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,

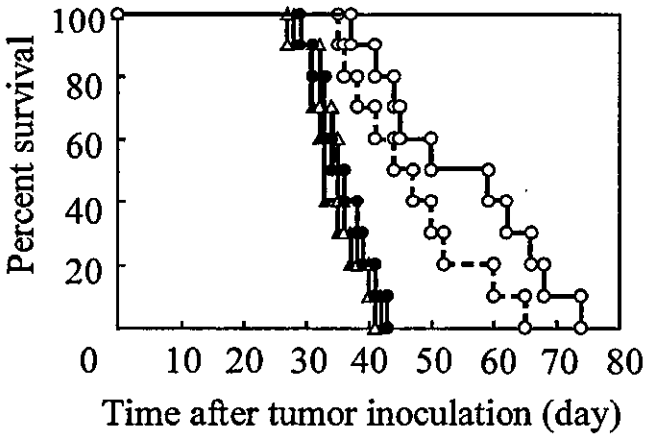

Fig. 2. Survival curves of tumor-bearing mice following the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue: cationized gelatin microspheres incorporating 100 (○---)* and 200 μg of NK4 plasmid DNA (○—)*, 200 μg of 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.

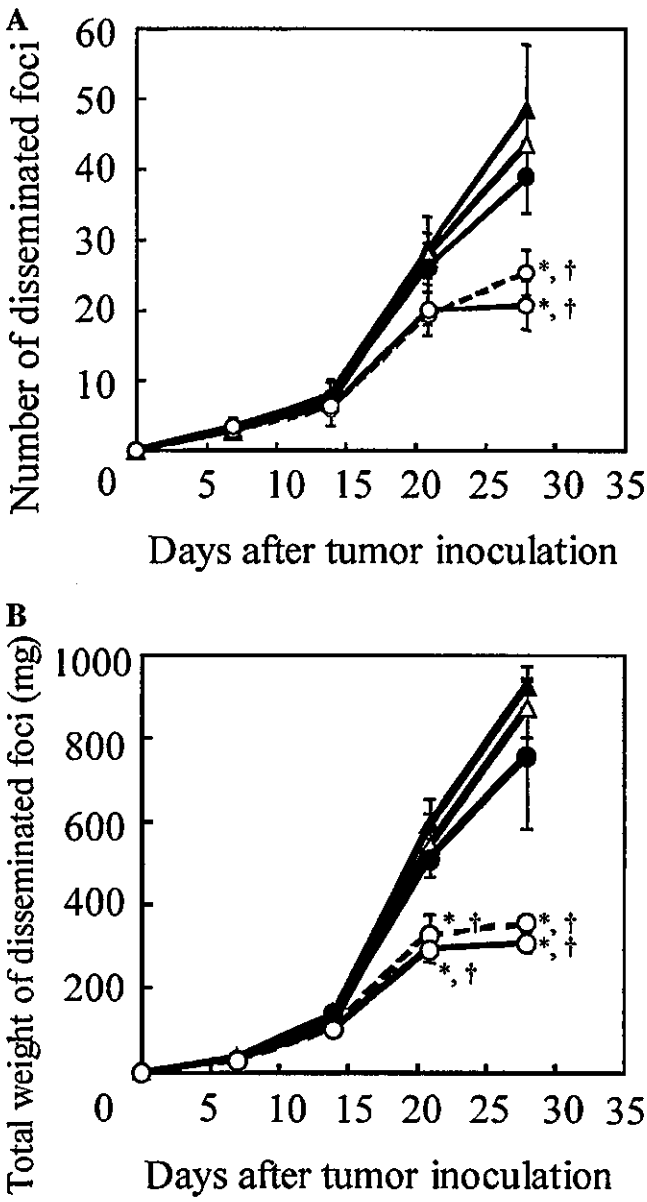

Fig. 3. *In vivo* tumor suppression effects of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA following the single injection into the subcutaneous tissue. (A) Time course of tumor number change: cationized gelatin microspheres incorporating 100 (○---) and 200 μg 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.

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).

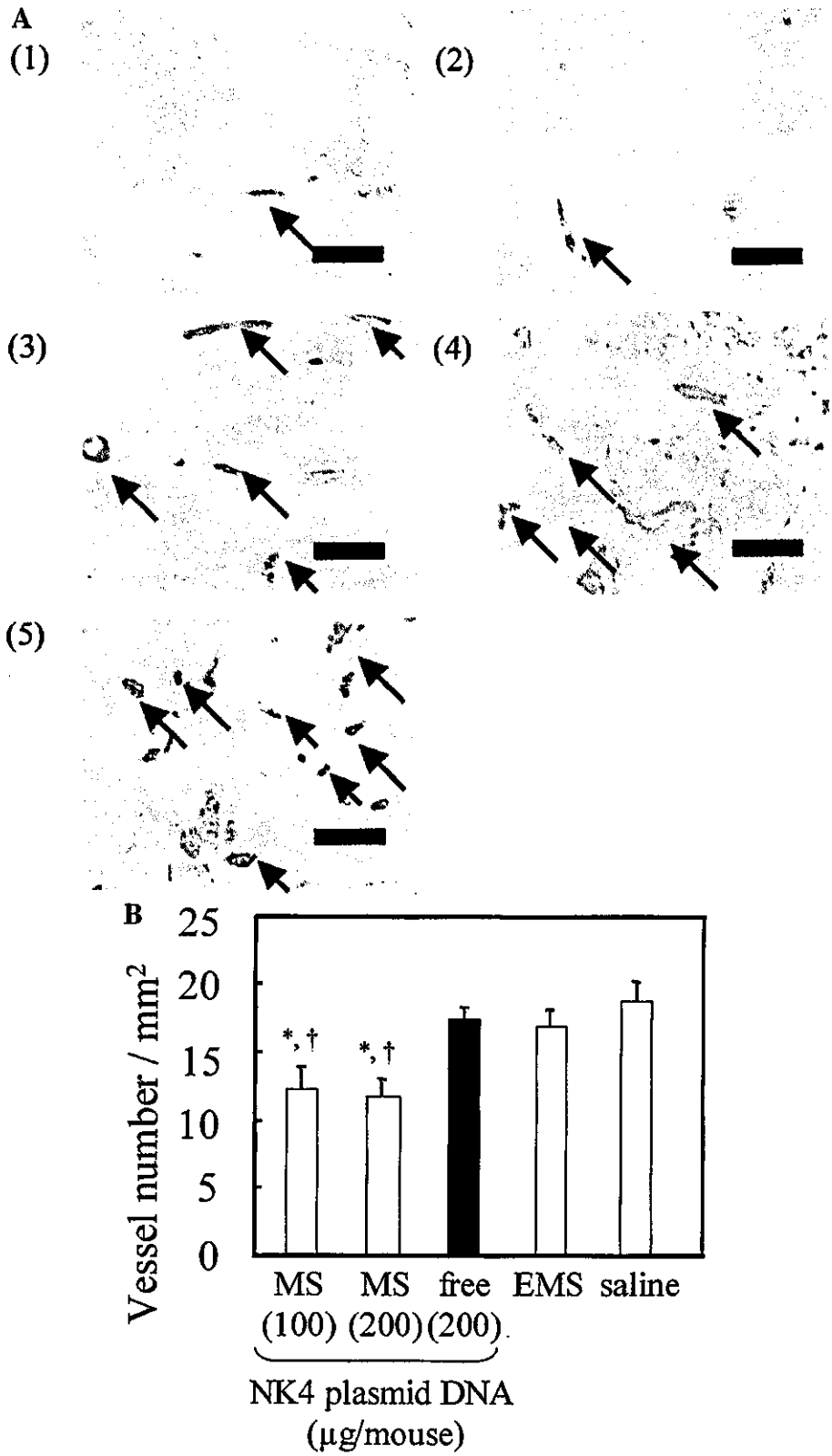

Fig. 4. (A) Immunohistochemical views of blood vessel formation of tumor tissues (arrows) 28 days after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue: cationized gelatin microspheres incorporating (1) 100 and (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.

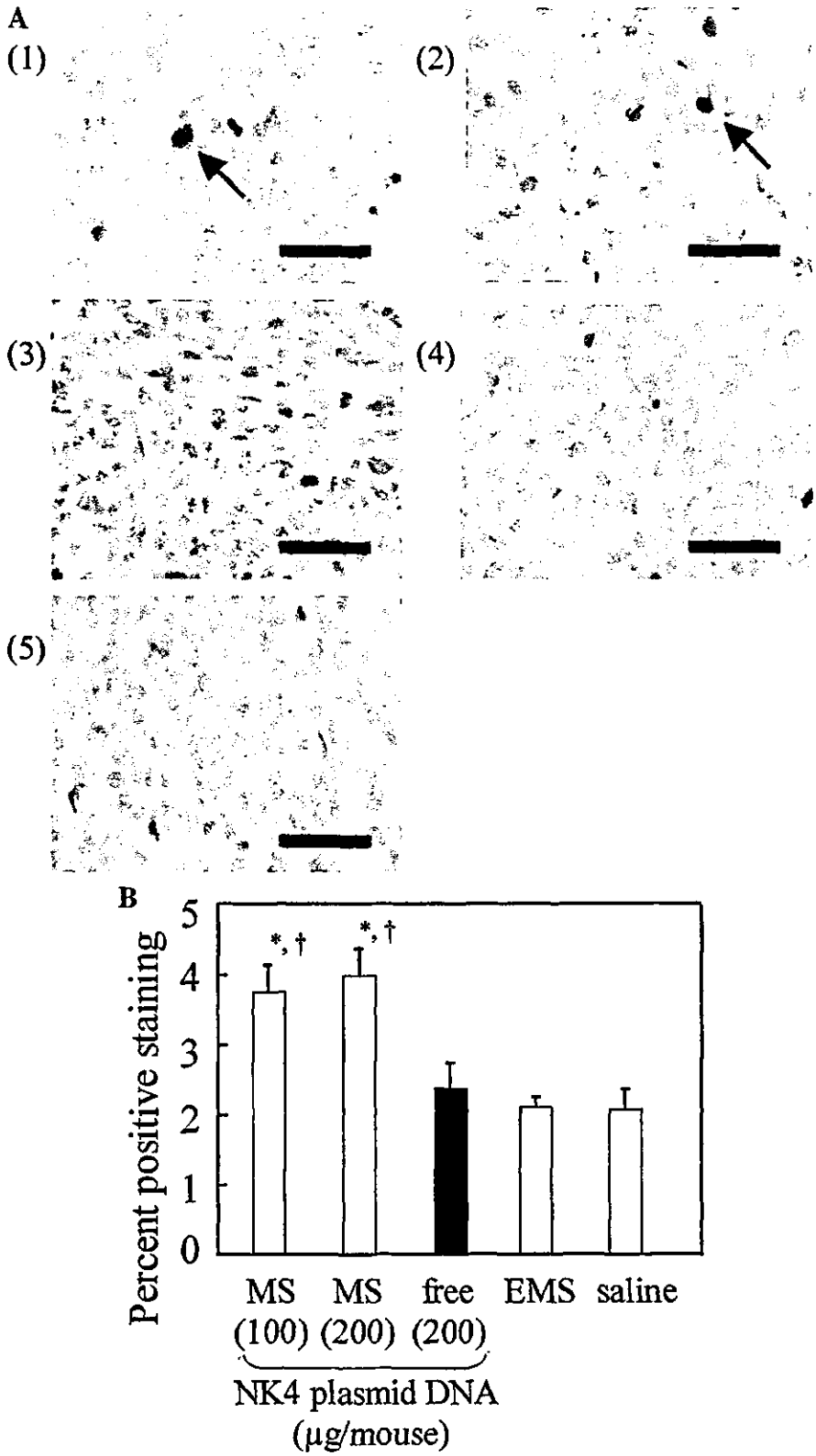

Fig. 5. (A) TUNEL staining of tumor tissues 28 days after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue: cationized gelatin microspheres incorporating (1) 100 and (2) 200 μg of NK4 plasmid DNA, (3) 200 μg of 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.

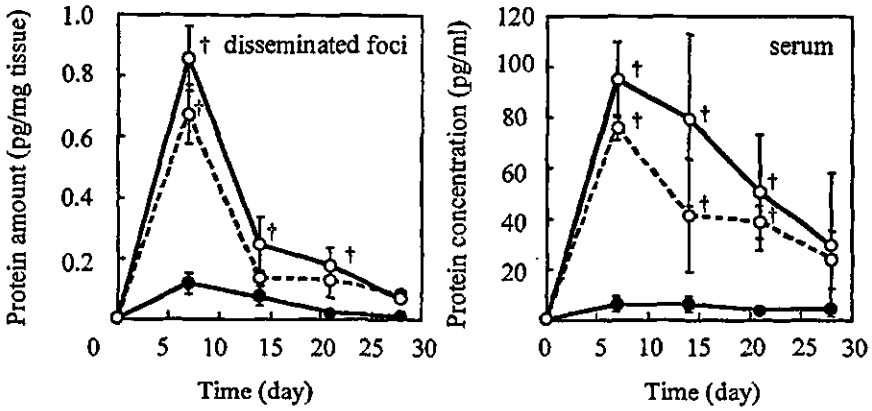

Fig. 6. Time course of amount of NK4 protein detected in the disseminated foci and serum of mice after the single injection of cationized gelatin microspheres incorporating 100 (○—) and 200 μg of NK4 plasmid DNA (○—) and 200 μg of free NK4 plasmid DNA (●) into the subcutaneous tissue. Irrespective of 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.

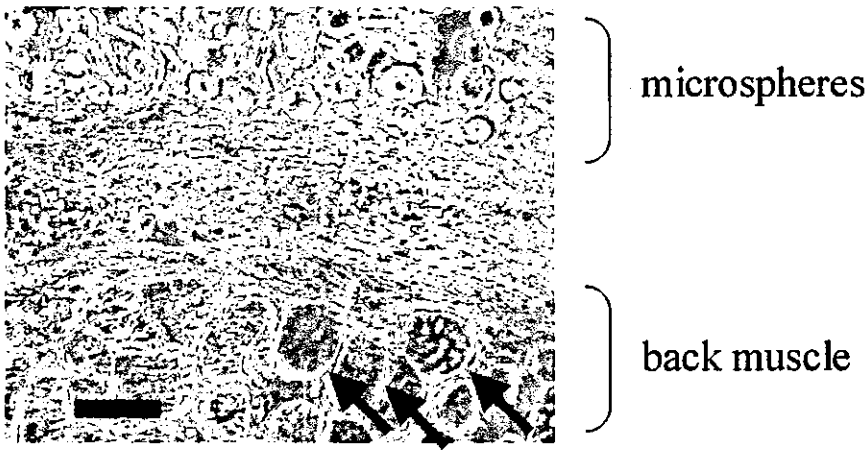

Fig. 7. Tissue localization of gene expression 7 days after the single injection of cationized gelatin microspheres incorporating lacZ plasmid DNA into the subcutaneous tissue. The microspheres injection enables lacZ plasmid DNA to express the β-galactosidase in the muscle around the microspheres (indicated 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.

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RESEARCH ARTICLE

Suppression of tumor metastasis by NK4 plasmid DNA released from cationized gelatin

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NK4, composed of the NH_2-terminal hairpin and subsequent four-kringle domains of hepatocyte growth factor (HGF), acts as an HGF-antagonist and angiogenesis inhibitor. This study is an investigation to evaluate the feasibility of controlled release formulation of NK4 plasmid DNA in suppressing the tumor growth, and lung metastasis. Biodegradable cationized gelatin microspheres were prepared for the controlled release of an NK4 plasmid DNA. The cationized gelatin microspheres incorporating NK4 plasmid DNA could continuously release plasmid DNA over 28 days as a result of microspheres degradation following the subcutaneous injection. The injection of cationized gelatin microspheres incorporating NK4 plasmid DNA into the subcutaneous tissue significantly prolonged the survival time period of the mice bearing Lewis lung carcinoma tumor. Increases in the tumor volume and the number of lung metastatic nodules of NK4 plasmid DNA release group were suppressed to a significantly greater extent than that of solution-injected group (77.4 and 64.0%, respectively). The number of blood vessels and the apoptosis cells in the tumor tissue were significantly suppressed (80.4%) and increased (127.3%) against free NK4 plasmid DNA-injected group. Thus, the controlled release of NK4 plasmid DNA augmented angiogenesis suppression and apoptosis of tumor cells, which resulted in suppressed tumor growth. We conclude that this controlled release technology is promising to enhance the tumor suppression achieved by gene expression of NK4.
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Keywords: *controlled release; cationized gelatin microspheres; NK4; tumor metastasis*

Introduction

Hepatocyte growth factor (HGF) has been noted as the signal molecule that plays an important role in development, morphogenesis, and regeneration of living systems.[1–4] Recently, some therapeutic trials of angiogenesis induction,[5,6] chronic fibrotic diseases,[7–9] and tissue regeneration[10,11] by HGF have been performed experimentally and clinically to demonstrate the potential efficacy. On the other hand, for malignant tumors, HGF plays a definitive role in invasive, angiogenic, and metastatic behavior of cancer cells by way of the c-Met receptor.[12–16] Therefore, it is highly expected that inhibition of interaction between HGF and the c-Met receptor effectively suppresses the malignant activity of tumors. Based on this concept, Date *et al*[17] prepared an antagonist for HGF. The antagonist (NK4) is composed of the NH_2-terminal hairpin domain and the subsequent four-kringle domains of α-subunit of HGF. The NK4 binds to the c-Met/HGF receptor, but does not induce tyrosine phosphorylation of c-Met.[17] NK4 competitively inhibits some biological events driven by the HGF-Met receptor binding, including the invasion and metastasis of distinct types of tumor cells.[17,18] Moreover, NK4 has antiangiogenic activity and the antiangiogenic action is independent of its activity as an HGF antagonist.[19] The recombinant protein of NK4 has been used for tumor animal models to demonstrate the *in vivo* therapeutic feasibility and the blocking effect on HGF functions.[18–20] In addition, antitumor effects of stable expression of NK4 in cancer cells and recombinant adenovirus-mediated gene expression of NK4 have been reported.[21–25]

Based on 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.[26] Presently, several human clinical trials are being carried out to treat the cancer by utilizing the viral vectors of retroviruses, adenoviruses, and adeno-associated viruses.[27] In spite of the high transfection efficiency, the trials are limited by the adverse effects of the virus itself, such as immunogenicity and toxicity or the possible mutagenesis of the cells transfected. Many types of cationized polymers[28] and cationized liposomes[29] have been explored and complexed with the plasmid DNA or antisense oligonucleotide for gene expression. 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, there are some problems to be solved, namely the transient and the low level of

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gene expression. One of the practically possible ways for better gene expression is to incorporate the plasmid DNA into an appropriate matrix for the controlled release over an extended time period. For the release matrix, we have taken advantage of a gelatin hydrogel prepared by the crosslinking of biodegradable polymer.[30] Since the polymer is positively charged, the plasmid DNA is likely to be immobilized in the hydrogel through the electrostatic interaction between the plasmid DNA and polymer molecule. The immobilized plasmid DNA could be released from the hydrogel if the hydrogel is degraded to generate the complex of water-soluble fragments of hydrogel polymer and plasmid DNA. This nonviral delivery system offers the advantages of biocompatibility of gelatin as a vector/carrier and the controllable biodegradability or achievement of local delivery and protection from rapid degradation of plasmid DNA by nucleases.

This study is the first report to demonstrate that the controlled release of NK4 plasmid DNA suppressed the tumor metastasis, in marked contrast to free plasmid DNA. 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.[31,32] We applied the cationized gelatin hydrogel to the controlled release of expression plasmid for human NK4, to evaluate the suppressive effects on tumor angiogenesis, growth, and metastasis in tumor-bearing mice. The results were compared to those with delivery of free plasmid to emphasize the efficacy of the release system in enhancing the biological activity of NK4.

Results

In vivo release profile of NK4 plasmid DNA from cationized gelatin microspheres

Figure 1 shows the time course of radioactivity remaining after the injection of cationized gelatin microspheres incorporating ^{125}I-labeled NK4 plasmid DNA and ^{125}I-labeled free NK4 plasmid DNA or ^{125}I-labeled cationized gelatin microspheres. The remaining radioactivity decreased with injection time for every case. For cationized gelatin microspheres, the radioactivity gradually decreased over the time period of 28 days, whereas the radioactivity of free ^{125}I-labeled NK4 plasmid DNA injected more rapidly reduced, and disappeared from the injected site within 7 days. A good correlation in the time profile of *in vivo* retention was observed between the NK4 plasmid DNA incorporated and microspheres, demonstrating the controlled release of NK4 plasmid DNA as a result of microsphere degradation.

Prolonged survival and suppression of tumor growth and metastasis by cationized gelatin microspheres incorporating NK4 plasmid DNA

In the present study, we attempted to elucidate the antitumor effect of NK4 in metastatic murine tumor model. When Lewis lung carcinoma (LLC) cells were inoculated subcutaneously into mice, tumor cells formed tiny nodules 4 days after implantation, and metastatic nodules on the lung surface became visible 28 days after implantation.

Figure 2 shows the survival curve of tumor-bearing mice after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA or other agents. When the mice were injected with saline, empty cationized gelatin microspheres, and free NK4 plasmid DNA solution, all the mice died within 30 days. In contrast, the injection of cationized gelatin microspheres incorporating NK4 plasmid DNA significantly prolonged the survival time period of mice, wherein the

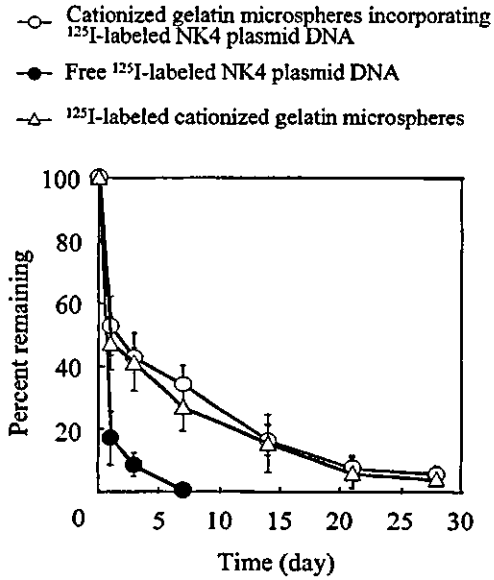

Figure 1 The time course of radioactivity of the remaining cationized gelatin microspheres incorporating ^{125}I-labeled NK4 plasmid DNA and free ^{125}I-labeled NK4 plasmid DNA or ^{125}I-labeled cationized gelatin microspheres after the subcutaneous injection into the backs of tumor-bearing mice. The 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 accordance with that of microspheres as the release carrier.

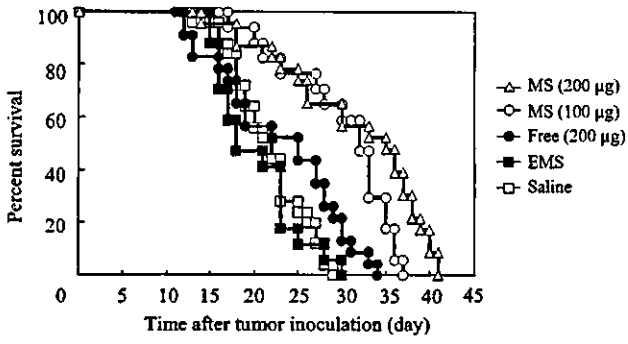

Figure 2 Survival curves of tumor-bearing mice following the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue around the tumor mass: cationized gelatin microspheres incorporating 100 (MS (100 μg)) and 200 μg of NK4 plasmid DNA (MS (200 μg))*, 200 μg of free NK4 plasmid DNA (free (200 μg)), empty cationized gelatin microspheres (EMS), and saline. *, $P<0.05$: significant against the survival curve of saline-injected, control mice. 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.*

higher dose of NK4 plasmid DNA allowed longer survival of mice.

Figure 3a and b shows changes in primary tumor growth and the number of metastatic nodules after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA or other agents. The increase in the tumor volume was significantly suppressed by the injection of cationized gelatin microspheres incorporating NK4 plasmid DNA, in marked contrast to free NK4 plasmid DNA. The tumor volume of mice receiving the injection of cationized gelatin microspheres incorporating 100 and 200 μg of NK4 plasmid DNA was 68.3 and 77.4% of that of free NK4 plasmid DNA solution-injected mice, respectively. Lung metastasis was also suppressed by cationized gelatin microspheres incorporating NK4 plasmid DNA to a significantly greater extent than other agents. The number of lung metastasis node of mice receiving the injection of cationized gelatin microspheres incorporating 100 and 200 μg of NK4 plasmid DNA was 62.3 and 64.0% of that of free NK4 plasmid DNA solution-injected mice, respectively. Injection of free NK4 plasmid DNA and empty cationized gelatin microspheres did not affect the number of lung metastasis, and the tumor volume and lung metastasis were similar to those of saline-injected, control mice.

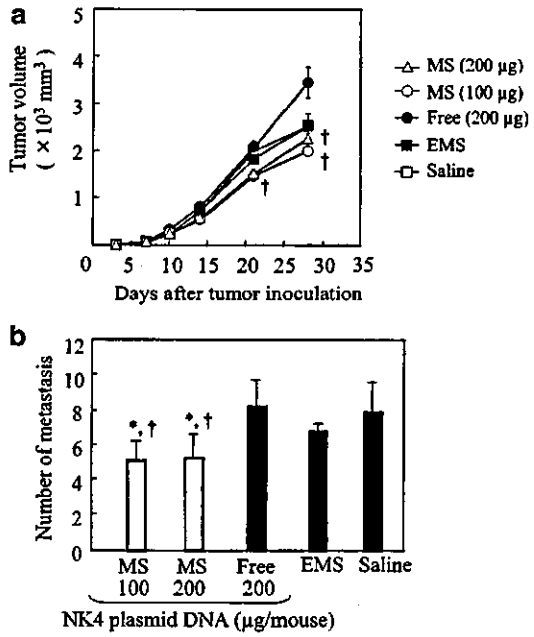

*Figure 3 In vivo tumor suppression effects of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA following the single injection into the subcutaneous tissue around the tumor mass. (a) Time course of tumor volume change: cationized gelatin microspheres incorporating 100 (MS (100 μg)) and 200 μg of NK4 plasmid DNA (MS (200 μg)), 200 μg of free NK4 plasmid DNA (free (200 μg)), empty cationized gelatin microspheres (EMS), and saline. *, $P<0.05$: significant against the tumor volume of saline-injected mice at the corresponding day. †, $P<0.05$: significant against the tumor volume of mice injected with 200 μg of free NK4 plasmid DNA. Irrespective of the NK4 plasmid DNA dose, the injection of microspheres incorporating NK4 plasmid DNA significantly suppressed the in vivo growth of tumor cells, in contrast to that of free NK4 plasmid DNA. (b) The nodule number of lung tumor metastasis 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 lung metastases of saline-injected mice. †, $P<0.05$: significant against the number of lung metastases of mice injected with 200 μg free NK4 plasmid DNA. The injection of microspheres incorporating both doses of NK4 plasmid DNA significantly decreased the nodule number of lung tumor metastasis, in contrast to that of free NK4 plasmid DNA.*

Angiogenesis and apoptosis in the tumor tissue

Figure 4a and b shows the immunohistochemical views and number of blood vessels of primary tumor tissue. The injection of cationized gelatin microspheres incorporating NK4 plasmid DNA significantly decreased the number of blood vessels in the tumor tissue, and the size of tumor blood vessels was smaller compared to that of other agents. 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 82.1 and 80.4% of that of NK4 plasmid DNA solution-injected mice, respectively.

Since inhibition of tumor growth is associated with the increase in apoptosis of tumor cells, we analyzed change in apoptosis of tumor cells by TUNEL staining after injection of NK4 plasmid DNA (Figure 5a and b). The number of tumor cells undergoing apoptosis in the tumor tissue was significantly increased by the injection of cationized gelatin microspheres. In contrast, delivery of free NK4 plasmid DNA had no significant effect on apoptosis of tumor cells. The number of apoptosis cells in the tumor tissue of mice receiving the injection of cationized gelatin microspheres incorporating 100 and 200 μg of NK4 plasmid DNA was 119.4 and 127.3% of that of free NK4 plasmid DNA solution-injected mice, respectively. Together with inhibition of tumor angiogenesis by delivery of NK4 plasmid DNA in cationized gelatin microspheres, these results suggest that inhibition of tumor growth might be caused by increased apoptosis of tumor cells associated with inhibition of tumor angiogenesis.

Figure 6a shows the time course of amount of NK4 protein expression in the primary tumor tissue, lung, and blood of tumor-bearing mice after injection of cationized gelatin microspheres incorporating 100 and 200 μg of NK4 plasmid DNA and 200 μg of free NK4 plasmid DNA. The NK4 concentration in the tumor and lung or blood increased with time up to day 7 to attain a maximum level, but thereafter decreased gradually. A low level of NK4 expression was detected in the lung tissue including metastatic nodules, whereas a high concentration of NK4 was observed in the blood. In contrast, no NK4 protein was detected at any sampling time and site for free NK4 plasmid DNA. A lacZ plasmid DNA was used to clarify the expression site. When cationized gelatin microspheres incorporating lacZ plasmid DNA were injected, gene expression was observed in the tumor mass around microspheres (Figure 6b).

Discussion

The gene delivery system is generally divided into two categories: viral and nonviral vectors. Although viral vectors such as retrovirus, adenovirus, and adeno-associated virus are potentially efficient, nonviral vectors have the advantages of less toxicity, less immunogenicity, and easier preparation. So far, several methods for delivering genes with nonviral carriers have been

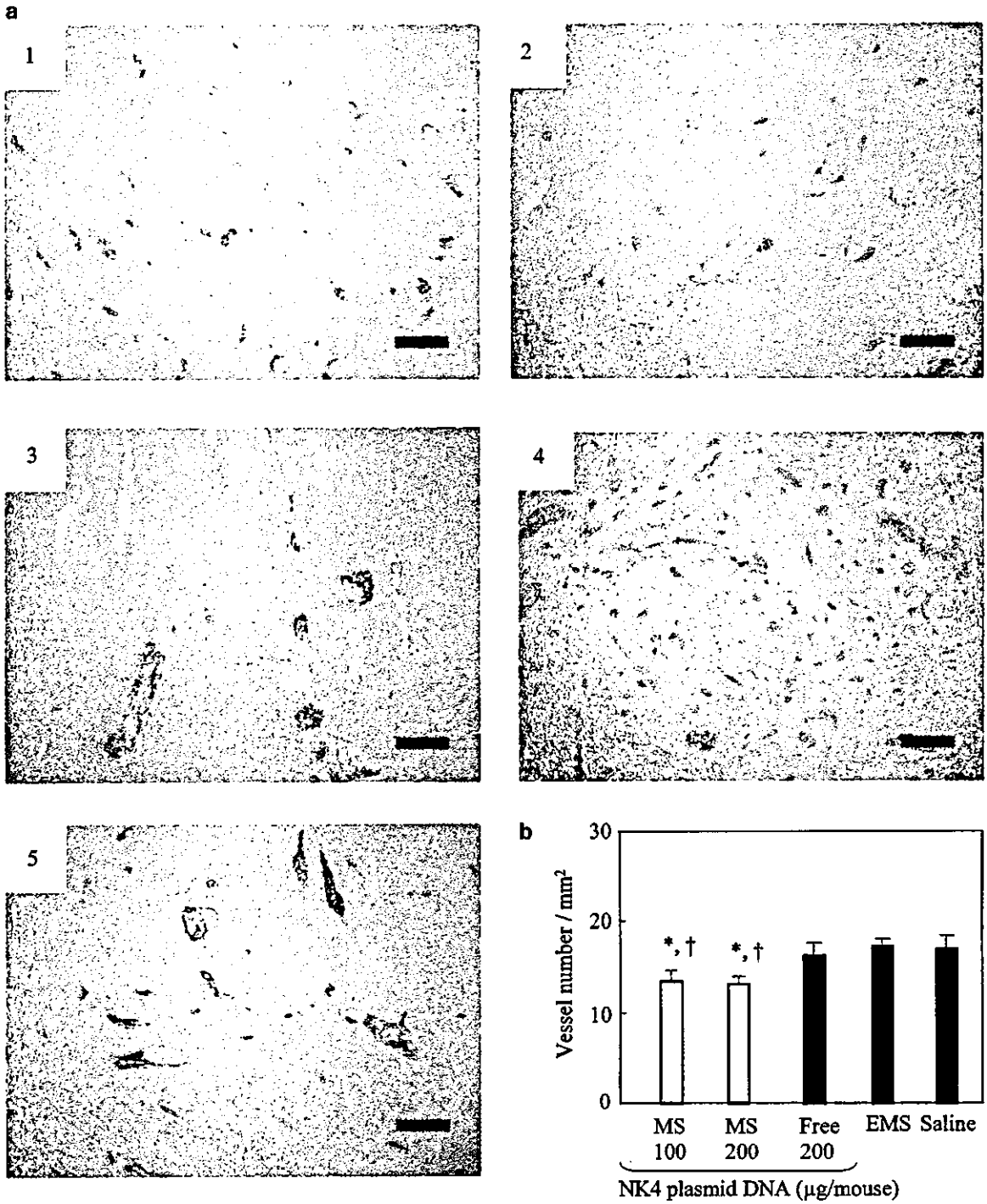

*Figure 4 (a) Immunohistochemical views of blood vessel formation of tumor tissues 28 days after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue around the tumor mass: cationized gelatin microspheres incorporating 100 (1) and 200 μg of NK4 plasmid DNA (2), 200 μg of free NK4 plasmid DNA (3), empty cationized gelatin microspheres (4), and saline (5) (magnification; ×100). The bar length is 200 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 doses of NK4 plasmid DNA significantly decreased the number of blood vessels formed around the tumor mass, in contrast to that of free NK4 plasmid DNA.*

developed, including naked plasmid DNA injection and complex formation with cationized polymers[28] or cationized liposomes.[29] However, there are several drawbacks with each nonviral vector, including a low efficiency of gene transfection compared with viral vectors and a transient gene expression. In this study, we introduce a system of prolonged gene expression based on the sustained release of plasmid DNA from cationized gelatin microspheres. The release mechanism driven by degradation of release carrier is quite different from that

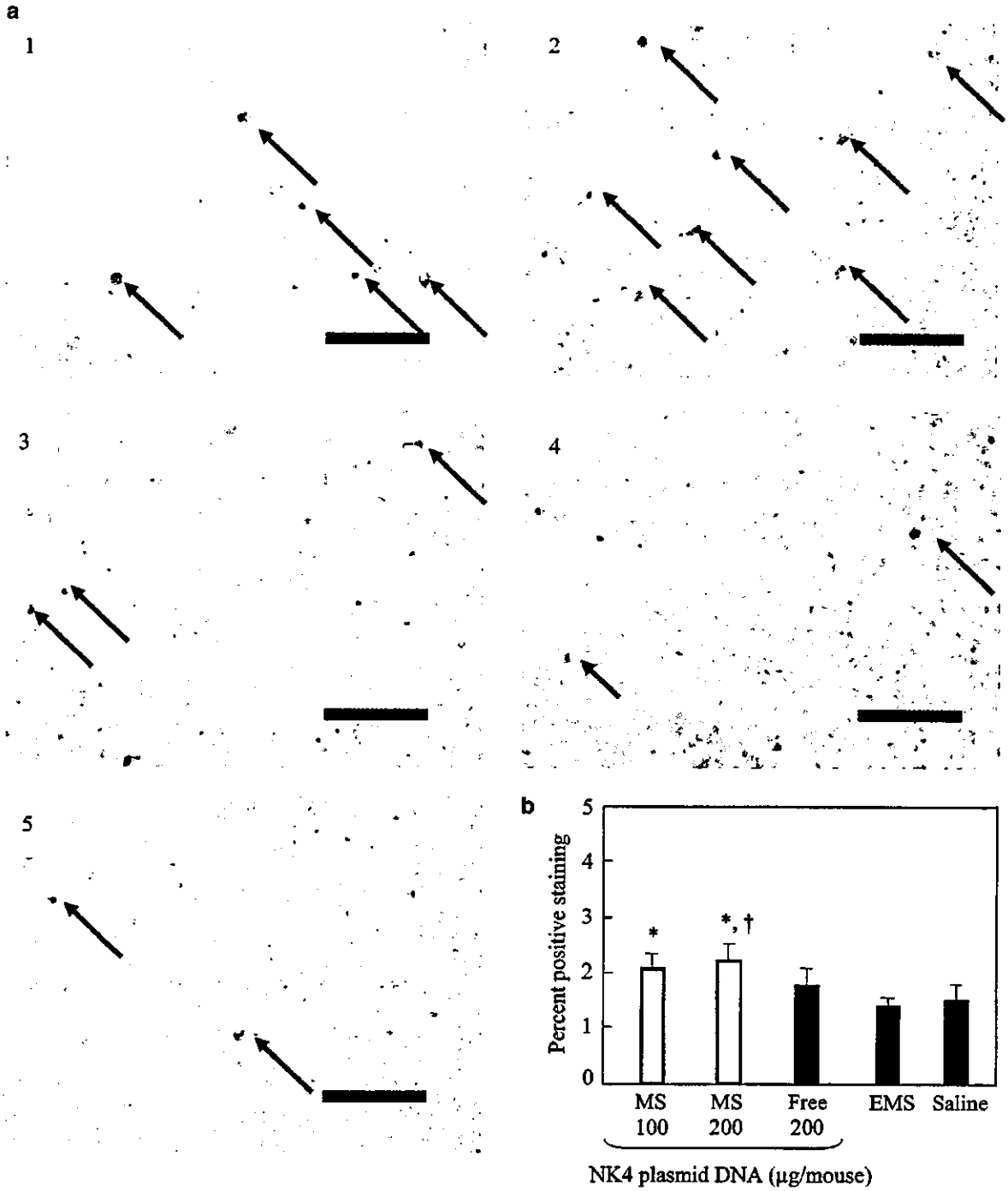

*Figure 5 (a) TUNEL staining of tumor tissues 28 days after the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue around the tumor mass: cationized gelatin microspheres incorporating 100 (1) and 200 μg of NK4 plasmid 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

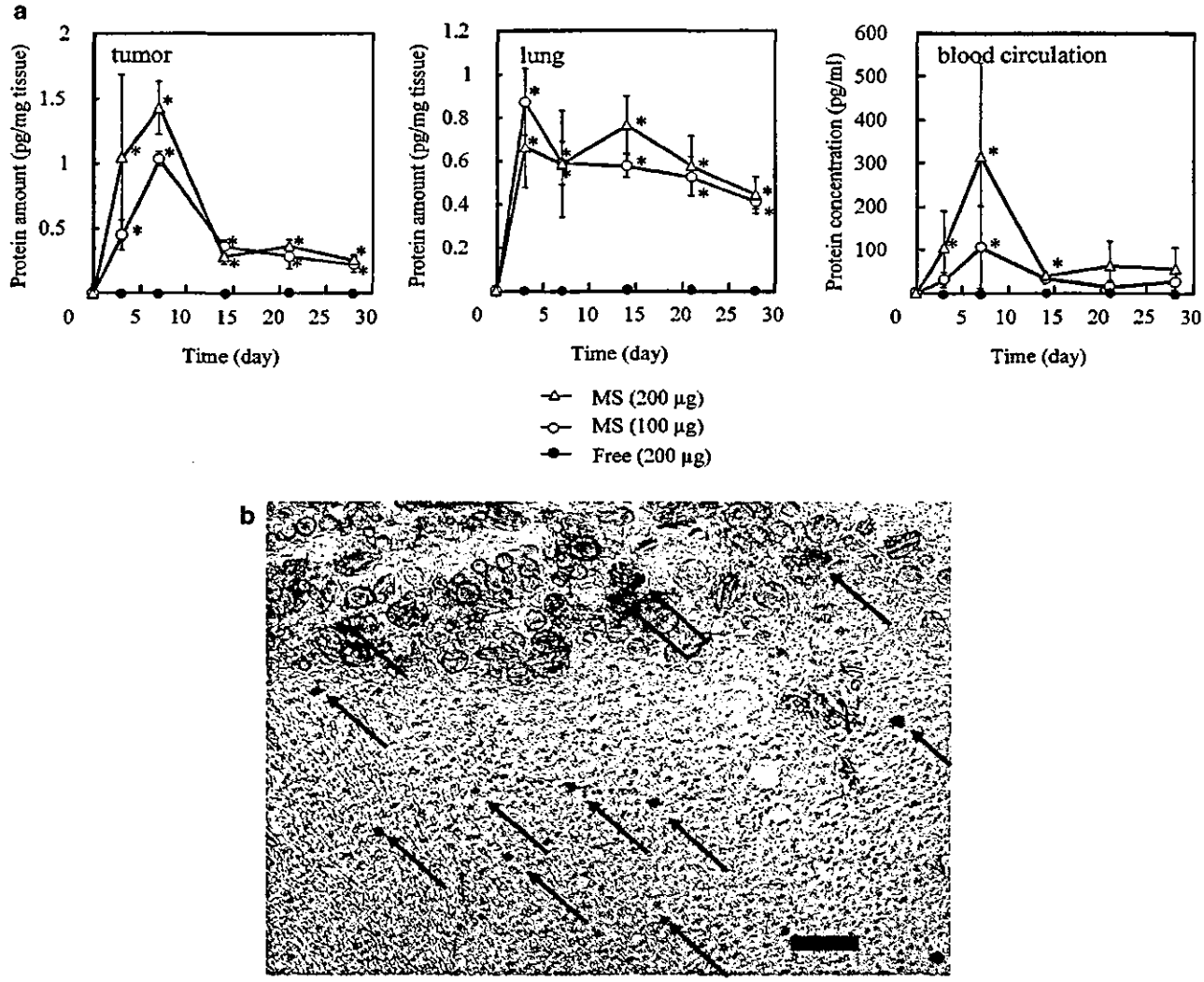

*Figure 6 (a) Time course of the amount of NK4 protein detected in the tumor, lung, and blood circulation of mice after the single injection of cationized gelatin microspheres incorporating 100 (MS (100 μg)) and 200 μg of NK4 plasmid DNA (MS (200 μg)) and 200 μg of free NK4 plasmid DNA (free (200 μg)) 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 width^2 × length × 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