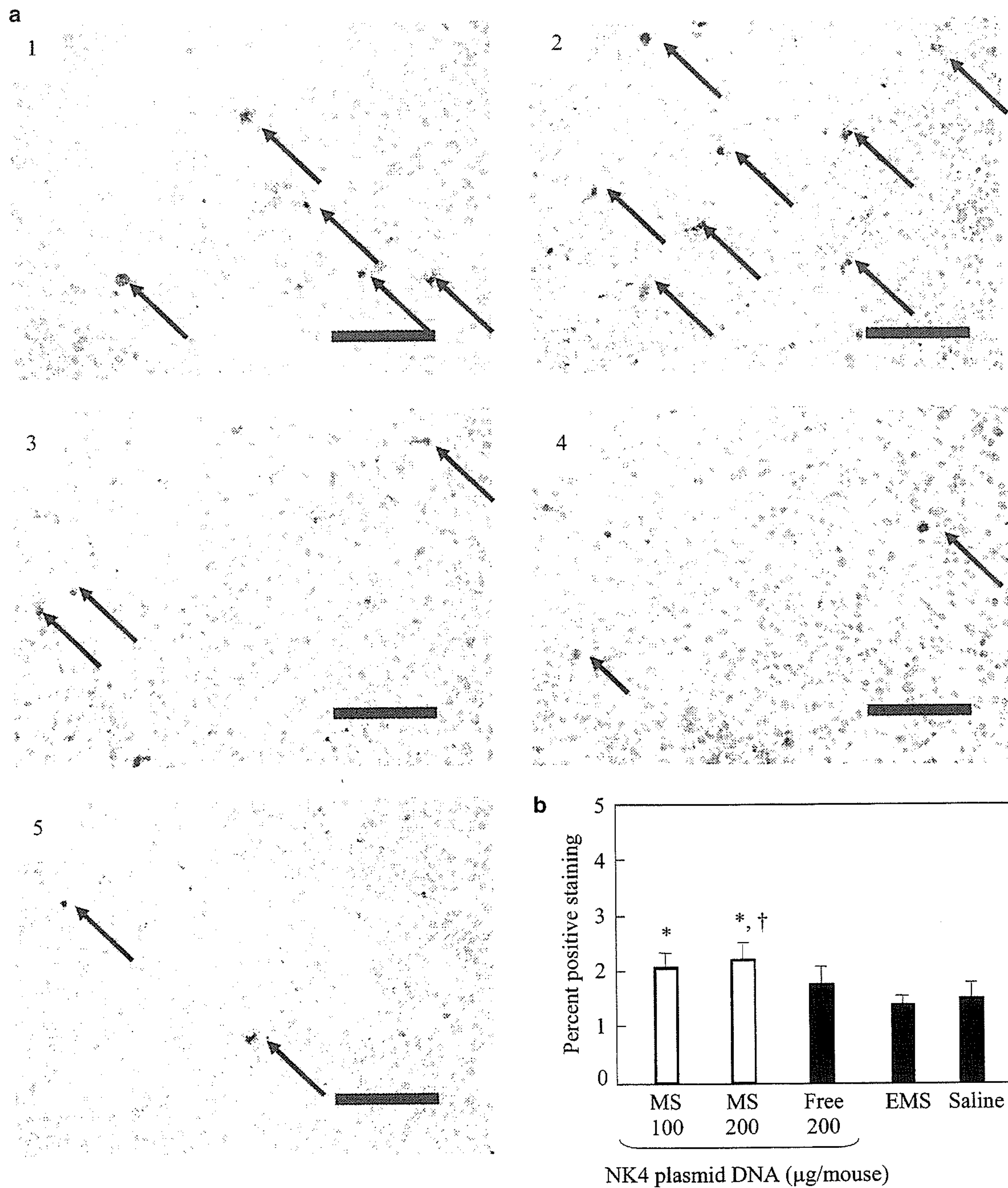
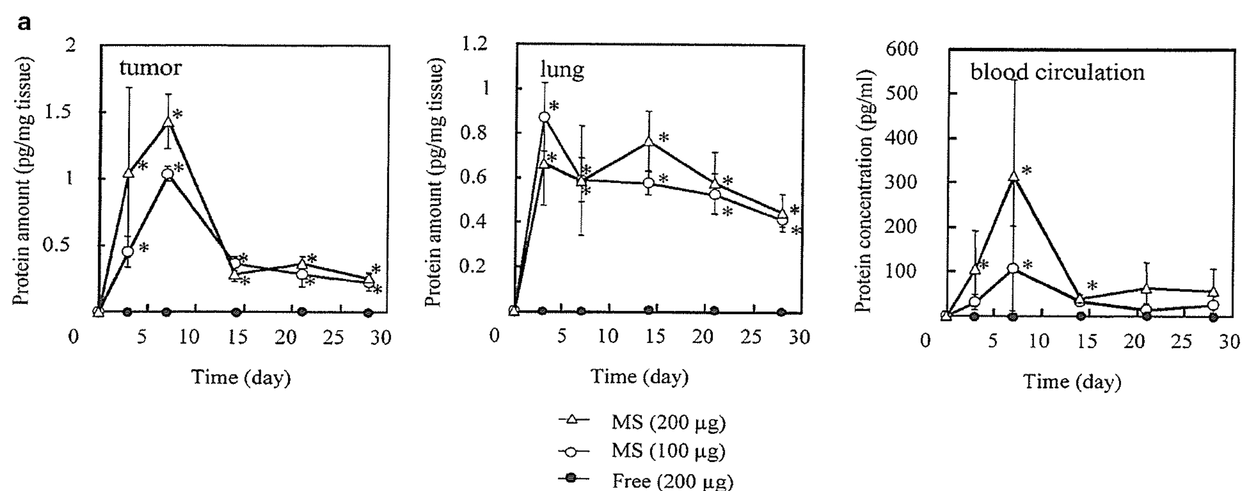


*Figure 5 (a) TUNEL staining of tumor tissues 28 days after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue around the tumor mass: cationized gelatin microspheres incorporating 100 (1) and 200 μg of NK4 plasmid DNA (2), 200 μg of free NK4 plasmid DNA (3), empty cationized gelatin microspheres (4), and saline (5) (magnification; × 400). The bar length is 100 μm. (b) The percent positive staining cells of tumor tissues 28 days after injection of cationized gelatin microspheres incorporating NK4 plasmid DNA (MS), free NK4 plasmid DNA (free), empty cationized gelatin microspheres (EMS), and saline. *, $P<0.05$: significant against the number of positive-stained cells of saline-injected mice. †, $P<0.05$: significant against the number of positive-stained cells of mice injected with 200 μg of free NK4 plasmid DNA. The injection of microspheres incorporating both doses of NK4 plasmid DNA significantly increased the number of apoptotic cells around the tumor mass, in contrast to that of free NK4 plasmid DNA.*

of plasmid DNA diffusion from the release carrier that has been reported as the conventional release system of plasmid DNA.[31,32]

The present release system seems to have advantages over other approaches that involve the direct injection of protein or plasmid DNA in the solution form. For instance, in the previous approach to evaluate antitumor effect of the direct injection of NK4 protein,[18–20] NK4 was administered daily to maintain effective level of NK4 for a long period because of the *in vivo* instability and/or clearance of NK4. In contrast, a single injection of plasmid DNA in cationized gelatin microspheres



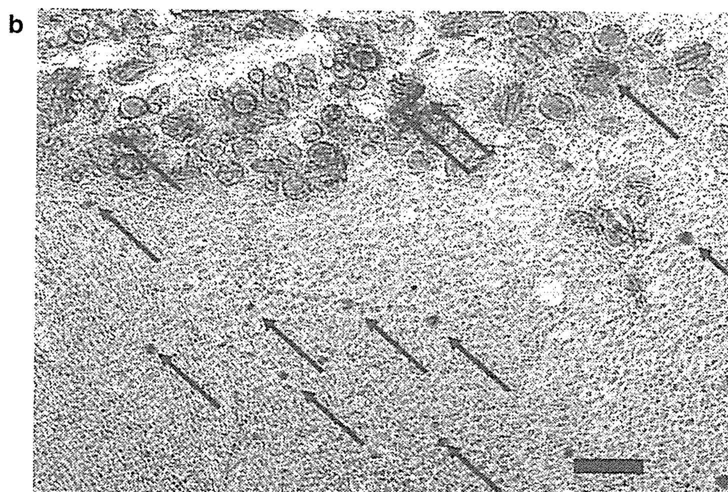


*Figure 6 (a) Time course of the amount of NK4 protein detected in the tumor, lung, and blood circulation of mice after the single injection of cationized gelatin microspheres incorporating 100 (MS (100 μg)) and 200 μg of NK4 plasmid DNA (MS (200 μg)) and 200 μg of free NK4 plasmid DNA (free (200 μg)) into the subcutaneous tissue around the tumor mass. *, $P<0.05$: significant against the NK4 protein detected in mice injected with 200 μg of free NK4 plasmid DNA at the corresponding day. Irrespective of the NK4 plasmid DNA dose, the NK4 protein was detected in the tumor, lung, and blood circulation by the injection of microspheres incorporating NK4 plasmid DNA but not by that of free NK4 plasmid DNA. (b) Tissue localization of gene expression 7 days after the single injection of cationized gelatin microspheres incorporating lacZ plasmid DNA into the subcutaneous tissue around the tumor mass. The bar length is 100 μm. The microspheres' injection enabled lacZ plasmid DNA to express the β-galactosidase in the tumor mass around the microspheres.*

allowed the continuous release of plasmid DNA and a prolonged expression of NK4. It is likely that the controlled release of the plasmid DNA prevents rapid degradation of DNA and facilitates exposure and transduction of plasmid DNA to cells, thereby increasing gene expression efficiency. Moreover, the *in vivo* enzymatic degradation of gelatin microspheres depends on their crosslinking extent, which can be regulated by changing the concentration of glutaraldehyde used for microsphere preparation.[30] In previous studies, we have reported that the time period of plasmid DNA release can be regulated only by changing that of cationized gelatin hydrogel degradation, which can be controlled by changing the crosslinking extent for hydrogel preparation.[33,34] In addition, the prolonged time period of gene expression was observed when the gelatin microspheres of slower degradation were used to achieve the longer-term release of plasmid DNA. There was a good correlation in the time period between the plasmid release and gene expression.[33,34] We have demonstrated that the time period of plasmid DNA expression can be prolonged with an increase in that of plasmid DNA release. This is because the controlled release prevents degradation of DNA by protection from DNase attack, and consequently facilitates it transfection into cells. Some researchers indicate that polyionical complexation prevents the plasmid DNA from enzymatic degradation by DNase attack.[35–37] In this study, the gene expression of NK4 induced by the cationized gelatin microspheres incorporating NK4 plasmid DNA disappeared approximately 28 days after injection (Figure 6a). At the same time, the carrier microspheres were completely degraded *in vivo* and the remaining amount of NK4 plasmid DNA was almost zero (Figure 1). These results suggest that the time period of the NK4 protein expression in the tumor tissue and blood circulation is controllable by the

changing of the degradation pattern of cationized gelatin microspheres.[33,34]

After injection of cationized gelatin microspheres incorporating NK4 plasmid DNA into the subcutaneous tissue around the tumor mass, NK4 protein was detected in the tumor tissue as well as in the blood circulation over a time period of 28 days. We consider it difficult to naturally move the cationized gelatin microspheres incorporating NK4 plasmid DNA themselves from the injected site to other sites. It is possible that only when the microspheres are enzymatically degraded to generate water-soluble complexes of cationized gelatin–NK4 plasmid DNA, the complexes may be distributed to other tissues. We performed the radiotracing test of NK4 plasmid DNA incorporated in cationized gelatin microspheres after subcutaneous injection into the backs of mice. As a result, no radioactive accumulation in the liver, kidney, thyroid grand, and other tissues was observed. These findings strongly suggest that cells transfected and expressed NK4 protein did not exist, except in the injected site.

The plasmid DNA ionically complexed with cationized gelatin will not be released from the hydrogel without water solubilization of degraded gelatin fragment accompanied by hydrogel degradation. It is possible that the plasmid DNA released is condensed because of the polyion complexation with the cationized gelatin of the degradation product. It has been demonstrated that plasmid DNA can be more readily taken up by cells through condensation in the molecular size of plasmid DNA through polyion complexation with cationized polymers.[38,39] This feature to induce the molecular condensation is also an advantage of the release system to enhance gene expression. It is likely that the condensed DNA-cationized gelatin complex of positive charge can electrostatically interact with the cell membrane for internalization. Taken together, we can say with certainty that the NK4 plasmid DNA was expressed at the injected site and secreted to the systemic circulation. We confirmed that the gene expression was observed in the tumor mass around microspheres (Figure 6b). In fact, following tumor growth, cationized gelatin microspheres were integrated in the tumor mass. Thus, because tumor cells were contacted with cationized gelatin microspheres incorporating NK4 plasmid DNA for a long time, the expression levels of NK4 protein were increased. The controlled release enables the plasmid DNA to increase and prolong the concentration over an extended time period around the cells when given. It is highly conceivable that the enhanced concentration increases the possibility of plasmid DNA exposure to cells, resulting in promoted gene expression. In addition, the NK4 protein amount expressed in the tumor tissue was higher than that in the lung including metastatic nodules. These findings suggest that the NK4 was expressed in the tumor cells and secreted into the blood circulation.

As angiogenesis is critical for tumor growth, increased angiogenesis coincides with increased tumor cell entry into the blood circulation and thus facilitates metastasis.[40] Therapeutic approach with angiogenesis inhibitors has gained much attention.[41] NK4 also suppresses the angiogenic effects of VEGF and bFGF.[19,42] On the basis of the bifunctional characteristics of NK4 to target both tumor angiogenesis and HGF-mediated invasion, it is highly expected that NK4 can function as a bioactive molecule effective for tumor therapy. In fact, we found here that the injection of cationized gelatin microspheres incorporating NK4 plasmid DNA significantly inhibited angiogenesis in tumor tissue while increasing the number of apoptotic tumor cells. These findings are consistent with previous studies in which angiogenesis inhibitors suppress the tumor growth by increasing apoptosis of tumor cells.[43,44] Thus, it is possible that NK4 suppresses primary tumor growth mainly through inhibition of tumor angiogenesis.

In conclusion, we propose that gene delivery system of controlled release of plasmid DNA with cationized gelatin microspheres is a promising technology to enhance the *in vivo* suppression of tumor metastasis activity of NK4. This release system is applicable to other types of plasmid DNA and antisense oligonucleotide for gene expression and inhibition.

Materials and methods

Materials

The gelatin sample with an isoelectric point of 9.0 (MW 100 000), prepared by an acid process of pig skin, was kindly supplied by Nitta Gelatin Inc., Osaka, Japan. Ethylenediamine was obtained from Wako Pure Chemical, Ltd., Osaka, Japan. 2,4,6-Trinitrobenzene sulfonic acid (TNBS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC), and 25 wt% glutaraldehyde aqueous solution were obtained from Nacalai Tesque, Kyoto, Japan.

Preparation of NK4 plasmid DNA

The NK4 plasmid DNA, consisting of a cytomegalovirus (CMV) promoter and the NK4 gene segments at the downstream, was prepared from a bacterial culture with the Qiagen Maxi kit (Qiagen, Tokyo, Japan). Briefly, the *Escherichia coli* transformants containing NK4 plasmid DNA were multiplied by incubating in Luria–Bertani (LB) medium (Invitrogen, Tokyo, Japan) at 37°C for 20 h. Following harvest of the bacterial cells by centrifugation (6000 *g* for 15 min at 4°C), the bacterial pellet was suspended in the solution of RNase (100 mg/ml) in resuspension buffer (50 mM Tris–HCl, 10 mM EDTA, pH 8.0) and lysed in a lysis buffer (200 mM NaOH, 1% sodiumdodecyl sulfate). The lysate was neutralized by the addition of 3.0 M potassium acetate solution (pH 5.5), filtered, and applied to a Qiagen syringe of anion-exchange resin. The Qiagen syringe was rinsed with a washing buffer containing 1 M NaCl to remove the remaining contaminants, such as traces of RNA and protein. The plasmid DNA was then eluted with an elution buffer (1.25 M NaCl at pH 8.5, 50 mM Tris–HCl and 15 vol% isopropyl alcohol) and precipitated by addition of isopropyl alcohol. After centrifugation at 15 000 *g* for 10 min at 4°C, the pellet was washed with 70 vol% ethanol aqueous solution to remove residual salts and to substitute the solvent. The DNA was air-dried and dissolved in a small volume of TE buffer (10 mM Tris–HCl and 1 mM EDTA). The absorbance ratio at a wavelength of 260–280 nm was measured for the evaluation of DNA purification to be between 1.8 and 2.0.

Preparation of cationized gelatin

The carboxyl groups of gelatin were chemically converted by introducing amino groups for cationization of gelatin.[33,34] Briefly, ethylenediamine and EDC were added into 250 ml of 100 mM phosphate-buffered solution (PBS) containing 5 g of gelatin. The molar ratio of ethylehediamine to the carboxyl groups of gelatin was 50. Immediately after this, the solution pH was adjusted to 5.0 by adding 5 M HCl aqueous solution. The reaction mixture was agitated at 37°C for 18 h and then dialyzed against double-distilled water (DDW) for 48 h at room temperature. The dialyzed solution was freeze-dried to obtain a cationized gelatin. When determined by the conventional TNBS method,[45] the percentage of amino groups introduced into gelatin was 50.9 mole percent per the carboxyl groups of gelatin.

Preparation of cationized gelatin microspheres incorporating NK4 plasmid DNA

Cationized gelatin microspheres were prepared by chemical crosslinking of gelatin in a water-in-oil emulsion state.[30] An aqueous solution of 10 wt% cationized gelatin (10 ml) was preheated at 40°C and then added dropwise into 375 ml of olive oil preheated at 40°C, while an impeller stirring at 420 rpm was performed for 10 min to yield a water-in-oil emulsion. The emulsion temperature was decreased to 4°C, followed by further stirring for 30 min for the natural gelation of gelatin aqueous solution. Cold acetone (100 ml) was added to the emulsion and stirring was continued for 10 min. The resulting microspheres were washed three times with cold acetone, collected by centrifugation (5000 rpm, 4°C, 5 min), fractionated in size by sieves with apertures of 70 and 100 μm, and air-dried at 4°C. The average diameter of microspheres used was 75 μm. The noncrosslinked and dried gelatin microspheres (50 mg) were placed in 25 ml of acetone/0.01 M HCl solution (7/3, vol/vol) containing 60 μl of 25 wt% glutaraldehyde solution and stirred at 4°C for 24 h to allow the cationized gelatin to crosslink. After washing by centrifugation (5000 rpm, 4°C, 5 min) with DDW, the microspheres were agitated in 25 ml of 100 mM aqueous glycine solution at room temperature for 1 h to block the residual aldehyde groups of unreacted glutaraldehyde. The resulting microspheres were washed three times with DDW by centrifugation and freeze-dried.

To impregnate NK4 plasmid DNA into cationized gelatin microspheres, 20 μl of 100 mM PBS solution (pH 7.4) containing 100 and 200 μg of NK4 plasmid DNA was dropped onto 2 mg of the freeze-dried cationized gelatin microspheres, and then was kept for 24 h at 4°C. A similar procedure, but excluding the use of PBS without NK4 plasmid DNA, was carried out to prepare empty cationized gelatin microspheres. The NK4 plasmid DNA was completely incorporated into cationized gelatin microspheres by this impregnation procedure since the volume of NK4 plasmid DNA solution was small enough compared with that theoretically incorporated into the microspheres.

Evaluation of in vivo degradation of cationized gelatin microspheres

Cationized gelatin microspheres were radioiodinated using [^{125}I] Bolton–Hunter reagent. Briefly, 100 μl of [^{125}I] Bolton–Hunter reagent solution in anhydrous benzene (NEN Research Products) was bubbled with dry nitrogen gas until benzene evaporation was completed. Then, 125 ml of 100 mM PBS (pH 7.4) was added to the dried reagent, followed by pipetting to prepare aqueous [^{125}I] Bolton–Hunter solution. The solution (20 μl) was dropped onto 2 mg of freeze-dried cationized gelatin microspheres for solution impregnation, and then it was kept at 4°C for 24 h to introduce ^{125}I residues into the amino groups of gelatin. The radioiodinated cationized gelatin microspheres were rinsed with DDW by periodically exchanging it at 4°C for 3 days to exclude noncoupled, free ^{125}I -labeled reagent from ^{125}I-labeled cationized gelatin microspheres. The radioactivity of DDW returned to the background level by rinsing for 3 days. No shape change of swollen microspheres was observed during radiolabeling and the subsequent rinsing process.

LLC cells were cultured in the Dulbecco's modified Eagle's medium supplemented with 100 U/l streptomycin, 100 μg/l penicillin, and 10 vol% fetal calf serum for proliferation. The LLC cells were subcutaneously inoculated into the back subcutis of male C57BL/6 mice, 6–8-week old (Japan SLC, Inc., Hamamatsu, Japan), at a concentration of 1×10^7 cells/400 μl serum-free medium. After 4 days of tumor inoculation, ^{125}I-labeled cationized gelatin microspheres (2 mg) were subcutaneously injected around the tumor at the injection volume of 200 μl. At 1, 3, 7, 14, 21, and 28 days after injection, the backs of mouse skin, muscle, and tumor containing the cationized gelatin microspheres injected were taken out to measure their radioactivity on a gamma counter (ARC-301B, Aloka, Tokyo, Japan). The radioactivity ratio of the sample to the cationized gelatin microspheres injected initially was measured to express the percentage of remaining activity in the cationized gelatin microspheres. The number of mice for each experiment at each sampling time was 6. All the animal experiments were carried out according to the Institutional Guidance of Kyoto University on Animal Experimentation.

Evaluation of in vivo NK4 plasmid DNA release from cationized gelatin microspheres incorporating NK4 plasmid DNA

NK4 plasmid DNA was radioiodinated according to the method of Chan *et al.*[46] Cationized gelatin microspheres incorporating ^{125}I-labeled NK4 plasmid DNA were subcutaneously injected to male C57BL/6 tumor-bearing mice at the injection volume of 200 μl. As control, the PBS solution of ^{125}I-labeled NK4 plasmid DNA (100 μg) was subcutaneously injected. At 1, 3, 7, 14, 21, and 28 days after injection, the radioactivity was measured on a gamma counter. The radioactivity ratio of the sample to the NK4 plasmid DNA injected initially was measured to express the percentage of remaining activity in the NK4 plasmid DNA. The number of mice for each experiment at each sampling time was 6.

In vivo experiments

The LLC cells were subcutaneously inoculated into the back subcutis of male C57BL/6 mice at a concentration of 1×10^7 cells/400 μl serum-free medium. For therapeutic treatment, 4 days later, 2 mg of cationized gelatin microspheres incorporating 100 and 200 μg of NK4

plasmid DNA, 200 μg of free NK4 plasmid DNA, 2 mg of empty cationized gelatin microspheres, and saline alone were subcutaneously injected around the tumor at the injection volume of 200 μl/mouse.

Firstly, the survival of treated mice was evaluated every day to prepare the survival curve. The size of the tumor mass was measured by a dial caliper to calculate the tumor volume by using the formula of $\text{width}^2 \times \text{length} \times 0.52$ (20–25 mice/group).[19] In another experiment, the tumor-bearing mice injected with free NK4 plasmid DNA were killed by cervical dislocation 28 days after tumor inoculation (10 mice/group). The primary tumor tissues were fixed in 10 wt% formalin aqueous solution at 4°C, embedded in paraffin, and cross-sectioned according to the standard histological procedure. The immunochemical section was stained with an antibody against the von-Willebrand factor (U-0034, Dako, Glostrup, Denmark) to recognize blood vessels in the tumor tissue, while it was pretreated with a solution of 5 mg/ml proteinase K in 100 mM PBS (pH7.4) at 37°C for 15 min. The stained section was viewed on a light microscope (AX-80, Olympus, Tokyo, Japan) to count the number of blood vessels from at least 20 fields randomly selected per section. For apoptosis evaluation, the tumor tissue was fixed in 70 wt% ethanol in water at 4°C, embedded in paraffin, and cross-sectioned according to the standard histological procedure. The immunochemical section was stained using the TUNEL method[47] by the Apoptosis Detection Kit (ApopTag®, Intergen, Canada) to recognize apoptosis cells in the tumor tissue. The number of positive-stained cells was counted under a light microscope from at least 20 fields randomly selected per section.

In vivo assessment of gene expression following injection of cationized gelatin microspheres incorporating NK4 plasmid DNA

Cationized gelatin microspheres incorporating 100 and 200 μg of NK4 plasmid DNA and 200 μg of free NK4 plasmid DNA were injected into tumor-bearing mice using a procedure similar to the one mentioned above. The mice were killed by cervical dislocation 3, 7, 14, 21, and 28 days after NK4 plasmid DNA treatment to evaluate gene expression. The gene expression of NK4 was measured using the HGF EIA kit (Institute of Immunology Co., Ltd., Tokyo, Japan). Briefly, the samples of primary tumor and lung including metastatic nodules were immersed and homogenized in a lysis buffer (Institute of Immunology Co., Ltd., Tokyo, Japan) at a buffer volume (μl)/sample weight (mg) ratio of 4:1 in order to normalize the influence of weight variance on the assay. The sample lysate (0.2 ml) was transferred to a centrifuge tube and centrifuged at 15 000 *g* at 4°C for 15 min. The supernatant (50 μl) and serum were applied to a well of the HGF EIA kit. One sample was taken from one mouse for each experiment.

Cationized gelatin microspheres incorporating 100 μg of lacZ plasmid DNA were injected into tumor-bearing mice using the same procedure mentioned above. The mice were killed by cervical dislocation to collect the tumor of mice 7 days later. In order to detect β-galactosidase protein, tissue samples were flash frozen, cut into 10 μm sections, and collected onto glass slides. The sections were fixed with 0.5% glutaraldehyde for 10 min, washed with PBS, and stained with X-gal using standard procedures. The sections were then washed with PBS and counterstained with eosin.

Statistical analysis

All the data were expressed as the mean ± the standard derivation of the mean. Statistical analyses were performed based on the unpaired Student's *t*-test (two-tailed), and the statistical difference between survival curves was determined with the generalized Wilcoxon test and significance was accepted at $P < 0.05$.

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A New Gene Delivery System Based on Controlled Release Technology

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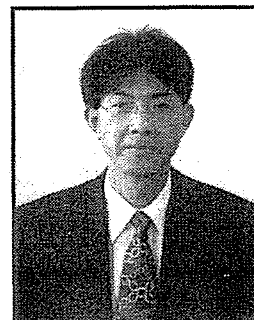

Abstract: The recent rapid development of molecular biology together with the steady progress of genome projects has given us some essential and revolutionary informations of gene to elucidate all the biological phenomena at the molecular level. Under these circumstances, gene transfection has become a fundamental technology indispensable to the basic research of medicine and biology. On the other hand, the technology of gene transfection is also important for gene therapy of several diseases. Some human gene therapies have been performed with a plasmid DNA alone or virus vectors but are clinically limited by the poor gene expression of plasmid DNA and the adverse effects of virus itself, such as immunogenicity and toxicity 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, the researches about non-viral vectors and recent research trials about the controlled release of plasmid DNA are briefly reviewed to emphasize the significance of gene delivery technology in basic biology and medicine as well as clinical medicine. A new system of gene release based on biodegradable hydrogel is introduced.

THE PRESENT NECESSITY OF GENE DELIVERY

The recent rapid development of molecular biology together with the steady progress of animal and plant genome projects has brought about some essential and revolutionary informations of gene to elucidate all the biological phenomena at the molecular level [1-4]. In this situation, gene transfection has been positioned as a key technology, which is indispensable to the research progress in molecular biology [5-11]. Based on the advent of genomics, new genes have been discovered and it is expected that they become therapeutically available for various diseases in the near future. In this connection, gene therapy will be one of the new and promising medical therapies [12-16]. From the viewpoint of pharmacokinetics, it is necessary for a successful gene therapy to deliver of genes to the target organ and tissue [17-20]. The objective of gene therapy is to allow a gene to express the coded protein in the target cells and consequently to treat disease by the protein secreted from the transfected cell. Especially, this is expected to be a new therapeutic way for refractory diseases, such as congenital diseases [21, 22], cancer [23-26], and AIDS [27, 28]. Thus, it is important to develop the technology and methodology of drug delivery system (DDS) to enhance the level of protein expression accompanied with gene transfection. For gene therapy, the 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 cells transfected. The new viral vector with less adverse effects has been explored [29, 30] while the non-viral vector is being investigated to enhance the transfection efficiency [6]. Comparable to the explosion and investigation, it will be important for *in vivo* gene therapy to control the body distribution of vector and consequently that of gene complexed. A plasmid DNA, only when complexed with the non-viral vector and given to cells or injected into the body in the solution form, degraded and inactivated by enzymes or cells with ease. It is known that the virus injected intravenously is often accumulated in the liver and lung [31]. 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 level of gene expression for cells transiently transfected decreases to disappear by cell division and the intracellular degradation. As one trial to overcome this shortcoming, repeated administration of viral or non-viral vector may be required. However, it is impossible to repeatedly administer the virus with immunogenicity. Gene is a new drug, a molecular medicine, containing genetic information. Therefore, using it properly will open, a new direction of drug therapy. However, to this end, it is of prime necessity to develop the DDS technology for gene drugs sufficiently considering the factors influencing their pharmacokinetics.

DDS TRIALS FOR PLASMID DNA

Several synthetic materials, including cationic liposomes [32-34] and cationic polymers like poly-L-lysine [35-38] and polyethyleneimine [39-44], have been molecularly designed and successfully used for transferring DNA into mammalian cells both *in vitro* and *in vivo*. Generally, since the plasmid DNA is a large and negatively charged molecule, it is

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impossible to make the plasmid DNA itself to internalize into cells following the attachment onto the cell membrane of negatively charge. When the plasmid DNA is complexed with the synthetic cationic polymers, it is well recognized that the molecular size of plasmid DNA decreases by the condensation due to the polyion complexation [45,46]. It is likely that the condensed DNA-vector complex of positive charge can electrostatically interact with the cell membrane for internalization. Among the cationic polymers, it is known that the protonable amine residues of polyethyleneimine could function as an endosomal buffering system, which suppresses the action of endosomal enzymes to protect the plasmid DNA from degradation, resulting in the enhanced transfection efficiency [47]. This is called "buffering effect".

Specific and efficient delivery of therapeutic genes into targeted cells is one of the most important objectives in gene therapy. Success of gene delivery to the specific cells is the basis of its therapeutic efficacy. The many challenges for tumor-specific targeting of non-viral gene delivery systems by the synthesis cationic polymer have been reported [48-59]. The folate receptor is known to be overexpressed in a large fraction of human tumors, but it is only minimally distributed in normal tissues [60]. Therefore, the folate receptor serves as an excellent tumor marker as well as a functional tumor-specific receptor. The complexation of plasmid DNA with cationic polymers covalently bound with folate was successfully accumulated in the tumor [57,58]. In addition, if it is possible to target a plasmid DNA to the parenchymal cells of liver, the therapeutic effect will be significantly enhanced. This liver targeting is achieved by the use of a ligand, which can be recognize as the asialoglycoprotein receptor specific for hepatocytes. The ligand is a glycoprotein, lactose, or galactose and coupled with the non-viral vector [61-67]. 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 Zn^{2+} ions to obtain the conjugate of pullulan derivative and plasmid DNA with Zn^{2+} coordination [67]. 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 rather at the liver parenchymal cells than non-parenchymal cells [67]. Moreover, delivery through the bloodstream represents the mean to gain access to disseminated and widespread disease targets. The development of long-circulating non-viral vectors for gene delivery can therefore facilitate a number of therapeutic strategies. Generally, the rapid uptake of colloidal drug carriers by the mononuclear phagocyte system (MPS) after intravenous administration is one of the major events, which often prevents drug injected from delivering to the sites other than the MPS tissue and organ. As one practical way to minimize the MPS uptake, the surface coating of drug carriers with Polyethylene Glycol (PEG) or PEG-like polymers is effective [68-70]. Since these delivery systems may be useful for prolonged gene expression following a single administration, PEG is widely used for non-viral gene carrier [71-75]. In order for gene therapy to be applicable in clinical medicine, it is imperative that a suitable method for stable controlled release of the required amount of the vector delivered over the desired period of time be developed.

Table 1 shows a list of research reports regarding the controlled release of plasmid DNA with different biodegradable biomaterials. The efficient delivery of growth-promoting genes locally in a sustained manner was important to effectively induce tissue regeneration. Mooney *et al.* reported that the *in vivo* 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 [76,77]. Plasmid DNA carrying a gene fragment of the human parathyroid hormone was released from a polymer matrix sponge called a Gene-activated Matrix (GAM) at the target tissue to induce tissue regeneration [95,96]. 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. However, these synthetic materials are often less efficient and are highly toxic after repeated use; as a result, prolonged *in vivo* usage is not allowed. The controlled release of plasmid DNA with a minipellet of atelocollagen has been reported by Ochiya *et al.* to demonstrate enhanced gene expression and therapeutic effects by using some disease model animals [91,92]. Atelocollagen of low immunogenicity [97], which is obtained by pepsin digestion of type I collagen [98] and free from telopeptides, is used as the carrier matrix, because it has been clinically employed for biomedical materials. The authors propose the mechanism of DNA release that the plasmid DNA incorporated in the collagen matrix is released accompanied with matrix degradation. However, it will be practically difficult to control the degradability of collagen matrix and consequently the time period of DNA release from the matrix unless collagen is chemically modified, such as crosslinking and cationization. Denatured collagen, gelatin is also used for the controlled release of plasmid DNA [93,94]. Compared with collagen, it is easy for gelatin to perform the chemical modification, such as derivation and crosslinking, because it is water-soluble and has a random coil structure. On the contrary, collagen has a 3-dimensional helical structure and is water-insoluble itself. Therefore, from the viewpoint of preparation of carrier matrix, it is difficult to artificially modify the chemical nature and the crosslinking extent of collagen, compared with the case of gelatin.

FEASIBILITY OF GELATIN AS THE RELEASE MATRIX

Gelatin has been extensively used for industrial, pharmaceutical and medical applications. The bio-safety has been proved through its long clinical usage as the surgical biomaterials and drug ingredients. Another unique advantage is the electrical nature of gelatin, which can be readily changed by the processing method of collagen for preparation [99]. For example, an alkaline processing allows collagen to structurally denature and hydrolyze the side chain of glutamine and asparagine residue. This results in the 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 hydrogels by crosslinking of the gelatin for the

Table 1. 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.* [76] Shea *et al.* [77] Wang *et al.* [78] Capan *et al.* [79] Luo *et al.* [80] Hedley *et al.* [81] Jang *et al.* [82]
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.* [83]
Poly(lactic acid)-poly(ethylene glycol) (PLA-PEG)	Luciferase	Release plasmid DNA from nanoparticles in a controlled manner.	Perez *et al.* [84]
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.* [85]
Poly(β-(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.* [86]
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.* [87]
Poly(ethylene-*co*-vinyl acetate) (EVAc)	Sperm-specific lactate dehydrogenase C4, 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.* [88]
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.* [89]
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.* [90]
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.* [91,92]
Gelatin	β-Galactosidase	Plasmid DNA release period can be regulated only by changing the hydrogel degradability.	Fukunaka *et al.* [93] Kushibiki *et al.* [94]

controlled release of growth factors. Growth factors with IEPs higher than 7.0, such as basic Fibroblast Growth Factor (bFGF) [100], bone morphogenetic protein-2 (BMP-2) [101], transforming growth factor beta1 (TGF-beta1) [102], and Hepatocyte Growth Factor (HGF) [103] are immobilized into the biodegradable hydrogels of "acidic" gelatin on the basis of the electrostatic interaction force between the growth factor and gelatin molecules. In this release system, the immobilized growth factor is not released from the gelatin hydrogel unless the hydrogel carrier is degraded to generate water-soluble gelatin fragments. The growth factor release could be controlled only by changing the hydrogel degradation [100]. Depending on the nature of growth factors to be released, we can achieve their controlled release

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

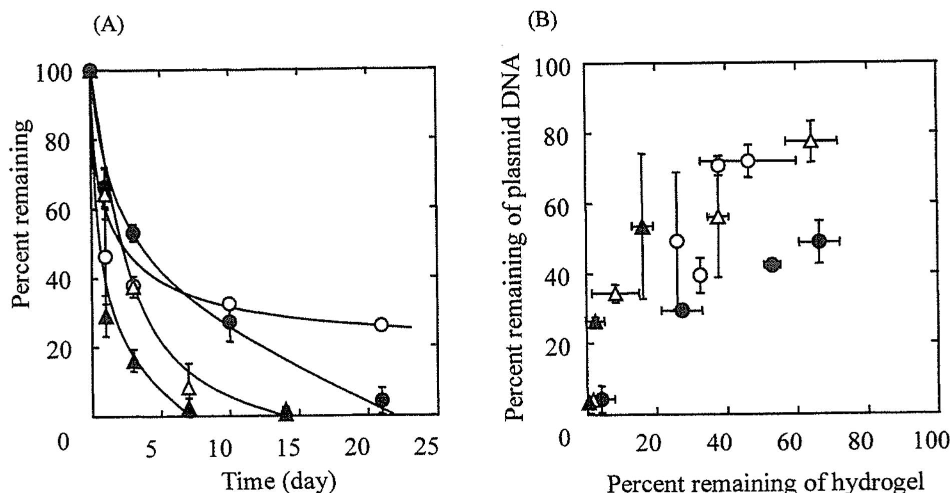

Fig. (1). (A) The time course of the radioactivity remaining of ^{125}I-labeled cationized gelatin hydrogels after implantation into the femoral muscle of mice (The wet weight of hydrogel implanted=0.2 g) (3 mice/group). The water content of cationized gelatin hydrogels is 96.4 (O), 97.4 (●), 98.3 (X),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

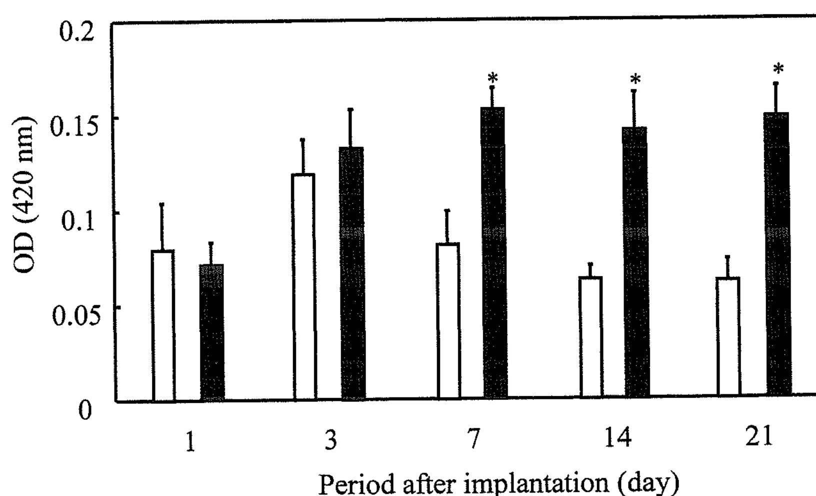

Fig. (2). The time course of lacZ gene expression after implantation of cationized gelatin hydrogels incorporating lacZ plasmid DNA into the femoral muscle of mice: free lacZ plasmid DNA (open bar) and lacZ plasmid DNA incorporated in cationized gelatin hydrogels (water content=97.4 wt%) (closed bar).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

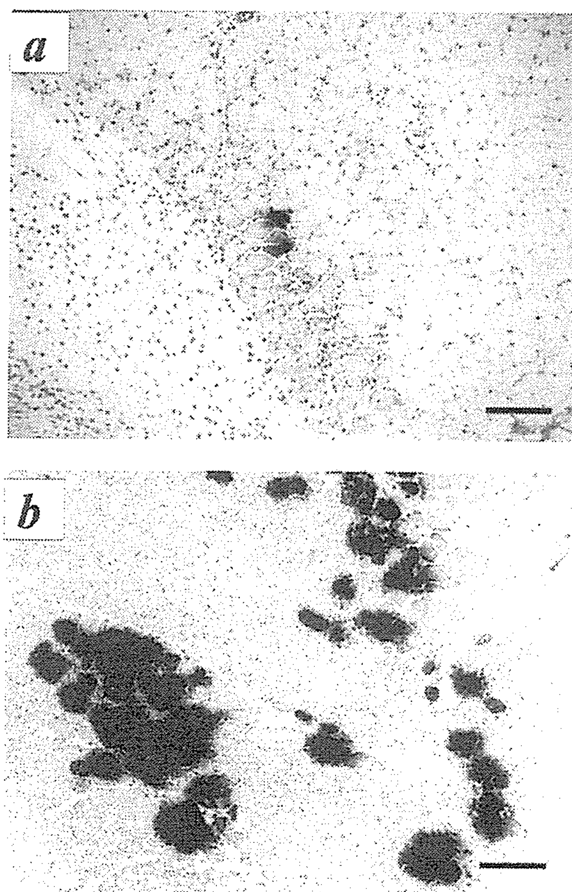

Fig. (3). Representative gene expression of lacZ in the ischemic adductor muscle of rabbits 17 days after treatment. Free lacZ plasmid DNA (a) or cationized gelatin microspheres incorporating lacZ plasmid DNA (b) was injected into the adductor muscle 10 days after the ischemic insultation (5 rabbits/group).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

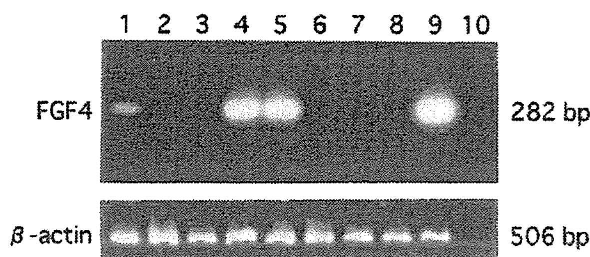

Fig. (4). Representative transgene expression demonstrated by reverse transcription-nested polymerase chain reaction (RT-nested PCR). The left adductor muscle of rabbits was injected with plasmid DNA of fibroblast growth factor 4 (FGF4) (lanes 1 to 3), cationized gelatin microspheres incorporating FGF4 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 Cc1/16 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).

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 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

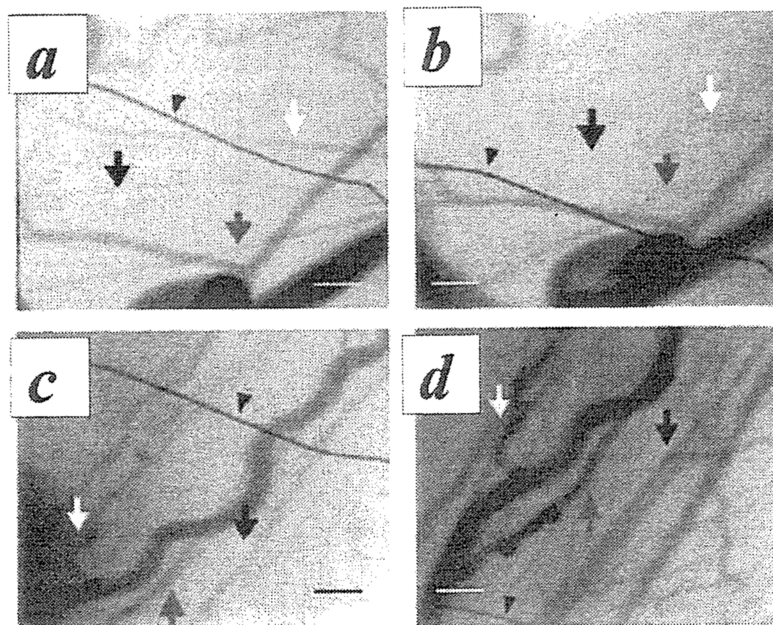

Fig. (5). Representative synchrotron radiation microangiograms of the rabbit hindlimb ischemia. Microangiograms were taken under baseline conditions (a and c) and after repeated adenosine administration (b and d) 38 days after injection of cationized gelatin microspheres incorporating lacZ (a and b) 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.

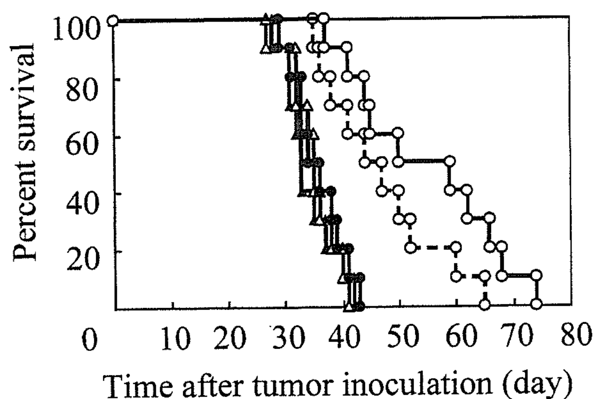

Fig. (6). Survival curves of tumor-bearing mice following the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue: cationized gelatin microspheres incorporating 100 (O---)* and 200 μg of NK4 plasmid DNA (O—)*, 200 μg 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

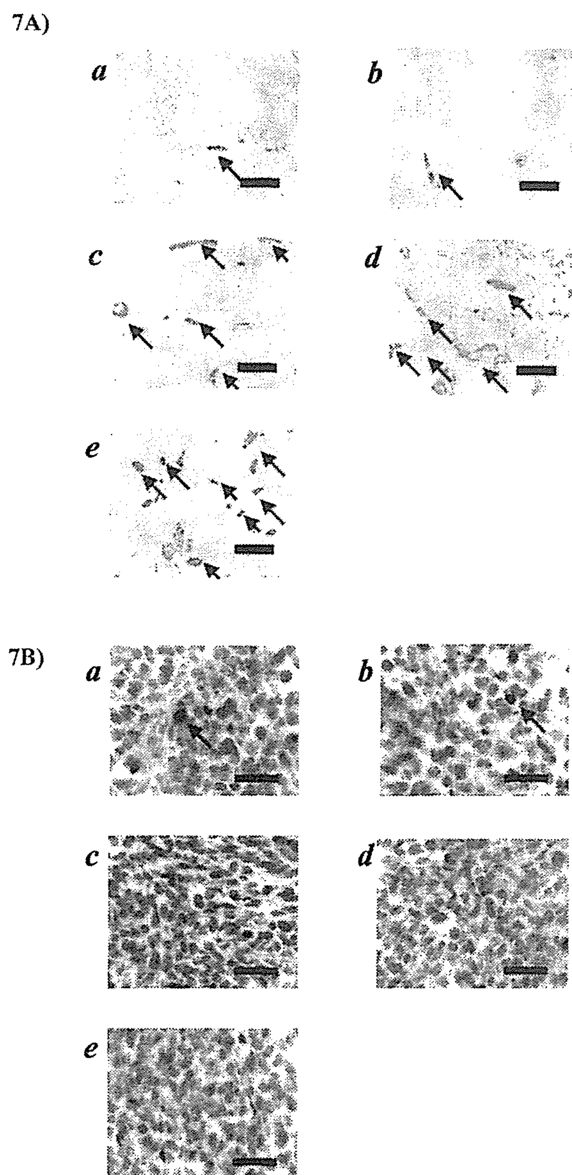

Fig. (7). (A) Immunohistochemical views of blood vessel formation of tumor tissues (*arrows*) 28 days after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue: cationized gelatin microspheres incorporating 100 (a) 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

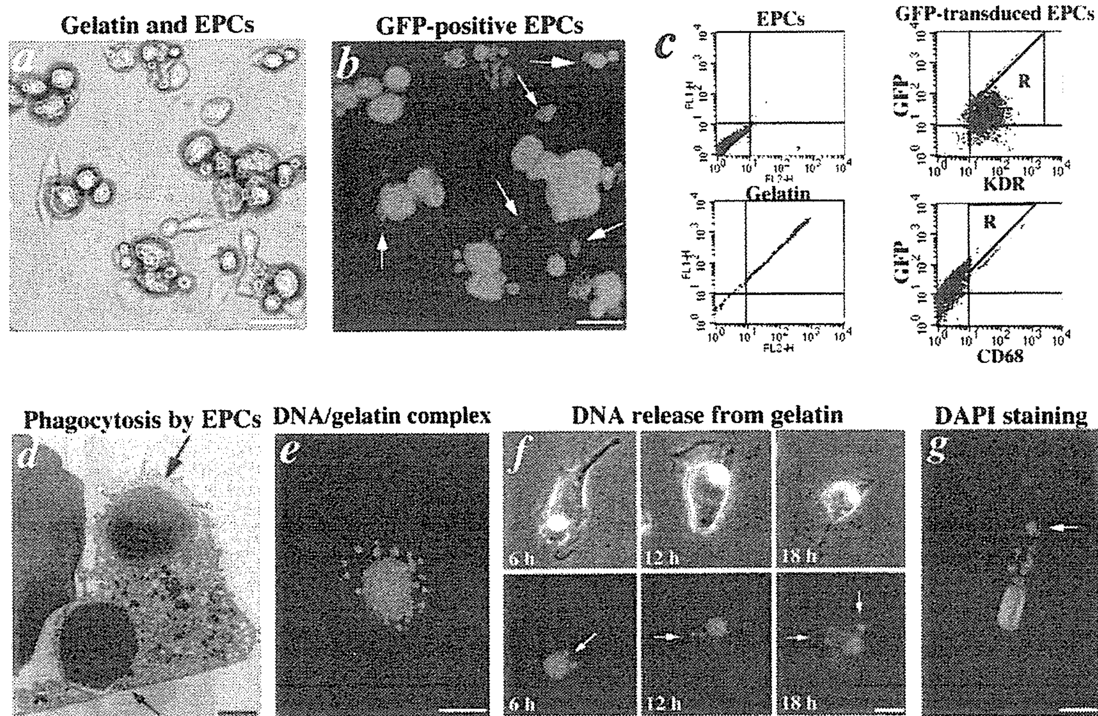

Fig. (8). Ex vivo gene transfer into endothelial progenitor cells (EPCs) based on phagocytosing action. (a) EPCs were cultured with cationized gelatin microspheres incorporating green fluorescent protein (GFP) plasmid DNA. (b) GFP was highly expressed in EPCs (arrows) in the same field as (a). (c) Flow 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).

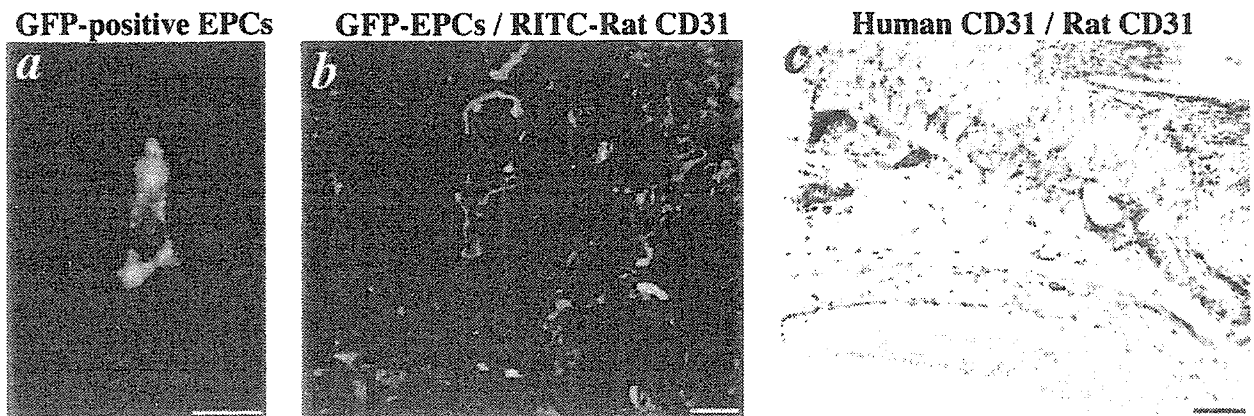

Fig. (9). Distribution of endothelial progenitor cells (EPCs) in the lungs of monocrotaline (MCT)-induced pulmonary hypertension model rats. (a) Intravenously administered green fluorescent protein (GFP)-expressing EPCs were incorporated into the walls of pulmonary arterioles. (b) Transplanted GFP-expressing 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.

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