Suppression of the Progress of Disseminated Pancreatic Cancer Cells by NK4 Plasmid DNA Released from Cationized Gelatin Microspheres

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Purpose. NK4, composed of the NH_2-terminal hairpin and subsequent four-kringle domains of hepatocyte growth factor (HGF), acts as a potent angiogenesis inhibitor. This study is an investigation to evaluate the feasibility of controlled release of NK4 plasmid DNA in suppressing the tumor growth. Controlled release by a biodegradable hydrogel enabled the NK4 plasmid DNA to exert the tumor suppression effects.

Methods. Biodegradable cationized gelatin microspheres were prepared for the controlled release of an NK4 plasmid DNA. The cationized gelatin microspheres incorporating NK4 plasmid DNA were subcutaneously injected to tumor-bearing mice to evaluate the suppressive effects on tumor angiogenesis and growth.

Results. The cationized gelatin microspheres incorporating NK4 plasmid DNA could release over 28 days as a result of microspheres degradation. The injection of cationized gelatin microspheres incorporating NK4 plasmid DNA into the subcutaneous tissue of mice inoculated with pancreatic cancer cells prolonged their survival time period. An increase in the tumor number was suppressed to a significantly greater extent than free NK4 plasmid DNA. The controlled release of NK4 plasmid DNA suppressed angiogenesis and increased the cell apoptosis in the tumor tissue while it enhanced and prolonged the NK4 protein level in the blood circulation.

Conclusions. We conclude that the controlled release technology is promising to enhance the tumor suppression effects of NK4 plasmid DNA.

KEY WORDS: cationized gelatin; controlled release; microspheres; NK4; pancreatic cancer.

INTRODUCTION

Pancreatic cancer ranks as the eighth most frequent type of solid tumor arising worldwide and represents the fourth most frequent cause of death. Although early tumor diagnosis, improved surgical treatment, and multimodal therapeutic concepts have helped to reduce the mortality of patients with pancreatic cancer, the prognosis is still extremely poor; the overall 5-year survival rate is only 1–4% after the diagnosis (1). A chance of cure exists only for a minority of patients with locally limited and surgically resectable tumor. However, of patients who are radically treated by surgical curative resection, 70–80% will suffer from an incurable local relapse, distant metastases, and peritoneal carcinosis. Although a local relapse might be caused by incomplete resection, distant metastases and peritoneal carcinosis depend on dissemination of malignant cells (2). Their elimination is the aim of various adjuvant therapy concepts that are currently under investigation, including chemotherapy, immunotherapy, and gene therapy.

Hepatocyte growth factor (HGF) has been noted as the signal molecule that plays an important role in development, differentiation, and morphogenesis of living systems (3–5). Recently, some therapeutic trials of angiogenesis (6,7), chronic fibrotic diseases (8,9), and tissue regeneration (10,11) by this HGF have been performed to demonstrate the potential efficacy. On the other hand, HGF often acts in an autocrine fashion to induce and enhance the invasive, angiogenic, and metastatic functions of malignant tumors by way of the c-Met/HGF receptor (12–15). Therefore, it is highly expected that the molecular blocking of c-Met/HGF receptor effectively suppresses the invasive, angiogenic, and metastatic functions of tumor cells. Based on this concept, Date *et al.* have prepared an antagonist for HGF, which is composed of the NH_2-terminal hairpin domain of HGF α-subunit and the subsequent four kringles domains (NK4) (16). The NK4 binds to the c-Met/HGF receptor but does not induce tyrosine phosphorylation of c-Met. NK4 competitively inhibits some biological events driven by the c-Met/HGF receptor binding, such as the invasion and metastasis of distinct types of tumor cells and angiogenesis (16–18). The recombinant protein of NK4 has been used for tumor animal models to demonstrate the *in vivo* efficacy in tumor therapy (17–19), and the plasmid DNA of NK4 exhibited similar antitumor effects *in vivo* (20–24).

Based on the recent advent of genomics, new genes have been discovered and will become therapeutically available for various diseases in the near future. In this connection, gene therapy is expected as a new and promising therapeutic choice. Currently, several human clinical trials are proceeding to treat the cancer by using the viral vectors of retroviruses, adenoviruses, and adeno-associated viruses. In spite of the high transfection efficiency, the trials are limited by the adverse effects of virus itself, such as immunogenicity and toxicity or the possible mutagenesis of cells transfected. As the nonviral vectors, many types of cationized polymers (25) and cationized liposomes (26) have been explored to use plasmid DNAs. This approach is to enable the plasmid DNA to neutralize the anionic charge as well as to reduce the molecular size for enhanced efficiency of plasmid DNA transfection, which causes an increase in the gene expression. However, the shorter duration and the lower level of gene expression than viral vectors are important issues to be technologically improved. Moreover, a plasmid DNA, only when complexed with the nonviral vector and given to cells or injected into the body in the naked form, is degraded and inactivated by enzymes or cells with ease. One of the possible ways to tackle the issues is to permit the controlled release of plasmid DNA by combining with an appropriate carrier.

Gelatin has extensively been used for industrial, pharmaceutical, and medical applications and the biosafety is proved through its long clinical usage as a surgical biomaterials and drugs ingredient. Another unique advantage of gelatin is variation in the electrical nature, while the electric nature can

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be readily changed by the processing method of collagen (27). We have designed and explored the controlled release system of drugs on the basis of drug release governed by degradation of drug carrier. Drugs are immobilized into the biodegradable hydrogel of gelatin on the way of physicochemical interaction forces between the drug and gelatin molecules. In this release system, the drug immobilized is not released from the hydrogel unless the hydrogel carrier is degraded to generate water-soluble gelatin fragments. The drug release can be controlled only by changing the hydrogel degradation (28). We have demonstrated that the hydrogel system enables growth factors to release in a bioactive state and consequently enhance their biological functions, in marked contrast to the growth factor in the solution form. The growth factor used includes basic fibroblast growth factor (bFGF) (28), bone morphogenetic protein-2 (BMP-2) (29), transforming growth factor beta1 (TGF-beta1) (30), and HGF (31). In addition, the cationized gelatin of positive charge can readily be prepared by introducing amine residues to the carboxyl groups of gelatin. The plasmid DNA polyionically immobilized in the cationized gelatin hydrogel is released from the hydrogel only if the hydrogel is degraded to generate the water-soluble gelatin fragments (32,33). This study indicates that the cationized gelatin hydrogel enabled a NK4 plasmid DNA to achieve the controlled release and consequently exert the tumor-suppressive effects that were not observed for the plasmid DNA solution.

In this study, we applied the cationized gelatin hydrogel to the controlled release of expression plasmid for human NK4 to evaluate the suppressive effects on tumor angiogenesis and growth in tumor-bearing mice. The release mechanism driven by degradation of release carrier is quite different from that of plasmid DNA diffusion from the release carrier, which has been reported as the conventional release system of plasmid DNA (34,35). The results were compared to those with delivery of free plasmid to emphasize efficacy of the release system in enhancing the biological activity of NK4.

MATERIALS AND METHODS

Preparation of Cationized Gelatin Microspheres Incorporating NK4 Plasmid DNA

The carboxyl groups of gelatin, with an isoelectric point of 9.0 (MW 100,000) prepared by an acid process of pig skin (Nitta Gelatin Inc., Osaka, Japan), were chemically converted by introducing amino groups for cationization of gelatin (32,33). Ethylenediamine was added at a molar ratio of 50 moles per mole of carboxyl groups of gelatin into 250 ml of 100 mM phosphate-buffered solution containing 5 g of gelatin. Immediately after that, the solution pH was adjusted to 5.0 by adding 5 M HCl aqueous solution. Further, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt was added at a molar ratio of 3 moles per mole of carboxyl groups of gelatin. 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 2,4,6-trinitrobenzene sulfonic acid (TNBS) method (36), the percentage of amino groups introduced into gelatin was 50.9 mol% per the carboxyl groups of gelatin. Gelatin microspheres were prepared by chemical cross-linking of gelatin in a water-in-oil emulsion state. 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 used for 10 min to yield a water-in-oil emulsion. The emulsion temperature was decreased to 4°C for the natural gelation of gelatin solution. 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 non–cross-linked 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 cross-link. After washing by centrifugation with DDW, the microspheres were agitated in 25 ml of 100 mM aqueous glycine solution at room temperature to block the residual aldehyde groups of glutaraldehyde. The resulting microspheres were washed with DDW by centrifugation and freeze-dried. The average diameter of microspheres prepared was 75 μm.

To impregnate NK4 plasmid DNA (6.2 kbp) containing the cytomegalovirus immediate early enhancer-chicken β-actin hybrid (CAG) promoter, into cationized gelatin microspheres, 20 μl of 100 mM phosphate-buffered saline solution (PBS; pH 7.4) containing 100 or 200 μg of NK4 plasmid DNA was dropped onto 2 mg of the freeze-dried cationized gelatin microspheres, followed by incubation for 24 h at 4°C. The similar procedure other than using PBS without NK4 plasmid DNA was done to prepare empty cationized gelatin microspheres. NK4 plasmid DNA was completely incorporated into cationized gelatin microspheres by this impregnation procedure, as the volume of plasmid DNA solution (20 μl) is much smaller than that theoretically impregnated into the freeze-dried microspheres and, in fact, was found to be sorbed into the microspheres. The observation of the cryosection of microspheres incorporating fluorescent-labeled plasmid DNA by fluorescent microscopy revealed that the fluorescent-labeled plasmid DNA was localized homogeneously throughout the microspheres but not on the surface (data not shown).

Evaluation of *in Vivo* Degradation of Cationized Gelatin Microspheres

Cationized gelatin microspheres were radioiodinated using [^{125}I] Bolton-Hunter reagent (37). The ^{125}I-labeled cationized gelatin microspheres (2 mg/200 μl PBS/mouse) were subcutaneously injected into the back of ddY mice, 6 to 8 weeks old (Japan SLC, Inc., Hamamatsu, Japan) (6 mice/group). At 1, 3, 7, 14, 21, and 28 days after injection, the mouse skin and muscle containing the cationized gelatin microspheres injected were taken out to measure their radioactivity on a gamma counter. The radioactivity ratio of the sample to the cationized gelatin microspheres injected initially was measured to express the percentage of remaining radioactivity in the cationized gelatin microspheres. 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.* (38). Cationized gelatin microspheres incorporating 100 μg of ^{125}I-labeled NK4 plasmid DNA were prepared similarly and subcutaneously injected to ddY mice at the injection volume of 200 μl (6 mice/group). As control, the PBS solution of ^{125}I-labeled NK4 plasmid DNA (100 μg/200 μl/mouse) was subcutaneously injected. The radioactivity was measured 1, 3, 7, 14, 21, and 28 days later. The percentage of remaining radioactivity in the NK4 plasmid DNA was similarly calculated.

In Vivo Experiments

The human pancreatic cancer cells, AsPC-1, suspension of 1×10^6 cells/200 μl, were transplanted to the peritoneal cavity of 6-week-old nude mice (BALBc nu/nu, Japan SLC, Inc.). For therapeutic treatment, 4 days later, 2 mg of cationized gelatin microspheres incorporating 100 or 200 μg of NK4 plasmid DNA, 200 μg of free NK4 plasmid DNA, 2 mg of empty cationized gelatin microspheres, and saline were subcutaneously injected into the back of nude mice (200 μl/mouse).

First, the survival of treated mice was evaluated every day to prepare the survival curve (10 mice/group). The number and weight of disseminated implants in the peritoneal cavity were recorded (5 mice/group per time point). The immunochemical section of disseminated nodule on day 28 was stained with an antibody against the von Willebrand factor (Dako, Glostrup, Denmark) to recognize blood vessels in the tumor tissue. The stained section was viewed on a light microscope to count the number of blood vessels from at least 10 fields randomly selected per section. The immunochemical section of disseminated nodule was stained with TdT (terminal deoxynucleotidyl transferase) mediated dUTP-biotin nick end labeling (TUNEL) method (39) by Apoptosis Detection Kit (ApopTag®, Intergren Company, NY, USA) to recognize apoptosis cells in the tumor tissue. The number of positive-stained cells was counted under a light microscope from at least 10 fields randomly selected per section.

In Vivo Assessment of Gene Expression Following Injection of Cationized Gelatin Microspheres Incorporating NK4 Plasmid DNA or lacZ Plasmid DNA

Cationized gelatin microspheres incorporating 100 or 200 μg of NK4 plasmid DNA and 200 μg of free NK4 plasmid DNA were injected into tumor-bearing mice by the similar procedure mentioned above. The mice were sacrificed by cervical dislocation 7, 14, 21, and 28 days after NK4 plasmid DNA treatment to evaluate gene expression. The level of NK4 protein expressed in the disseminated nodule and serum was measured by use of HGF EIA kit (Institute of Immunology Co., Ltd., Tokyo, Japan) (5 samples/group). Briefly, the samples of tumor were immersed and homogenized in a lysis buffer (Institute of Immunology Co., Ltd., Tokyo, Japan) at the 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 the well of HGF EIA kit.

The tumor-bearing mice were sacrificed by cervical dislocation to collect the tissue around microspheres 7 days after injection of cationized gelatin microspheres incorporating 100 μg of lacZ plasmid DNA. The tissue samples were frozen and cut into 10-μm sections. The sections were fixed with 0.5% gluteraldehyde for 10 min, washed with PBS, and stained with X-gal using the standard procedure to detect β-galactosidase protein, while they were 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,; significance was accepted at $p < 0.05$.

RESULTS

In Vivo Release Profile of NK4 Plasmid DNA from Cationized Gelatin Microspheres

Radiotracing experiment (Fig. 1) revealed that the NK4 plasmid DNA was retained around the injected site of cationized gelatin microspheres incorporating NK4 plasmid DNA over the time period of 28 days, whereas free NK4 plasmid DNA injected was excreted more rapidly. A good correlation in the time profile of *in vivo* retention was observed between the NK4 plasmid DNA incorporated and mi-

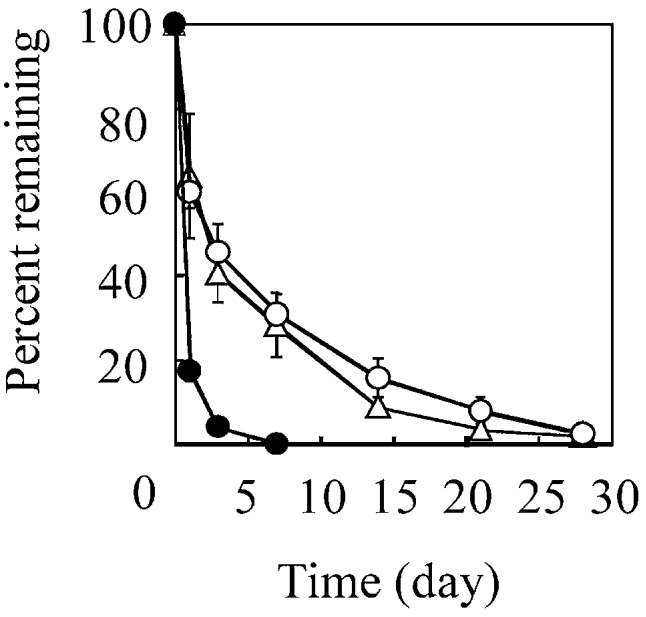

Fig. 1. The time course of radioactivity remaining of cationized gelatin microspheres incorporating ^{125}I-labeled NK4 plasmid DNA (○) and free ^{125}I-labeled NK4 plasmid DNA (●) or ^{125}I-labeled cationized gelatin microspheres (△) after subcutaneous injection into the back of mice. The microspheres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

crospheres, demonstrating the controlled release of NK4 plasmid DNA as a result of microsphere degradation. No radioactivity in thyroid gland and lymph nodes was detected over the time range studied. In addition, no inflammation, macrophage accumulation, and granuloma formation around the injected site was histologically observed.

Prolonged Survival and Tumor Suppression Effects

When the nude mice receiving peritoneal implantation of human pancreatic cancer cells, AsPC-1, were subcutaneously injected with saline, empty cationized gelatin microspheres, and free NK4 plasmid DNA, all the mice died within 50 days. On the contrary, the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA significantly prolonged the survival time period of mice (Fig. 2). The number and total weight increase of the disseminated implants in the peritoneal cavity were significantly suppressed by the cationized gelatin microspheres incorporating NK4 plasmid DNA, in remarkable contrast to free NK4 plasmid DNA (Fig. 3A). The total weight of disseminated nodule was smaller by the cationized gelatin microspheres incorporating NK4 plasmid DNA than by the free NK4 plasmid DNA (Fig. 3B). To examine how the growth of disseminated nodules was suppressed by the NK4 plasmid DNA incorporated in cationized gelatin microspheres, microvessel density and apoptotic index in the disseminated tumors were immunohistochemically quantified. The injection of cationized gelatin microspheres incorporating NK4 plasmid DNA significantly decreased the number of blood vessels in the tumor tissue and the blood vessel diameter compared with that of free NK4 plasmid DNA (Fig. 4A). The number of blood vessels in the tumor tissue of mice receiving the injection of cationized gelatin microspheres incorporating 100 and 200 μg of NK4 plasmid DNA was 65.6% and 62.4% of that of saline-injected mice, respectively (Fig. 4B). The cell apoptosis in the tumor tissue was significantly increased by the cationized gelatin microspheres, in marked contrast to free NK4 plasmid DNA (Figs. 5A and 5B).

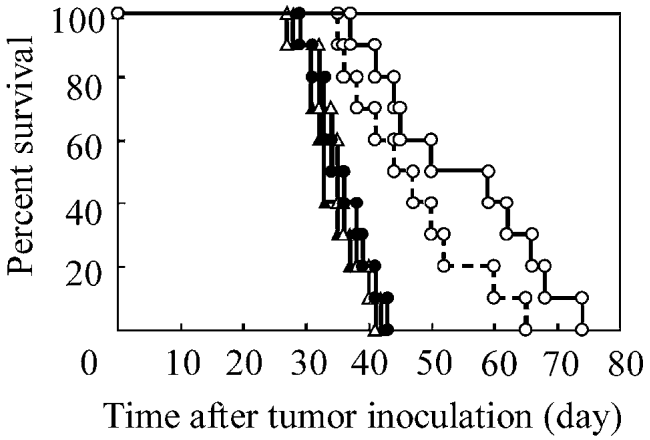

Fig. 2. Survival curves of tumor-bearing mice following the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue: cationized gelatin microspheres incorporating 100 (○---)* and 200 μg of NK4 plasmid DNA (○—)*, 200 μg of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

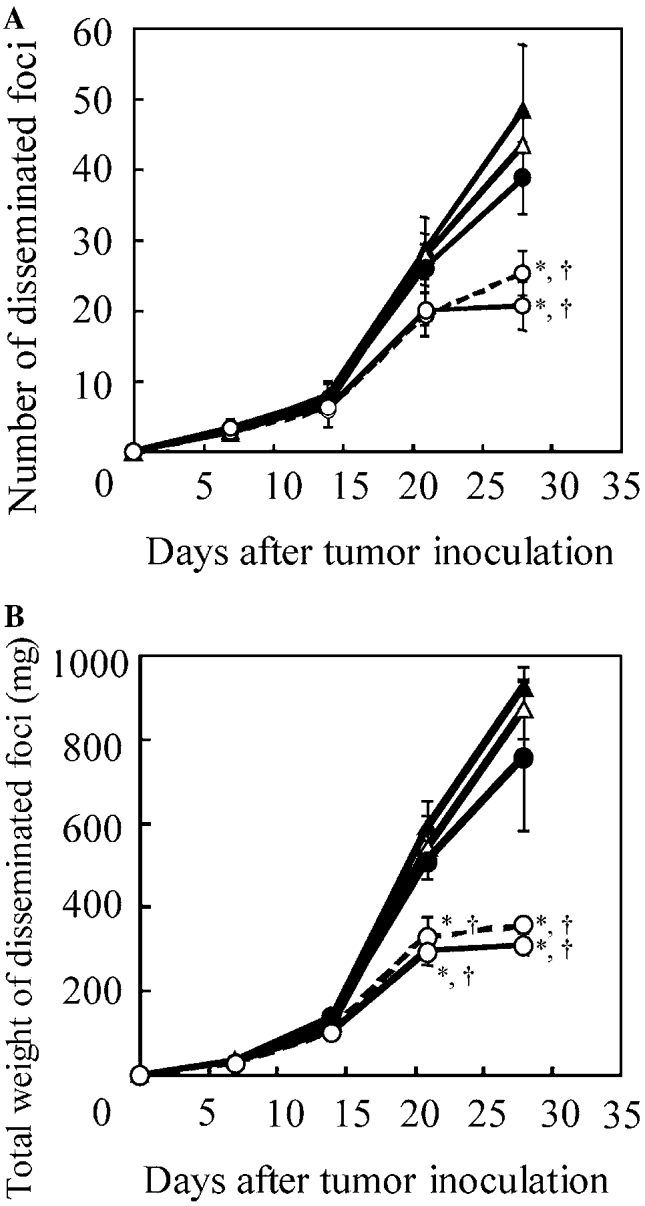

Fig. 3. *In vivo* tumor suppression effects of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA following the single injection into the subcutaneous tissue. (A) Time course of tumor number change: cationized gelatin microspheres incorporating 100 (○---) and 200 μg of NK4 plasmid DNA (○—), 200 μg of free NK4 plasmid DNA (●), empty cationized gelatin microspheres (△), and saline (▲). *$p < 0.05$: significant against the tumor number of saline-injected mice at the corresponding day. †$p < 0.05$: significant against the tumor number of mice injected with 200 μg of free NK4 plasmid DNA at the corresponding day. (B) Time course of total weight of disseminated nodule: cationized gelatin microspheres incorporating 100 (○---) and 200 μg of NK4 plasmid DNA (○—), 200 μg of free NK4 plasmid DNA (●), empty cationized gelatin microspheres (△), and saline (▲). *$p < 0.05$: significant against the tumor weight of saline-injected mice at the corresponding day. †$p < 0.05$: significant against the tumor weight of mice injected with 200 μg of free NK4 plasmid DNA at the corresponding day.

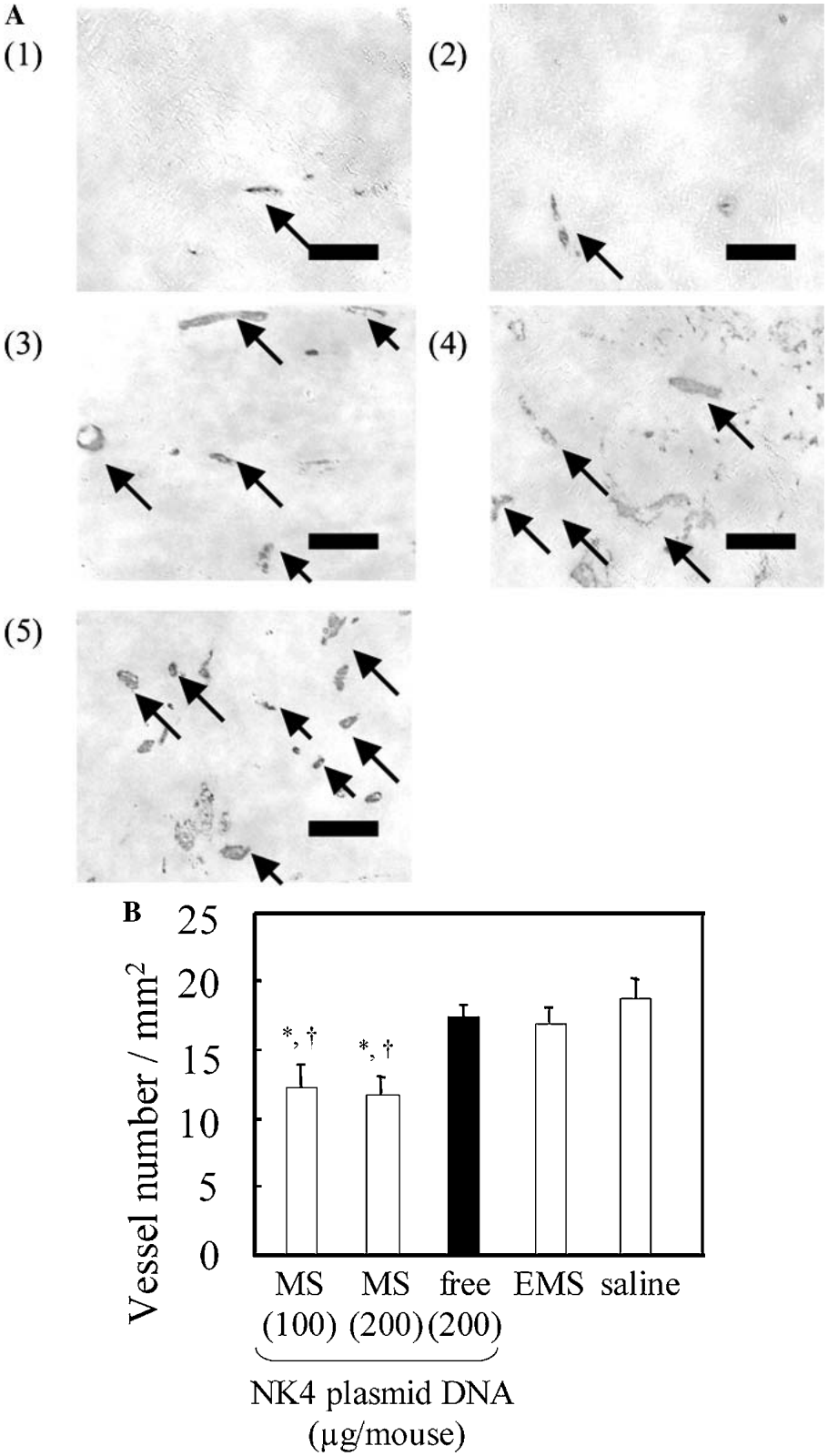

Fig. 4. (A) Immunohistochemical views of blood vessel formation of tumor tissues (arrows) 28 days after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue: cationized gelatin microspheres incorporating (1) 100 and (2) 200 μg of NK4 plasmid DNA, (3) 200 μg of free NK4 plasmid DNA, (4) empty cationized gelatin microspheres, and (5) saline (magnification, ×200). The bar length is 100 μm. (B) The vessel number 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 blood vessels formed of saline-injected mice. †$p < 0.05$: significant against the number of blood vessels formed of mice injected with 200 μg of free NK4 plasmid DNA. The injection of microspheres incorporating both the NK4 plasmid DNA doses significantly decreased the number of blood vessels formed around the tumor mass, in contrast to that of free NK4 plasmid DNA.

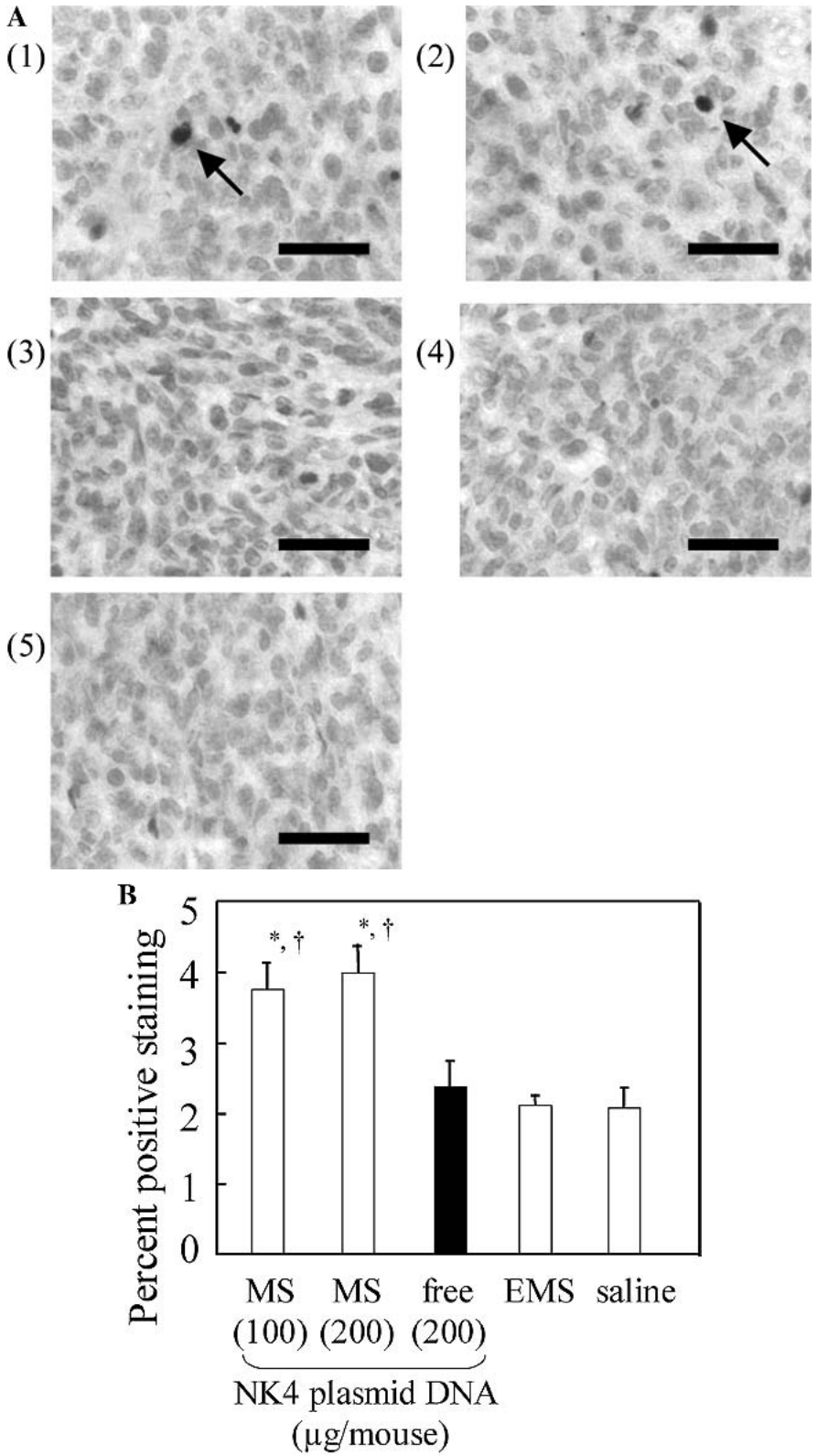

Fig. 5. (A) TUNEL staining of tumor tissues 28 days after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue: cationized gelatin microspheres incorporating (1) 100 and (2) 200 μg of NK4 plasmid DNA, (3) 200 μg of free NK4 plasmid DNA, (4) empty cationized gelatin microspheres, and (5) saline (magnification, ×400). The bar length is 50 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 dose of NK4 plasmid DNA significantly increased the number of apoptotic cells, in contrast to that of free NK4 plasmid DNA.

After injection of cationized gelatin microspheres incorporating 100 or 200 μg of NK4 plasmid DNA, the amount of NK4 protein in the tumor and blood circulation significantly increased with time up to day 7 to attain a maximum level, but thereafter decreased gradually. On the contrary, NK4 protein was hardly detected at any sampling time and site for free NK4 plasmid DNA (Fig. 6). A lacZ plasmid DNA with the same CAG promoter was used to clarify the expression site. When cationized gelatin microspheres incorporating a plasmid DNA of lacZ were injected, the β-galactosidase expression was observed around microspheres (Fig. 7).

DISCUSSION

We have demonstrated here that the subcutaneous injections of cationized gelatin microspheres incorporating NK4 plasmid DNA significantly suppressed the tumor progression of the pancreatic cancer cells disseminated in the peritoneal cavity of nude mice and prolonged their survival. There is often difference in the proangiogeneic state of tumor vessels between the animal and human tumors. However, the current data revealed that the controlled release of NK4 plasmid DNA from cationized gelatin microspheres was therapeutically positive to tumor regression. Generally, it is known that the vessel formation in the tumor tissue is in progress after metastasis of tumor cells. Continuous exposure of NK4 protein to the tumor cells is effective in suppressing the vessel formation. We believe that suppression of angiogenesis at least enables tumor to maintain the dormant state rather than to eradicate biologically. Therefore, in terms of tumor dormancy, tumor gene therapy based on continuous release of NK4 plasmid DNA from cationized gelatin microspheres may be an attractive new approach for treatment of advanced tumor patients. A clinical setting of this experiment was postoperative peritoneal dissemination of pancreatic cancer without other clinical or radiological evidence of the disease. It was reported that minimal residual disease was detected in 29% of the peritoneal cavity in the patients who underwent curative resection of pancreatic cancer, and the occurrence of isolated tumor cells correlated with a poor prognosis (2). We set up the early treatment, beginning on day 4 after peritoneal seeding of cancer cells, supposing this as an adjuvant therapy after the surgery. In this study, there are no distant metastases to the liver, spleen, lung, pancreas, or kidney. The tumor cells injected are mainly proliferated at greater omentum or mesenterium (23).

It is known that NK4 retains the binding capacity to the HGF receptor, c-Met, competing with HGF and inhibits the migration-facilitating activity of HGF (16, 17). NK4 also suppresses the angiogenic effects of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (18,40). Namely, NK4 is a bifunctional molecule: it acts as not only an HGF antagonist but also as an angiogenesis inhibitor. In our model, the controlled release of NK4 plasmid DNA significantly suppressed the progression of AsPC-1 cells peritoneally inoculated. The expression of c-Met receptor in AsPC-1 was relatively strong compared to other pancreatic cancer cell lines (22), and HGF stimulated the migration and invasion of AsPC-1 cells, although the proliferation of AsPC-1 was not stimulated by HGF. In addition, angiogenesis is indispensable for tumor growth, and it has been reported that any foci larger than 2 mm require new tumor vessels for their growth (41). The angiogenic properties of pancreatic cancer remain unclear, partially because pancreatic cancers have been considered hypovascular, based on roentgenographic findings. However, it has been shown that tumor angiogenesis is implicated in the rapid growth and metastasis of pancreatic cancer (42), and some angiogenesis inhibitors efficiently suppressed tumor growth and metastasis of pancreatic cancer in experimental models (43).

The prolonged expression of NK4 results in significant suppression of increase in the number and total weight of disseminated nodules (Figs. 3A and 3B). Although it has been reported that HGF and NK4 have no direct suppressive effect on the proliferation of tumor cells (16–18), this can be explained in terms of angiogenesis. The injection of cationized gelatin microspheres incorporating NK4 plasmid DNA decreased the number of blood vessels in the tumor tissue and the vessel diameter compared with that of other agents (Figs.

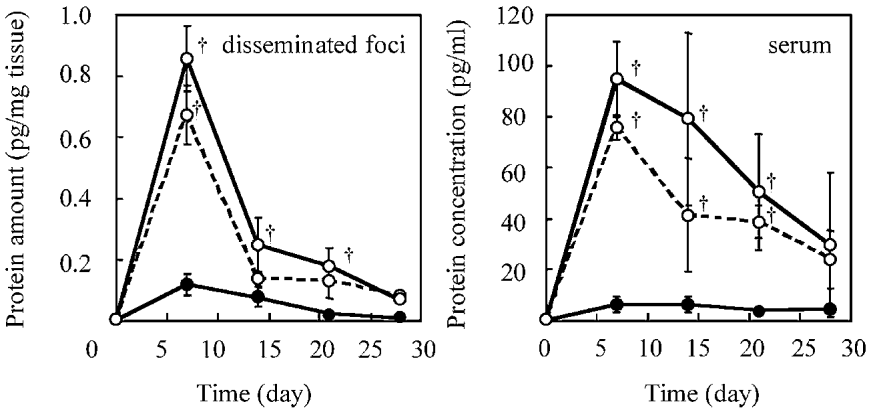

Fig. 6. Time course of amount of NK4 protein detected in the disseminated foci and serum of mice after the single injection of cationized gelatin microspheres incorporating 100 (○---) and 200 μg of NK4 plasmid DNA (○—) and 200 μg of free NK4 plasmid DNA (●) into the subcutaneous tissue. Irrespective of the NK4 plasmid DNA dose, the NK4 protein was detected in the tumor and blood by the injection of cationized gelatin microspheres incorporating NK4 plasmid DNA. †$p < 0.05$: significant against the protein amount of mice injected with 200 μg of free NK4 plasmid DNA at the corresponding day.

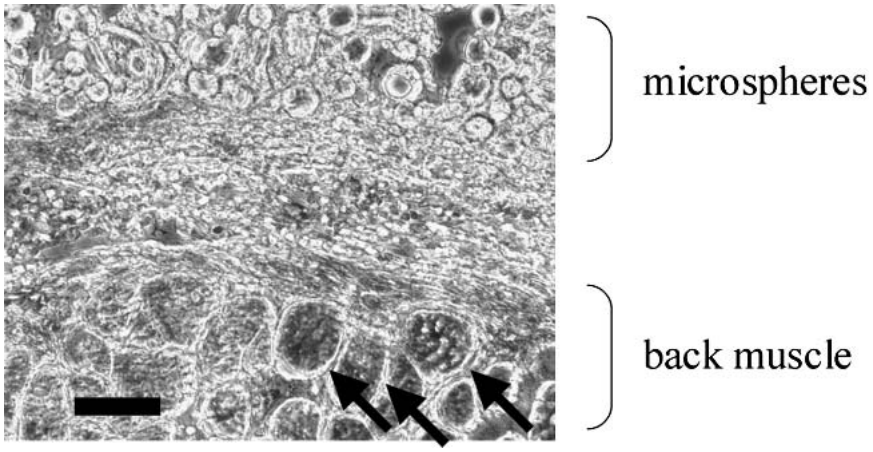

Fig. 7. Tissue localization of gene expression 7 days after the single injection of cationized gelatin microspheres incorporating lacZ plasmid DNA into the subcutaneous tissue. The microspheres injection enables lacZ plasmid DNA to express the β-galactosidase in the muscle around the microspheres (indicated by arrows) (magnification, ×200). The bar length is 100 μm.

4A and 4B). Additionally, the microspheres injection was effective in increasing the number of apoptotic cells (Figs. 5A and 5B). These findings are consistent with previous studies in which angiogenesis inhibitors suppress the tumor growth based on the increasing apoptosis of tumor cells (44,45). We supposed that NK4 prevented the progression of disseminated tumor cells as an angiogenesis inhibitor in addition to an HGF antagonist, and the improvement of survival in the NK4 incorporating cationized gelatin microspheres–treated mice implicated that NK4 also inhibited the further extension of peritoneal dissemination.

Gene delivery system is generally divided into two categories: viral and nonviral vectors. However, the viral vectors currently used have some problems for their clinical trials, such as the immunogenicity and toxicity or the possible mutagenesis of cells transfected. Therefore, the nonviral vector system with the gene expression capacity comparable to viral vectors should be developed. In this study, we introduce a system of prolonged gene expression based on the controlled release of plasmid DNA from cationized gelatin microspheres (Fig. 1). The plasmid DNA immobilized in the microspheres is released only when the microspheres are degraded to make cross-linked cationized gelatin soluble in water. The plasmid DNA release can be controlled only by changing the gelatin hydrogel degradation (32,33). In this hydrogel system, the release of plasmid DNA is driven by enzymatic degradation of gelatin microspheres as the release carrier. This mechanism is quite different from that based on plasmid DNA diffusion from the release carrier, which has been reported for the conventional system of plasmid DNA release (34,35). Whenever any particle is injected, normally inflammatory reaction is observed although the extent depends on the type and size of particle injected. However, following the subcutaneous injection of cationized gelatin microspheres, no inflammation, macrophage accumulation, and granuloma formation around the injected site were histologically detected during the experiment. In addition, the interleukin 1 concentration, the indicator of inflammatory response, at the injected site of cationized gelatin microspheres was not increased either (data not shown). The microspheres are degraded within a short time period, and the cationized gelatin is biocompatible compared with other polymer materials. In this radiotracing study of plasmid DNA, ^{125}I was continuously excreted in the urine, not accumulated in any organs or the thyroid gland or lymph nodes. Because the molecular size of plasmid DNA with or without complexation with the cationized gelatin is too large to permeate the blood vessel wall, it is unlikely that the plasmid DNA is detected in the urine without degradation of plasmid DNA. Taken together, we believe that the ^{125}I excreted in urine is due to the degradation products of plasmid DNA after transfection to cells. In addition, this release system is advantageous compared with other approaches that involve the direct injection of protein or plasmid DNA in the solution form. A traditional approach to achieve tumor dormancy is the direct injection of protein. However, by this approach, it is difficult to induce the biological function expected and maintain it for a long time period because of the *in vivo* protein instability and immunogenicity (46). On the other hand, the plasmid DNA may achieve a prolonged biological effect by the transfected cells, although the low transfection efficiency by plasmid DNA should be improved (47). The controlled release enables the plasmid DNA to increase the concentration in the tissue over an extend time period. It is highly conceivable that the enhanced concentration increases the exposure possibility of plasmid DNA to cells, resulting in promoted gene expression. It is likely that the controlled release of the plasmid DNA prevents rapid degradation of DNA and facilitates exposure and transduction of plasmid DNA to cells, thereby increasing gene expression efficiency. In this study, the radioactivity remaining was evaluated only at the injected site and the urine, but not in lymph nodes. It is recognized that metastasis of tumor cells is often achieved via the lymph system. We cannot deny the possibility that NK4 protein expressed is accumulated in lymph nodes, resulting in suppression of tumor metastasis via the lymph system. Although the mechanism of NK4-induced suppression effect of tumor metastasis is not fully explored, the current study indicates the therapeutically positive effect of NK4 plasmid DNA release on tumor suppression.

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 cross-linking extent for hydrogel preparation (32,33). 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 (32,33). 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 facilitate its transfection into cells. Some researchers indicate that polyionical complexation prevents the plasmid DNA from enzymatic degradation by DNase attack (48–50). In this study, the gene expression of NK4 induced by the cationized gelatin microspheres incorporating NK4 plasmid DNA disappeared approximately 28 days after injection (Fig. 6A). At the same time, the carrier microspheres were completely degraded *in vivo,* and the remaining amount of NK4 plasmid DNA was almost zero (Fig. 1A). In fact, gene expression was observed around the injected site of cationized gelatin microspheres incorporating lacZ plasmid DNA (Fig. 7). In the preliminary experiments, we expected that intraperitoneal (i.p.) administration of NK4 plasmid DNA was effective to suppress the progression of disseminated tumor cells because the NK4 expressed protein had directly affected the inoculated tumor cells. However, in fact, there were no significant differences on the survival rate of mice between the cationized gelatin microspheres incorporating NK4 plasmid DNA i.p. injected group and saline i.p. injected group. As it is well-known, there are many immunocompetent cells like macrophages in the peritoneal cavity. We suggest that cationized gelatin microspheres injected to peritoneal cavity were degraded by them faster than those subcutaneously injected to the back of mice. By the injection of cationized gelatin microspheres incorporating NK4 plasmid DNA, NK4 protein was detected in the tumor tissue and in the blood circulation over the time period of 28 days (Fig. 6). It is difficult to naturally move the cationized gelatin microspheres incorporating NK4 plasmid DNA themselves from the injected site to other sites. Only when the microspheres are degraded to generate water-soluble complexes of cationized gelatin-NK4 plasmid DNA will the complexes 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 the result, no radioactive accumulation in the liver, kidney, thyroid grand, and other tissues was observed. These findings strongly suggest that gene expression will not be expected except for the injected site. The plasmid DNA ionically complexed with the cationized gelatin constituting a hydrogel will not be released from the hydrogel without fragmentation and the consequent water-solubilization of cationized gelatin accompanied with hydrogel degradation. It is possible that the plasmid DNA released is condensed because of the polyion complexation with the cationized gelatin of degradation product. It has been demonstrated that plasmid DNA can be more readily taken up by cells through condensation in the molecular size of plasmid DNA through polyion complexation with cationized polymers (51,52). This feature to induce the molecular condensation is also an advantage of the release system to enhance gene expression. Taken together, we can say with certainty that the NK4 plasmid DNA was expressed around the injection site and secreted to the systematic circulation. Our research results demonstrated that it was important for successful tumor therapy to expose NK4 to tumor cells for a long time even at a low concentration by a controlled release system. Although the mechanism of the effect of long term exposure of NK4 at a low concentration on tumor suppression is not fully clear, we believe that the current result indicates the positive effect of the release system on tumor suppression.

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

REFERENCES

1. J. E. Niederhuber, M. F. Brennan, and H. R. Menck. The national cancer data base report on pancreatic cancer. *Cancer* **76**:1671–1677 (1995).
2. I. Vogel, U. Kruger, J. Marxsen, E. Soeth, H. Kalthoff, D. Henne-Bruns, B. Kremer, and H. Juhl. Disseminated tumor cells in pancreatic cancer patients detected by immunocytology: a new prognostic factor. *Clin. Cancer Res.* **5**:593–599 (1999).
3. T. Nakamura, T. Nishizawa, M. Hagiya, T. Seki, M. Shimonishi, A. Sugimura, K. Tashiro, and S. Shimizu. Molecular cloning and expression of human hepatocyte growth factor. *Nature* **342**:440–443 (1989).
4. R. Zarnegar and G. K. Michalopoulos. The many faces of hepatocyte growth factor: from hepatopoiesis to hematopoiesis. *J. Cell Biol.* **129**:1177–1180 (1995).
5. K. Matsumoto and T. Nakamura. Hepatocyte growth factor (HGF) as a tissue organizer for organogenesis and regeneration. *Biochem. Biophys. Res. Commun.* **239**:639–644 (1997).
6. E. Van Belle, B. Witzenbichler, D. Chen, M. Silver, L. Chang, R. Schwall, and J. M. Isner. Potentiated angiogenic effect of scatter factor/hepatocyte growth factor via induction of vascular endothelial growth factor, the case for paracrine amplification of angiogenesis. *Circulation* **97**:381–390 (1998).
7. Y. Taniyama, R. Morishita, M. Aoki, H. Nakagami, K. Yamamoto, K. Yamazaki, K. Matsumoto, T. Nakamura, Y. Kaneda, and T. Ogihara. Therapeutic angiogenesis induced by human hepatocyte growth factor gene in rat and rabbit hindlimb ischemia models: preclinical study for treatment of peripheral arterial disease. *Gene Ther.* **8**:181–189 (2001).
8. Y. Matsuda, K. Matsumoto, T. Ichida, and T. Nakamura. Hepatocyte growth factor suppresses the onset of liver cirrhosis and abrogates lethal hepatic dysfunction in rats. *J. Biochem.* **118**:634–639 (1995).
9. S. Mizuno, T. Kurosawa, K. Matsumoto, Y. Mizuno-Horikawa, M. Okamoto, and T. Nakamura. Hepatocyte growth factor prevents renal fibrosis and dysfunction in a mouse model of chronic renal disease. *J. Clin. Invest.* **101**:1827–1834 (1998).
10. Y. Ishiki, H. Ohnishi, Y. Muto, K. Matsumoto, and T. Nakamura. Direct evidence that hepatocyte growth factor is a hepatotrophic factor for liver regeneration and has a potent antihepatitis effect in vivo. *Hepatology* **16**:1227–1235 (1992).
11. H. Ohmichi, K. Matsumoto, and T. Nakamura. *In vivo* mitogenic action of HGF on lung epithelial cells: pulmotrophic role in lung regeneration. *Am. J. Physiol.* **270**:L1031–L1039 (1996).
12. J. Cortner, G. F. Vande Woude, and S. Rong. The Met-HGF/SF autocrine signaling mechanism is involved in sarcomagenesis. *EXS* **74**:89–121 (1995).
13. M. Jeffers, S. Rong, and G. F. Vande Woude. Hepatocyte growth factor/scatter factor-Met signaling in tumorigenicity and invasion/metastasis. *J. Mol. Med.* **74**:505–513 (1996).

14. C. T. To and M. S. Tsao. The roles of hepatocyte growth factor/scatter factor and met receptor in human cancers. *Oncol. Rep.* **5**:1013–1024 (1998).
15. W. G. Jiang, S. Hiscox, K. Matsumoto, and T. Nakamura. Hepatocyte growth factor/scatter factor, its molecular, cellular and clinical implications in cancer. *Crit. Rev. Oncol. Hematol.* **29**:209–248 (1999).
16. K. Date, K. Matsumoto, H. Shimura, M. Tanaka, and T. Nakamura. HGF/NK4 is a specific antagonist for pleiotrophic actions of hepatocyte growth factor. *FEBS Lett.* **420**:1–6 (1997).
17. K. Date, K. Matsumoto, K. Kuba, H. Shimura, M. Tanaka, and T. Nakamura. Inhibition of tumor growth and invasion by a four-kringle antagonist (HGF/NK4) for hepatocyte growth factor. *Oncogene* **17**:3045–3054 (1998).
18. K. Kuba, K. Matsumoto, K. Date, H. Shimura, M. Tanaka, and T. Nakamura. 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).
19. D. Tomioka, N. Maehara, K. Kuba, K. Mizumoto, M. Tanaka, K. Matsumoto, and T. Nakamura. Inhibition of growth, invasion, and metastasis of human pancreatic carcinoma cells by NK4 in an orthotopic mouse model. *Cancer Res.* **61**:7518–7524 (2001).
20. Y. Saga, H. Mizukami, M. Suzuki, M. Urabe, A. Kume, T. Nakamura, I. Sato, and K. Ozawa. Expression of HGF/NK4 in ovarian cancer cells suppresses intraperitoneal dissemination and extends host survival. *Gene Ther.* **8**:1450–1455 (2001).
21. S. Hirao, Y. Yamada, F. Koyama, H. Fujimoto, Y. Takahama, M. Ueno, K. Kamada, T. Mizuno, M. Maemondo, T. Nukiwa, K. Matsumoto, T. Nakamura, and Y. Nakajima. Tumor suppression effect using NK4, a molecule acting as an antagonist of HGF, on human gastric carcinomas. *Cancer Gene Ther.* **9**:700–707 (2002).
22. N. Maehara, E. Nagai, K. Mizumoto, N. Sato, K. Matsumoto, T. Nakamura, K. Narumi, T. Nukiwa, and M. Tanaka. Gene transduction of NK4, HGF antagonist, inhibits in vitro invasion and in vivo growth of human pancreatic cancer. *Clin. Exp. Metastasis* **19**:417–426 (2002).
23. M. Saimura, E. Nagai, K. Mizumoto, N. Maehara, H. Okino, M. Katano, K. Matsumoto, T. Nakamura, K. Narumi, T. Nukiwa, and M. Tanaka. Intraperitoneal injection of adenovirus-mediated NK4 gene suppresses peritoneal dissemination of pancreatic cancer cell line AsPC-1 in nude mice. *Cancer Gene Ther.* **9**:799–806 (2002).
24. M. Maemondo, K. Narumi, Y. Saijo, K. Usui, M. Tahara, R. Tazawa, K. Hagiwara, K. Matsumoto, T. Nakamura, and T. Nukiwa. Targeting angiogenesis and HGF function using an adenoviral vector expressing the HGF antagonist NK4 for cancer therapy. *Mol. Ther.* **5**:177–185 (2002).
25. T. Merdan, J. Kopecek, and T. Kissel. Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. *Adv. Drug Deliv. Rev.* **54**:715–758 (2002).
26. M. Matsuura, Y. Yamazaki, M. Sugiyama, M. Kondo, H. Ori, M. Nango, and N. Oku. Polycation liposome-mediated gene transfer in vivo. *Biochim. Biophys. Acta* **1612**:136–143 (2003).
27. A. Veis. The physical chemistry of gelatin. *Int. Rev. Connect. Tissue Res.* **3**:113–200 (1965).
28. Y. Tabata and Y. Ikada. Protein release from gelatin matrices. *Adv. Drug Deliv. Rev.* **31**:287–301 (1998).
29. M. Yamamoto, Y. Tabata, and Y. Ikada. Ectopic bone formation induced by biodegradable hydrogels incorporating bone morphogenetic protein. *J. Biomater. Sci. Polym. Ed.* **9**:439–458 (1998).
30. M. Yamamoto, Y. Tabata, L. Hong, S. Miyamoto, N. Hashimoto, and Y. Ikada. Bone regeneration by transforming growth factor beta1 released from a biodegradable hydrogel. *J. Controlled Release* **64**:133–142 (2000).
31. M. Ozeki, T. Ishii, Y. Hirano, and Y. Tabata. Controlled release of hepatocyte growth factor from gelatin hydrogels based on hydrogel degradation. *J. Drug Target.* **9**:461–471 (2001).
32. Y. Fukunaka, K. Iwanaga, K. Morimoto, M. Kakemi, and Y. Tabata. Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation. *J. Controlled Release* **80**:333–343 (2002).
33. T. Kushibiki, R. Tomoshige, Y. Fukunaka, M. Kakemi, and Y. Tabata. In vivo release and gene expression of plasmid DNA by hydrogels of gelatin with different cationization extents. *J. Controlled Release* **90**:207–216 (2003).
34. H. Cohen, R. J. Levy, J. Gao, I. Fishbein, V. Kousaev, S. Sosnowski, S. Slomkowski, and G. Golomb. Sustained delivery and expression of DNA encapsulated in polymeric nanoparticles. *Gene Ther.* **7**:1896–1905 (2000).
35. C. Perez, A. Sanchez, D. Putnam, D. Ting, R. Langer, and M. J. Alonso. Poly(lactic acid)-poly(ethylene glycol) nanoparticles as new carriers for the delivery of plasmid DNA. *J. Controlled Release* **75**:211–224 (2001).
36. S. L. Snyder and P. Z. Sobocinski. An improved 2,4,6-trinitroimproving benzenesulfonic acid method for the determination of amines. *Anal. Biochem.* **64**:284–288 (1975).
37. A. E. Bolton and W. M. Hunter. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. *Biochem. J.* **133**:529–539 (1973).
38. H. C. Chan, W. T. Ruyechan, and J. G. Wetmur. *In vitro* iodination of low complexity nucleic acids without chain scission. *Biochemistry* **15**:5487–5491 (1976).
39. Y. Gavrieli, Y. Sherman, and S. A. Ben-Sasson. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**:493–501 (1992).
40. K. Kuba, K. Matsumoto, K. Ohnishi, T. Shiratsuchi, M. Tanaka, and T. Nakamura. Kringle 1-4 of hepatocyte growth factor inhibits proliferation and migration of human microvascular endothelial cells. *Biochem. Biophys. Res. Commun.* **279**:846–852 (2000).
41. J. Folkman. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* **1**:27–31 (1995).
42. N. Ikeda, M. Adachi, T. Taki, C. Huang, H. Hashida, A. Takabayashi, M. Sho, Y. Nakajima, H. Kanehiro, M. Hisanaga, H. Nakano, and M. Miyake. Prognostic significance of angiogenesis in human pancreatic cancer. *Br. J. Cancer* **79**:1553–1563 (1999).
43. Y. Kawarada, H. Ishikura, T. Kishimoto, K. Saito, T. Takahashi, H. Kato, and T. Yoshiki. Inhibitory effects of the antiangiogenic agent TNP-470 on establishment and growth of hematogenous metastasis of human pancreatic carcinoma in SCID beige mice in vivo. *Pancreas* **15**:251–257 (1997).
44. L. Holmgren, M. S. O'Reilly, and J. Folkman. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat. Med.* **1**:149–153 (1995).
45. M. S. O'Reilly, L. Holmgren, C. Chen, and J. Folkman. Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nat. Med.* **2**:689–692 (1996).
46. R. Langer. Drug delivery and targeting. *Nature* **392S**:5–10 (1998).
47. F. D. Ledley. Pharmaceutical approach to somatic gene therapy. *Pharm. Res.* **13**:1595–1614 (1996).
48. P. M. Mullen, C. P. Lollo, Q. C. Phan, A. Amini, M. G. Banaszczyk, J. M. Fabrycki, D. Wu, A. T. Carlo, P. Pezzoli, C. C. Coffin, and D. J. Carlo. Strength of conjugate binding to plasmid DNA affects degradation rate and expression level in vivo. *Biochim. Biophys. Acta* **1523**:103–110 (2000).
49. I. Moret. J. Esteban Peris, V. M. Guillem, M. Benet, F. Revert, F. Dasi, A. Crespo, and S. F. Alino. Stability of PEI-DNA and DOTAP-DNA complexes: effect of alkaline pH, heparin and serum. *J. Controlled Release* **76**:169–181 (2001).
50. S. Park and K. E. Healy. Nanoparticulate DNA packaging using terpolymers of poly(lysine-g-(lactide-b-ethylene glycol)). *Bioconjug. Chem.* **14**:311–319 (2003).
51. W. Zauner, A. Kichler, W. Schmidt, A. Sinski, and E. Wagner. Glycerol enhancement of ligand-polylysine/DNA transfection. *Biotechniques* **20**:905–913 (1996).
52. P. Midoux and M. Monsigny. Efficient gene transfer by histidylated polylysine/pDNA complexes. *Bioconjug. Chem.* **10**:406–411 (1999).