[CANCER RESEARCH 61, 7518–7524, October 15, 2001]

Inhibition of Growth, Invasion, and Metastasis of Human Pancreatic Carcinoma Cells by NK4 in an Orthotopic Mouse Model[1]

Daisaku Tomioka,[2] Naoki Maehara, Keiji Kuba,[2] Kazuhiro Mizumoto, Masao Tanaka, Kunio Matsumoto, and Toshikazu Nakamura[3]

Division of Molecular Regenerative Medicine, Course of Advanced Medicine, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871 [D. T., K. K., K. Ma., T. N.] and Department of Surgery and Oncology, Kyushu University Graduate School of Medical Science, Maidashi, Fukuoka 812-8582 [N. M., K. Mi., M. T.], Japan

ABSTRACT

Hepatocyte growth factor (HGF) is involved in malignant behavior of cancers as a mediator in tumor-stromal interactions through enhancing tumor invasion and metastasis. We found recently that NK4, a four-kringle fragment of HGF, functions as both an HGF-antagonist and an angiogenesis inhibitor. We have now determined whether blockade of the HGF-c-Met/HGF receptor pathway and tumor angiogenesis by administration of recombinant NK4 would inhibit growth, invasion, and metastasis of human pancreatic carcinoma implanted into the pancreas of nude mice. When treatment with NK4 or anti-HGF neutralizing antibody was initiated from the third day after orthotopic injection of SUIT-2 human pancreatic cancer cells, both NK4 and anti-HGF antibody suppressed the conversion of orthotopic pancreatic tumors from carcinoma *in situ* to aberrantly invading cancers during days 3–14. On the other hand, when the treatment was begun on day 10, a time when cancer cells were already invading surrounding tissues, NK4 but not anti-HGF antibody inhibited tumor growth, peritoneal dissemination, and ascites accumulation at 4 weeks after the inoculation. Antitumor effects of NK4 correlated with decreased microvessel density in pancreatic tumors thereby indicating that the antiangiogenic activity of NK4 may have mainly contributed to its antitumor effects. Moreover, although NK4-treatment was initiated from the end stage (day 24 after tumor inoculation), NK4 prolonged survival time of mice, and the suppression of peritoneal dissemination, ascites accumulation, and invasion of metastasized cancer cells into the peritoneal wall were remarkable. We propose that simultaneous targeting of both tumor angiogenesis and the HGF-mediated invasion-metastasis may prove to be a new approach to treating patients with pancreatic cancer.

INTRODUCTION

Pancreatic cancer is one of the major causes of cancer-related deaths in industrialized countries (1, 2). At the time of diagnosis, >80% of patients with this cancer have locally advanced or metastatic disease (3, 4), and only 1–4% of all of the patients with pancreatic adenocarcinoma survive 5 years after the diagnosis (2). Even if patients are correctly diagnosed at an early stage, pancreatic cancer frequently exhibits highly malignant phenotypes characterized by extensive invasion into surrounding tissues and metastasis to distant organs, even at an early stage; hence, the prognosis of patients is poor (3, 5, 6). Thus, elucidation of molecular mechanisms related to invasion-metastasis of pancreatic cancers and the novel therapeutic approaches to pancreatic cancer treatment are urgently required.

HGF,[4] originally identified and cloned as a potent mitogen for hepatocytes (7, 8), can potently enhance cell motility and extracellular matrix breakdown in a variety of cancer cells and leads to the invasion and metastasis of cancer cells (9–12). Previous reports indicated that the HGF-c-Met receptor system is involved in the malignant behavior of pancreatic cancer cells. The c-Met receptor is overexpressed frequently in pancreatic cancer cells (13–20). On the other hand, HGF is rarely expressed by pancreatic cancer cells, but tumor-associated fibroblasts do produce HGF in a paracrine manner (17, 19, 20). *In vitro*, HGF stimulates growth, locomotion, and invasion of pancreatic cancer cell lines (16, 17, 20). Hence, HGF-c-Met coupling and the subsequent activation of c-Met may play a definite role in invasion and metastasis of pancreatic cancer. However, data to directly address the significance of HGF-c-Met coupling in the development and progression of pancreatic cancer *in vivo* have heretofore not been documented.

We prepared previously an antagonist for HGF by proteolytic digestion of HGF (21). This HGF antagonist, which we termed NK4, is composed of the NH_2-terminal hairpin domain and four kringle domains in the α chain of HGF. NK4 binds to the c-Met receptor and competitively antagonizes HGF-induced tyrosine phosphorylation of the c-Met receptor, the result being inhibition of HGF-mediated invasion of some distinct types of human cancer cells (21–24). On the other hand, we recently found that NK4 is an angiogenesis inhibitor and has inhibitory effects on angiogenesis driven by basic fibroblast growth factor and vascular endothelial growth factor, as well as HGF (25). Because angiogenesis in malignant tumors (including pancreatic cancer) is involved in tumor growth and metastasis (26, 27), bifunctional properties of NK4, which is an HGF-antagonist and an angiogenesis inhibitor, raised the potential therapeutic value of NK4 for treatment of patients with pancreatic cancer.

We now report that host stroma-derived HGF plays a critical role in acquisition of the invasive phenotype of pancreatic cancer cells *in vivo* and that the administration of recombinant NK4 to tumor-bearing mice leads to inhibition of growth, invasion, and disseminating metastasis of pancreatic cancer and prolongs the life span of these mice.

MATERIALS AND METHODS

Materials. Polyclonal antirat HGF and antihuman HGF antibodies were prepared, respectively, as described elsewhere (21, 22, 28, 29). This antirat HGF antibody cross-reacts with murine HGF but not with human HGF (28). Antirat HGF IgG (1 μg) neutralizes biological activity of at least 5 ng of murine HGF. Recombinant human NK4 was purified from culture medium of CHO cells, which stably secrete human NK4, using three-step chromatographies.[5] The purity of NK4 was 96.4% as determined by SDS-PAGE and protein staining. The purified NK4 protein and antirat HGF antibody were analyzed and determined to be negligible for endotoxin levels using a *Limulus* Amebocyte Lysate kit (BioWhittaker) as described elsewhere (25).

Received 5/14/01; accepted 8/15/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by a research grant for science and cancer from the Ministry and Education, Science, Technology, Sports, and Culture of Japan; a research grant for cancer research from the Ministry of Welfare of Japan; and research grants from the Princess Takamatsu Cancer Research Fund, The Tokyo Biochemical Research Foundation, Foundation for Promotion of Cancer Research in Japan, Takeda Science Foundation, Nissan Science Foundation, and Sagawa Cancer Research Promotion Foundation.

[2] D. T. and K. K. are recipients of research fellowships of the Japan Society for the Promotion of Science.

[3] To whom requests for reprints should be addressed, at Division of Molecular Regenerative Medicine, Course of Advanced Medicine, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-3783; Fax: 81-6-6879-3789; E-mail: nakamura@onbich.med.osaka-u.ac.jp.

[4] The abbreviations used are: HGF, hepatocyte growth factor; EtOH, ethanol; ECM, extracellular matrix; PECAM, platelet/endothelial cell adhesion molecule; PCNA, proliferating cell nuclear antigen.

[5] K. Matsumoto, K. Kuba, and T. Nakamura, manuscript in preparation.

Orthotopic Inoculation of Tumor Cells. Human pancreatic cancer cell lines (SUIT-2, KP-3, and MiaPaCa-2) were donated by Dr. H. Iguchi (National Kyushu Cancer Center, Fukuoka, Japan). SUIT-2 cells were cultured at 37°C in RPMI 1640 supplemented with streptomycin, penicillin, and 10% fetal bovine serum. c-Met receptor expression and effects of HGF and NK4 on growth and invasion of SUIT-2 cells were described elsewhere (20). Cell suspension (50 μl) at 2×10^7 cells/ml was injected into the pancreas of 6-week-old male nude mice (BALB/c-*nu/nu*; Japan SLC, Inc., Hamamatsu, Japan).

Administration of NK4. Experimental schedules for NK4-treatment of mice implanted with pancreatic cancer cells are described in Fig. 1. Mice were i.p. administered twice daily with 1.5 mg/kg/day NK4 or BSA in saline. When the mice were treated with antibody, antirat HGF IgG or normal rabbit IgG (8.7 mg/kg/day) was administered twice daily. At autopsy, size of the tumors in all of the mice was measured using a dial caliper, and the tumor volume was determined using the formula width $\times$ (length)^2 $\times$ 0.52. The number of macroscopically metastatic nodules >1 mm in diameter in the peritoneal cavity was counted, and the volume of ascites was measured. For histological procedures, tissues were fixed in formalin or EtOH, embedded in paraffin, and tissue sections were stained with H&E unless otherwise mentioned. Survival analysis was computed by the Kaplan-Meier method and compared by the log-rank test.

Immunohistochemistry and Measurement for Tissue HGF Levels. For blood vessel staining, tumor tissues were fixed in EtOH and embedded in paraffin. These tissue sections were then quenched with 3% hydrogen peroxide in PBS for 5 min, washed in PBS, and treated with 0.1% trypsin at room temperature for 20 min. The sections were exposed for 30 min to 10% normal rabbit serum and incubated with the anti-CD31/PECAM-1 antibodies (diluted 1:50; PharMingen) overnight at 4°C. Next, the sections were incubated with biotinylated horseradish peroxidase-conjugated rabbit antirat IgG antibodies (diluted 1:200; DAKO) for 30 min. The reaction was observed by incubating the sections with substrate solution containing diaminobenzidine and hydrogen peroxide. The sections were then washed in PBS and stained with hematoxylin. The number of blood vessels was counted under a light microscope at a 200-fold magnification using $\geq$10 randomly selected fields per each sample. To detect proliferating and apoptotic cells, tissue sections were respectively analyzed by immunohistochemistry for PCNA and terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling assay, as described elsewhere (22, 25, 28).

To analyze the expression of HGF and human c-Met, tissues were fixed in EtOH and formalin, respectively. The tissue sections were incubated overnight with 10 μg/ml antirat HGF IgG, antihuman HGF IgG, or antihuman c-Met (C-12; Santa Cruz Biotechnology) at 4°C then were incubated with the DAKO rabbit Envision plus visualization system for 30 min. The sections were counterstained with hematoxylin.

For measurement of tissue HGF levels, pancreatic primary tumors were excised on day 28, and tissue extracts were prepared as described elsewhere (28, 29). The protein levels of human HGF and murine HGF were determined in ELISAs, respectively using IMMUNIS human HGF enzyme immunoassay and rat HGF EIA (Institute of Immunology, Tokyo, Japan). These ELISA kits have no cross-reactivity between human and rodent HGF.

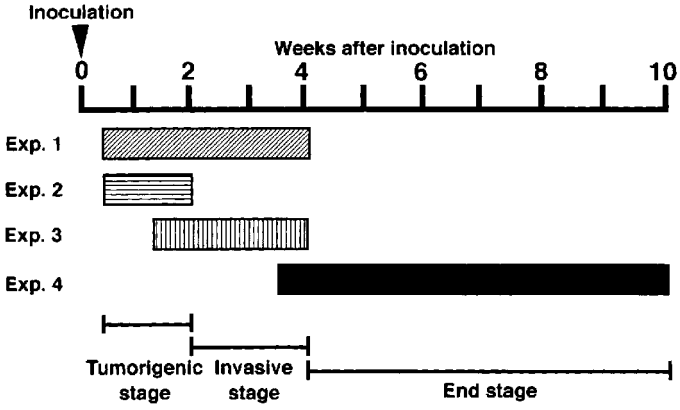

Fig. 1. Schedules for NK4 or anti-HGF antibody treatment of mice with pancreatic cancer. Mice were i.p. administrated twice daily with 0.75 mg/kg NK4 or BSA in saline (vehicle). Anti-HGF IgG or normal IgG was administrated twice daily at 4.35 mg/kg. Stages of tumor progression were defined by histological and pathological analysis in this model. *Exp. 1*, tumorigenic and invasive stage (days 3–28); *Exp. 2*, tumorigenic stage (days 3–14); *Exp. 3*, invasive stage (days 10–28); *Exp. 4*, end stage (days 24–70, until moribund).

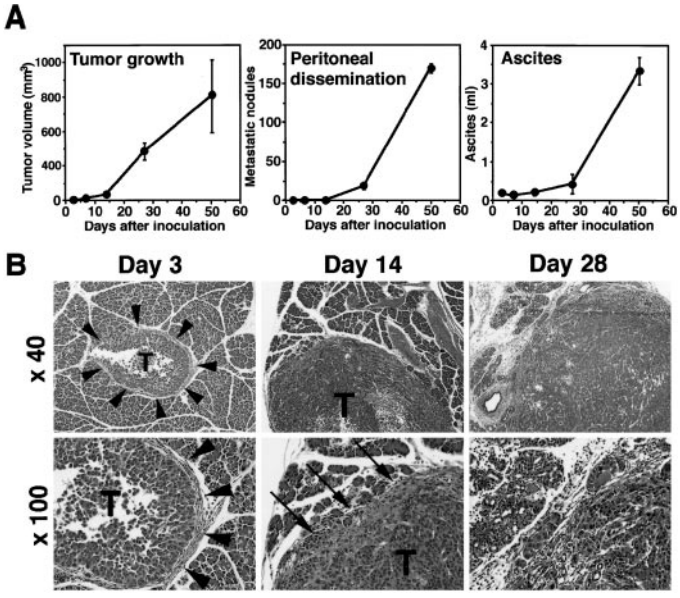

Fig. 2. Progression of pancreatic cancer in the pancreas after orthotopic inoculation of SUIT-2 cells into nude mice. *A*, tumor growth, peritoneal dissemination, and ascites accumulation after orthotopic tumor inoculation. *B*, microphotographs of xenografts of human pancreatic cancer in mice. Three days after inoculation, tumor cells were evident at the capsular interface of the pancreas (*arrowheads*). Fourteen days later, SUIT-2 cells had invaded vigorously the surrounding pancreatic tissue (*arrows*). On day 28, the mouse normal pancreas was virtually replaced by tumor cells. Original magnification, $\times$40 (*top*) and $\times$100 (*bottom*). *T*, tumor tissue.

Invasion Score. To semiquantitate the invasiveness of the implanted pancreatic cancer, we defined the invasion score based on histological observations as follows: score 0, invasion was undetectable, and the tumor was surrounded by a capsule; score 1, invasion was undetectable, but the tumor was not surrounded by a capsule; score 2, invasion was partial; score 3, invasion was extensive, and normal pancreatic and tumor regions could not be distinguished.

Data Analysis. For statistical analyses, we used unpaired Student's *t* test (two-tailed) unless otherwise mentioned. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Possible Involvement of HGF-c-Met Interaction in the Progression of Pancreatic Cancer. On the basis of our previous report (20), we initially implanted two c-Met-positive human pancreatic cancer cell lines (SUIT-2 and KP-3) or c-Met-negative MiaPaCa-2 human pancreatic cancer cells into the pancreas of nude mice. Orthotopically implanted c-Met-negative MiaPaCa-2 cells did not form tiny nodules in the pancreas of nude mice for up to 4 weeks after tumor inoculation. In contrast, two c-Met-positive cells led to invasive tumors in a reproducible manner (100% tumor uptake in both lines). Because SUIT-2 pancreatic tumors showed a more aggressive and malignant phenotype than KP-3 pancreatic tumors, we used SUIT-2 cells in the orthotopic transplantation model of human pancreatic cancer.

Three days after tumor inoculation, SUIT-2 cells were engrafted in the pancreas in all of the mice, and the tiny mass of cancer cells was encapsulated by multilayers of host stromal cells (Fig. 2*B*, *Day 3*). At this stage, these SUIT-2 tumors did not invade surrounding tissues and showed localized tumor growth resembling adenoma or carcinoma *in situ*. Two weeks after tumor inoculation, SUIT-2 pancreatic tumors

showed the invasive phenotype. Multilayers of host stromal cells had largely disappeared, and cancer cells were focally invading surrounding pancreatic tissue thereby disorganizing pancreatic acinar structures. In the cancer mass, fibroblast-like stromal cells were integrated within clusters of cancer cells, and angiogenesis in the tumor periphery was apparent. Increase in tumor volume became remarkable >2 weeks after inoculation (Fig. 2*A*). At 4 weeks after inoculation, the pancreatic tumor was large and occupied the entire area of the pancreas, penetrated the capsule of the pancreas, and spontaneously disseminated into the peritoneal cavity. Visible metastatic nodules in the mesentery and peritoneal wall appeared, and the accumulation of cancerous ascites was apparent (Fig. 2*A*). Therefore, in this pancreatic cancer model, we designated the 3–14 days as tumorigenic stage, the 2–4 weeks as invasive stage, and 4–10 weeks as end stage (Fig. 1).

To examine the potential involvement of HGF and the c-Met/HGF receptor in malignant behavior of SUIT-2 pancreatic tumors, expression of the human c-Met receptor and murine HGF were immunohistochemically analyzed (Fig. 3). Human c-Met immunoreactivity was observed to be specific in cancer cells but not so in host stromal cells and ductal cells (Fig. 3*a*). Human HGF was not detected in normal regions or in cancerous lesions (Fig. 3*b*), consistent with the finding that SUIT-2 cells do not produce HGF *in vitro* (20). Although expression of murine HGF was not detectable in cancer cells or in host ductal cells, it was detected in stromal cells (vascular endothelial cells and fibroblasts), located in border regions between cancer tissue and pancreatic acinar structures (Fig. 3, *c* and *d*). It is noteworthy that expression of HGF was scarcely detectable in cells distant from tumor tissue (data not shown), suggesting that HGF expression in these cells might be regulated through interaction with tumor cells. These observations were confirmed by measurement of human and murine HGF levels in tumor tissues. Pancreatic tumors contained significant levels of murine HGF (0.63 ± 0.11 ng/mg protein) but not human HGF (<0.005 ng/mg protein). Together with the finding that HGF potently stimulated invasion of SUIT-2 cells *in vitro* (20), these results strongly suggest that stroma-derived HGF may play a role in pancreatic cancer cell behavior such as invasion, particularly in cancer cells located in peripheral regions interactive with host cells.

Inhibition of Pancreatic Cancer Growth and Peritoneal Dissemination by NK4. We then determined whether the malignant behavior of SUIT-2 pancreatic tumors in the pancreas of nude mice would be suppressed by NK4. Three days after the inoculation of SUIT-2 cells into the pancreas of nude mice, the mice were randomized into two treatment groups ($n = 5$ in each group), and recombinant NK4 or BSA (1.5 mg/kg/day) in saline was administered i.p. twice daily for 25 days (Fig. 1, *Exp. 1*), then all of the mice were killed on day 28.

NK4 had no significant effects on body weight or general well-being of the animals (data not shown). NK4 treatment significantly suppressed the growth of SUIT-2 pancreatic tumors by 60.7% (Fig. 4*A*; $P < 0.05$). Control tumors aggressively invaded the neighboring spleen and penetrated the pancreatic capsule, whereas NK4-treated tumors neither invaded the spleen nor penetrated the capsule of the pancreas, and macroscopically the tumors had a smooth surface. The effect of NK4 on peritoneal dissemination was assessed by counting the number of metastatic nodules in the mesentery and peritoneal wall. NK4-treatment resulted in 84.1% decrease in the number of metastatic nodules in the mesentery, diaphragm, and peritoneum (Fig. 4*B*; $P < 0.05$). These results suggest that HGF plays a role in the malignant behavior of human pancreatic cancers. However, because NK4 functions as an angiogenesis inhibitor independently of its HGF-antagonist activity (25), the antiangiogenic activity of NK4 might have participated in the antitumor effects. To define mechanisms related to the antitumor effects of NK4 on malignant behavior of pancreatic cancers, the potential participation of HGF-antagonist activity and antiangiogenic activity of NK4 should be defined additionally.

Inhibition of Tumorigenesis and Invasive Growth by NK4. To determine whether NK4 affects tumorigenesis and invasive growth of SUIT-2 pancreatic tumors, NK4 was administered for 11 days from day 3 after inoculation (Fig. 1, *Exp. 2*). In vehicle-treated mice, cancer cells extensively invaded pancreatic tissue; thus, the border between tumor lesions and intact normal pancreatic tissues was not obvious (Fig. 5*A*, *panels a* and *d*). In contrast, in NK4-treated mice, tumor lesions were still largely encapsulated by host stromal cells, and the tumor lesions were apparently separated from normal pancreatic tissues, thus, indicating that invasion of cancer cells was inhibited by NK4 (Fig. 5*A*, *panels b* and *e*). When tumor invasion seen in histological observations was evaluated semiquantitatively, the tumor-invasive score in control mice reached 2.3 ± 0.1 (Fig. 5*B*), whereas tumor invasion was significantly inhibited to 1.3 ± 0.2 in NK4-treated

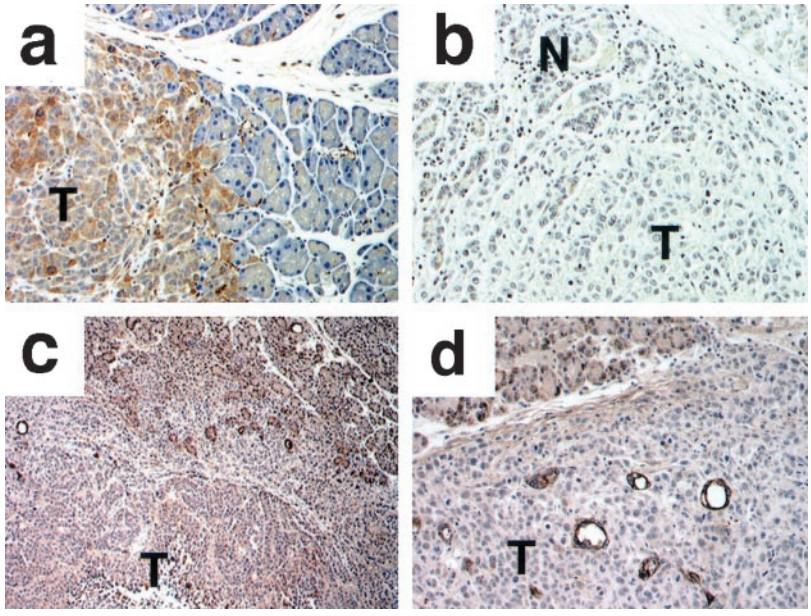

Fig. 3. Immunohistochemistry for human c-Met (*a*), human HGF (*b*), and murine HGF (*c* and *d*) in SUIT-2 pancreatic tumor at day 14 after inoculation. Murine HGF expression was detected in stromal cells (endothelial cells and fibroblasts) but not in tumor cells (*c* and *d*). Human HGF was undetectable in tumor tissues (*b*). Original magnification, ×40 (*c*), ×100 (*a*, *b*, and *d*). *T*, tumor tissue; *N*, normal pancreatic tissue. Faint staining in tumor cells in *c* and *d* was attributable to nonspecific background staining, because weak background staining was still seen in case of use of the preneutralized antibody (data not shown).

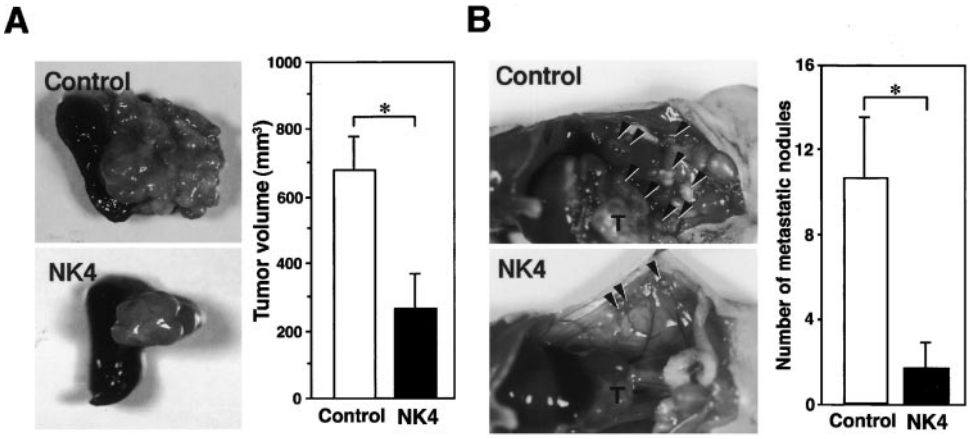

Fig. 4. Suppression of pancreatic cancer growth and peritoneal dissemination by NK4-treatment. *A*, inhibition of primary tumor growth by NK4. Photographs show appearance of the primary pancreatic cancer. *B*, suppression of peritoneal dissemination. Photographs show metastatic nodules on the peritoneum. Mice were treated with vehicle or NK4 from day 3 after tumor inoculation for 25 days (Fig. 1, *Exp. 1*). On day 28 after tumor inoculation, the mice were killed. *Arrowheads*, metastatic nodules. *T*, primary tumor. Five mice were used in each group. *, $P < 0.05$. Each value represents the mean; *bars*, ± SE.

mice ($P < 0.01$). Because HGF potently stimulated invasion of SUIT-2 cells *in vitro* (20), we speculated that the suppression of invasion of SUIT-2 tumors by NK4-treatment might be attributable to antagonistic actions of NK4 for HGF. To address this possibility, mice were administered neutralizing anti-HGF IgG instead of NK4. Similar to the case of NK4, anti-HGF IgG inhibited tumor invasion (Fig. 5*A*, *panels c* and *f*). Administration of normal rabbit IgG had no inhibitory effect on invasive behavior of cancer cells in the control mice (data not shown). Histological estimation of tumor invasion indicated that the invasive score in normal IgG-treated mice reached 2.3 ± 0.2, whereas anti-HGF IgG treatment significantly inhibited tumor invasion to 1.3 ± 0.2 (Fig. 5*B*; $P < 0.01$). On the other hand, tumor growth was not significantly affected by either NK4 or anti-HGF IgG, and the poor vasculature at this early stage made it difficult to quantify microvessel density (data not shown). Together with the expression of murine HGF in host stromal cells (Fig. 3, *c* and *d*), these results strongly suggest that host stroma-derived HGF promoted tumorigenesis and played a major role in acquisition of the invasive phenotype of SUIT-2 pancreatic tumors. NK4 may have inhibited SUIT-2 cell invasion through its potential to antagonize the biological actions of HGF as an HGF antagonist.

NK4-induced Inhibition of Tumor Angiogenesis, Growth, and Peritoneal Dissemination. We next determined whether the HGF-c-Met interaction system plays a role in the malignant behavior of SUIT-2 pancreatic tumors at the invasive stage (advanced cancers already invading or metastasizing during days 14–28 after inoculation). NK4 was administered daily from day 10 after inoculation until day 28. Administration of NK4 significantly decreased (55.6%) tumor volume as compared with findings in control mice (Fig. 6*A*; 230 ± 37 mm^3 and 518 ± 108 mm^3, respectively; $P < 0.05$). In contrast, treatment with anti-HGF IgG had no significant effect on tumor growth. Therefore, we speculated that NK4 inhibited tumor growth through the inhibitory actions of NK4 on tumor angiogenesis.

For analysis of the tumor vasculature, blood vessels in tumor tissues were stained with anti-CD31/PECAM-1 antibodies. The number of CD31/PECAM-1-positive blood vessels in NK4-treated tumors (9.0 ± 1.4 vessels/field) was suppressed by 56.5% when compared with findings in control mice (20.7 ± 3.5 vessels/field; $P < 0.05$; Fig. 6*B*). In contrast, treatment with anti-HGF IgG had no significant effect on angiogenesis in tumor tissues. Furthermore, measurement of the number of proliferating and apoptotic cells in tumor tissues, as respectively determined by PCNA staining and terminal deoxynucle-

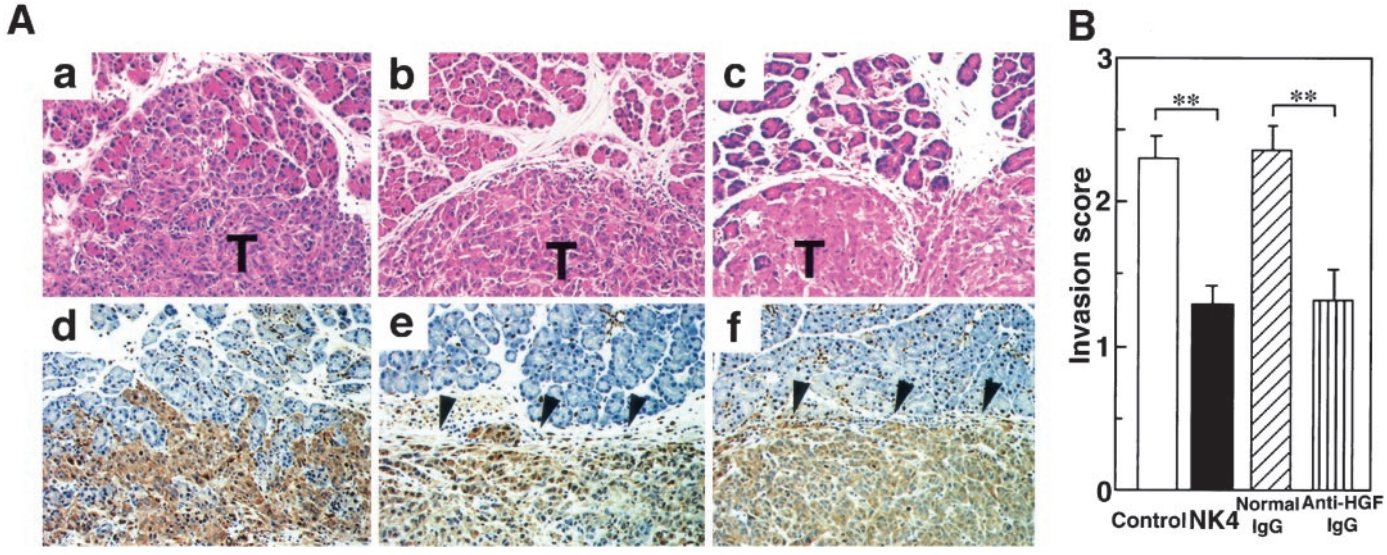

Fig. 5. Inhibition of SUIT-2 pancreatic cancer invasion by NK4. *A*, histological appearances of pancreatic cancer in mice treated with vehicle (*a* and *d*), NK4 (*b* and *e*), or anti-HGF IgG (*c* and *f*). *B*, invasive potentials of pancreatic cancer in mice, evaluated by histological scoring (described in "Materials and Methods"). Mice were treated with vehicle, NK4, anti-HGF IgG, or normal IgG from day 3 after tumor inoculation for 11 days (Fig. 1, *Exp. 2*). On day 14 after tumor inoculation, the mice were killed. Tissue sections were stained with H&E (*a–c*) or antihuman Met (*d–f*). Original magnification, ×100. Human c-Met-positive cells reveal SUIT-2 human pancreatic cancer cells. Seven mice were used in each group. **, $P < 0.01$. Data represent the mean; *bars*, ± SE.

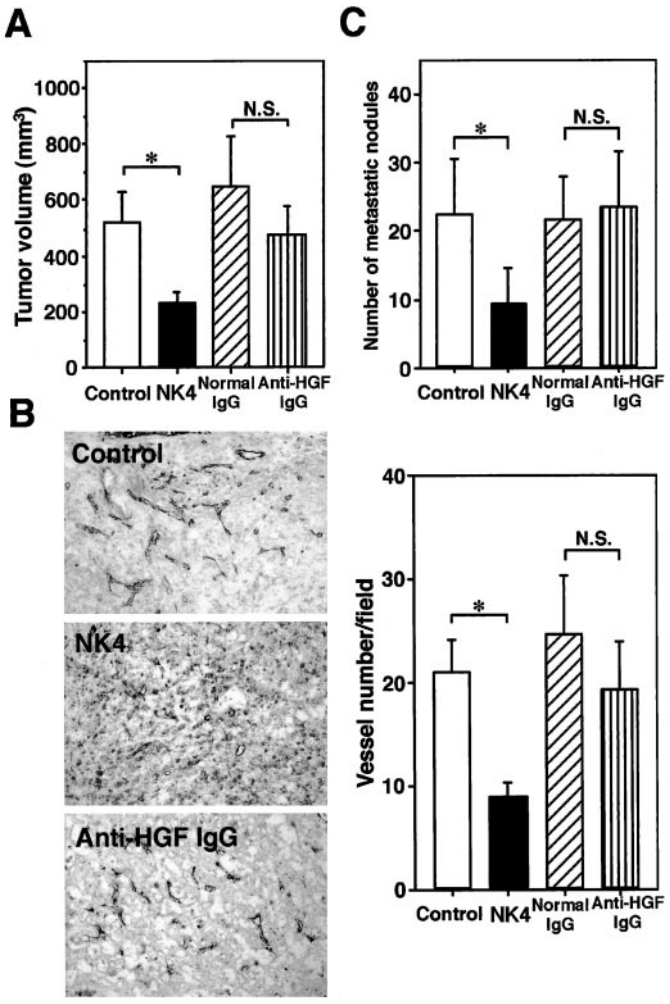

Fig. 6. Inhibition of tumor angiogenesis and peritoneal dissemination in pancreatic cancer by NK4. *A*, inhibition of primary tumor growth by NK4. *B*, immunohistological analysis of vascularization of tumors from vehicle-, NK4-, and anti-HGF IgG-treated mice. Mice were treated with vehicle, NK4, or anti-HGF IgG from day 10 for 18 days (Fig. 1, *Exp. 3*). On day 28 after tumor inoculation, the mice were killed, and tissue sections were stained with anti-CD31/PECAM-1 antibodies. Microvessels in a 10 randomly selected field (×200) were counted in tumors in each group. *C*, suppression of peritoneal dissemination by NK4. Six mice were used in each group. *, $P < 0.05$. Each value represents the mean; *bars*, ± SE.

otidyl transferase (Tdt)-mediated nick end labeling assay, revealed that NK4 did not affect the number of PCNA-positive tumor cells (64.6 ± 2.3% in control mice *versus* 64.1 ± 0.9% in NK4-treated mice; $P = 0.83$) but did increase the number of apoptotic tumor cells (4.5 ± 0.3% in control mice *versus* 10.3 ± 0.4% in NK4-treated mice; $P < 0.0001$). Because HGF hardly stimulates the proliferation of SUIT-2 cells *in vitro* (20), NK4 seems to suppress tumor growth mainly by inhibiting tumor angiogenesis instead of by inhibiting HGF-c-Met signaling.

Twenty-eight days after inoculation, SUIT-2 pancreatic tumors spontaneously metastasized to the peritoneum. The number of metastatic nodules >1 mm in diameter reached 22.6 ± 7.9 (Fig. 6*C*). NK4-treatment strongly inhibited disseminated metastasis by 58.4% of the control value (9.4 ± 5.2; $P < 0.05$). Treatment with anti-HGF IgG had no significant effect on disseminated metastasis. Therefore, the inhibitory effect of NK4 on peritoneal dissemination might be predominantly achieved by antiangiogenic activity and not by HGF-antagonist activity.

Prolonged Survival of Mice by NK4 Treatment. We next investigated the therapeutic potential of NK4 for treatment of end-stage pancreatic cancers. We initiated NK4-treatment from day 24 after tumor inoculation (Fig. 1, *Exp. 4*), a time when peritoneal dissemination and accumulation of cancerous ascites became apparent, yet all of the mice survived. Control mice died of pancreatic cancer only after 26 days, and all of the mice ($n = 15$) died within 69 days after the inoculation (Fig. 7). In mice treated with NK4 ($n = 10$), 4 mice died within 65 days, whereas 6 mice survived for ≥70 days after tumor inoculation ($P < 0.01$). Even on day 70 after tumor inoculation, 6 surviving mice appeared active without anorexia, cachexia, and accumulation of ascites, although pancreatic tumors were palpable. Consistent with this observation, NK4-treated mice maintained their weight significantly better than control mice (data not shown). When moribund control mice were examined, peritoneum, diaphragm, and mesentery were occupied with numerous metastatic nodules (Fig. 8*C*, *panels a* and *b*). In contrast, metastatic nodules in NK4-treated mice were apparent but much less numerous than in control mice (Fig. 8*C*, *panels c* and *d*). The mean number of disseminated metastatic nodules reached 180 ± 12 in control mice but remained at 29 ± 14 in NK4-treated mice (Fig. 8*A*; $P < 0.05$). Surface of the peritoneum was covered by a relatively thick layer of cancer cells, and cancer cells had extensively invaded muscular tunics (Fig. 8*C*, *panels e* and *g*). A similar histological appearance was noted in large areas of the peritoneum and the diaphragm. In contrast, in NK4-treated mice, large areas of peritoneum were free of cancer cells (data not shown). Even in the region with metastatic cancer cells, the cancer cells were more tightly associated than in the control, and invasion of cells into muscular tissues was remarkably inhibited by NK4-treatment (Fig. 8*C*, *panels f* and *h*). In control mice, ascites accumulation reached 4.0 ± 0.3 ml, but NK4-treatment inhibited ascites accumulation to 24.5% of the control levels (Fig. 8*B*; 1.0 ± 0.8 ml; $P < 0.05$).

DISCUSSION

In the orthotopic pancreatic cancer model, blockade of the HGF-c-Met receptor coupling by NK4 or anti-HGF antibody led to inhibition of invasion of pancreatic primary tumors at the tumorigenesis stage, a time when pancreatic tumors underwent phenotypic changes from carcinoma *in situ* to advanced invasive carcinomas. Because the antirat HGF antibody used here does not cross-react with human HGF and SUIT-2 cells do not express human HGF both *in vitro* (20) and *in vivo* (Fig. 3, *b*), the phenotypic changes in this pancreatic cancer

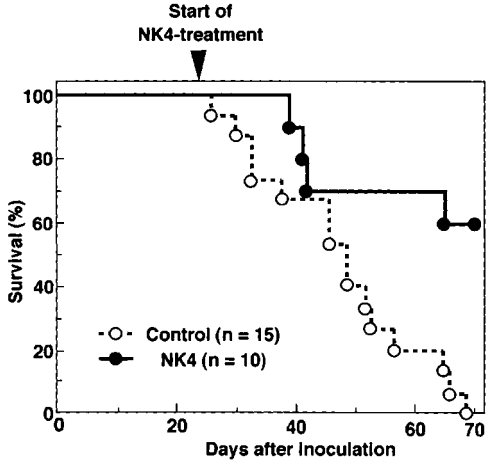

Fig. 7. Prolonged survival of tumor-bearing mice treated with NK4. Mice were treated with vehicle or NK4 from day 24 after tumor inoculation. When mice were moribund or NK4-treated mice survived until day 70, they were killed for analysis. A statistically significant difference was evident in the long-rank analysis of a Kaplan-Meier survival curve ($P < 0.01$).

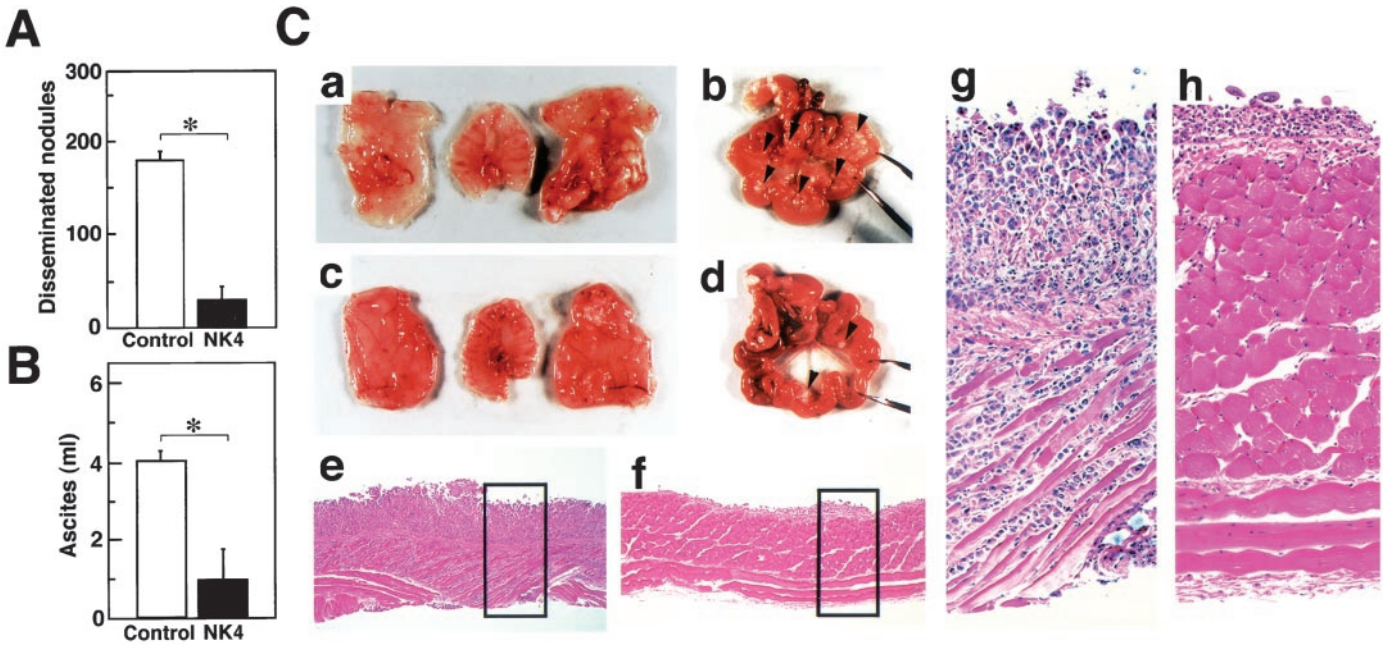

Fig. 8. Suppression of peritoneal dissemination, ascites accumulation, and tumor invasion in peritoneal wall by NK4. *A*, suppression of disseminating metastasis by NK4. Mice were treated with vehicle or NK4 from day 24 after inoculation. *B*, inhibition of ascites accumulation by NK4. *, $P < 0.05$. *C*, macroscopic and histological appearance of peritoneal walls in mice treated with vehicle (*a*, *b*, *e*, and *g*) or NK4 (*c*, *d*, *f*, and *h*). Control mice had a large number of disseminated nodules in the peritoneum (*a*, *both sides*), diaphragm (*a*, *center*), and mesentery (*b*, *arrowheads*). NK4-treated mice that survived at least until day 70 had much fewer disseminated nodules in the peritoneum (*c*, *both sides*), diaphragm (*c*, *center*), and mesentery (*d*, *arrowheads*) compared with findings in control mice. Peritoneal walls from control mice had invasive tumor cells in muscular bundles and a thickened layer of mesothelial cells (*e* and *g*). Peritoneal walls from NK4-treated mice were largely free from metastatic tumors but were partially occupied with tumor cells (*f*). In metastatic nodules in NK4-treated mice, tumor cells on peritoneal wall had a much less invasive phenotype, and muscular tissue was mostly free of invasive cancer cells (*h*). Original magnifications, ×40 (*e* and *f*) and ×100 (*g* and *h*).

model seem to be the result of coupling of stroma-derived murine HGF and c-Met receptor in cancer cells. In contrast, when SUIT-2 pancreatic cancers had once acquired the invasive phenotype and aberrantly invaded host surrounding tissues (invasive stage; day 10–28), anti-HGF antibody had no significant effects on tumor growth, angiogenesis, and disseminating metastasis. Taken together with the finding that c-Met expressing pancreatic cancer cells but not c-Met-negative cells was successfully transplantable to the pancreas of nude mice, we postulate that HGF may play a major role in acquisition of the invasive phenotype of pancreatic cancers rather than in the tumor growth, angiogenesis, and peritoneal dissemination.

Although the precise role of HGF-c-Met signaling in pancreatic carcinogenesis remains to be additionally addressed in other experimental models, there are reports suggesting the involvement of HGF-c-Met coupling in pancreatic carcinogenesis. Mutations of codon 12 in the K-*ras* gene were frequently detected in cancer tissues from patients with pancreatic cancer, and accumulation of K-*ras* mutations was observed in metastases obtained from early passage of orthotopic xenografts (30, 31). When the mutationally activated form of Ras was transfected into nontumorigenic cells, these cells overexpressed both HGF and c-Met and acquired tumorigenic and metastatic potential *in vivo*, suggesting that HGF-c-Met signaling is necessary for Ras-mediated tumorigenesis and for metastasis (32, 33). Furthermore, immunohistochemical analysis revealed that the expression of HGF and c-Met in human pancreatic cancers correlated with clinical and histological grades (15). These data support our proposal for the potential role of HGF in the development of human pancreatic cancers.

In addition to the involvement of c-Met receptor-mediated signals in invasive growth of pancreatic cancers, another aspect of tumor-stromal interaction necessary for local growth and metastasis is neovascularization in pancreatic cancers (26, 27). Findings of few vessels seen in pancreatic tumors in the angiography plus the scirrhous-type appearance in the histology of human pancreatic cancers sometimes characterizes pancreatic cancer as a less angiogenesis-dependent tumor compared with other well-vascularized tumors such as hepatomas, gliomas, or melanomas. However, tumor angiogenesis is apparent in the periphery and invasive front of pancreatic tumors, and some angiogenesis inhibitors efficiently inhibited tumor growth and metastasis of pancreatic cancer in experimental models (34–36). Thus, an antiangiogenic molecule may well be a candidate for treating patients with pancreatic cancers. We recently reported that NK4 inhibits the angiogenesis mediated by basic fibroblast growth factor and vascular endothelial growth factor, as well as HGF, and that the antiangiogenic activity of NK4 seems to be independent of HGF-antagonist activity (25, 37). Because NK4 but not anti-HGF antibody inhibited tumor-associated angiogenesis in pancreatic cancers, the inhibitory effect of NK4 on growth of cancer in the pancreas seems attributable to its action as an angiogenesis inhibitor rather than as an HGF-antagonist.

When rapid accumulation of ascites occurred in mice at the end stage of the disease, mice died within a few days, and the volume of ascites correlated with the number of disseminated metastatic nodules. Primary tumors in the NK4-treated mice at the end of treatment had grown larger than seen in mice before the treatment but were smaller than matched-control tumors (albeit not significantly so; data not shown), which indicates that NK4 at the dosage used here weakly suppressed the growth of end-stage primary tumors but had no significant impact on tumor growth at this stage. The potential for prolonged survival of mice given NK4-treatment might be associated with the remarkable reduction in the number of disseminated metastases in peritoneum and accumulation of ascites rather than with suppression of growth of the primary tumor.

The formation of metastatic nodules in the peritoneum seems to depend on the invasiveness, growth, and the breakdown of pancreatic-capsule of primary tumor, and subsequent growth of colonized micrometastases. Bifunctional properties of NK4 (HGF antagonist/

angiogenesis inhibitor) make it difficult to clearly address the precise mechanism for the antimetastatic effect of NK4 in this model. However, the failure of anti-HGF antibody to suppress peritoneal dissemination indicates that antiangiogenic activity of NK4 contributed mainly to its antimetastatic action. We therefore speculate that: (*a*) NK4 suppressed the local invasion and angiogenesis-dependent primary tumor growth, thereby inhibiting metastatic spreading, and/or; (*b*) NK4 inhibited angiogenesis-dependent growth of newly colonized metastases in the peritoneal cavity. On the other hand, accumulation of ascites is attributable to enhanced vascular permeability and plasma leakage influenced by cancer cells and/or impaired reabsorption of ascitic fluid attributable to massive invasion of cancer cells into lymphatic vessels (38). Because NK4 remarkably suppressed the penetration of cancer cells into muscle layers of the peritoneal wall (Fig. 8*C*), antagonistic activities of NK4 for HGF may have participated to some extent in suppressing the accumulation of ascites.

Because effective systemic therapy for pancreatic cancer is currently not available, and diagnosing pancreatic cancer in its early stages is difficult, the highly invasive and metastatic behavior of pancreatic cancer lead to difficulty in attaining a long-term survival and a recurrence-free status. Targeting tumor angiogenesis and blockade of HGF-mediated invasion of cancer cells may prove to be potential therapy for patients with pancreatic cancer.

ACKNOWLEDGMENTS

We thank Dr. S. Mizuno (Department of Molecular Regenerative Medicine, Osaka University) for technical advice on the immunohistochemistry and M. Ohara for helpful comments.

REFERENCES

1. Bramhall, S. R., Allum, W. H., Jones, A. G., Allwood, A., Cummins, C., and Neoptolemos, J. P. Treatment and survival in 13,560 patients with pancreatic cancer, and incidence of the disease, in the West Midlands: an epidemiological study. Br. J. Surg., *82:* 111–115, 1995.
2. Niederhuber, J. E., Brennan, M. F., and Menck, H. R. The National Cancer Database report on pancreatic cancer. Cancer (Phila.), *76:* 1671–1677, 1995.
3. Warshaw, A. L., and Fernandez-del Castillo, C. Pancreatic carcinoma. N. Engl. J. Med., *326:* 455–465, 1992.
4. Wanebo, H. J., and Vezeridis, M. P. Pancreatic carcinoma in perspective. A continuing challenge. Cancer (Phila.), *78:* 580–591, 1996.
5. Poston, G. J., Gillespie, J., and Guillou, P. J. Biology of pancreatic cancer. Gut, *32:* 800–812, 1991.
6. Kern, S., Hruban, R., Hollingsworth, M. A., Brand, R., Adrian, T. E., Jaffee, E., and Tempero, M. A. A white paper: the product of a pancreas cancer think tank. Cancer Res., *61:* 4923–4932, 2001.
7. Nakamura, T., Nawa, K., and Ichihara, A. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. Biochem. Biophys. Res. Commun., *122:* 1450–1459, 1984.
8. Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K., and Shimizu, S. Molecular cloning and expression of human hepatocyte growth factor. Nature (Lond.), *342:* 440–443, 1989.
9. Vande Woude, G. F., Jeffers, M., Cortner, J., Alvord, G., Tsarfaty, I., and Resau, J. Met-HGF/SF: tumorigenesis, invasion and metastasis. Ciba Found. Symp., *212:* 119–130, discussion 130–132, 148–154, 1997.
10. Nakamura, T., Matsumoto, K., Kiritoshi, A., Tano, Y., and Nakamura, T. Induction of hepatocyte growth factor in fibroblasts by tumor-derived factors affects invasive growth of tumor cells: *in vitro* analysis of tumor-stromal interactions. Cancer Res., *57:* 3305–3313, 1997.
11. Jiang, W., Hiscox, S., Matsumoto, K., and Nakamura, T. Hepatocyte growth factor/scatter factor: its molecular, cellular and clinical implications in cancer. Crit. Rev. Oncol. Hematol., *29:* 209–248, 1999.
12. Matsumoto, K., and Nakamura, T. HGF-c-Met receptor pathway in tumor invasion-metastasis and potential cancer treatment with NK4. *In:* W. G. Jiang, K. Matsumoto, and T. Nakamura (eds.), Growth Factors and their Receptors in Cancer Metastasis. London: Kluwer Academic Publishers, 2001, *in press*.
13. Ebert, M., Yokoyama, M., Friess, H., Buchler, M. W., and Korc, M. Coexpression of the *c-met* proto-oncogene and hepatocyte growth factor in human pancreatic cancer. Cancer Res., *54:* 5775–5778, 1994.
14. Vila, M. R., Nakamura, T., and Real, F. X. Hepatocyte growth factor is a potent mitogen for normal human pancreas cells *in vitro*. Lab. Investig., *73:* 409–418, 1995.
15. Furukawa, T., Duguid, W. P., Kobari, M., Matsuno, S., and Tsao, M. S. Hepatocyte growth factor and Met receptor expression in human pancreatic carcinogenesis. Am. J. Pathol., *147:* 889–895, 1995.
16. Kiehne, K., Herzig, K. H., and Folsch, U. R. c-met expression in pancreatic cancer and effects of hepatocyte growth factor on pancreatic cancer cell growth. Pancreas, *15:* 35–40, 1997.
17. Paciucci, R., Vila, M. R., Adell, T., Diaz, V. M., Tora, M., Nakamura, T., and Real, F. X. Activation of the urokinase plasminogen activator/urokinase plasminogen activator receptor system and redistribution of E-cadherin are associated with hepatocyte growth factor-induced motility of pancreas tumor cells overexpressing Met. Am. J. Pathol., *153:* 201–212, 1998.
18. Perugini, R. A., McDade, T. P., Vittimberga, F. J., Jr., and Callery, M. P. The molecular and cellular biology of pancreatic cancer. Crit. Rev. Eukaryot. Gene Expr., *8:* 377–393, 1998.
19. Di Renzo, M. F., Poulsom, R., Olivero, M., Comoglio, P. M., and Lemoine, N. R. Expression of the Met/hepatocyte growth factor receptor in human pancreatic cancer. Cancer Res., *55:* 1129–1138, 1995.
20. Maehara, N., Matsumoto, K., Kuba, K., Mizumoto, K., Tanaka, M., and Nakamura, T. NK4, a four-kringle antagonist of HGF, inhibits spreading and invasion of human pancreatic cancer. Br. J. Cancer, *84:* 864–873, 2001.
21. Date, K., Matsumoto, K., Shimura, H., Tanaka, M., and Nakamura, T. HGF/NK4 is a specific antagonist for pleiotrophic actions of hepatocyte growth factor. FEBS Lett., *420:* 1–6, 1997.
22. Date, K., Matsumoto, K., Kuba, K., Shimura, H., Tanaka, M., and Nakamura, T. Inhibition of tumor growth and invasion by a four-kringle antagonist (HGF/NK4) for hepatocyte growth factor. Oncogene, *17:* 3045–3054, 1998.
23. Hiscox, S., Parr, C., Nakamura, T., Matsumoto, K., Mansel, R. E., and Jiang, W. G. Inhibition of HGF/SF-induced breast cancer cell motility and invasion by the HGF/SF variant, NK4. Breast Cancer Res. Treat., *59:* 245–254, 2000.
24. Parr, C., Hiscox, S., Nakamura, T., Matsumoto, K., and Jiang, W. G. NK4, a new HGF/SF variant, is an antagonist to the influence of HGF/SF on the motility and invasion of colon cancer cells. Int. J. Cancer, *85:* 563–570, 2000.
25. Kuba, K., Matsumoto, K., Date, K., Shimura, H., Tanaka, M., and Nakamura, T. HGF/NK4, a four-kringle antagonist of hepatocyte growth factor, is an angiogenesis inhibitor that suppresses tumor growth and metastasis in mice. Cancer Res., *60:* 6737–6743, 2000.
26. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat. Med., *1:* 27–31, 1995.
27. Hanahan, D., and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell, *86:* 353–364, 1996.
28. Mizuno, S., Matsumoto, K., Kurosawa, T., Mizuno-Horikawa, Y., and Nakamura, T. Reciprocal balance of hepatocyte growth factor and transforming growth factor-β 1 in renal fibrosis in mice. Kidney Int., *57:* 937–948, 2000.
29. Nakamura, T., Mizuno, S., Matsumoto, K., Sawa, Y., Matsuda, H., and Nakamura, T. Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. J. Clin. Investig., *106:* 1511–1509, 2000.
30. Reyes, G., Villanueva, A., Garcia, C., Sancho, F. J., Piulats, J., Lluis, F., and Capella, G. Orthotopic xenografts of human pancreatic carcinomas acquire genetic aberrations during dissemination in nude mice. Cancer Res., *56:* 5713–5719, 1996.
31. Capella, G., Farre, L., Villanueva, A., Reyes, G., Garcia, C., Tarafa, G., and Lluis, F. Orthotopic models of human pancreatic cancer. Ann. N.Y. Acad. Sci., *880:* 103–109, 1999.
32. Ivan, M., Bond, J. A., Prat, M., Comoglio, P. M., and Wynford-Thomas, D. Activated *ras* and *ret* oncogenes induce over-expression of c-met (hepatocyte growth factor receptor) in human thyroid epithelial cells. Oncogene, *14:* 2417–2423, 1997.
33. Webb, C. P., Taylor, G. A., Jeffers, M., Fiscella, M., Oskarsson, M., Resau, J. H., and Vande Woude, G. F. Evidence for a role of Met-HGF/SF during Ras-mediated tumorigenesis/metastasis. Oncogene, *17:* 2019–2025, 1998.
34. Kawarada, Y., Ishikura, H., Kishimoto, T., Saito, K., Takahashi, T., Kato, H., and Yoshiki, T. Inhibitory effects of the antiangiogenic agent TNP-470 on establishment and growth of hematogenous metastasis of human pancreatic carcinoma in SCID beige mice *in vivo*. Pancreas, *15:* 251–257, 1997.
35. Bruns, C. J., Solorzano, C. C., Harbison, M. T., Ozawa, S., Tsan, R., Fan, D., Abbruzzese, J., Traxler, P., Buchdunger, E., Radinsky, R., and Fidler, I. J. Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. Cancer Res., *60:* 2926–2935, 2000.
36. Schwarte-Waldhoff, I., Volpert, O. V., Bouck, N. P., Sipos, B., Hahn, S. A., Klein-Scory, S., Luttges, J., Kloppel, G., Graeven, U., Eilert-Micus, C., Hintelmann, A., and Schmiegel, W. Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. Proc. Natl. Acad. Sci. USA, *97:* 9624–9629, 2000.
37. Kuba, K., Matsumoto, K., Ohnishi, K., Shiratsuchi, T., Tanaka, M., and Nakamura, T. Kringle 1–4 of hepatocyte growth factor inhibits proliferation and migration of human microvascular endothelial cells. Biochem. Biophys. Res. Commun., *279:* 846–852, 2000.
38. Dvorak, H. F., Brown, L. F., Detmar, M., and Dvorak, A. M. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am. J. Pathol., *146:* 1029–1039, 1995.

Inhibition of Growth, Invasion, and Metastasis of Human Pancreatic Carcinoma Cells by NK4 in an Orthotopic Mouse Model

Daisaku Tomioka, Naoki Maehara, Keiji Kuba, et al.

Cancer Res 2001;61:7518-7524.