

Tumor Suppression through Angiogenesis Inhibition by SUIT-2 Pancreatic Cancer Cells Genetically Engineered to Secrete NK4¹

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ABSTRACT

NK4, composed of the N-terminal hairpin and subsequent four-kringle domains of hepatocyte growth factor (HGF), acts not only as a competitive antagonist of HGF but also as an inhibitor of angiogenesis. By studying the antitumor effect of NK4, we evaluated the potential of gene therapy with NK4 as a treatment for pancreatic cancer. Expression vector pcDNA3-NK4 containing NK4 cDNA was used to transfect human pancreatic cancer cell line SUIT-2. Although the established NK4 transfectant continuously expressed NK4 protein, the expression was shown by migration assay to be insufficient to antagonize HGF *in vitro*. Proliferation of the NK4 transfectant did not differ significantly from that of a mock transfectant. *In vivo*, we used models of orthotopic implantation and liver metastasis to transplant NK4-transfected clone or mock-transfected clone into nude mice. Cell proliferation *in vivo*, evaluated by immunohistochemical staining of proliferating cell nuclear antigen, did not differ between NK4 and mock transfectants, and this was also the finding in the *in vitro* assay. However, the NK4-transfected clone showed significant inhibition of tumor progression in both the orthotopic implantation and liver metastasis models. The number of vessels within tu-

mors was significantly decreased, and the apoptotic tumor cells were increased in number. The results of these experiments show that genetic modification of tumor cells with NK4 cDNA yields a significant antitumor effect and that this effect is mainly obtained by NK4's function as an angiogenesis inhibitor rather than as an HGF antagonist. We conclude that the potent angiogenesis inhibitor NK4 may be a promising molecule for gene therapy of pancreatic cancer.

INTRODUCTION

Pancreatic cancer is one of the most difficult neoplasms to treat curatively (1, 2). Peritoneal dissemination, a frequent mode of recurrence after surgical resection (3, 4), invariably culminates in death and is often associated with intractable ascites leading to substantial impairment of the patient's quality of life. Moreover, liver metastasis via the portal vein also occurs frequently in patients with pancreatic cancer before and after surgical removal of the primary tumor and constitutes a major cause of death from the disease (2). These conditions are generally refractory to existing chemotherapy, radiotherapy, endocrinotherapy, or immunotherapy (2, 5, 6). Thus, development of a new treatment modality for peritoneal dissemination and liver metastasis of pancreatic cancer has been eagerly awaited (5).

NK4 is composed of the N-terminal hairpin and subsequent four-kringle domains of HGF³ and acts as a competitive HGF antagonist devoid of its own HGF-related activities (7–9). NK4 inhibits mitogenic, motogenic, and morphogenic activities of HGF (8, 9). The NK4 binding to the c-Met receptor is 10-fold lower than the HGF binding, whereas tyrosine phosphorylation of the c-Met receptor is almost completely inhibited by NK4 at 1000-fold higher concentration than that of HGF (8–10). Competitive inhibitory effects of NK4 on HGF and c-Met receptor interaction have been reported in some particular types of human cancer cells (9–12). NK4 dose dependently inhibits pancreatic cancer cell migration and invasion, coincident with the competitive inhibition of HGF-binding to the c-Met receptor by NK4 (13). A recent study showed that NK4 is an angiogenesis inhibitor as well as an HGF antagonist and that the antiangiogenic action of NK4 is independent of its activity as an HGF antagonist (10). The study also showed that NK4 inhibited tumor growth and metastasis, acting as an angiogenesis inhibitor and an HGF antagonist, in Lewis lung carcinoma and Jyg-MC(A) mammary carcinoma implanted s.c. into mice (10).

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³ The abbreviations used are: HGF, hepatocyte growth factor; FBS, fetal bovine serum; MVD, microvessel density; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

Therefore, both functions have been considered important to the therapeutic potential of NK4.

Before NK4 can be used clinically for cancer therapy, an appropriate delivery system must be developed. In previous experiments, prolonged administration and high doses of recombinant NK4 suppressed tumor progression *in vivo* (9, 10, 14). However, the administration of recombinant NK4 is impractical and difficult in clinical settings because a large amount of NK4 is required, and the cost of its preparation is prohibitive.

To examine the potential role of NK4 in the gene therapy of pancreatic cancer, we transfected human pancreatic cancer cell line SUIT-2 with NK4 cDNA. The clone of SUIT-2 tumor cells expressing NK4 was isolated, and *in vivo* growth characteristics of the NK4 transfectant were examined in experimental orthotopic implantation and liver metastasis models. We report here a dramatic antitumor effect in the NK4-transfected SUIT-2 cells. Mainly through reduction of tumor angiogenesis, NK4 inhibited pancreatic tumor growth, peritoneal dissemination, and liver metastasis in both models.

MATERIALS AND METHODS

Plasmid Construction and Preparation. Plasmid pcDNA3-NK4 was constructed by inserting human NK4 cDNA (8) into the *Hind*III and *Xba*I restriction sites of pcDNA3; this construction was driven by the cytomegalovirus promoter. Plasmids were grown under ampicillin selection in *Escherichia coli* and purified with the use of the Qiagen Plasmid Maxi kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's recommendations. The quantity of plasmid DNA was assessed by determining absorbance at 260 and 280 nm, and the DNA was dissolved in PBS, aliquoted, and stored at -20°C until use.

Cells and Culture Conditions. Human pancreatic cancer cell line SUIT-2 was generously donated by Dr. Haruo Iguchi (National Kyushu Cancer Center, Fukuoka, Japan). SUIT-2 cells were cultured in RPMI supplemented with streptomycin, penicillin, and 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2 .

Electroporation and Establishment of Stable Transfectants. Twenty μg of pcDNA3-NK4 or pcDNA3 was mixed with 1×10^6 SUIT-2 cells and then electroporated at 250 mV and 950 μF (GenePulser II and Capacitance Extender; Bio-Rad, Hercules, CA). The cells were immediately transferred to complete medium prewarmed at 37°C . G418 (Promega, Madison, WI) selection was started 24 h after the transfection, and individual clones derived from G418-resistant single cells were pooled and grown for NK4 expression analyses.

Confirmation of NK4 Expression in the Stable Transfectants. ELISA was carried out to quantify the expression of NK4 protein secreted by the G418-resistant clones. pcDNA3-NK4 and pcDNA3-transfected clones (NK4 transfectants and mock transfectants) were cultured in 10-cm tissue culture dishes at 1×10^6 cells/well for 24 h. The conditioned media were collected, and NK4 concentration in the media was assayed with a human HGF ELISA kit (Immunis HGF EIA; Institute of Immunology, Tokyo, Japan) according to the manufacturer's recommendations.

Determination of *In Vitro* Cell Proliferation. Each selected transfectant was plated onto 6-well tissue culture dishes at

1×10^5 cells/well (day 0) and on days 1, 3, 5, and 7, cell numbers in each well were counted in duplicate with a particle distribution counter (CDA500; Sysmex, Kobe, Japan).

***In Vitro* Migration Assay.** *In vitro* migration of each transfectant was measured with the use of Costar Transwell cell culture chamber inserts (Corning, Inc., Corning, NY). The transfectants were suspended in RPMI containing 2% FBS and added to the inner cup of the chamber at a density of 5×10^4 cells/ cm^2 . HGF (1 or 10 ng/ml) was added to the medium of the outer cup. After a 24-h cultivation, cells that migrated through the 8- μm -pore filter membrane were stained with H&E. Ten microscopic fields ($\times 200$) were randomly selected for cell counting.

Animals and Orthotopic Implantation of the Stable Transfectants. Six-week-old female athymic nude mice (BALBc nu/nu) were purchased from Japan SLC (Hamamatsu, Japan). The mice were housed in laminar flow cabinets under specific pathogen-free conditions in facilities approved by Kyushu University. Cell suspensions of NK4 transfectant (NK4 group, $n = 5$) and of mock transfectant (mock group, $n = 5$) at 1×10^6 cells/0.1 ml were injected into the pancreas of mice under anesthesia. All mice were killed when one was moribund (at 3 weeks). The size and weight of the primary pancreatic tumors and the size and number of disseminated peritoneal tumor nodules with the volume of ascites were measured. Tissue samples of the tumors were subjected to additional analyses.

Liver Metastasis of the Stable Transfectants. The ability of the transfectants to metastasize to the liver was evaluated by slowly injecting 1×10^6 cells/0.2 ml of each transfectant into the spleen via 27-gauge needle (NK4 group, mock group; $n = 5$ /group). All mice were killed at 4 weeks. The liver was excised and weighed, and metastases were observed macroscopically.

NK4 Expression in Tumors. Tissue samples were homogenized in ice-cold lysis buffer consisting of 1% Triton X-100, 10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.1% mM sodium orthovanadate, and 0.1% bovine albumin. The supernatants were collected by centrifugation at 12,000 rpm for 10 min. Expression of NK4 was checked by ELISA analysis as described above for the *in vitro* assay.

Immunohistochemical Staining and Counting. For microvessel staining, the peroxidase-conjugated avidin-biotin complex method was used with a Vectastain Elite ABC kit (Vector, Burlingame, CA). Mouse monoclonal CD31 antibody JC/70A (NeoMarkers, Fremont, CA) was used at a dilution of 1:50, followed by the application of biotinylated antimouse IgG (1:100; Vector). MVD was assessed in tumor areas showing high staining density. The number of vessels was counted in 10 fields/section, 1 field was magnified 200-fold (0.739 mm^2 /field), and the mean counts were recorded. Proliferating cells were detected with antibody against PCNA PC10 (DAKO, A/S, Glostrup, Denmark). To quantify PCNA expression in the tumors, we counted the number of positive cells in 10 random fields/section at $\times 200$. Apoptotic cells within the tumor nodules were detected by TUNEL assay (*In situ* Apoptosis Detection kit; Takara, Shiga, Japan). The number of positive cells was counted in 10 random fields/section at $\times 200$.

Statistical Analysis. Statistical significance was evaluated with Fisher's exact test or nonparametric Mann-Whitney U

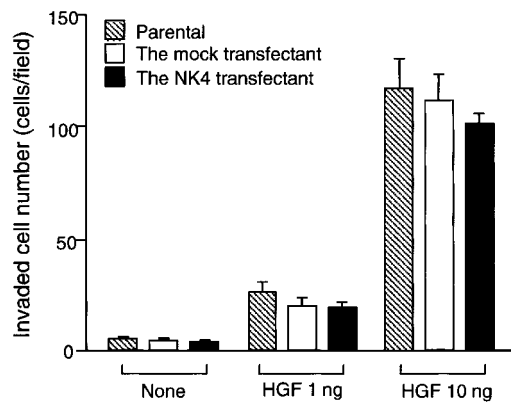


Fig. 1 Ability of autocrine NK4 of the NK4 transfectant to antagonize HGF. The migration potential of parental SUI-2 (human pancreatic cancer cell line) cells and the stable transfectants (mock and NK4) were evaluated in the absence and presence of HGF (1 or 10 ng/ml). Mean \pm SD values are shown.

test. All tests were two-tailed, and a $P < 0.05$ was considered to indicate statistical significance.

RESULTS

NK4 Expression and Cell Proliferation of the Stable Transfectants. Three individual clones in which expression of NK4 was at a high level were selected from among the G418-resistant clones obtained by the transfection of pcDNA3-NK4. Expression levels of NK4 in the three transfectants were 6.87, 1.96, and 0.25 ng/ 1×10^6 cells/24 h. The clone that showed the highest expression of NK4 was analyzed further. A mock transfectant that expressed no detectable NK4 protein was used for control. We examined whether cell proliferation of the NK4 transfectant was affected by autocrine NK4. *In vitro* proliferation of the NK4 transfectant did not differ from that of the mock transfectant (data not shown), indicating that autocrine NK4 had no effect on proliferation of the NK4 transfectant *in vitro*.

Ability of Autocrine NK4 to Antagonize HGF. We conducted an *in vitro* migration assay to determine whether autocrine NK4 of the NK4 transfectant had an antagonistic effect on the biological activities of HGF. HGF stimulated migration of these tumor cells dose dependently, and ~ 30 -fold more cells migrated through the membrane in the presence of 10 ng/ml HGF than in the absence of HGF (Fig. 1). Although autocrine NK4 in the NK4 transfectant was expected to antagonize the migration induced by HGF, this migration was in fact scarcely inhibited. The estimated concentration of autocrine NK4 of the NK4 transfectant was about 0.1 ng/well/24 h, and autocrine NK4 at that level was incapable of antagonizing even 1 ng/ml HGF because a 1000-fold greater concentration of NK4 than of HGF is required to completely inhibit the HGF-induced migration. Therefore, the NK4 transfectant used in this study only slightly inhibited the HGF-induced migration by autocrine NK4.

Suppression of Pancreatic Tumor Growth and Peritoneal Dissemination by Autocrine NK4 of the NK4 Transfectant in the Orthotopic Implantation Model. Orthotopic implantation into the back side of the pancreas was performed

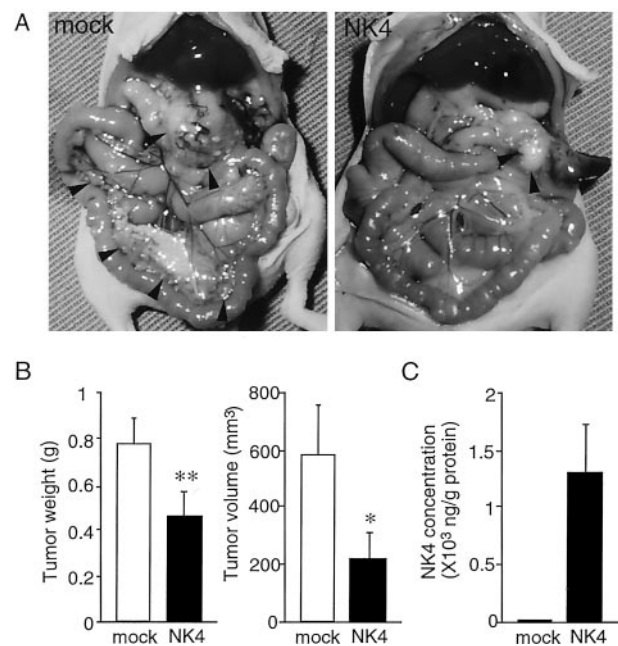


Fig. 2 Inhibitory effect of autocrine NK4 on the primary tumor and peritoneal dissemination 3 weeks after orthotopic implantation of each stable transfectant into the pancreas ($n = 5$ /group). **A**, macroscopic appearance of the pancreatic tumor and peritoneal dissemination. Arrowheads indicate the pancreatic tumor and disseminated nodules. **B**, volume and weight of the pancreatic tumor. Mean \pm SD values are shown; *, $P < 0.05$; **, $P < 0.01$. **C**, NK4 expression in the pancreatic tumors formed by each stable transfectant.

carefully to prevent spillage of tumor cells into the abdominal cavity. Three separate experiments were done. On day 21 after implantation, pancreatic tumors were macroscopically confirmed in all mice, and peritoneal dissemination had developed to various degrees. Macroscopic metastasis was not detected in other organs. Growth of the pancreatic tumors and peritoneal dissemination were remarkably inhibited in the NK4 group in comparison to growth and dissemination in the mock group (Fig. 2A). The pancreatic tumor was significantly heavier ($P = 0.005$) in the mock group (0.8 ± 0.04 g) than in the NK4 group (0.5 ± 0.03 g), and the volume was significantly greater ($P = 0.012$) in the mock group (1100 ± 50 mm³) than in the NK4 group (400 ± 24 mm³; Fig. 2B). NK4 expression in the pancreatic tumor was 1288.7 ± 442.1 ng/g protein in the NK4 group and below the threshold of 0.15 ng/g protein in the mock group (Fig. 2C). Because neither the normal pancreatic tissues nor parental SUI-2 cells expressed NK4 (data not shown), these results suggest that the NK4 transfectant continued to express the NK4 protein and that the autocrine NK4 suppressed the development of pancreatic tumor. Disseminated tumors in the mesentery, hepatic hilum, diaphragm, and retroperitoneum were evaluated on the basis of maximum size and number, as shown in Table 1. Disseminated peritoneal nodules were fewer in number and smaller in the NK4 group than in the mock group, and the degree of peritoneal dissemination was significantly less ($P < 0.05$) in the NK4 group than in the mock group. Although we cannot rule out the possibility of tumor cell leakage into the

Table 1 Suppression of peritoneal dissemination by autocrine NK4 of the NK4 transfectant

Peritoneal dissemination and ascites 3 weeks after orthotopic implantation of each stable transfectant into the pancreas.

	Peritoneal dissemination ^a				Ascites (ml)
	Mesentery	Hepatic hilum	Diaphragm	Retroperitoneum	
Mock	+++	++	–	++	4.8
	+++	++	–	++	4.7
	+++	++	–	++	3.9
	+++	++	++	++	0.1
	+	+	–	–	0
	4/5 ^b	4/5 ^c	1/5 ^c	4/5 ^c	
NK4	+	–	–	–	3.8
	+	–	+	+	3.2
	++	+	–	+	3.7
	–	–	–	–	0
	–	–	–	–	0
	0/5 ^d	0/5 ^d	0/5	0/5 ^d	

^a Disseminated tumors in the mesentery, hepatic hilum, diaphragm, and retroperitoneum were evaluated on the basis of maximum size and number as follows: +, <1 mm in diameter and <50 in number; ++, 1–2 mm in diameter or 50–100 in number; +++, >2 mm in diameter or >100 in number.

^b Number of mice with disseminated tumors >+++ per total.

^c Number of mice with disseminated tumors >+ per total.

^d The degree of peritoneal dissemination was significantly less in the NK4 group than in the mock group (χ^2 ; $P < 0.05$).

peritoneum at implantation, progression of the disseminated tumors was consistently inhibited in the NK4 group. Peritoneal ascites did not differ between groups.

Inhibition of Liver Metastasis after Splenic Injection of the NK4 Transfectant. At 28 days after transplantation, macroscopic metastases in the liver were examined for evaluation of the between-group difference in metastatic development in solid organs (Fig. 3A). Metastatic nodules in the mock group were numerous and large, occupying the entire liver. In contrast, only a few, small liver metastases were seen in the NK4 group. The mean liver weight was 1.611 ± 0.271 g in the NK4 group and 2.434 ± 0.501 g in the mock group. The NK4 transfectant inhibited liver metastasis significantly more than the mock transfectant did ($P = 0.022$; Fig. 3B). NK4 protein expression in the metastatic tumors was 68.3 ± 40.4 ng/g in the NK4 group and below the threshold of 0.15 ng/g in the mock group (Fig. 3C). Normal liver tissues expressed no detectable NK4 protein (data not shown).

Histological and Immunohistochemical Examination of the Primary Pancreatic Tumor. Histologically, the primary tumors were moderately to poorly differentiated adenocarcinomas that were similar in both groups. As to PCNA staining, the difference between groups was not significant; that is, the proliferation index was not changed by NK4 expression, and this was consistent with the *in vitro* results. Whereas many TUNEL-positive apoptotic tumor cells were seen in the center of the tumor region in the NK4 group ($4.6 \pm 1.5\%$), no such apoptosis was observed in the mock group ($2.6 \pm 1.1\%$); this between-group difference was significant ($P = 0.046$). Blood vessels in the tumor tissues, as evaluated by staining with anti-CD31

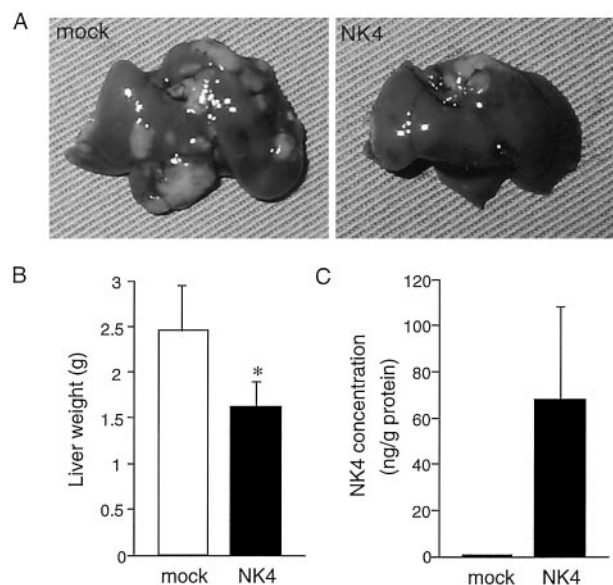


Fig. 3 Inhibitory effect of autocrine NK4 on liver metastasis 4 weeks after splenic injection of each stable transfectant ($n = 5$ /group). **A**, macroscopic appearance of liver metastasis. **B**, total liver weight. Mean \pm SD values are shown; *, $P < 0.05$. **C**, NK4 expression in the metastatic tumors formed by each stable transfectant.

antibodies, were fewer and smaller in the NK4 group than in the mock group. Significantly fewer ($P < 0.0001$) vessels/field were detected in the tumors of the NK4 group (7.8 ± 1.9) than in those of the mock group (16.6 ± 1.9 ; Fig. 4).

Histological and Immunohistochemical Examination of Metastatic Tumors in the Liver. Examination of H&E-stained sections revealed that the metastatic tumors were moderately to poorly differentiated adenocarcinomas. No clear histological differences were observed between the groups. PCNA staining did not differ between the groups. The apoptotic index determined by TUNEL indicated a higher incidence of apoptosis in the NK4 group ($4.4 \pm 1.5\%$) than in the mock group ($2.2 \pm 1.3\%$; $P = 0.039$). MVD in the metastatic tumors was significantly lower ($P = 0.0002$) in the NK4 group (9.6 ± 2.1 vessels/field) than in the mock group (19.7 ± 3.1 ; Fig. 5).

DISCUSSION

We have shown here the antitumor effect of NK4 as a potent angiogenesis inhibitor and its potential for antiangiogenic gene therapy against pancreatic cancer. We designed a gene therapy model by transfecting pancreatic cancer cell line SUIT-2 with NK4 cDNA, with the aim of obtaining a continuous local source of NK4. Characteristics of the NK4 transfectant were that the autocrine NK4 had no effect on proliferation of these tumor cells and only slightly antagonized HGF for the c-Met receptor because the expression of NK4 was weak. In contrast, NK4 transfectants implanted in the pancreas or spleen of nude mice showed a remarkable antitumor effect on the pancreatic tumor, peritoneal dissemination, and liver metastasis. The MVD in the tumors was low, and the number of apoptotic cells was high. These results indicate that autocrine NK4 sup-

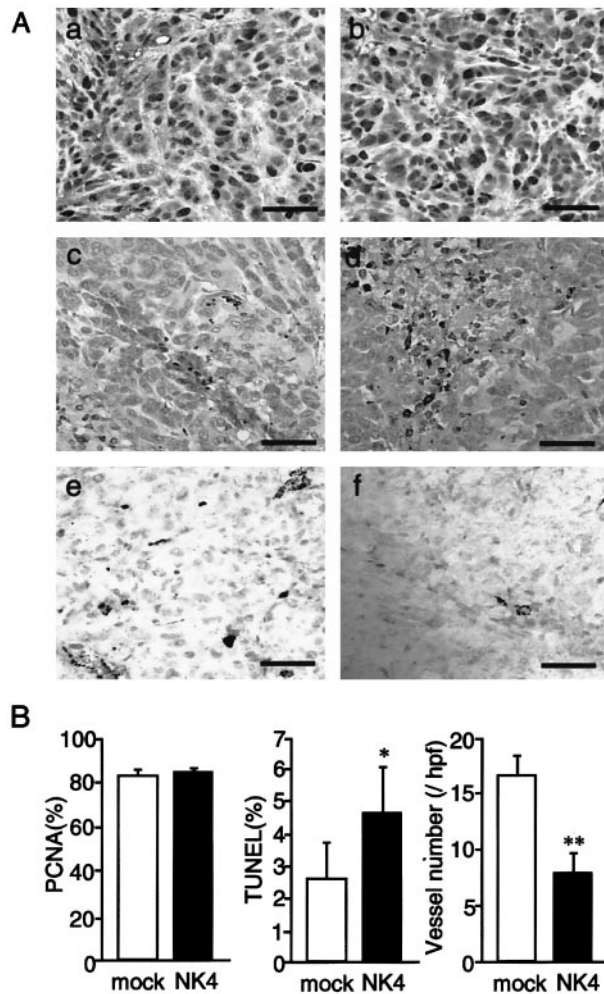


Fig. 4 Immunohistochemical analysis of the pancreatic tumor formed by the mock transfectant (*a, c, and e*) and the NK4 transfectant (*b, d, and f*). *A*, proliferation (*a and b*), apoptosis (*c and d*), and angiogenesis (*e and f*) were determined by use of anti-PCNA antibody, the TUNEL method, and anti-CD31 antibody, respectively. Bars, 50 μ m. *B*, changes in PCNA-positive cells, TUNEL-positive cells, and number of blood vessels. Mean \pm SD values are shown; *, $P < 0.05$; **, $P < 0.01$.

presses tumor progression mainly through the inhibition of tumor angiogenesis.

NK4 was originally prepared as a competitive receptor-antagonist devoid of its own HGF-related activities (8). HGF has strong motogenic activity in various types of cancer cells (15–21). We reported that HGF potently stimulated dissociation and the invasive potential of pancreatic cancer cells and that NK4 dose dependently inhibited pancreatic cancer cell migration and invasion induced by HGF (13). However, for complete inhibition of HGF-binding to the c-Met receptor, a large amount of NK4 (~1000-fold higher concentration than that of HGF) is required. Recent reports suggested that NK4 inhibits angiogenesis through mechanisms distinct from HGF antagonism (10, 22). The bifunctional properties of NK4 as an angiogenesis inhibitor and HGF antagonist mean that NK4 may prove therapeutic for cancer patients (10, 22).

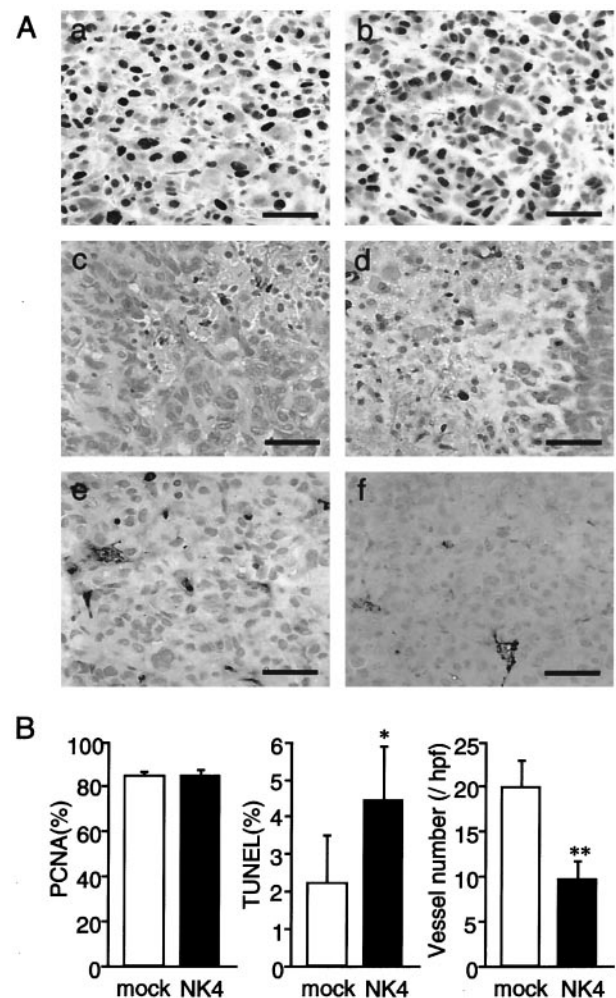


Fig. 5 Immunohistochemical analysis of the metastatic tumor in the liver after splenic injection of the mock transfectant (*a, c, and e*) and the NK4 transfectant (*b, d, and f*). *A*, proliferation (*a and b*), apoptosis (*c and d*), and angiogenesis (*e and f*) were determined by use of anti-PCNA antibody, the TUNEL method, and anti-CD31 antibody, respectively. Bars, 50 μ m. *B*, changes in PCNA-positive cells, TUNEL-positive cells, and number of blood vessels. Mean \pm SD values are shown; *, $P < 0.05$; **, $P < 0.01$.

In this study, we used a stable transfectant expressing a small amount of NK4 to examine the individual effect of NK4 as an angiogenesis inhibitor. Notably, the constitutive expression of NK4 in tumors, at a level not expected to be effective as an HGF antagonist, was associated with a potent inhibition of angiogenesis and thus of tumor development. Why did such a low level of NK4 have an apparent antitumor effect *in vivo*? One possibility is that the primary tumor growth and the process of peritoneal dissemination and liver metastasis may be more dependent on angiogenesis than on HGF-mediated invasion or migration of cancer cells. There are several reports that aggressive angiogenesis in primary tumors correlates well with a high frequency of distant metastasis in cancer patients (23, 24). Besides, although small tumors (1–2 mm in diameter) can receive all nutrients by diffusion, additional growth depends on

the development of an adequate blood supply through angiogenesis (25, 26). In this study, NK4 inhibited tumor angiogenesis and consequently prevented the growth of tumor cells, at both primary and secondary sites, and the emergence of metastases. Second, local delivery of NK4 within the tumors should alter the angiogenic balance more effectively and more persistently than other means of delivery such as systemic administration. This advantage could be accomplished by gene therapy, and selective gene transduction in the tumor site would attain the effects of NK4 most efficiently.

Antiangiogenic therapy is an important, potent method for limiting tumor growth, and some investigators recently described antiangiogenic therapy for peritoneal dissemination and for liver metastasis of pancreatic cancer (27–29). For other malignancies, gene therapy with various angiogenic inhibitors has been reported: *e.g.*, angiostatin (30–32); platelet factor 4 (33); endostatin (31, 34, 35); antisense mRNA against vascular endothelial growth factor (31); and soluble flt-1 (36). Studies on antiangiogenic NK4 gene therapy for pancreatic cancer are currently being carried out in our laboratory. Such gene therapy could be a promising new strategy for the treatment, not only of primary but also of metastatic pancreatic cancer.

In summary, using NK4-transfected pancreatic cancer cells, we showed the dramatic antitumor effect of NK4. The inhibition of primary tumor growth, peritoneal dissemination, and liver metastasis of the NK4 transfectant was mediated mainly by the antiangiogenic effect of NK4. This finding will contribute to the development of a new NK4 gene therapy delivery system that can eliminate the need for continuous administration of recombinant protein and can provide for a local source of the molecule.

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