatin samples which are prepared by various treatments (acidic, basic and emzymatic) from collagen of different sites and animals. Although the χ parameter of gelatin is reported to be 0.495 [39], the method to assess the cross-linking density from the hydrogel swelling may be inaccurate. Therefore, in this study, we have chosen to determine the cross-linking density of hydrogels from their tensile modulus [28]. Gelatin hydrogels behave as a rubber-like material. From the stress–strain behavior of gelatin hydrogels swollen, the number of cross-links can be assessed. For this assessment to calculate the cross-linking level of gelatin hydrogels by classical rubber theories, the ideality of chains is basically assumed. In this study, since the strain of hydrogels was small, it is theoretically possible that the hydrogels deform in an entropic modulus manner. When the degree of cross-linking in gelatin hydrogel is lower, hydrogel is so fragile that the tensile test cannot be performed. In that case, the compressive test can be substituted for the tensile test [40]. Therefore, this method is useful to assess the cross-linking density, such as the number of cross-links of gelatin hydrogels.

Mechanical testing and enzymatic degradation of gelatin hydrogel have been performed in the past [41–43]. One paper reported that cross-linked gelatin was degraded in an alkaline protease solution [41]. As that study, some other studies reported only one kind of gelatin type, a few kinds of cross-linking methods and *in vitro* enzymatic degradation. However, this study used various kinds of gelatin types and various kinds of cross-linking methods and performed in the back subcutis of mice.

Gelatin hydrogels are degraded *in vivo* not by simple hydrolysis, but by proteolysis. Therefore, in the present study, several radioiodinated gelatin hydrogels were implanted into the mouse subcutis to evaluate the time profile of hydrogel degradation. We have demonstrated that the time profile of radioactivity remaining in ^{125}I-labeled gelatin hydrogels in the back subcutis of mice was in good accordance with that of hydrogel weight [44]. As shown in Fig. 6, there is a good correlation between the number of cross-links in gelatin hydrogels and the degradability of gelatin hydrogels. This can be explained in terms of susceptibility to protease attack. It is likely that the cross-linking density of hydrogels stereochemically reduces the molecular approach of protease to gelatin chains, resulting in less susceptibilty of hydrogels to proteolysis. The hydrogels prepared through GA cross-linking of fish-scale gelatin were degraded more slowly than other hydrogels (Fig. 6). This may be due to the molecular hydrophobicity of GA and the transition temperature of gelatin. GA is a cross-linker with a hydrophobic moiety of methylene chain. It is well recognized that the transition temperature of fish collagen is higher than that of other animal collagens. Therefore, the fish-scale gelatin is likely to form a helix structure compared with other types of gelatin. Based on this feature, it is possible that the molar percentage of helix–helix interaction of gelatin molecules in the hydrogel of fish-scale gelatin is higher than that of other hydrogels, resulting

in reduced approach of proteases to gelatin chains. In addition, the GA hydrophobicity also suppresses the protease approach to gelatin chains. These natures of fish-scale gelatin, different from gelatin of other animal species, would cause the lower degradability of fish-scale gelatin hydrogels. When the *in vivo* biodegradability of hydrogels is compared by the measure of hydrogel water content, a big dependence of the gelatin type and cross-linking method on the degradation was observed. This implies that the water content is only one measure to express the density of hydrogel cross-linking for one type of gelatin and cross-linking method and not universal. The present study indicates that the biodegradability of gelatin hydrogels can be determined by the number of cross-links per gelatin molecule, irrespective of the gelatin type and cross-linking method.

Acknowledgements

The authors gratefully acknowledge Mr. Fukano and Mr. Ishiguro (Nichiban Co., Ltd., Tokyo, Japan) for preparing gelatin hydrogels cross-linked by electron beam irradiation.

REFERENCES

1. D. L. Stocum, *Science* **276**, 59 (1997).
2. Y. Tabata, *Tissue Eng.* 9, S5 (2003).
3. Y. Tabata and Y. Ikada, *Adv. Drug Deliv. Rev.* **31**, 287 (1998).
4. D. Zekorn, *Bibl. Haematol.* **33**, 30 (1969).
5. N. Chakfe, Y. Marois, R. Guidoin, X. Deng, M. Marios, R. Roy, M. King and Y. Douville, *Polym. Polym. Composit.* **1**, 229 (1993).
6. Y. Marois, N. Chakfe, X. Deng, M. Marois, T. How, M. W. King and R. Guidoin, *Biomaterials* **16**, 1131 (1995).
7. G. A. Digenis, T. B. Gold and V. P. Shah, *J. Pharm. Sci.* **83**, 915 (1994).
8. R. Narayani and K. P. Rao. *J. Biomater. Sci. Polym. Edn.* **7**, 39 (1995).
9. R. Guidoin, D. Marceau, T. J. Rao, M. King, Y. Merhi, P. E. Roy, L. Martin and M. Duval, *Biomaterials* **8**, 433 (1987).
10. R. A. Jonas, G. Ziemer, F. J. Schoen, L. Briton and A. R. Castaneda, *J. Vasc. Surg.* **7**, 414 (1988).
11. Y. Marois, N. Chakfe, X. Deng, M. Morois. T. How, M. W. King and R. Guidon, *Biomaterials* **16**, 1131 (1995).
12. J. Bachet and D. Guilmet, *Cardiol. Clin.* **17**, 779 (1999).
13. J. Bachet and D. Guilmet, *Cardiol. Clin.* **17**, ix (1999).
14. Y. Otani, Y. Tabata and Y. Ikada, *Biomaterials* **19**, 2091 (1998).
15. Y. Tabata, K. Uno, T. Yamaoka, Y. Ikada and S. Muramatsu, *Cancer Res.* **51**, 5532 (1991).
16. P. Kallinteri and S. G. Antimisiaris, *Int. J. Pharm.* **221**, 219 (2001).
17. M. M. Welz and C. M. Ofner, *J. Pharm. Sci.* **81**, 85 (1992).
18. M. Muniruzzaman, Y. Tabata and Y. Ikada, *J. Biomater. Sci. Polymer Edn* **9**, 4593 (1998).
19. A. J. Kuijpers, P. B. van Wachem, M. J. van Luyn, J. A. Plantinga, G. H. Engbers, J. Krijgsveld, S. A. Zaat, J. Dankert and J. Feijen, *J. Biomed. Mater. Res.* **51**, 136 (2000).
20. L. Di Silvio, N. Gurav, M. V. Kayser, M. Traden and S. Downes, *Biomaterials* **15**, 931 (1994).
21. A. Veis, *The Macromolecular Chemistry of Gelatin*. Academic Press, New York, NY (1964).
22. Y. Tabata, S. Hijikata and Y. Ikada, *J. Control. Rel.* **31**, 189 (1994).