CHAPTER 13

Future Direction of Gene Therapy in Tissue Engineering

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Summary

Tissue Engineering is a general name of biomedical fields to enable cells to enhance their proliferation, differentiation, and morphological organization for induction of tissue regeneration, resulting in regenerative medical therapy of diseases. For this purpose, it is important that a local environment suitable for the cell-induced regeneration is created by functionally combining various biomaterials, protein, and gene. In addition to research development of basic biology and medicine regarding development and regeneration phenomena, the biomedical technology and methodology to apply the research results in clinics is important for successful tissue regeneration. The recent rapid advent of molecular biology together with the steady progress of genome projects has provided us some essential and revolutionary information of gene which may elucidate several biological phenomena at a molecular level. Based on the genetic information, gene manipulation has become one of the key technologies indispensable to the basic research of medicine and biology, while it also will open a new field for gene therapy of several diseases and tissue engineering. Gene therapy by use of virus vectors and cell therapy with cells genetically engineered have been performed. Although their biological and therapeutic results by virus vectors are practically promising, their research use and clinical therapy are often limited by difficulty in the handling and the adverse effects of virus vector itself, such as immunogenicity and toxicity or the possible mutagenesis of cells transfected. Therefore, it

is of prime importance for future development of the research and clinical fields to create the non-viral vectors of synthetic materials for enhanced transfection efficiency of gene into mammalian cells both in vitro and in vivo. In this paper, briefly overreviewing several researches about non-viral vectors, recent research trials about drug delivery system (DDS) of gene are introduced to show significance and future direction of gene delivery technology in tissue engineering.

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Introduction

A variety of patients suffer from injured and deficient tissues or damaged organ functions. In this case, there are only two therapeutic choices, reconstructive surgery and organ or/and tissue transplantation. However, they encounter several clinical issues to be resolved, such as poor biocompatibility of biomaterials and artificial organs and the shortage of tissue or/and organ donors or the adverse effects of immunosuppressive agents eventually taken. To break through the problems, it is necessary to develop a new therapeutic strategy. One promising strategy is called regeneration medical therapy where disease is cured based on the natural healing potential of patients themselves. Tissue engineering is a biomedical technology or methodology which enables cells to enhance the proliferation and differentiation, resulting in natural promotion of tissue regeneration for disease therapy (1-4). In tissue engineering, cells and the scaffold or biosignal molecules to accelerate their proliferation and differentiation are combined and used to induce tissue regeneration. Among the signaling molecules, growth factor and the related gene are promising in the cell-based tissue regeneration. It has been demonstrated that growth factors are efficiently used to realize the regeneration therapy of various tissues (5). With the recent advent of basic molecular biology and genomics, gene has been considered as one candidate of therapeutic agents. Gene therapy has been experimentally and clinically tried mainly aiming at the therapy of tumor and immunologic disease (http://www.wiley.co.uk/genmed/clinical/). However, it will be therapeutically applicable to different types of disease. For example, it is expected that genes which codes biosignal molecules to promote the proliferation and differentiation of cells, play an important role in tissue engineering to induce tissue regeneration. Some researchers have reported on tissue regeneration with some plasmid DNAs of growth factor to demonstrate their therapeutic feasibility (6). There are two carrier systems for gene therapy, viral and non-viral carriers (7). The former has been mainly used because of the high transfection efficiency. However, the inherent toxic and safety issues should be considered. Viral vectors, such as adenovirus, retrovirus, and adenoassociated virus,

have been mainly used because of the high efficiency of gene transfection although the clinical trials are quite limited by the adverse effects of virus itself, such as immunogenicity and toxicity or the possible mutagenesis of transfected cells. On the other hand, one large problem of the latter is low efficiency of gene transfection. Therefore, several trials have been performed to improve the poor efficiency of non-viral carriers. In order to do so, the DNA to be transferred must escape the processes that affect the disposition of macromolecules. These processes include the interaction with blood components and uptake by the reticuloendothelial system. Furthermore, the degradation of therapeutic DNA by nucleases is also a potential obstacle for functional delivery to the target cell. The objective of this review was to address the state of the art in gene therapy using synthetic and natural polycations. In addition, recent researches of non-viral carrier systems are overviewed while several concrete examples are introduced to emphasize importance of gene therapy in tissue engineering to induce tissue regeneration.

Necessity of DDS technology in gene delivery

Over the last decade, rapid development of molecular biology together with the steady progress of animal and plant genome projects has been brought about some essential and revolutionary informations of gene to elucidate biological phenomena at the molecular level (8-10). Under this circumstance, gene transfection has been positioned to be a key technology which is indispensable to research progress in molecular biology (11-17). In addition, it is expected that gene therapy will become one of the promising medical therapies (18-22). From the viewpoint of pharmacokinetics, it is necessary for successful gene therapy to achieve the delivery of genes to the target organ and tissue (23-26). The objective of gene therapy is to allow a gene to express the protein coded in the target cells and consequently to treat disease by the protein secreted from cells transfected. So far, gene therapy has been applied to refractory diseases, such as

congenital diseases (27, 28), cancer (29-32), and acquired immuno-deficiency syndrome (AIDS) (33, 34). Several new viral vectors has been explored for these diseases (35, 36). Those papers reported that adeno-associated virus (AAV) is a promising viral vector in treating many kinds of hereditary diseases. The broad host range, low level of immune response, and longevity of gene expression observed with this vector have enabled the initiation of a number of clinical trials using this gene delivery system. Another potential benefit of AAV vectors is their ability to integrate site-specifically in the presence of Rep proteins. However, this virus is not well characterized. Unlike the viral vector, it does not have any inherent ability to allow plasmid DNA to internalize into cells for gene transfection. Moreover, the DNA to be transferred must escape the processes that affect the disposition of macromolecules. These processes include the interaction with blood components and uptake by the reticuloendothelial system. Furthermore, the degradation of therapeutic DNA by serum nucleases is also a potential obstacle for functional delivery to the target cell. Cationic polymers have a great potential for DNA complexation and may be useful as non-viral vectors for gene therapy applications. Thus, it is important to add the technology and methodology of drug delivery system (DDS) to the research and design of non-viral vectors. A plasmid DNA, only when complexed with the non-viral vector and given to cells or injected into the body in the solution form, is readily degraded and inactivated by enzymes or cells. It is also known that the plasmid DNA does not have any properties to accumulate in a certain tissue or organ. Therefore, the plasmid DNA should be established or targeted to the site of action by making use of DDS. Moreover, if the gene expression is transient, this is not suitable to therapeutically treat disease for which long-term gene expression over several weeks or more is required. For example, the time period of gene expression can be prolonged by the controlled release technology of plasmid DNA. Thus, as far as water-soluble gene is used as a drug, DDS technology and methodology are required to expect the biological effects of gene.

DDS trials for plasmid DNA

Several synthetic materials, including cationic liposomes (37-39) and cationic polymers like poly-L-lysine (40-43) and polyethyleneimine (44-49), have been molecularly designed and as the transfection vector of plasmid DNA for mammalian cells both *in vitro* and *in vivo*. Generally, since plasmid DNA is a large and negatively charged molecule, it is impossible to allow the plasmid DNA itself to interact with the cell membrane of negatively charge and consequently internalize into cells for gene transfection. When the plasmid DNA is complexed with the cationic materials, it is well recognized that the molecular size of plasmid DNA decreases by the condensation due to the polyion complexation (50, 51). It is likely that the condensed plasmid DNA-vector complex with a positive charge electrostatically interacts with the cell membrane, resulting in the internalization. Among the cationic polymers, polyethyleneimine with secondary amine residues functions to buffer the endosomal pH of cells, which protects the plasmid DNA from enzymatic degradation in endosome, resulting in the enhanced transfection efficiency (52). This is called “buffering effect”.

Efficient and specific delivery of a therapeutic gene into the targeted cells is one of key technologies for gene therapy. Successful gene delivery to the specific tissues or cells results in high therapeutic efficacy. For example, tumor-specific targeting with non-viral vector of synthetic cationic polymers has been reported (53-63). A folate receptor is known to be overexpressed on the surface of several human tumors, where as it is only minimally distributed in normal tissues (64). Therefore, the folate receptor serves as an excellent tumor marker as well as a functional tumor-specific receptor. The complexation with cationic polymers covalently bound with folate enabled plasmid DNAs to efficiently accumulate in the tumor (61, 62). HM. Vriesendorp *et al.* reported that an indium 111-labeled antiferritin targeted 95 % of Hodgkin's disease lesions with the diameter of 1 cm or more. In addition, treatment with a yttrium 90-labeled antiferritin showed a high therapeutic response to patients with recurrent Hodgkin's disease (65). Targeting of plasmid DNA to the parenchymal cells of liver has been also

investigated. For the liver targeting, glycoprotein, lactose, or galactose which is a ligand recognizable by the asialoglycoprotein receptor specific for hepatocytes, is coupled with the non-viral vector (66-79). For instance, pullulan, which is a natural polysaccharide with a high affinity for the asialoglycoprotein receptor, has been used to target a plasmid DNA to the liver. Pullulan derivatives with metal chelating residues were mixed with a plasmid DNA in aqueous solution containing Zn2+ ions to obtain the conjugate of pullulan derivative and plasmid DNA with Zn2+ coordination (79). Metal coordinate conjugation with the pullulan derivatives enabled the plasmid DNA to target the liver for gene expression and the level of gene expression was enhanced at the liver parenchymal cells rather than non-parenchymal cells (79). On the other hand, delivery technology through systemic bloodstream will permit gene therapy for disseminated and widespread disease targets. The research and development of long-circulating non-viral vectors for gene delivery have been intensively performed. Generally, the rapid uptake of colloidal drug carriers by the mononuclear phagocyte system (MPS) after the intravenous administration is one of the major events, which often prevents a drug injected from delivering to the site of action other than the MPS tissue and organ. As one practical way to minimize the MPS uptake, the surface modification of drug carriers with polyethylene glycol (PEG) or PEG-like polymers is effective (80-90). Based on this feature, PEG has been widely used for the material of non-viral gene carrier to demonstrate efficient gene expression following a single administration (91-95). PEG-conjugated copolymers have advantages for gene delivery. First, the PEG-conjugated copolymers show low cytotoxicity to cells *in vitro* and *in vivo*. Second, PEG increases water-solubility of the polymer/DNA complex. Third, PEG reduces the interaction of the polymer/DNA complex with serum proteins and increases circulation time of the complex.

In addition to the *in vivo* targetability and stability of plasmid DNA, stable controlled release of plasmid DNA at a required amount and right place over the desired period of time is practically important in terms of the regulation of gene expression period.

Table 1 (96-115) summarizes researches about the controlled release of plasmid DNA with different biodegradable biomaterials. The purpose is to enhance the level of gene transfection and prolong the transfection period. D. Mooney *et al.* have reported that the *in vivo* sustained release of a plasmid DNA encoding platelet-derived growth factor (PDGF) gene with the carrier matrix of poly(lactide-co-glycolide) enhanced matrix deposition and blood vessel formation (96, 97). Plasmid DNA carrying a gene fragment of human parathyroid hormone was released from a polymer matrix sponge called a gene-activated matrix (GAM) to induce tissue regeneration (116, 117). Implantation of GAM at a bone injury site achieved the retention and expression of plasmid DNA for a longer time period, resulting in reproducible and high regeneration of bone tissue. The controlled release of plasmid DNA with an atelocollagen minipellet has been reported by T. Ochiya *et al.* to demonstrate enhanced level of gene expression and high therapeutic effects for some model animal diseases (111, 112). Atelocollagen obtained by pepsin digestion of type I collagen is of low immunogenicity (118, 119) and free from telopeptides while it has been clinically employed for biomedical materials. Atelocollagen carrying plasmid DNA may enhance the clinical potency of plasmid-based gene transfer, facilitating a more effective and long-term use of naked plasmid DNAs for gene therapy (111, 112).

Carrier material	Plasmid DNA	Biological function	References
Poly(D,L-lactic acid-*co*-glycolic acid) (PLGA)	β-Galactosidase, Platelet-derived growth factor (PDGF)	Deliver intact and functional plasmid DNA at controlled rates. The ability to create porous polymer scaffolds capable of controlled release rates may provide a means to enhance and regulate gene transfer within a developing tissue, which will increase their utility in tissue engineering.	Murphy *et al*. [96] Shea *et al*. [97] Wang *et al*. [98] Capan *et al*. [99] Luo *et al*. [100] Hedley *et al*. [101] Jang *et al*. [102]
Polymethacrylic acid (PMA) and polyethylene glycol (PEG), hydroxypropylmethylcellulose-carbopol	Luciferase	The in situ gelling systems can be considered as a valuable injectable controlled-delivery system for plasmid DNA in their role to provide protection from DNase degradation.	Ismail *et al*. [103]
Poly(lactic acid)-poly(ethylene glycol) (PLA-PEG)	Luciferase	Release plasmid DNA from nanoparticles in a controlled manner.	Perez *et al*. [104]
Poly(2-aminoethyl propylene phosphate)	β-Galactosidase	Enhanced β-galactosidase expression in anterior tibialis muscle in mice, as compared with naked DNA solution injections.	Wang *et al*. [105]
Poly(α-(4-aminobutyl)-L-glycolic acid) (PAGA)	β-Galactosidase	The complexes showed about 2-fold higher transfection efficiency than DNA complexes of poly-L-lysine (PLL) which is the most commonly used poly-cation for gene delivery.	Lim *et al*. [106]
Poloxamers	β-Galactosidase	The use of *in situ* gelling and mucoadhesive polymer vehicles could effectively and safely improve the nasal retention and absorption of plasmid DNA. Moreover, the rate and extent of nasal absorption could be controlled by choice of polymers and their contents.	Park *et al*. [107]
Poly(ethylene-*co*-vinyl acetate) (EVAc)	Sperm-specific lactate dehydrogenase C_4, β-Galactosidase	The EVAc disks are efficient and convenient vehicles for delivering DNA to the vaginal tract and providing long-term local immunity.	Shen *et al*. [108]
Silk-elastinlike polymer (SELP)	Luciferase	The ability to precisely customize the structure and physicochemical properties of SELP using recombinant techniques, coupled with their ability to form injectable, *in situ* hydrogel depots that release DNA, renders this class of polymers an interesting candidate for controlled gene delivery.	Megeed *et al*. [109]
Denatured collagen-PLGA	β-Galactosidase	Increase the level of gene expression because of integrin-related mechanisms and associated changes in the arterial smooth muscle cell actin cytoskeleton.	Perlstein *et al*. [110]
Atelocollagen	Green fluorescent protein (GFP), Fibroblast growth factor 4 (FGF4)	Increased serum and muscle FGF4 levels and long-term release and localization of plasmid DNA *in vivo*.	Ochiya *et al*. [111,112]
Gelatin	β-Galactosidase	Plasmid DNA release period can be regulated only by changing the hydrogel degradability.	Fukunaka *et al*. [113] Kushibiki *et al*. [114,115]

Table 1. Research reports on the controlled release of plasmid DNA.

Feasibility of gelatin as the release matrix

Gelatin is a naturally occurring polymer of biodegradability which has been extensively used for industrial, pharmaceutical, and medical applications. The bio-safety of gelatin 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 (120). 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 prepared biodegradable hydrogels by chemical crosslinking of the gelatin and succeeded in the controlled release of various growth factors with the biological activity remaining. For example, growth factors with IEPs higher than 7.0, such as basic fibroblast growth factor (bFGF) (121), transforming growth factor beta1 (TGF-beta1) (122), and hepatocyte growth factor (HGF) (123), are immobilized into the biodegradable hydrogels of "acidic" gelatin mainly through the electrostatic interaction force between the growth factor and gelatin molecules. In this release system, the growth factor immobilized is released from the gelatin hydrogel only when the hydrogel carrier is degraded to generate water-soluble gelatin fragments. Therefore, the time profile of growth factor release could be controlled only by changing that of hydrogel degradation which can be modified by the extent of hydrogel crosslinking (121). The key point is to give gelatin the chemical property which can physicochemically interact with the growth factor to be released. Chemical derivatization enables gelatin to interact with different factors. Hydrogels prepared from the gelatin derivatives have achieved the controlled release of bioactive substance, such as growth factors and plasmid DNA.

Controlled release of plasmid DNA from cationized gelatin hydrogels of gelatin derivatives

Gelatin was cationically derivatized to allow to polyionically interact with plasmid DNA of anionic nature. We have demonstrated the enhanced expression of plasmid DNA by polyion complexation with the cationized gelatin by ultrasound *in vitro* and *in vivo* (124-126). We have prepared cationized gelatin by chemically introducing amine residues to the carboxyl groups of gelatin and demonstrated that as expected, the hydrogel of cationized gelatin achieved the controlled release of plasmid DNA as a result of hydrogel degradation following intramuscular implantation (113-115). The controlled release of plasmid DNA enhanced the level of gene expression to a significantly greater extent than the plasmid DNA solution injected, while it also could prolong the duration period of gene expression. Since the time period of plasmid DNA release was prolonged, the time period of gene expression became longer (113-115). The plasmid DNA release is driven by degradation of release carrier. This release mechanism is quite different from that of diffusional release of plasmid DNA from the conventional release carrier of plasmid DNA reported (96-112). Another key point is the physicochemical structure of plasmid DNA released. Since the plasmid DNA is incorporated into the hydrogel being polyionically complexed with the hydrogel-constituted cationized gelatin, it is likely that the plasmid DNA released is always complexed with the fragment of gelatin degraded. From the viewpoint of gene transfection, such the plasmid DNA complexed is preferable in terms of the plasmid DNA size condensed and the positive charged character. This hydrogel release system has these advantageous points over the direct injection of free plasmid DNA or the conventional release system of plasmid DNA itself. Based on the points, the hydrogel for controlled release enabled the plasmid DNA to increase and prolong the concentration over an extend time period around the target cells or tissue when injected. It is highly conceivable that the locally enhanced concentration of plasmid DNA increases possibility of the exposure to cells, resulting in promoted gene expression. The plasmid DNA is complexed with the cationized gelatin when incorporated in the hydrogel of release carrier or after released (113-115). This

complexation prevents the plasmid DNA from the enzymatic degradation by DNase. Some researches have indicated that polyionic complexation effectively suppresses the DNase degradation of plasmid DNA (127-129). Thus, it is likely that the plasmid DNA is biologically stabilized by the incorporation into the hydrogel and the controlled release enhances the local concentration of plasmid DNA around cells to be transfected, consequently increasing the efficiency of gene transfection. As expected from the release mechanism of hydrogel system, the time profile of plasmid DNA release was in good accordance with that of cationized gelatin hydrogels degradation which can be controlled by changing the reaction conditions of crosslinking for hydrogel preparation. The retained time period of gene expression became longer when the cationized gelatin hydrogel of slower degradation was used for the longer-term release of plasmid DNA. Generally, gelatin is not degraded by simple hydrolysis, but by proteolysis. This phenomenon was observed for cationized gelatin hydrogels (113-115). The water content of hydrogel is one of the factors affecting the crosslinking extent of hydrogels; the higher water content of hydrogels, the smaller their crosslinking extent. Hydrogel with smaller crosslinking extents or higher water contents is more susceptible to enzymatic digestion, resulting in faster degradation. For example, a cationized gelatin hydrogel with a water content of 98.3 wt% was degraded with time to completely disappear in the femoral muscle of mice 14 days after implantation. The time period of complete degradation for the cationized gelatin hydrogels with water contents of 97.4 and 99.7 wt% were 21 and 7 days (114). This indicates that *in vivo* degradation of gelatin hydrogels could be controlled by their water content (Fig. 1A). When a plasmid DNA was incorporated into the cationized gelatin hydrogel with different water contents and implanted into the mouse muscle, the *in vivo* remaining of plasmid DNA decreased with time although the time profile depended on the type of hydrogels. The plasmid DNA remained in the muscle for longer time periods as the water content of hydrogels used became lower. The time profile of plasmid DNA remaining was correlated with that of hydrogel remaining, irrespective of the hydrogel water content (Fig. 1B). This finding indicates that as expected, the lacZ plasmid DNA was released from the cationized gelatin hydrogels of release carrier in the body accompanied with the biodegradation of

hydrogels. It is likely that the lacZ plasmid DNA molecules ionically complexed with the cationized gelatin cannot be released from the cationized gelatin hydrogel unless hydrogel degradation takes place to generate water-soluble cationized gelatin fragments. Based on this release mechanism, it is conceivable that the lacZ plasmid DNA molecules are released from the hydrogels with being complexed with degraded gelatin fragments of positive charge. If the lacZ plasmid DNA-cationized gelatin complex has a positive charge, the charge will enable the lacZ plasmid DNA to promote the internalization into cells because the complex easily interacts ionically with the cell surface of negative charge. Moreover, it is expected that the continuous presence of complex at a certain body site and close to cells by the controlled release enhances frequency of plasmid DNA transfection, resulting in promoted gene expression thereat. From the cationized gelatin hydrogel, the lacZ plasmid DNA is released as a result of hydrogel biodegradation. Figure 2 shows the time period of gene expression induced by lacZ plasmid DNA in the solution or hydrogel-incorporated form. The time period of gene expression induced by lacZ plasmid DNA incorporated in hydrogel was significantly longer than that of lacZ plasmid DNA in the solution form. It is possible that an extended release enables the plasmid DNA to maintain the concentration at the implanted site for a longer time period, resulting in prolonged gene transfection. This study is the first report to experimentally confirm that the time period of gene expression can be regulated by altering that of plasmid DNA release.

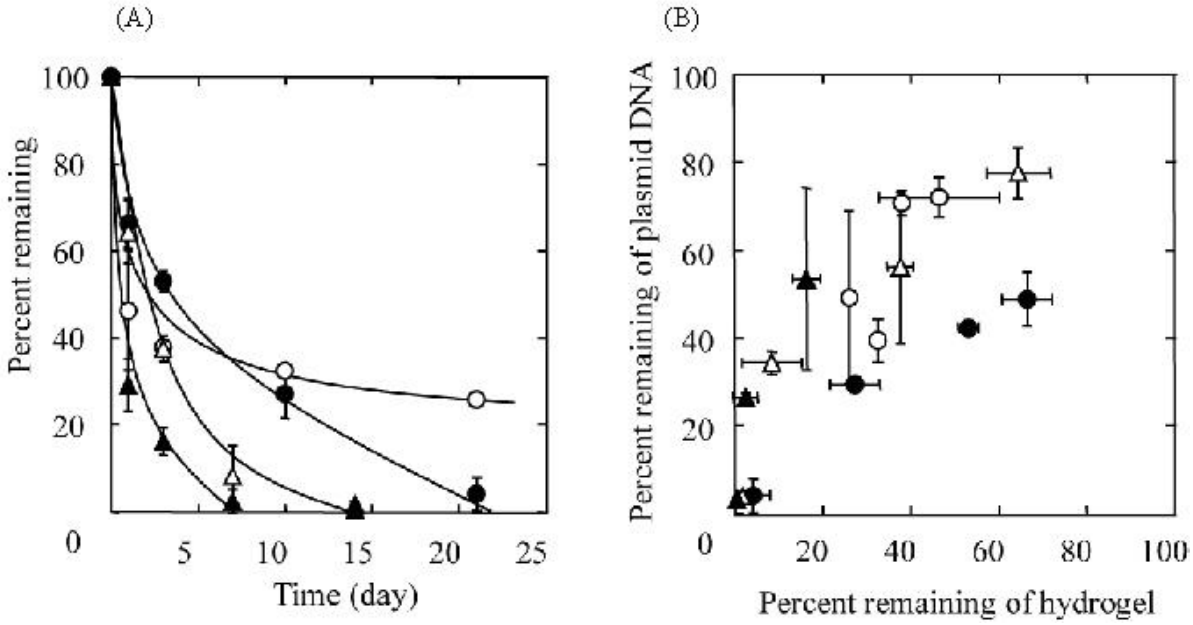

Fig. 1:
(A) The time course of the radioactivity remaining of 125I-labeled cationized gelatin hydrogels after implantation into the femoral muscle of mice (The wet weight of hydrogel implanted=0.2 g) (3 mice/group). The water content of cationized gelatin hydrogels is 96.4 (○), 97.4 (●), 98.3 (△), or 99.7 wt% (▲).
(B) The radioactivity remaining of cationized gelatin hydrogels incorporating 125I-labeled lacZ plasmid DNA plotted against that of 125I -labeled cationized gelatin hydrogels after implantation into the femoral muscle of mice (3 mice/group): The water content of cationized gelatin hydrogels is 96.4 (○), 97.4 (●), 98.3 (△), or 99.7 wt% (▲).

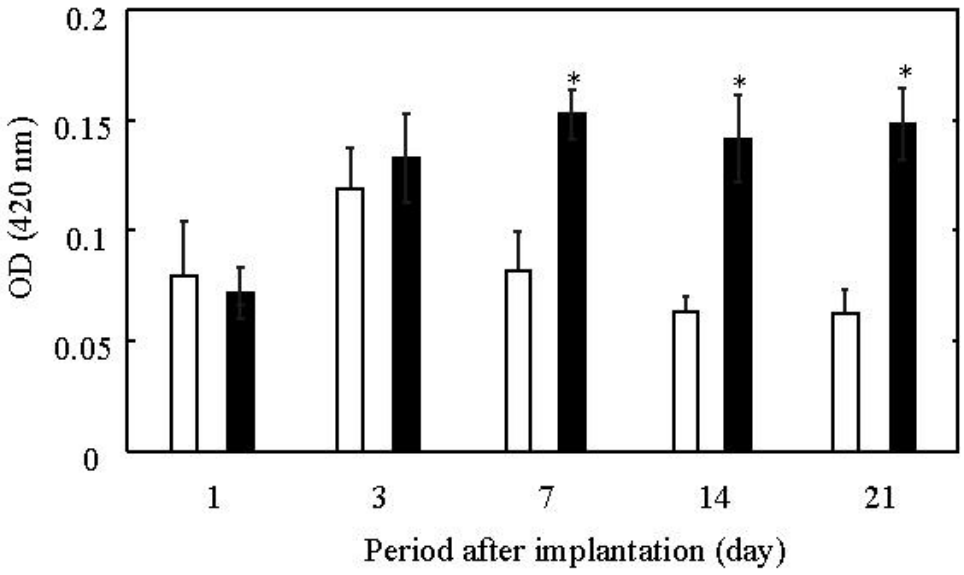

Fig. 2: The time course of lacZ gene expression after implantation of cationized gelatin hydrogels incorporating lacZ plasmid DNA into the femoral muscle of mice: free lacZ plasmid DNA (open bar) and lacZ plasmid DNA incorporated in cationized gelatin hydrogels (water content=97.4 wt%) (closed bar). The lacZ plasmid DNA dose is 100 μg/mouse muscle (3 mice/group). *, P<0.05: significant against the OD value of free plasmid DNA injected group.

Controlled release of plasmid DNA from cationized gelatin microspheres

Another superior point of the hydrogel release system is that the hydrogel shape has no influence on the release profile of plasmid DNA. Since the plasmid DNA release is governed only by the degradation of the release carrier, but not by simple diffusion from the carrier, it is possible to achieve the controlled release even if the surface area of

hydrogel carrier per the volume is large like injectable microspheres (130). Hydrogel microspheres of cationized gelatin were enabled a plasmid DNA of fibroblast growth factor 4 (FGF4) to enhance the angiogenesis effect, in remarked contrast to the plasmid DNA solution, which is similar to the case of cationized gelatin hydrogels incorporating plasmid DNA described previously (131). The *in vivo* experiment with a lacZ plasmid DNA indicated that the intramuscular injection of cationized gelatin microspheres incorporating the plasmid DNA into the ischemic hindlimb of rabbits augmented both the number of myocytes transfected and the degree of gene expression, and induced gene expression spatially expanded around the injected site, which is in remarked contrast to that of plasmid DNA solution. When the microspheres incorporating FGF4 plasmid DNA were injected into the femoral muscle of rabbit hindlimb ischemia, the gene expression widely expanded around the injected site was observed. Superior angiogenesis by FGF4 plasmid DNA incorporated in cationized gelatin microspheres at the hindlimb ischemia to free FGF4 plasmid DNA was achived. The cationized gelatin microspheres incorporating FGF4 plasmid DNA did not induce severe tissue damage in the ischemic limb. The blood vessel newly formed by the plasmid DNA released normally responded to a vasoresponsive agent, adenosine, in contrast to that by the plasmid DNA in the solution form. Such a normal responsiveness clearly indicates the functional recovery of vascular segments angiogenically formed and their physiological maturation.

Applications of plasmid DNA release technology to tissue engineering

We introduce a new tissue engineering which is different from the "surgical" tissue engineering in which tissue regeneration is induced by surgically adding cells, scaffold, and growth factor to the site to be regenerated. That can be named "physical" tissue engineering of internal medicine from the viewpoint of therapeutic way. The idea is to

therapeutically treat chronic fibrotic diseases based on the natural healing potential of healthy tissue around the fibrous tissue following loosening and digestion of the fibrotic tissue by drug treatment of internal medicine. In other words, natural healing capability at a disease site is induced by removing the pathogenic cause to therapeutically cure the fibrosis or delay the deterioration of chronic disease. In general, chronically injured tissue is gradually repaired by the excessive formation of fibrous tissues (scar formation), which eventually suppresses natural tissue regeneration. If such fibrosis can be suppressed or excluded by drug treatment, it is physically expected that the fibrotic tissue is repaired by the regeneration potential of the surrounding tissue. For example, matrix metalloproteinase-1 (MMP-1) digestion allows a fibrotic tissue to convert the tissue to a state that the natural process of tissue regeneration can function to heal fibrosis. Iimuro and co-workers demonstrated that transfection of pro-MMP-1 gene using an adenovirus vector, histologically improved tissue fibrosis at the liver of rat cirrhosis model (132). It is suggested that the possible healing mechanism is associated with the suppression of hepatic stellate cells and proliferation of hepatocytes. Another research has reported on experimental evidence of liver cirrhosis reversion using a MMP-8 gene and adenovirus vector (133).

Cationized gelatin microspheres incorporating a MMP-1 plasmid DNA were injected into the subcapsule of mouse kidney in advance, and then the mice received streptozotocin (STZ) to induce diabetic renal disease. It is reported that the advanced lesion of STZ-induced diabetic kidney mimics some findings of early-stage clinical diabetic nephropathy. Figure 3 shows histological renal sections of mice preadministered microspheres incorporating MMP-1 plasmid DNA 28 days after STZ injection. Renal fibrosis was histologically suppressed by the application of cationized gelatin microspheres incorporating MMP-1 plasmid DNA, compared with that of free MMP-1 plasmid DNA. The administration of cationized gelatin microspheres was not effective and the tissue appearance was similar to that of the saline-administered control group (134).

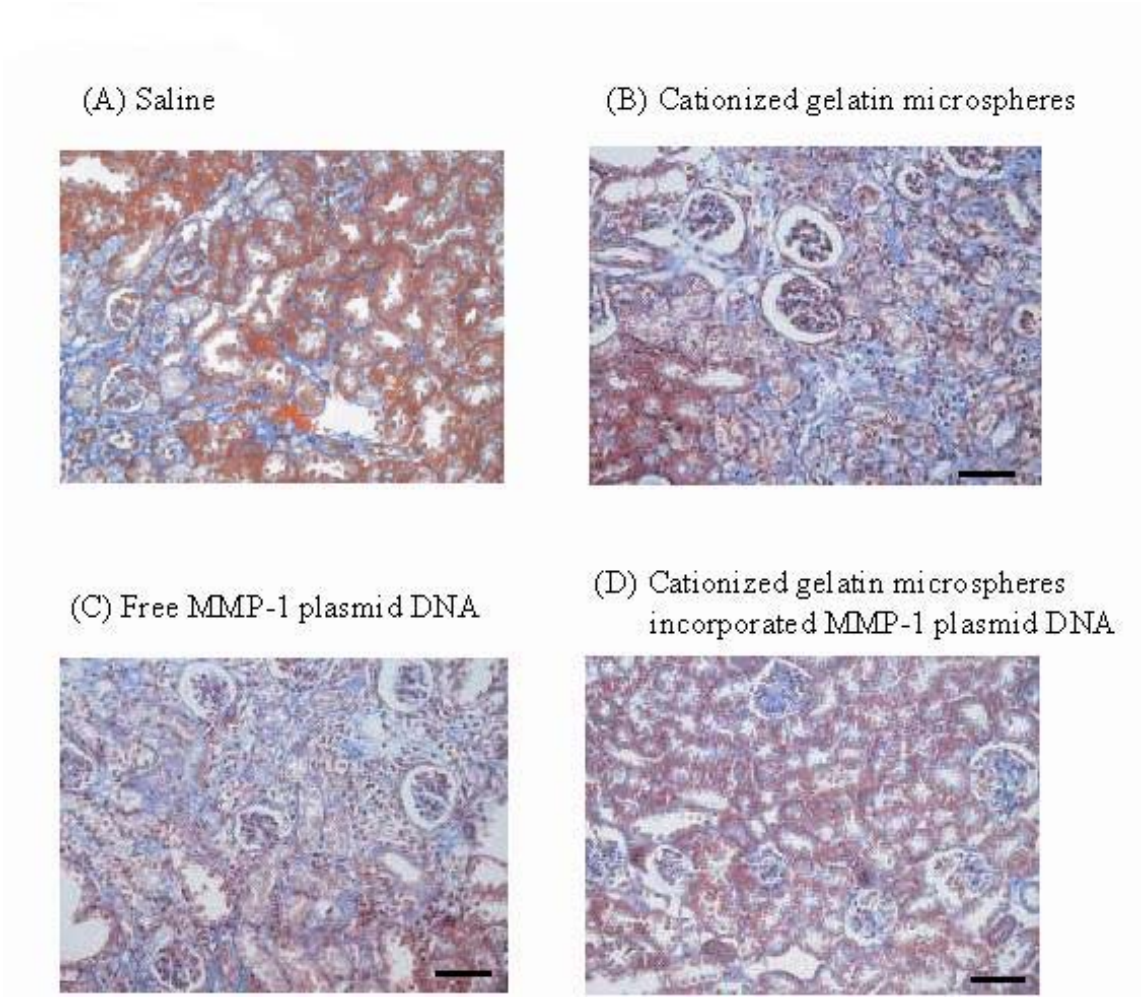

Fig. 3: The time course of lacZ gene expression after implantation of cationized gelatin hydrogels incorporating lacZ plasmid DNA into the femoral muscle of mice: free lacZ plasmid DNA (open bar) and lacZ plasmid DNA incorporated in cationized gelatin hydrogels (water content=97.4 wt%) (closed bar). The lacZ plasmid DRenal histological sections of mice preadministered gelatin microspheres incorporating MMP-1 plasmid DNA or other agents into the renal subcapsule 28 days after STZ injection: (A) saline, (B) cationized gelatin microspheres, (C) free MMP-1 plasmid DNA (50 µg/site), and (D) cationized gelatin microspheres incorporating MMP-1 plasmid DNA (50 µg/site). Masson trichrome stain; original magnification, ×200.

In addition to the enhanced transfection efficacy of plasmid DNA in gene therapy, the controlled release system is effective in genetically manipulating stem cells. With the recent development of stem cells researches, various stem cells of highly proliferation and differentiation potentials have been available to cell therapy for some incurable disease. Stem cell therapy is promising, but there are some cases where the cells are not always powerful for disease therapy. In such cases, it is necessary to genetically modify and activate the biological function of stem cells. So far, virus has been used to manipulate cells for activation because of the high efficiency of gene transfection. However, we cannot apply the viral cell manipulation to clinical therapy since the toxicity and immunogenicity of viruses themselves cannot be ruled out completely. Thus, it is of prime importance to develop a non-viral system suitable for the genetic manipulation of cells. In case the stem cells have a phagocytic property, the cationized gelatin microspheres incorporating plasmid DNA were readily taken up by the cells to achieve the sustained release of plasmid DNA inside the cells. Interestingly, this intracellular controlled release enabled the plasmid DNA to enhance the level of gene expression significantly higher than that of virus system (135). This system will break through the virus-related problems to be resolved for clinical applications. Here, we introduce a new therapeutic concept for cell-based gene delivery. This concept worked very well to therapeutically treat pulmonary hypertension for which there is no effective clinical treatment at present. Endothelial progenitor cells (EPC) of phagocytic property were isolated and incubated with cationized gelatin microspheres incorporating plasmid DNA of angiogenic adrenomedullin to genetically activate through the intracellular controlled release of plasmid DNA. Next, the gene-modified EPC were injected intravenously into monocrotaline (MCT)-induced pulmonary hypertension model rats. This novel gene delivery system has great advantages over the conventional gene therapy in terms of non-viral or non-invasive therapy and the usage of natural targeting vehicle to the ischemic site of disease, that is cells. The system causes taking advantage of the inherent ability of EPC to phagocytose cationized gelatin microspheres capable for plasmid DNA release and to positively migrate to the sites of injured endothelium. When incubated with cationized gelatin microspheres incorporating green fluorescent

protein (GFP) plasmid DNA and the GFP plasmid DNA solution, EPC, not monocytes/macrophages, were strongly transfected to express the GFP protein by the former, in remarked contrast to the latter. Fluorescent imaging studies indicated that the DNA molecules incorporated in cationized gelatin microspheres was continuously released in the cytoplasm of EPC after phagocytosis and the cationized gelatin-DNA complexes released were transferred to the nucleus although the Rhodamine B isothiocyanate-labeled DNA molecules were mainly distributed to the cytoplasm rather than nucleus. This unique intracellular traffic may be one of the reasons why the microspheres incorporating plasmid DNA enhanced the level of DNA expression. Other reasons why the DNA release was effective, should be considered. It is possible that polyion complexation with the cationized gelatin prevents the plasmid DNA from the enzymatic degradation in the cytoplasm. Moreover, the GFP-expressing EPC intravenously administered were incorporated into pulmonary arterioles and capillaries in MCT rats and differentiated into mature endothelial cells. Taking the findings together, it is highly possible that as expected, the EPC injected circulate in the blood and target to pulmonary endothelia injured in MCT rats. Thus, EPC serve not only as a vehicle for gene delivery to injured pulmonary endothelia, but also as a tissue-engineering tool in restoring intact pulmonary endothelium. The injection of genetically modified EPC by the transfection of plasmid DNA of adrenomedullin significantly improved the therapeutic efficacy in the pulmonary hypertension compared with that of non-genetically modified EPC (135).

Conclusions

Gene delivery system is generally divided into two categories: viral and non-viral vectors. From the viewpoint of the clinical application, the non-viral vector will be superior. Therefore, several non-viral vectors have been explored aiming at the capacity of gene expression comparable to that of viral vectors. However, little concept of

plasmid DNA release has been introduced to develop the non-viral vector. Cationized gelatin microspheres permitted the controlled release of plasmid DNA and consequently offered several advantages as a new gene delivery system: 1) The system increases the local concentration of plasmid DNA around the site applied, resulting in enhanced gene expression; 2) The plasmid DNA is ionically complexed with cationized gelatin or the fragment, resulting in enhanced transfection efficiency of plasmid DNA; 3) The time period of gene expression can be regulated by changing that of the microspheres; 4) The system is applicable to the controlled release of biologically active substances with negative charges other than plasmid DNA, such as protein and nucleic acid drugs. The substance to be released is immobilized into the hydrogel of release carrier based on the physicochemical intermolecular forces between the substance and hydrogel material. The coulombic interaction force is used for the present gene delivery system of gelatin hydrogel. The controlled release of substance immobilized is achievable only by the degradation of release carrier. It is possible for substance immobilization to make use of other intermolecular interaction forces. We believe that this release concept will open a new direction for the research and development of tissue engineering.

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