only if a biodegradable hydrogel is prepared from gelatin or the derivative which can physicochemically interact with the growth factor molecule.

CONTROLLED RELEASE OF PLASMID DNA FROM CATIONIZED GELATIN HYDROGELS

Plasmid DNA is a negatively macromolecule charged. Based on this concept, we have reported the enhanced expression of plasmid DNA-cationized gelatin molecule polyion complex by ultrasound *in vitro* and *in vivo* [104-106]. Therefore, it is possible to allow the plasmid DNA to release from a hydrogel of cationized gelatin, which is capable to form polyion complex with the plasmid DNA. 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 based on the hydrogel degradation following intramuscular implantation [93,94]. The cationized gelatin hydrogels incorporating a plasmid DNA not only enhanced the level of gene expression to a significantly greater extent than the plasmid DNA injected in the solution form, but also allowed to prolong the duration of gene expression. The period of gene expression became longer as that of plasmid DNA release prolonged [93,94]. The mechanism that the plasmid DNA release is driven by the degradation of release carrier is quite different from that of diffusional release of plasmid DNA from the release carrier by which the conventional release system of plasmid DNA reported so far has been attempted. Another advantage is the physicochemical structure of the released plasmid DNA. Since the plasmid DNA is incorporated into the hydrogel being polyionically complexed with the cationized gelatin, it is likely that the plasmid DNA is complexed upon release. From the viewpoint of gene transfection, the polyion complexation will be preferable. This release system has several advantages points over the direct injection of free plasmid DNA. The controlled release enables the plasmid DNA to increase and prolong the concentration over an entended time period around the cells when given, or around the tissue when injected. It is highly conceivable that the enhanced concentration increases the possibility of plasmid DNA 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 released [93]. This complexation prevents the plasmid DNA from degradation by DNase attack. Some researches have indicated that polyionic complexation effectively suppresses the DNase degradation of plasmid DNA [107-109]. Thus, it is likely that the plasmid DNA is biologically stabilized by the incorporation into the hydrogel and the controlled release enhances the concentration of plasmid DNA around cells, consequently increasing the efficiency of gene transfection. As expected from the release mechanism of hydrogel system, the time period of plasmid DNA release was in good accordance with that of cationized gelatin hydrogels degradation, which can be controlled by changing the condition of crosslinking reaction 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 [93,94]. The water content of hydrogel is one of the factors reflecting the crosslinking extent of hydrogels; the higher the water content of hydrogels, the smaller their crosslinking extent. The smaller crosslinking extent of hydrogels with higher water contents is more susceptible to enzymatic digestion, resulting in faster hydrogel 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 was 21 and 7 days for the cationized gelatin hydrogels with water contents of 97.4 and 99.7 wt% respectively [93]. This indicated that *in vivo* degradation of gelatin hydrogels could be controlled by their water content (Fig. 1A). When a plasmid DNA was incorporated into cationized gelatin hydrogels 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 are not 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 complexed with the positively charged degraded gelatin fragments. 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 it is easy to ionically interact the complex with the cell surface of negative charge. Moreover, it is expected that the continuous presence of the 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. Fig. (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 long 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. Another superior point of the plasmid DNA release system is no influence of the hydrogel shape 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

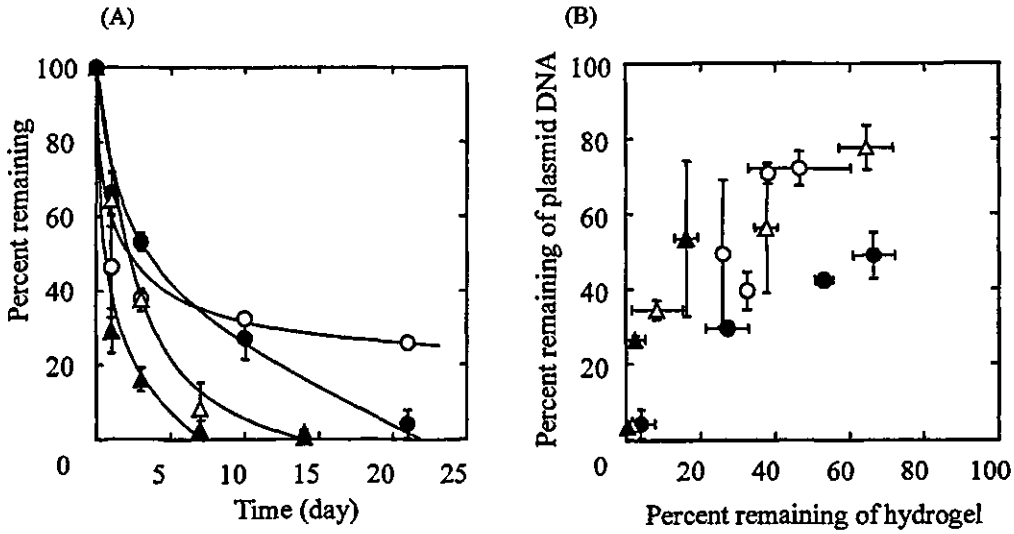


Fig. (1). (A) The time course of the radioactivity remaining of ^{125}I-labeled cationized gelatin hydrogels after implantation into the femoral muscle of mice (The wet weight of hydrogel implanted=0.2 g) (3 mice/group). The water content of cationized gelatin hydrogels is 96.4 (○), 97.4 (●), 98.3 (×),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

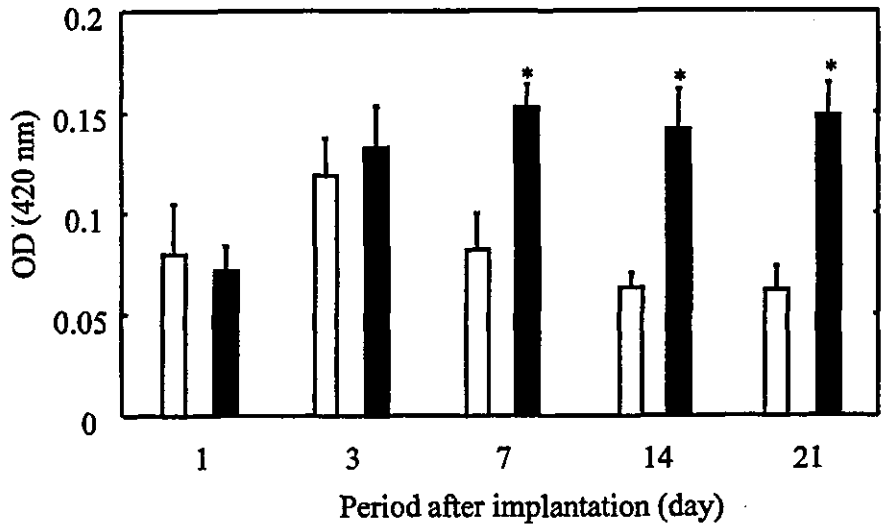


Fig. (2). The time course of lacZ gene expression after implantation of cationized gelatin hydrogels incorporating lacZ plasmid DNA into the femoral muscle of mice: free lacZ plasmid DNA (open bar) and lacZ plasmid DNA incorporated in cationized gelatin hydrogels (water content=97.4 wt%) (closed bar). 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.

possible to achieve the controlled release even if the hydrogel carrier is as small as injectable microspheres.

CONTROLLED RELEASE OF PLASMID DNA FROM CATIONIZED GELATIN MICROSPHERES

Microspheres prepared from cationized gelatin enabled a plasmid DNA of fibroblast growth factor 4 (FGF4) to enhance the angiogenesis effect based on the mechanism of plasmid DNA release, similarly to that of cationized gelatin hydrogels incorporating plasmid DNA described previously [110]. The *in vivo* experiment with a lacZ plasmid DNA of reporter gene indicated that the intramuscular injection of cationized gelatin microspheres incorporating plasmid DNA into a hindlimb ischemia model 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 marked contrast to that of plasmid DNA solution (Fig. 3). 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 (Fig. 4). Superior angiogenesis by FGF4 plasmid DNA incorporated in cationized gelatin microspheres at the

Fig. (3). Representative gene expression of lacZ in the ischemic adductor muscle of rabbits 17 days after treatment. Free lacZ plasmid DNA (a) or cationized gelatin microspheres incorporating lacZ plasmid DNA (b) was injected into the adductor muscle 10 days after the ischemic insultation (5 rabbits/group). magnification; X20, bar = 200 μm.

hindlimb ischemia to free FGF4 plasmid DNA was achieved (Fig. 5). 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 released plasmid DNA normally responded to a vasoresponsive agent, adenosine, in contrast to that by the plasmid DNA in the solution form (Fig. 5). Such vascular responsiveness to the adenosine administration indicates the recovery of fundamental function in angiogenic vascular segments and their physiological maturation.

The controlled release technology also promoted the anti-tumor activity of plasmid DNA. When the cationized gelatin microspheres incorporating a plasmid DNA of NK4, which is a protein composed of the NH_2-terminal hairpin and the subsequent four-kringle domains of HGF, were subcutaneously injected into nude mice with ascitic AsPC-1 tumor cells, they significantly prolonged the mice survival compared with the NK4 plasmid DNA in the solution form (Fig. 6). It is known that NK4 has a binding capacity to the HGF receptor, c-Met, competing with HGF and inhibits the

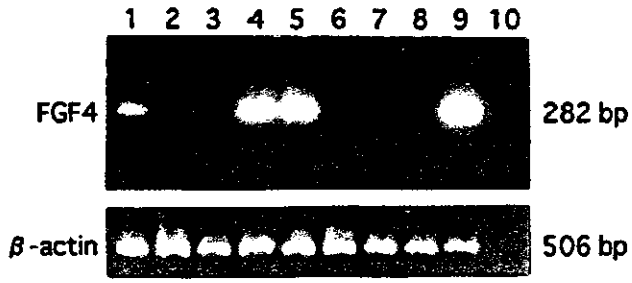


Fig. (4). Representative transgene expression demonstrated by reverse transcription-nested polymerase chain reaction (RT-nested PCR). The left adductor muscle of rabbits was injected with plasmid DNA of fibroblast growth factor 4 (FGF4) (lanes 1 to 3), cationized gelatin microspheres incorporating FGF4 plasmid DNA (lanes 4 to 6), and cationized gelatin microspheres incorporating lacZ plasmid DNA (lanes 7 and 8). Each sample was obtained from the injection site (lanes 1, 4, and 7) and the adjacent region 10 mm apart from the injection site (lanes 2, 5 and 8) in the left adductor muscle, and from the contralateral adductor muscle (lanes 3 and 6). The RT-nested PCR products from ribonucleic acid of each sample were analyzed on agarose gel; FGF4 expressed Cc1/l6 cells as a positive control (lane 9) and no DNA template as a negative control (lane 10). A housekeeping beta-actin gene was amplified as a complementary DNA loading control (5 rabbits/group).

cell migration-facilitating activity of HGF [111,112], while it also suppresses the angiogenic effects of VEGF and bFGF [113,114]. Namely, NK4 is a bifunctional molecule: it acts not only as an HGF-antagonist, but also as an angiogenesis inhibitor. In the model of tumor metastasis to the abdominal peritoneum, the controlled release of NK4 plasmid DNA significantly suppressed the progression of AsPC-1 tumor cells in the peritoneal cavity. In addition, it was effective in significantly suppressing increase in the number and total weight of metastatic nodules. For the tumor-bearing mice receiving the injection of cationized gelatin microspheres incorporating NK4 plasmid DNA, the number of blood vessels in the tumor tissue and the vessel diameter decreased to a significantly greater extent than that of other agents (Fig. **7A**). Moreover, the injected microspheres increased the number of apoptotic cells (Fig. **7B**). It is likely that the controlled release of NK4 plasmid DNA enhanced the NK4 gene expression and prolonged the time period of expression. The NK4 plasmid DNA was expressed around the injected site and the NK4 protein was secreted thereat to the systemic blood circulation (data not shown). Some previous studies revealed that angiogenesis inhibitors suppress the tumor growth based on increase in the apoptosis of tumor cells [115,116]. Taken together, we can say with certainty that the NK4 protein efficiently induced the plasmid DNA released and thus prevented the progression of metastatic tumor cells due to the biological function as an angiogenesis inhibitor, in addition to an HGF antagonist, resulting in prolonged survival of tumor-bearing mice. The present results demonstrate that it is important for successful tumor therapy with plasmid DNA to expose NK4 to tumor cells for a long time period by making use of the controlled release system. It is concluded from our research data that controlled release with cationized gelatin microspheres was a promising

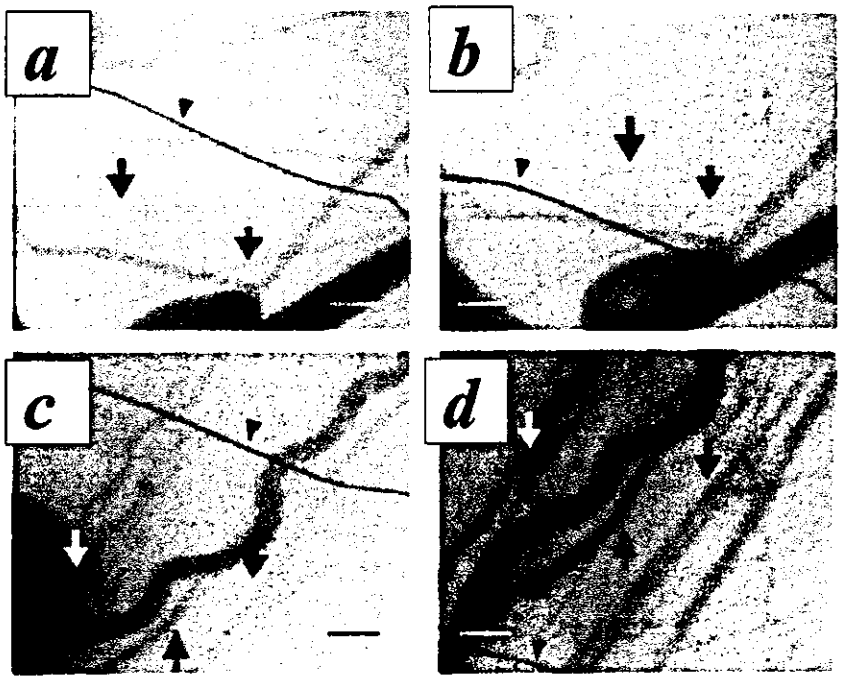


Fig. (5). Representative synchrotron radiation microangiograms of the rabbit hindlimb ischemia. Microangiograms were taken under baseline conditions (a and c) and after repeated adenosine administration (b and d) 38 days after injection of cationized gelatin microspheres incorporating lacZ (a and b) or FGF 4 plasmid DNA (c and d) (4 rabbits/group). Arrows indicate the same point in the vessels. An arrowhead indicates a reference copper wire with a diameter of 130 μm; bar = 1 mm.

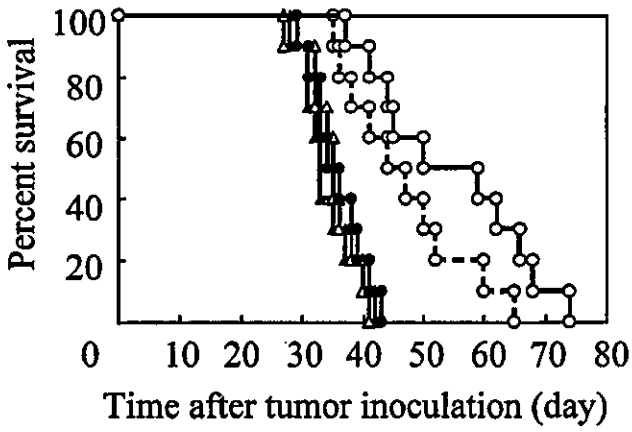
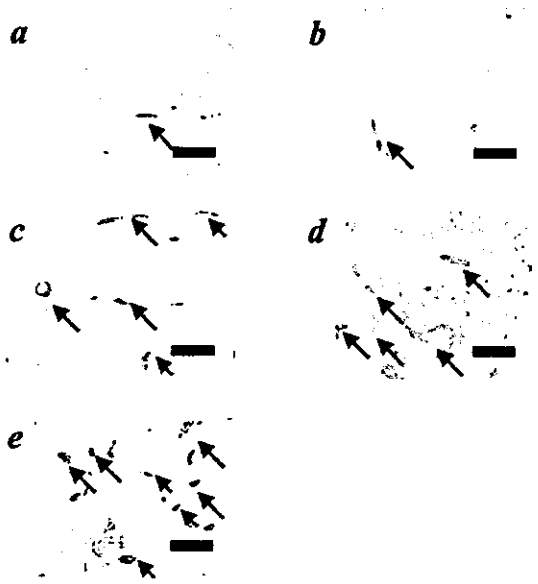


Fig. (6). Survival curves of tumor-bearing mice following the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue: cationized gelatin microspheres incorporating 100 (○—)* and 200 μg of NK4 plasmid DNA (○—)*, 200 μg of free NK4 plasmid DNA (●—), empty cationized gelatin microspheres (Δ), and saline (▲) (10 mice/group). Irrespective of the NK4 plasmid DNA dose, the injection of microspheres incorporating NK4 plasmid DNA significantly prolonged the survival time period of tumor-bearing mice, in contrast to that of free NK4 plasmid DNA. *, $P<0.05$: significant against the survival curve of saline-injected, control mice.

technology to enable plasmid DNA to enhance the *in vivo* biological effects.

In addition to the enhanced efficacy in gene therapy, the controlled release system is effective in genetically manipulating stem cells. Based on 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. [117,118]. However, we cannot apply the viral cell manipulation to clinical therapy since we cannot rule out the toxicity and immunogenicity of viruses themselves. Thus, it is of prime importance to develop a non-viral system capable of the genetic manipulation of cells. When the stem cells have 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 phenomenon enabled the plasmid DNA to enhance the level of gene expression significantly higher than that of virus system. This system will break through the virus-related problems to be resolved for clinical applications. Here, we introduced a new therapeutic concept for cell-based gene delivery. This concept worked very well to therapeutically treat pulmonary hypertension [119] for which there is no effective clinical treatment at present. Endothelial progenitor cells (EPCs) of phagocytic property were isolated and incubated with cationized gelatin microspheres incorporating plasmid DNA of angiogenic adrenomedullin to genetically modify through the

7A)



7B)

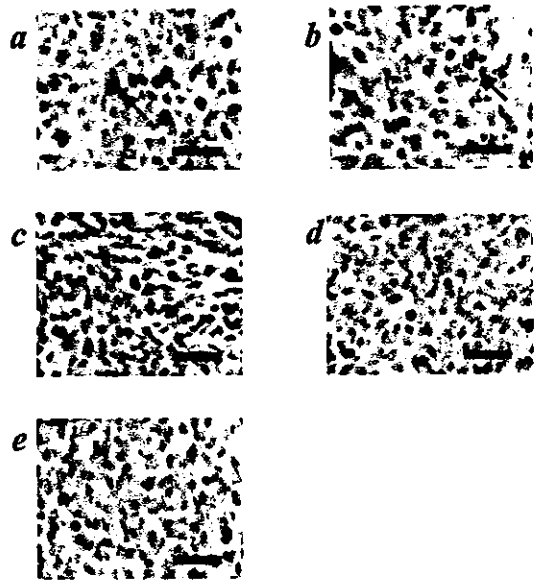


Fig. (7). (A) Immunohistochemical views of blood vessel formation of tumor tissues (*arrows*) 28 days after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue: cationized gelatin microspheres incorporating 100 (a) and 200 μg of NK4 plasmid DNA (b), 200 μg of free NK4 plasmid DNA (c), empty cationized gelatin microspheres (d), and saline (e) (5 mice/group); (magnification; X200); bar = 100 μm.
(B) TUNEL staining of tumor tissues 28 days after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue: cationized gelatin microspheres incorporating 100 (a) and 200 μg of NK4 plasmid DNA (b), 200 μg of free NK4 plasmid DNA (c), empty cationized gelatin microspheres (d), and saline (e) (5 mice/group); (magnification; X400); bar = 50 μm.

transfection of plasmid DNA. Next, the gene-modified EPCs 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 system and highly efficient gene targeting to the ischemic site of disease. The system benefits are due to the ability of EPCs to phagocytose cationized gelatin microspheres capable of plasmid DNA release (Fig. 8) and of positively migrating to the sites of injured endothelium (Fig. 9). When incubated with cationized gelatin microspheres incorporating Green Fluorescent Protein (GFP) plasmid DNA and the GFP plasmid DNA solution, EPCs, not monocytes/macrophages, were strongly transfected to express the GFP protein by the former, a marked contrast to the latter (Fig. 8). Although the Rhodamine B isothiocyanate (RITC)-labeled DNA molecules were mainly distributed rather to the cytoplasm than nucleus, the DNA molecules incorporated in cationized gelatin microspheres were continuously released in the cytoplasm of EPCs after phagocytosis and the cationized gelatin-DNA complexes released were transferred to the nucleus. This is because the microspheres incorporating plasmid DNA enhanced the level of DNA expression. There are several possible reasons why the DNA release was effective. It is possible that polyion complexation with cationized gelatin prevents the plasmid DNA from the enzymatic degradation in the cytoplasm. Moreover, the GFP-expressing EPCs 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 injected EPCs circulate in the blood and target injured pulmonary endothelia in MCT rats. Thus, EPCs 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 EPCs genetically modified by the plasmid DNA of adrenomedullin significantly improved the therapeutic efficacy in the pulmonary hypertension compared with that of original EPCs [119].

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

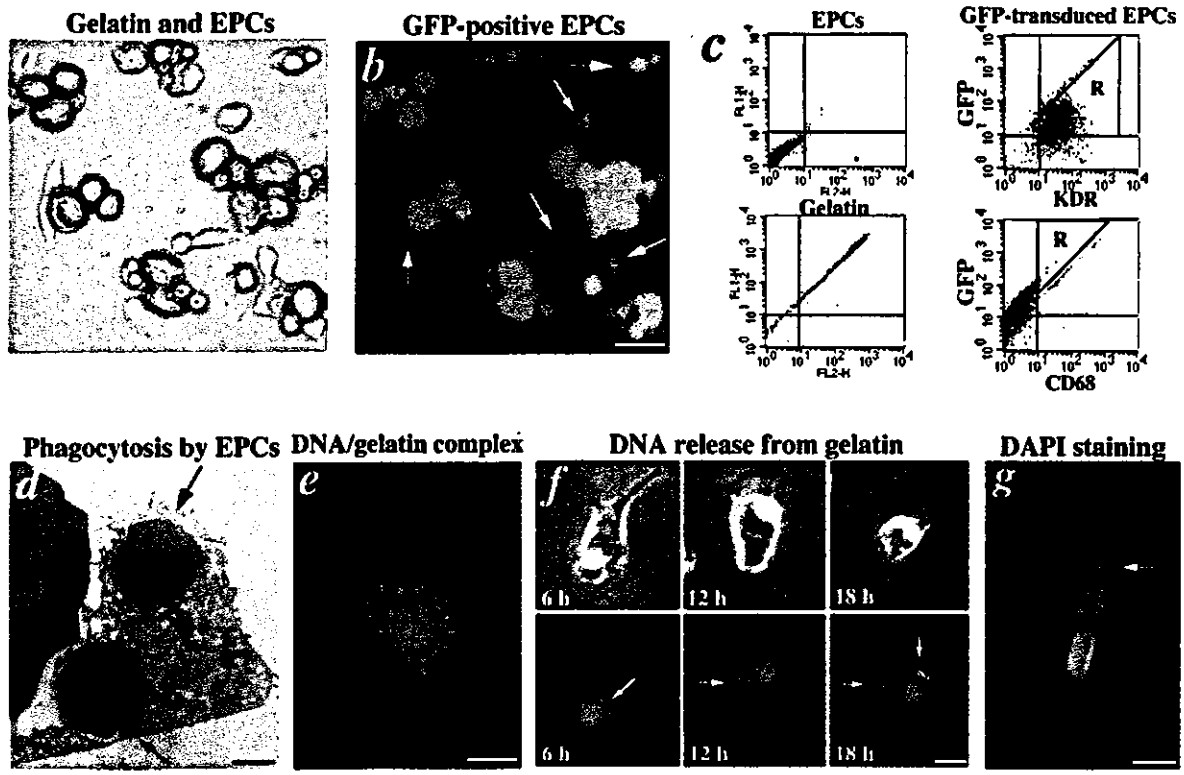


Fig. (8). Ex vivo gene transfer into endothelial progenitor cells (EPCs) based on phagocytosing action. (a) EPCs were cultured with cationized gelatin microspheres incorporating green fluorescent protein (GFP) plasmid DNA. (b) GFP was highly expressed in EPCs (arrows) in the same field as (a). (c) Flow cytometric analyses of EPCs cultured with cationized gelatin microspheres incorporating GFP plasmid DNA. Negative controls (EPC and gelatin background) are shown in left panels. (d) Transmission electron microscopy revealed that EPCs had phagocytosed cationized gelatin microspheres incorporating GFP plasmid DNA (arrows). (e) Rhodamine B isothiocyanate (RITC)-labeled DNA particles were incorporated in cationized gelatin microspheres. (f) RITC-labeled DNA particles (arrows) were released from cationized gelatin microspheres through its degradation. (g) RITC-labeled DNA particles released from cationized gelatin microspheres (arrow) were distributed in the cytoplasm of EPCs. The nuclei of EPCs were identified by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. bar = 10 μm (a and b); 2 μm (d and e); 5 μm (f and g).

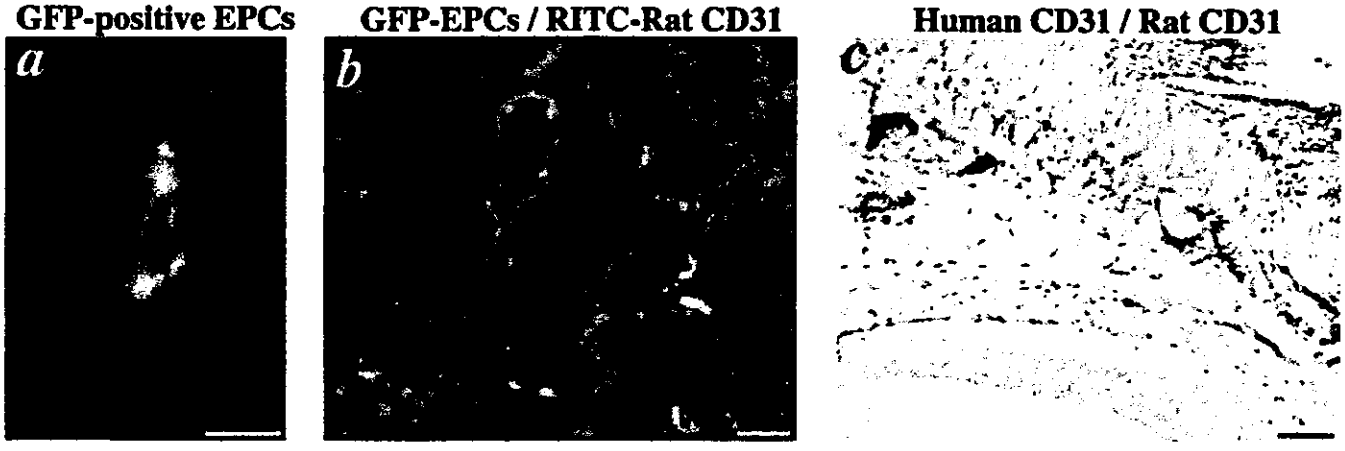


Fig. (9). Distribution of endothelial progenitor cells (EPCs) in the lungs of monocrotaline (MCT)-induced pulmonary hypertension model rats. (a) Intravenously administered green fluorescent protein (GFP)-expressing EPCs were incorporated into the walls of pulmonary arterioles. (b) Transplanted GFP-expressing EPCs were distributed on lung tissues. Pulmonary vasculature was detected by Rhodamine B isothiocyanate (RITC)-conjugated anti-rat CD31. (c) Immunohistochemistry for human CD31 (peroxidase) and rat CD31 (alkaline phosphatase). (8 rats/group); bar = 50 μm.

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 drug delivery.

REFERENCE

[1] Roses, A.D. *Life Sci.*, **2002**, *70*, 1471-80.
[2] Abuin, A.; Holt, K.H.; Platt, K.A.; Sands, A.T.; Zambrowicz, B.P. *Trends Biotechnol.*, **2002**, *20*, 36-42.
[3] Tsai, Y.J.; Hoyme, H.E. *Clin. Genet.*, **2002**, *62*, 257-64.
[4] Hood, L.; Galas, D. *Nature*, **2003**, *421*, 444-8.
[5] Merdan, T.; Kopecek, J.; Kissel, T. *Adv. Drug Deliv. Rev.*, **2002**, *54*, 715-58.
[6] Niidome, T.; Huang, L. *Gene Ther.*, **2002**, *9*, 1647-52.
[7] Tomita, N.; Azuma, H.; Kaneda, Y.; Ogihara, T.; Morishita, R. *Curr. Drug Targets*, **2003**, *4*, 339-46.
[8] Hosseinkhani, H.; Aoyama, T.; Ogawa, O.; Tabata, Y. *Curr. Pharm. Biotechnol.*, **2003**, *4*, 109-22.
[9] Kumar, V.V.; Singh, R.S.; Chaudhuri, A. *Curr. Med. Chem.*, **2003**, *10*, 1297-306.
[10] Lu, Q.L.; Bou-Gharios, G.; Partridge, T.A. *Gene Ther.*, **2003**, *10*, 131-42.
[11] Herweijer, H.; Wolff, J.A. *Gene Ther.*, **2003**, *10*, 453-8.
[12] Scherman, D.; Bigey, P.; Bureau, M.F. *Technol. Cancer Res. Treat.* **2002**, *1*, 351-4.
[13] Houdebine, L.M., *Biochimie*, **2002**, *84*, 1137-41.
[14] Gelse, K.; von der Mark, K.; Schneider, H. *Curr. Gene Ther.*, **2003**, *3*, 305-17.
[15] Buning, H.; Ried, M.U.; Perabo, L.; Gerner, F.M.; Huttner, N.A.; Enssle, J.; Hallek, M. *Gene Ther.*, **2003**, *10*, 1142-51.
[16] Yla-Herttuala, S.; Alitalo, K. *Nat. Med.*, **2003**, *9*, 694-701.
[17] Rainov, N.G.; Kramm, C.M. *Curr. Gene Ther.*, **2001**, *1*, 367-83.
[18] Pooga, M.; Langel, U. *Curr. Cancer Drug Targets*, **2001**, *1*, 231-9.
[19] Gosselin, M.A.; Lee, R.J. *Biotechnol. Annu. Rev.*, **2002**, *8*, 103-31.
[20] Wickham, T.J. *Nat. Med.*, **2003**, *9*, 135-9.
[21] Stacpoole, P.W.; Owen, R.; Flotte, T.R. *Curr. Gene Ther.*, **2003**, *3*, 239-45.
[22] Martin-Rendon, E.; Blake, D.J. *Trends Pharmacol. Sci.*, **2003**, *24*, 178-83.
[23] Lotze, M.T.; Kost, T.A. *Cancer Gene Ther.*, **2002**, *9*, 692-9.
[24] Green, N.K.; Seymour, L.W. *Cancer Gene Ther.*, **2002**, *9*, 1036-42.
[25] Ciardiello, F.; Tortora, G. *Eur. J. Cancer*, **2003**, 39, 348-54.
[26] Theys, J.; Barbe, S.; Landuyt, W.; Nuyts, S.; Van Mellaert, L.; Wouters, B.; Anne, J.; Lambin, P. *Curr. Gene Ther.*, **2003**, *3*, 207-21.
[27] Chang, L.J.; He, J. *Curr. Opin. Mol. Ther.*, **2001**, *3*, 468-75.
[28] Palu, G.; Li Pira, G.; Gennari, F.; Fenoglio, D.; Parolin, C.; Manca, F. *Gene Ther.*, **2001**, *8*, 1593-600.
[29] Fanning, G.; Amado, R.; Symonds, G.J. *Gene Med.*, **2003**, *5*, 645-53.
[30] Zhao, N.; Liu, D.P.; Liang, C.C. *Mol. Biotechnol.*, **2001**, *19*, 229-37.
[31] Grimm, D. *Methods*, **2002**, 28, 146-57.
[32] Groot-Wassink, T.; Aboagye, E.O.; Glaser, M.; Lemoine, N.R.; Vassaux, G. *Hum. Gene Ther.*, **2002**, 13, 1723-35.
[33] Audouy, S.A.; de Leij, L.F.; Hoekstra, D.; Molema, G. *Pharm. Res.*, **2002**, *19*, 1599-605.
[34] Voinea, M.; Simionescu, M. *J. Cell Mol. Med.*, **2002**, *6*, 465-74.
[35] Pedroso de Lima, M.C.; Neves, S.; Filipe, A.; Duzgunes, N.; Simoes, S. *Curr. Med. Chem.*, **2003**, *10*, 1221-31.
[36] Nah, J.W.; Yu, L.; Han, S.O.; Ahn, C.H.; Kim, S.W. *J. Control. Release*, **2002**, *78*, 273-84.
[37] Lee, H.; Jeong, J.H.; Park, T.G. *J. Control. Release*, **2002**, *79*, 283-91.
[38] Molas, M.; Bartrons, R.; Perales, J.C. *Biochim. Biophys. Acta*, **2002**, *1572*, 37-44.
[39] Jeon, E.; Kim, H.D.; Kim, J.S. *J. Biomed. Mater. Res.*, **2003**, *66A*, 854-9.
[40] Remy-Kristensen, A.; Clamme, J.P.; Vuilleumier, C.; Kuhry, J.G.; Mely, Y. *Biochim. Biophys. Acta*, **2001**, *1514*, 21-32.
[41] Wightman, L.; Kircheis, R.; Rossler, V.; Carotta, S.; Ruzicka, R.; Kursa, M.; Wagner, E. *J. Gene Med.*, **2001**, *3*, 362-72.
[42] Benns, J.M.; Maheshwari, A.; Furgeson, D.Y.; Mahato, R.I.; Kim, S.W. *J. Drug Target.*, **2001**, *9*, 123-39.
[43] Sagara, K.; Kim, S.W. *J. Control. Release*, **2002**, *79*, 271-81.
[44] Lee, H.; Kim, T.H.; Park, T.G. *J. Control. Release*, **2002**, *83*, 109-19.
[45] Kursa, M.; Walker, G.F.; Roessler, V.; Ogris, M.; Roedl, W.; Kircheis, R.; Wagner, E. *Bioconjug. Chem.*, **2003**, *14*, 222-31.
[46] Gonzalez, H.; Hwang, S.J.; Davis, M.E. *Bioconjug. Chem.* **1999**, *10*, 1068-74.
[47] Rungsardthong, U.; Ehtezazi, T.; Bailey, L.; Armes, S.P.; Garnett, M.C.; Stolnik, S. *Biomacromolecules*, **2003**, *4*, 683-90.
[48] Cotton, M.; Wagner, E. *Curr. Opin. Biotechnol.*, **1993**, *4*, 705-10.
[49] Choi, Y.H.; Liu, F.; Choi, J.S.; Kim, S.W.; Park, J.S. *Hum. Gene Ther.*, **1999**, *10*, 2657-65.
[50] Li, S.; Tan, Y.; Viroonchatapan, E.; Pitt, B.R.; Huang, L. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **2000**, *278*, L504-11.
[51] Orson, FM.; Kinsey, BM.; Hua, P.J.; Bhogal, B.S.; Densmore, C.L.; Barry, M.A. *J. Immunol.*, **2000**, *164*, 6313-21.
[52] Fortunati, E.; Ehlert, E.; van Loo, N.D.; Wyman, C.; Eble, J.A.; Grosveld, F.; Scholte, B.J. *Gene Ther.*, **2000**, *7*, 1505-15.
[53] Schatzlein, A.G. *Anticancer Drugs*, **2001**, *12*, 275-304.
[54] Mastrobattista, E.; Kapel, R.H.; Eggenhuisen, M.H.; Roholl, P.J.; Crommelin, D.J.; Hennink, W.E.; Storm, G. *Cancer Gene Ther.*, **2001**, *8*, 405-13.
[55] Guillem, V.M.; Tormo, M.; Revert, F.; Benet, I.; Garcia-Conde, J.; Crespo, A.; Alino, S.F. *J. Gene Med.*, **2002**, 4, 170-82.
[56] Backer, M.V.; Aloise, R.; Przekop, K.; Stoletov, K.; Backer, J.M. *Bioconjug. Chem.*, **2002**, 13, 462-7.
[57] Hofland, H.E.; Masson, C.; Iginla, S.; Osetinsky, I.; Reddy, J.A.; Leamon, C.P.; Scherman, D.; Bessodes, M.; Wils, P. *Mol. Ther.* **2002**, 5, 739-44.
[58] Dauty, E.; Remy, J.S.; Zuber, G.; Behr, J.P. *Bioconjug. Chem.*, **2002**, *13*, 831-9.
[59] Kursa, M.; Walker, G.F.; Roessler, V.; Ogris, M.; Roedl, W.; Kircheis, R.; Wagner, E. *Bioconjug. Chem.* **2003**, *14*, 222-31.
[60] Ross, J. F.; Chaudhuri, P.K.; Ratnam, M. *Cancer*, **1994**, *73*, 2432-43.
[61] Zanta, M.A.; Boussif, O.; Adib, A.; Behr, J.P. *Bioconjug. Chem.*, **1997**, *8*, 839-44.
[62] Bandyopadhyay, P.; Ma, X.; Linehan-Stieers, C.; Kren, B.T.; Steer, C.J. *J. Biol. Chem.*, **1999**, *274*, 10163-72.
[63] Hisayasu, S.; Miyauchi, M.; Akiyama, K.; Gotoh, T.; Satoh, S.; Shimada, T. *Gene Ther.*, **1999**, *6*, 689-93.
[64] Nishikawa, M.; Yamauchi, M.; Morimoto, K.; Ishida, E.; Takakura, Y.; Hashida, M. *Gene Ther.*, **2000**, *7*, 548-55.
[65] Lim, D.W.; Yeom, Y.I.; Park, T.G. *Bioconjug. Chem.*, **2000**, *11*, 688-95.
[66] Wu, J.; Nantz, M.H.; Zern, M.A. *Front. Biosci.*, **2002**, 7, d717-25.
[67] Hosseinkhani, H.; Aoyama, T.; Ogawa, O.; Tabata, Y. *J. Control. Release*, **2002**, *83*, 287-302
[68] Klibanov, A.L.; Maruyama, K.; Torchilin, V.P.; Huang,L. *FEBS Lett.* **1990**, *268*, 235–238.
[69] Torchilin, V.P.; Omelyanenko, V.G.; Papisov, M.I.; Bogdanov, A.A. Jr.; Trubetskoy, V.S.; Herron, J.N.; Gentry, C.A. *Biochim. Biophys. Acta* **1994**, *1195*, 11-20.
[70] Torchilin, V.P.; Shtilman, M.I.; Trubetskoy, V.S.; Whiteman, K.; Milstein, A.M. *Biochim. Biophys. Acta* **1994**, *1195*, 181-184.
[71] Zhang, Y.P.; Sekirov, L.; Saravolac, E.G.; Wheeler, J.J.; Tardi, P.; Clow, K.; Leng, E.; Sun, R.; Cullis, P.R.; Scherrer, P. *Gene Ther.*, **1999**, *6*, 1438-47.
[72] Monck, M.A.; Mori, A.; Lee, D.; Tam, P.; Wheeler, J.J.; Cullis, P.R.; Scherrer, P. *J. Drug Target.*, **2000**, *7*, 439-52.
[73] Song, L.Y.; Ahkong, Q.F.; Rong, Q.; Wang, Z.; Ansell, S.; Hope, M.J.; Mui, B. *Biochim. Biophys. Acta*, **2002**, *1558*, 1-13.
[74] Oupicky, D.; Ogris, M.; Howard, K.A.; Dash, P.R.; Ulbrich, K.; Seymour, L.W. *Mol. Ther.*, **2002**, *5*, 463-72.
[75] Roy, K.; Wang, D.; Hedley, M.L.; Barman, S.P. *Mol. Ther.*, **2003**, *7*, 401-8.
[76] Murphy, W.L.; Mooney, D.J. *J. Periodontal Res.*, **1999**, 34, 413-9.
[77] Shea, L.D.; Smiley, E.; Bonadio, J.; Mooney, D.J. *Nat. Biotechnol.*, **1999**, *17*, 551-54.

[78] Wang, D.; Robinson, D.R.; Kwon, G.S.; Samuel, J. *J. Control. Release*, **1999**, *57*, 9-18.
[79] Capan, Y.; Woo, B.H.; Gebrekidan, S.; Ahmed, S.; DeLuca, P.P. *Pharm. Res.* **1999**, *16*, 509-13.
[80] Luo, D.; Woodrow-Mumford, K.; Belcheva, N.; Saltzman, W.M. *Pharm. Res.*, **1999**, *16*, 1300-8.
[81] Hedley, M.L. *Expert. Opin. Biol. Ther.*, **2003**, *3*, 903-10.
[82] Jang, J.H.; Shea, L.D. *J. Control. Release*, **2003**, *86*, 157-68.
[83] Ismail, F.A.; Napaporn, J.; Hughes, J.A.; Brazeau, G.A. *Pharm. Dev. Technol.*, **2000**, *5*, 391-7.
[84] Perez, C.; Sanchez, A.; Putnam, D.; Ting, D.; Langer, R.; Alonso, M.J. *J. Control. Release*, **2001**, *75*, 211-24.
[85] Wang, J.; Zhang, P.C.; Mao, H.Q.; Leong, K.W. *Gene Ther.* **2002**, *9*, 1254-61.
[86] Lim, Y.B.; Han, S.O.; Kong, H.U.; Lee, Y.; Park, J.S.; Jeong, B.; Kim, S.W. *Pharm. Res.*, **2000**, *17*, 811-6.
[87] Park, J.S.; Oh, Y.K.; Yoon, H.; Kim, J.M.; Kim, C.K. *J. Biomed. Mater. Res.*, **2002**, *59*, 144-51.
[88] Shen, H.; Goldberg, E.; Saltzman, W.M. *J. Control. Release*, **2003**, *86*, 339-48.
[89] Megeed, Z.; Cappello, J.; Ghandehari, H. *Pharm. Res.*, **2002**, *19*, 954-9.
[90] Perlstein, I.; Connolly, J.M.; Cui, X.; Song, C.; Li, Q.; Jones, P.L.; Lu, Z.; DeFelice, S.; Klugherz, B.; Wilensky, R.; Levy, R.J. *Gene Ther.* **2003**, *10*, 1420-8.
[91] Ochiya, T.; Takahama, Y.; Nagahara, S.; Sumita, Y.; Nishida, A.; Itoh, H.; Nagai, Y.; Terada, M. *Nat. Med.* **1999**, *5*, 707-10.
[92] Ochiya, T.; Nagahara, S.; Sano, A.; Itoh, H.; Terada, M. *Curr. Gene Ther.* **2001**, *1*, 31-52.
[93] Fukunaka, Y.; Iwanaga, K.; Morimoto, K.; Kakemi, M.; Tabata, Y. *J. Control. Release*, **2002**, *80*, 333-43.
[94] Kushibiki, T.; Tomoshige, R.; Fukunaka, Y.; Kakemi, M.; Tabata, Y. *J. Control. Release*, **2003**, *90*, 207-16.
[95] Bonadio, J. *J. Mol. Med.*, **2000**, *78*, 303-11.
[96] Bonadio, J.; Smiley, E.; Patil, P.; Goldstein, S. *Nat. Med.*, **1999**, *5*, 753-9.
[97] Stenzel, K.H.; Miyata, T.; Rubin, A.L. *Annu Rev Biophys Bioeng.*, **1974**, *3*, 231-53.
[98] DeLustro, F.; Condell, R.A.; Nguyen, M.A.; McPherson, J.M. *J. Biomed. Mater. Res.*, **1986**, *20*, 109-20.
[99] Veis, A. *Int. Rev. Connect. Tissue Res.* **1965**, *3*, 113-200.
[100] Tabata, Y.; Ikada, Y. *Adv. Drug Deliv. Rev.* **1998**, *31*, 287-301.
[101] Yamamoto, M.; Tabata, Y.; Ikada, Y. *J. Biomater. Sci. Polym. Ed.* **1998**, *9*, 439-458.
[102] Yamamoto, M.; Tabata, Y.; Hong, L.; Miyamoto, S.; Hashimoto, N., Ikada, Y. *J. Control. Release*, **2000**, *64*, 133-42.
[103] Ozeki, M.; Ishii, T.; Hirano, Y.; Tabata, Y. *J. Drug Target.* **2001**, *9*, 461-71.
[104] Aoyama, T.; Hosseinkhani, H.; Yamamoto, S.; Ogawa, O.; Tabata, Y. *J. Control. Release*, **2002**, *80*, 345-56.
[105] Hosseinkhani, H.; Aoyama, T.; Ogawa, O.; Tabata, Y. *J. Drug Target.*, **2002**, *10*, 193-204.
[106] Hosseinkhani, H.; Aoyama, T.; Yamamoto, S.; Ogawa, O.; Tabata, Y. *Pharm. Res.*, **2002**, *19*, 1471-9.
[107] Mullen, P.M.; Lollo, C.P.; Phan, Q.C.; Amini, A.; Banaszczyk, M.G.; Fabrycki, J.M.; Wu, D.; Carlo, A.T.; Pezzoli, P.; Coffin, C.C.; Carlo, D.J. *Biochim. Biophys. Acta* **2000**, *1523*, 103-10.
[108] Moret, I.; Esteban Peris, J.; Guillem, V.M.; Benet, M.; Revert, F.; Dasi, F.; Crespo, A.; Alino, S.F. *J. Control. Release*, **2001**, *76*, 169-81.
[109] Park, S.; Healy, K.E. *Bioconjug Chem.* **2003**, *14*, 311-9.
[110] Kasahara, H.; Tanaka, E.; Fukuyama, N.; Sato, E.; Sakamoto, H.; Tabata, Y.; Ando, K.; Iseki, H.; Shinozaki, Y.; Kimura, K.; Kuwabara, E.; Koide, S.; Nakazawa, H.; Mori, H. *J. Am. Coll. Cardiol.* **2003**, *41*, 1056-62.
[111] Date, K.; Matsumoto, K.; Shimura, H.; Tanaka, M.; Nakamura, T. *FEBS Lett.* **1997**, *420*, 1-6.
[112] Date, K.; Matsumoto, K.; Kuba, K.; Shimura, H.; Tanaka, M.; Nakamura, T. *Oncogene* **1998**, *17*, 3045-54.
[113] Kuba, K.; Matsumoto, K.; Date, K.; Shimura, H.; Tanaka, M.; Nakamura, T. *Cancer Res.* **2000**, *60*, 6737-43.
[114] Kuba, K.; Matsumoto, K.; Ohnishi, K.; Shiratsuchi T.; Tanaka, M.; Nakamura, T. *Biochem. Biophys. Res. Commun.* **2000**, *279*, 846-52.
[115] Holmgren, L.; O'Reilly, M.S.; Folkman, J. *Nat. Med.* **1995**, *1*, 149-53.
[116] O'Reilly, M.S.; Holmgren, L.; Chen, C.; Folkman, J. *Nat. Med.* **1996**, *2*, 689-92.
[117] Orwig, K.E.; Avarbock, M.R.; Brinster, R.L. *Biol. Reprod.* **2002**, *67*, 874-9.
[118] Lee, J.H.; Klein, H.G. *Hematol. Oncol. Clin. North. Am.* **1995**, *9*, 91-113.
[119] Nagaya, N.; Kangawa, K.; Kanda, M.; Uematsu, M.; Horio, T.; Fukuyama, N.; Hino, J.; Harada-Shiba M.; Okumura, H.; Tabata, Y.; Mochizuki, N.; Chiba, Y.; Nishioka, K.; Miyatake, K.; Asahara, T.; Hara, H.; Mori, H. *Circulation* **2003**, *108*, 889-895.

Received: September 24, 2003 Accepted: November 04, 2003

Mini Review

生体吸収性カチオン化ゼラチンハイドロゲルを用いたプラスミドDNAの徐放化とその生物活性の増強
ーNK4プラスミドDNAの腫瘍転移抑制効果を例としてー

櫛引俊宏, 友重龍治, 田畑泰彦

京都大学再生医科学研究所 生体組織工学研究部門生体材料学分野

Controlled release of plasmid DNA by cationized gelatin hydrogel

Recent rapid development of molecular biology together with the steady progress of genome projects has been given us some essential and revolutionary informations of gene to elucidate all the biological phenomena at the molecular level. Under these circumstances, gene transfection has become one of the fundamental technologies indispensable to the basic research of medicine and biology. Human gene therapy has been performed with plasmid DNA alone or the virus vector constructs. However, there are clinical limitations, low gene expression of plasmid DNA and the immunogenicity and toxicity of virus itself or the possible mutagenesis of cells transfected. Therefore, several non-viral vectors of synthetic materials have been explored to enhance the transfection efficiency of gene into mammalian cells both *in vitro* and *in vivo*. In this paper, as one research trial, the controlled release of plasmid DNA is overviewed. A new system of plasmid DNA release with a biodegradable hydrogel is explained while the biological activity of a plasmid DNA of hepatocyte growth factor antagonist, NK4, is augmented by use of the release system.

Rec.3/18/2004, Acc.5/21/2004, pp634-641

Toshihiro Kushibiki, Ryuji Tomoshige, Yasuhiko Tabata

Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University

Key words controlled release, plasmid DNA, cationized gelatin, gene expression, NK4

近年の遺伝子治療に関する技術の進展は目覚しいものがある．1980年代の遺伝性疾患に始まり，悪性腫瘍・自己免疫性疾患に対して遺伝子治療の臨床応用が開始され，欠損遺伝子や変異遺伝子を補充する治療や生体が本来有している治癒力を補う治療も行われている[1]．これら遺伝子治療において，生体内で細胞へ安全に，かつ効率的に遺伝子を導入する方法の開発は重要な課題である．また，遺伝子導入は，遺伝子治療のみならず，遺伝子改変細胞を用いた細胞療法[2]や再生医療[3,4]，分子生物学など，広い分野に対しても必要な研究技術である． 遺伝子の細胞内への導入方法としては，ベクターと呼ばれる遺伝子の運搬体を用いるのが一般的であり，ウイルスを用いる方法と非ウイルスを用いる方法とに大別できる．前者はアデノウイルスやレトロウイルスなどが主として用いられ，遺伝子導入効率が高いという利点があるが，その免疫原性や体内動態をコントロールできないという問題がある[5]．これに対して，プ

ラスミドDNA水溶液を直接投与する方法は，遺伝子導入効率においてはウイルスに劣るものの，安全性の点で優れている[5]．しかしながら，水溶液投与されたプラスミドDNAは，細胞に導入される前に生体内に存在する核酸分解酵素などによって分解，失活するため，プラスミドDNAを大量投与しなければ期待する効果を得ることができないことも多い．また，プラスミドDNAなどの核酸分子は，その分子がマイナスに帯電しているので，そのままではマイナスの電荷を有している細胞表面に付着できず，細胞内にも導入されにくいと考えられている．

そこでこれらの問題を解決する方法の一つとして，cationic liposomeやpolyethyleneimineなどのカチオン性の合成高分子を用いた遺伝子導入方法が数多く研究，報告されている[5]．カチオン性高分子とのポリイオンコンプレックスにより，プラスミドDNAの見かけの分子サイズが減少するとともに，コンプレックスの表面電荷がプラスになり，プラスミドDNAの細胞表面への付着，その結果として起こる細胞内への導入が容易になると考えられている[6]．さらに，エンドサイトーシスにより細胞内のエンドソーム内に導入された塩基性のコンプレックスを中和するために，エンドソーム内へ水分子とともにプロトンが供給される結果，エンドソームが破壊され，プラスミドDNAが細胞質内に放出される（buffering効果）ことでプラスミドDNAの核内への導入を促進することが報告されている[7]．

遺伝子治療を臨床的に有用な治療手段とするためには，細胞内に導入された遺伝子が長期間にわたり安定にタンパク質を発現することが必要である．例えば，これまでの研究報告から，ウイルス性ベクターを用いてプラスミドDNAを細胞内に導入した場合にも，ある程度のタンパク質の持続的な発現が認められるものの，その発現期間を人為的にコントロールすることは難しいことがわかる．遺伝子発現レベルとその期間とをコントロールする一つの方法として，Drug Delivery System (DDS)の徐放技術の利用が考えられる．例えば，高分子担体内にプラスミドDNAを包含，徐放することによって，遺伝子発現レベルやその発現期間をコントロールできることが報告されている(表)[8-26]．これらの徐放システムにより，プラスミドDNAの徐放が達成されている．しかしながら，これらのシステムでは，上述したような遺伝子導入効率を高めるためのプラスミドDNAの分子サイズとその電荷についての工夫は加えられていない．

そこで，私たちは，これらの点を考慮したプラスミドDNAの徐放システムを考案している[25,26]．すなわち，プラスミドDNAとポリイオンコンプレックス形成ができるカチオン化高分子からプラスミドDNAの徐放化担体を作る．担体の分解によりプラスミドDNAは徐放される．このシステムでは，プラスミドDNAはカチオン化高分子とコンプレックスを形成していることから，その分子サイズは低下し，かつ負電荷が中和された状態でプラスミドDNAは徐放される．徐放担体のための高分子としてゼラチンを用い，それにアミノ基を導入することでカチオン化ゼラチンを作製した．

ゼラチンは，長年にわたり外科用材料や医薬品添加物として医療に用いられてきた材料であり，その生体安全性と適合性とは立証されている．また，ゼラチンは化学修飾によりその物理化学的性質を変化させ，種々の生理活性物質と親和性を持たせることができる．これまでに，私たちは，ある種類のゼラチンを架橋して作製したハイドロゲルを利用することで，塩基性線維芽細胞増殖因子(bFGF)[27]，肝細胞増殖因子(HGF)[28]や形質転換成長因子β(TGFβ)[29]などの生理活性物質を生物活性を持った状態で徐放できることを報告している．このシステムでは，生理活性物質がゼラチン分子との物理的相互作用力によってゼラチンハイドロゲル内に固定化されている．ハイドロゲルが分解され，それに伴うゼラチン分子の水可溶化によって，固定化生理活性物質は徐放される[27]．すなわち，このハイドロゲルシステムでは，徐放担体の分解性を変化させることによって，生理活性物質の徐放性をコントロールすることができる[27]．

本稿では，このハイドロゲルシステムによるプラスミドDNAの徐放化について概説するとともに，具体例を示しながらプラスミドDNAの徐放化が，その生理活性物質の発現に有効であることを強調する．まず，プラスミドDNAとポリイオンコンプレックスを形成させるために，ゼラチンをカチオン化する．このカチオン化ゼラチンからなるハイドロゲルを作製し，そのハイドロゲル担体内にプラスミドDNAを物理化学的に固定化した．ハイドロゲル徐放担体の分解に伴うカチオン化ゼラチン分子の水可溶化により，固定化プラスミドDNAは徐放する．このプラスミドDNAの徐放化システムのアイデアと，このシステムを用いたプラスミドDNAの徐放がその発現に与える効果について紹介する．

プラスミドDNAとして，lac ZとNK4のプラスミドDNAを用いた．NK4はHGFのN末端ヘアピン構造と4つのkringle構造からなるHGFのアンタゴニストである．すでに，NK4にはHGFにより促進される腫瘍細胞の浸潤と転移を抑制する作用のあることが報告されている[30]．しかしながら，体内で不安定なNK4タンパク質あるいはその遺伝子を水溶液として体内に投与するだけではその生物作用は必ずしも期待できない．そこで，NK4の生物活性

表 Research reports on the controlled release of plasmid DNA

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[8] Shea et al[9] Wang et al[10] Capan et al[11] Luo et al[12] Hedley et al[13] Jang et al[14]
Polymethacrylic acid (PMA) and polyethylene glycol (PEG), hydroxypropylmethylcellulose-carbopol		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[15]
Poly(lactic acid)-poly(ethylene glycol) (PLA-PEG)	Luciferase	Release plasmid DNA from nanoparticles in a controlled manner.	Perez et al[16]
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[17]
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 polycation for gene delivery.	Lim et al[18]
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[19]
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[20]
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[21]
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[22]
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[23,24]
Gelatin	β-Galactosidase	Plasmid DNA release period can be regulated only by changing the hydrogel degradability.	Fukunaka et al[25] Kushibiki et al[26]

を発現させるための一つの方法として，カチオン化ゼラチンハイドロゲルからのNK4プラスミドDNAの徐放化を行い，その生物活性について検討した[31]．

カチオン化ゼラチンハイドロゲルからのプラスミドDNAの徐放

ゼラチン分子のカルボキシル基にエチレンジアミンを化学的に導入することによりカチオン化ゼラチンを作製した[25,26]．このカチオン化ゼラチンをグルタルアルデヒドにて化学架橋することでハイドロゲルを作製した．その際，グルタルアルデヒド濃度を変化させることでハイドロゲルの含水率を変化させることができる．このカチオン化ゼラチンハイドロゲルをマウス大腿筋組織内に埋入後，生体内における残存率を経時的に測定した結果，初期の分解速度

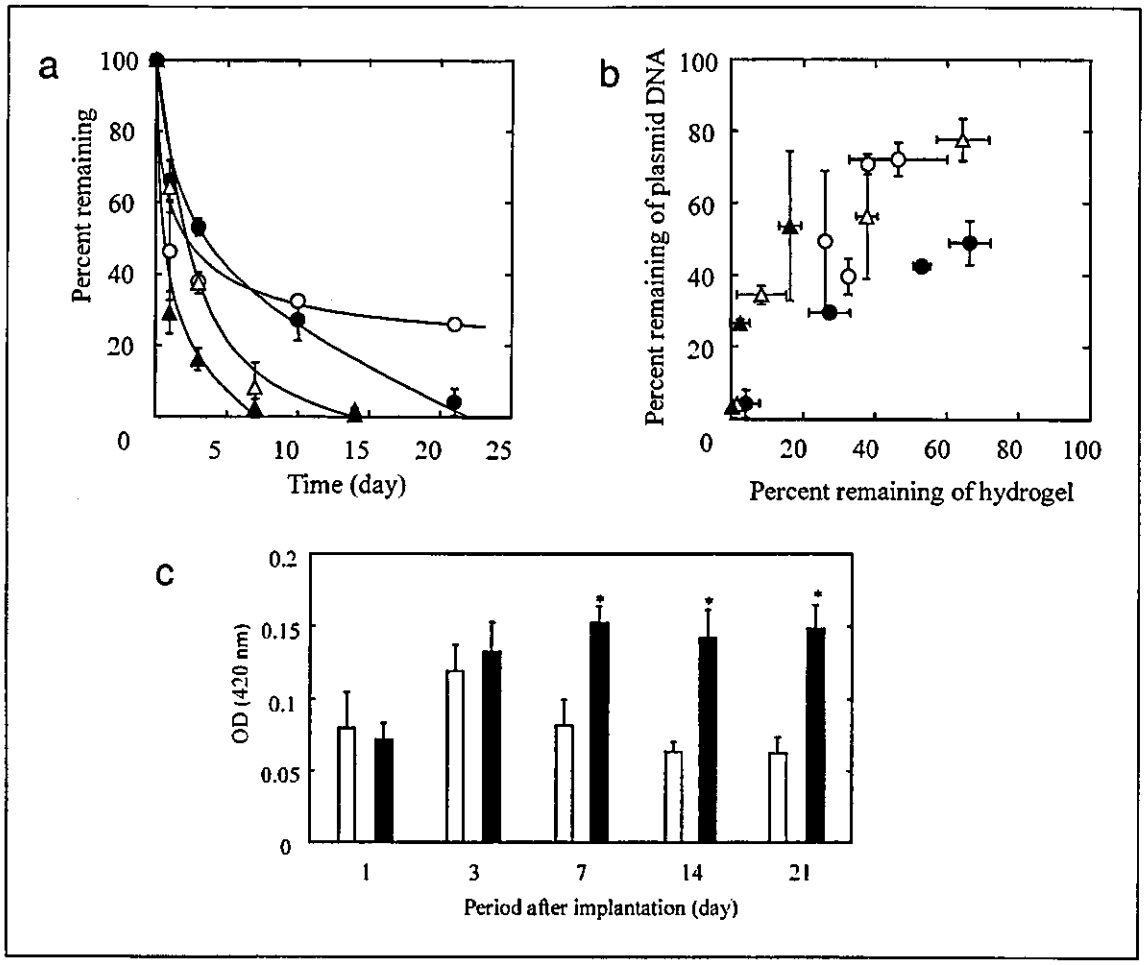


図1

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

は同じであるものの，カチオン化ゼラチンハイドロゲルの含水率を変化させることで，生体内残存期間をコントロールすることができることがわかった（図1a）．また，このカチオン化ゼラチンハイドロゲルにlac Z プラスミドDNAを含浸固定した後，マウス大腿筋組織内に埋入した．lac Z プラスミドDNAの筋組織内残存性を評価したところ，カチオン化ゼラチンハイドロゲル担体の分解とともにlacZ プラスミドDNAは徐放され，lac Z プラスミドDNA水溶液投与と比較して，その生体内残存期間が延長した．また，カチオン化ゼラチンハイドロゲルの含水率，つまり生体残存性をコントロールすることでlac Z プラスミドDNAの徐放期間をコントロールすることができた（図1b）．さらに，lac Z プラスミドDNAを徐放することによって，lac Z プラスミドDNA水溶液投与時と比較して，発現効率の増加と発現期間の延長が認められた（図1c）[25]．

lac Z プラスミドDNA分子はハイドロゲル内でカチオン

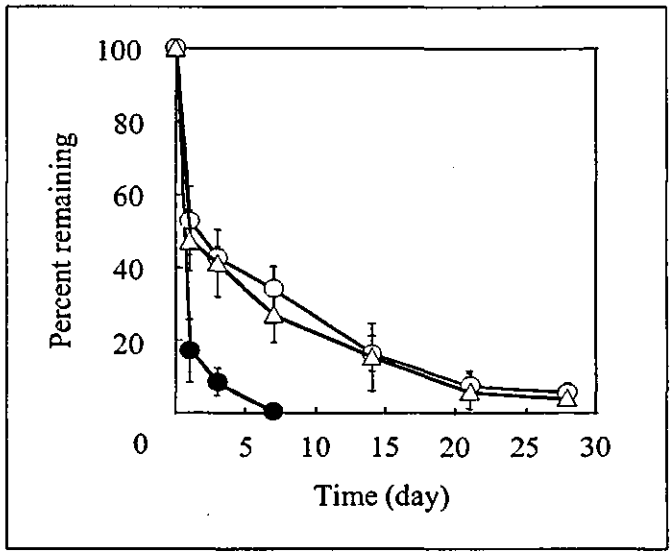


図2
The time course of radioactivity remaining of cationized gelatin microspheres incorporating ^{125}I-labeled NK4 plasmid DNA (○) and free ^{125}I-labeled NK4 plasmid DNA (●) or ^{125}I-labeled cationized gelatin microspheres (△) after the subcutaneous injection into the back of mice.

化ゼラチン分子とポリイオンコンプレックスを形成しているため，プラスミドDNAはハイドロゲルが分解しない状態では徐放されない．すなわち，担体ハイドロゲルが分解されることにより生じた水可溶性のカチオン化ゼラチンフラグメントとコンプレックスされた状態でプラスミドDNAは徐放されていると考えられる．また，蛍光試薬により各々を標識したカチオン化ゼラチン分子とプラスミドDNA分子のコンプレックスが，コンプレックスを形成した状態で細胞内へ移行していることも確認された[25]．徐放されたプラスミドDNA分子がカチオン化ゼラチン分子とコンプレックスを形成しているため，プラスミドDNA分子の負電荷は中和され，プラスミドDNA自身と比べて負電荷を持つ細胞表面と相互作用しやすくなり，プラスミドDNAの細胞内への導入も容易になっていると考えられる[26]．また，エンドサイトーシスにより細胞内のエンドソーム内に導入されたカチオン化ゼラチン分子とプラスミドDNA分子のコンプレックスがbuffering効果を誘導することでプラスミドDNAの核内への導入を促進することも報告している[27]．さらに，徐放することにより，細胞周辺局所でのプラスミドDNA濃度が維持される結果，プラスミドDNAの細胞内への導入効率も増加すると考えられる．

このカチオン化ゼラチンハイドロゲルを用いたプラスミドDNA徐放システムは，これまで報告されている徐放担体からの拡散によるプラスミドDNAの徐放とは異なっている．つまり，徐放されたプラスミドDNAがポリイオンコンプレックスを形成していること，徐放期間および発現期間のコントロールができるという点において新しい徐放システムである．また，ハイドロゲルの形によらず，injectableなカチオン化ゼラチンマイクロスフェアを用いてもプラスミドDNAを徐放することができる．

カチオン化ゼラチンマイクロスフェアを用いた徐放化NK4プラスミドDNAによる抗腫瘍効果の増強

肺に転移することが知られているLewis Lung Carcinoma (LLC)細胞をマウス背部皮下に接種し，4日後，放射ラベル化したカチオン化ゼラチンマイクロスフェアおよび放射ラベル化NK4プラスミドDNAを含浸固定したカチオン化ゼラチンマイクロスフェアを腫瘍塊近傍に投与した．皮下投与されたNK4プラスミドDNA水溶液は投与部位より速やかに消失した．これに対して，カチオン化ゼラチンマイクロスフェアおよびそれに含浸されたNK4プラスミドDNAの放射活性は，時間とともに4週間にわたって投与部位から徐々に減少した（図2）．カチオン化ゼラチンマイクロスフェアおよびNK4プラスミドDNAの消失パターンが時間的によく一致したことから，NK4プラスミドDNAがマイクロスフェアの分解とともに*in vivo*で徐放されたことを示している．また，NK4プラスミドDNAを含有するカチオン化ゼラチンマイクロスフェア投与群のマウス生存期間は，他のコントロール群と比較して，有意に増加した（図3）．HGFのアンタゴニストとして発現したNK4タンパク質が腫瘍細胞表面に存在するHGF receptor (c-Met)に結合することで，HGF依存性の腫瘍細胞の浸潤・転移を抑制したと考えられた．NK4プラスミドDNAを含有するカチオン化ゼラチンマイクロスフェア投与群の腫瘍組織中の血管数および血管径においても，他のコントロール群と比較して有意な減少が認められた（図4a）．

NK4は，HGF依存性の腫瘍細胞の浸潤・転移を抑制す

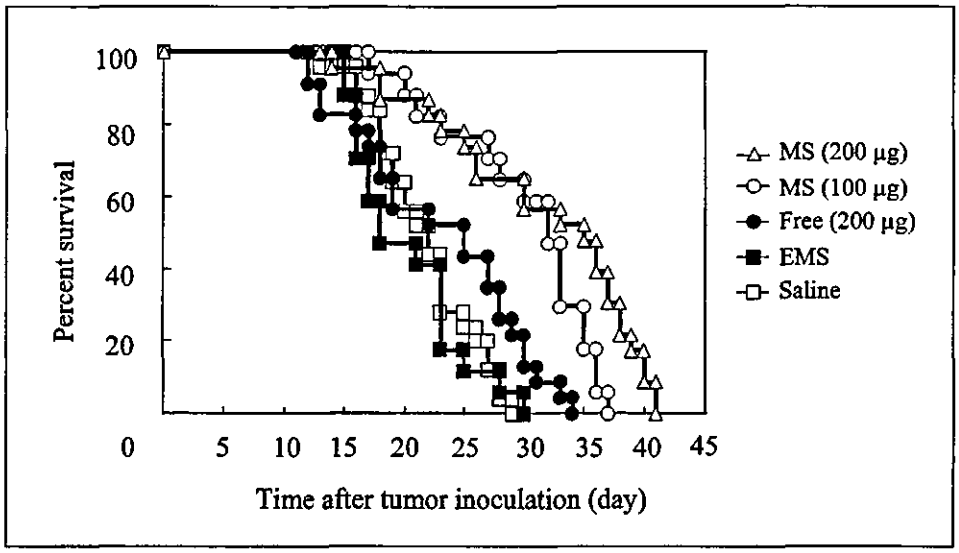


図3

Survival curves of tumor-bearing mice following the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue around the tumor mass: cationized gelatin microspheres incorporating 100 [MS (100 μg)]* and 200 μg of NK4 plasmid DNA [MS (200 μg)]*, 200 μg of free NK4 plasmid DNA [free (200 μg)], empty cationized gelatin microspheres (EMS), and saline. *$p < 0.05$: significant against the survival curve of saline-injected, control mice (Kaplan-Mayer method).

ることだけではなく，bFGFや血管内皮増殖因子(VEGF)による新生血管の形成を阻害することが知られている[33]．このNK4の作用により，腫瘍組織中の血管数および血管径の減少が認められたと考えられた．NK4プラスミドDNAを含有するカチオン化ゼラチンマイクロスフェア投与群では腫瘍組織中のアポトーシス細胞数も有意に増加した(図4b)．発現したNK4タンパク質による腫瘍組織中の新生血管形成が抑制された結果，腫瘍細胞のアポトーシスが誘導されたと考えられた．

NK4プラスミドDNAを徐放しているカチオン化ゼラチンマイクロスフェアを腫瘍塊近傍に投与することで，長期間，NK4プラスミドDNAが腫瘍細胞近傍に存在している．このことは，腫瘍細胞内へプラスミドDNAの導入効率が増加した理由の一つであると考えられる．

おわりに

カチオン化ゼラチンハイドロゲルまたはカチオン化ゼラチンマイクロスフェアを用いてプラスミドDNAを徐放化することにより，プラスミドDNAの発現を持続させることが可能となった．このカチオン化ゼラチンを用いた徐放システムは，プラスミドDNAの種類に関係なく応用可能であることから，種々の疾病の遺伝子治療に有用なDDS手段になると考えられる．

謝　辞

本研究に際して，ご指導ご鞭撻をいただきました大阪大学大学院医学研究科 中村敏一教授，松本邦夫助教授に深甚なる謝意を表します．さらに，種々の貴重なご助言を賜りました京都大学再生医科学研究所 生体材料学分野研究室員の諸氏に深く感謝いたします．

文　献

1) 遺伝子医学別冊：これだけは知っておきたい遺伝子医学の基礎知識，本庶 佑監修，メディカルドゥ，大阪，2003.
2) Nabel EG, Plautz G, Boyce FM, Stanley JC, Nabel GJ: Recombinant gene expression in vivo within endothelial cells of the arterial wall. Science, 244: 1342-1344, 1989.
3) Deasy BM, Huard J: Gene therapy and tissue engineering based on muscle-derived stem cells. Curr Opin Mol, Ther, 4: 382-389, 2002.
4) Iwaguro H, Yamaguchi J, Kalka C, Murasawa S, Masuda H, Hayashi S, Silver M, Li T, Isner JM, Asahara T: Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. Circulation, 105: 732-738, 2002.
5) Niidome T, Huang L: Gene therapy progress and prospects: Nonviral vectors. Gene Ther, 9: 1647-1652, 2002.

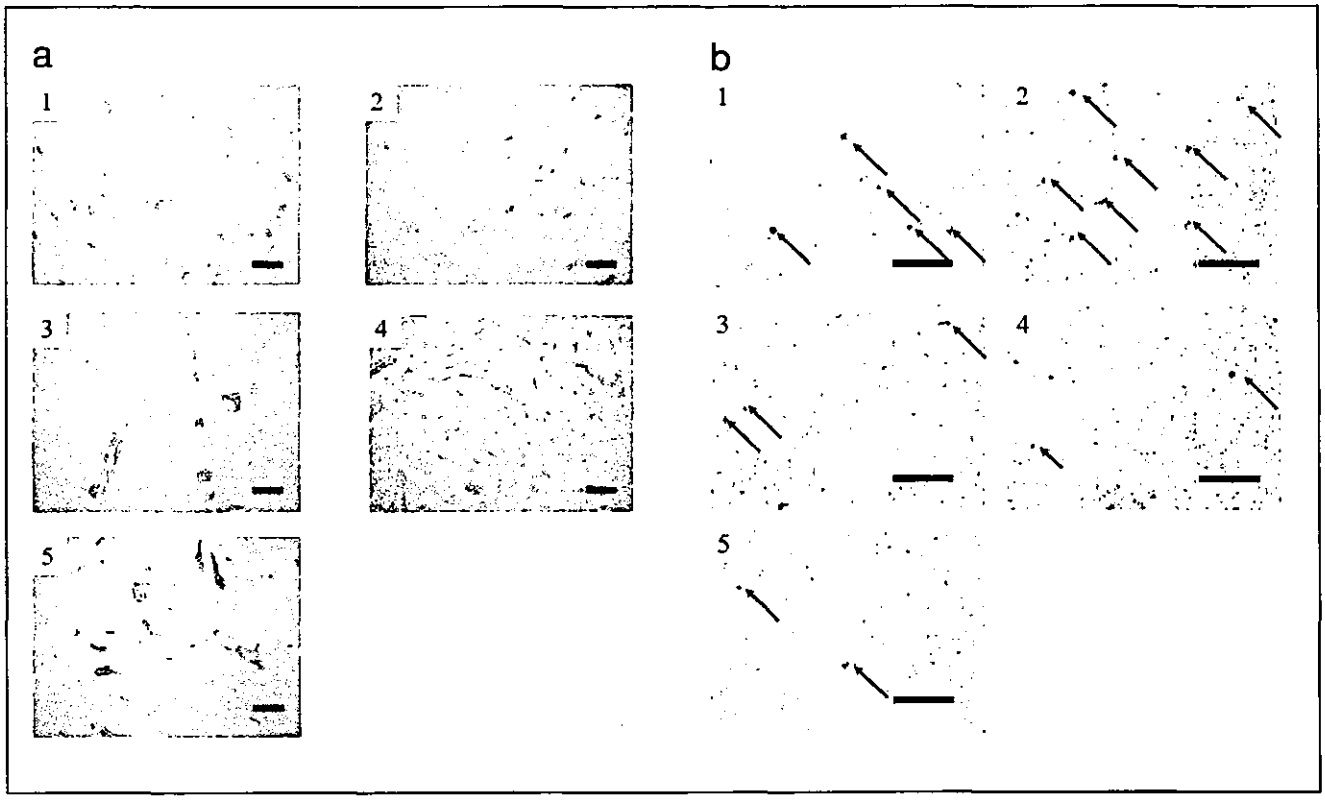


図4

(a) Immunohistochemical views of blood vessel formation of tumor tissues 28 days after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue around the tumor mass: cationized gelatin microspheres incorporating 100 (1) and 200 μg of NK4 plasmid DNA (2), 200 μg of free NK4 plasmid DNA (3), empty cationized gelatin microspheres (4), and saline (5) (magnification; X100). The bar length is 200 μm.
(b) TUNEL staining of tumor tissues 28 days after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue around the tumor mass: cationized gelatin microspheres incorporating 100 (1) and 200 μg of NK4 plasmid DNA (2), 200 μg of free NK4 plasmid DNA (3), empty cationized gelatin microspheres (4), and saline (5) (magnification; X400). The bar length is 100 μm.

6) Kushibiki T, Tabata Y: A new gene delivery system based on controlled release technology. Curr Drug Deliv, in press.
7) Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP: A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci USA, 92: 7297-7301, 1995.
8) Murphy WL, Mooney DJ: Controlled delivery of inductive proteins, plasmid DNA and cells from tissue engineering matrices. J Periodontal Res, 34: 413-419, 1999.
9) Shea LD, Smiley E, Bonadio J, Mooney DJ: DNA delivery from polymer matrices for tissue engineering. Nat Biotechnol, 17: 551-554, 1999.
10) Wang D, Robinson DR, Kwon GS, Samuel J: Encapsulation of plasmid DNA in biodegradable poly(D, L-lactic-co-glycolic acid) microspheres as a novel approach for immunogene delivery. J Control Release, 57: 9-18, 1999.
11) Capan Y, Woo BH, Gebrekidan S, Ahmed S, DeLuca PP: Preparation and characterization of poly (D,L-lactide-co-glycolide) microspheres for controlled release of poly (L-lysine) complexed plasmid DNA. Pharm Res, 16: 509-513, 1999.
12) Luo D, Woodrow-Mumford K, Belcheva N, Saltzman WM: Controlled DNA delivery systems. Pharm Res, 16: 1300-1308, 1999.
13) Hedley ML: Formulations containing poly (lactide-co-glycolide) and plasmid DNA expression vectors. Expert Opin Biol, Ther, 3: 903-910, 2003.
14) Jang JH, Shea LD: Controllable delivery of non-viral DNA from porous scaffolds. J Control Release, 86: 157-168, 2003.

15) Ismail FA, Napaporn J, Hughes JA, Brazeau GA: In situ gel formulations for gene delivery: release and myotoxicity studies. Pharm Dev Technol, 5: 391-397, 2000.

16) Perez C, Sanchez A, Putnam D, Ting D, Langer R, Alonso MJ: Poly(lactic acid)-poly(ethylene glycol) nanoparticles as new carriers for the delivery of plasmid DNA. J Control Release, 75: 211-224, 2001.

17) Wang J, Zhang PC, Mao HQ, Leong KW: Enhanced gene expression in mouse muscle by sustained release of plasmid DNA using PPE-EA as a carrier. Gene Ther, 9: 1254-1261, 2002.

18) Lim YB, Han SO, Kong HU, Lee Y, Park JS, Jeong B, Kim SW: Biodegradable polyester, poly [alpha-(4-aminobutyl)-L-glycolic acid], as a non-toxic gene carrier. Pharm Res, 17: 811-816, 2000.

19) Park JS, Oh YK, Yoon H, Kim JM, Kim CK: In situ gelling and mucoadhesive polymer vehicles for controlled intranasal delivery of plasmid DNA. J Biomed Mater Res, 59: 144-151, 2002.

20) Shen H, Goldberg E, Saltzman WM: Gene expression and mucosal immune responses after vaginal DNA immunization in mice using a controlled delivery matrix. J Control Release, 86, 339-348, 2003.

21) Megeed Z, Cappello J, Ghandehari H: Controlled release of plasmid DNA from a genetically engineered silk-elastinlike hydrogel. Pharm Res, 19: 954-959, 2002.

22) Perlstein I, Connolly JM, Cui X, Song C, Li Q, Jones PL, Lu Z, DeFelice S, Klugherz B, Wilensky R, Levy RJ: DNA delivery from an intravascular stent with a denatured collagen-polylactic-polyglycolic acid-controlled release coating: mechanisms of enhanced transfection. Gene Ther, 10: 1420-1428, 2003.

23) Ochiya T, Takahama Y, Nagahara S, Sumita Y, Nishida A, Itoh H, Nagai Y, Terada M: New delivery system for plasmid DNA in vivo using atelocollagen as a carrier material: the Minipellet. Nat Med, 5: 707-710, 1999.

24) Ochiya T, Nagahara S, Sano A, Itoh H, Terada M: Biomaterials for gene delivery: atelocollagen-mediated controlled release of molecular medicines. Curr Gene Ther, 1: 31-52, 2001.

25) Fukunaka Y, Iwanaga K, Morimoto K, Kakemi M, Tabata Y: Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation. J Control Release, 80, 333-343, 2002.

26) Kushibiki T, Tomoshige R, Fukunaka Y, Kakemi M, Tabata Y: In vivo release and gene expression of plasmid DNA by hydrogels of gelatin with different cationization extents. J Control Release, 90, 207-216, 2003.

27) Tabata Y, Ikada Y: Protein release from gelatin matrices. Adv Drug Deliv Rev, 31: 287-301, 1998.

28) Ozeki M, Ishii T, Hirano Y, Tabata Y: Controlled release of hepatocyte growth factor from gelatin hydrogels based on hydrogel degradation. J Drug Target, 9: 461-471, 2001.

29) Yamamoto M, Tabata Y, Hong L, Miyamoto S, Hashimoto N, Ikada Y: Bone regeneration by transforming growth factor beta1 released from a biodegradable hydrogel. J Control Release, 64: 133-142, 2000.

30) Date K, Matsumoto K, Shimura H, Tanaka M, Nakamura T: HGF/NK4 is a specific antagonist for pleiotrophic actions of hepatocyte growth factor. FEBS Lett, 420: 1-6, 1997.

31) Kushibiki T, Matsumoto K, Nakamura T, Tabata Y: Suppression of tumor metastasis by NK4 plasmid DNA released from cationized gelatin. Gene Ther, in press.

32) Hosseinkhani H, Aoyama T, Yamamoto S, Ogawa O, Tabata Y: In vitro transfection of plasmid DNA by amine derivatives of gelatin accompanied with ultrasound irradiation. Pharm Res, 19: 1471-1479, 2002.

33) Kuba K, Matsumoto K, Date K, Shimura H, Tanaka M, Nakamura T: HGF/NK4, a four-kringle antagonist of hepatocyte growth factor, is an angiogenesis inhibitor that suppresses tumor growth and metastasis in mice. Cancer Res, 60: 6737-6743, 2000.